MILENA SERENATO KLEPA

TAXONOMIA BACTERIANA NA ERA GENÔMICA E A DESCRIÇÃO DE DEZ NOVAS ESPÉCIES DE *BRADYRHIZOBIUM*

Londrina
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Tese apresentada ao Programa de Pós-graduação em Microbiologia da Universidade Estadual de Londrina - UEL, como requisito parcial para a obtenção do título de Doutor.

Orientador: Prof. Mariangela Hungria

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KLEPA, Milena Serenato. **Taxonomia bacteriana na era genômica e a descrição de dez novas espécies de *Bradyrhizobium***. 2022. 131 f. Tese (Doutorado em Microbiologia) – Universidade Estadual de Londrina, Londrina, 2022.

RESUMO

Os rizóbios são bactérias amplamente difundidas nos solos e contribuem significativamente para o aporte de nitrogênio em ambientes naturais e agrícolas pela simbiose com plantas leguminosas. No entanto, a magnitude da diversidade desses microrganismos, juntamente com suas propriedades metabólicas e evolutivas, ainda está longe de ser totalmente decifrada. Identificar, classificar e nomear bactérias seguindo critérios específicos é tarefa da taxonomia, uma ciência que é realizada desde a década de 1870, mas que tem sido revolucionada com o progresso tecnológico. Os objetivos deste trabalho foram estudar como a taxonomia bacteriana evoluiu e as implicações na taxonomia de rizóbios, além da descrição de dez novas espécies de *Bradyhrizobium*, um gênero amplamente conhecido pela interação bem-sucedida com a soja, além de várias outras espécies de leguminosas, particularmente nos trópicos. O capítulo I se refere a uma revisão bibliográfica na qual são detalhados os caminhos que a taxonomia bacteriana percorreu até chegar à era genômica. São abordados ainda os requisitos necessários para a descrição de novas espécies bacterianas, desde as técnicas comumente utilizadas, até as regras estabelecidas por comitês de taxonomia bacteriana. Para finalizar, analisa-se como isso impactou no número de espécies bacterianas e na compreensão da diversidade e evolução dos rizóbios. Os capítulos II, III e IV envolvem a descrição de dez novas espécies de *Bradyrhizobium,* nomeadas como *B. archetypum, B. australiense, B. murdochi, B. agreste, B. diversitatis, B. glycinis, B. australafricanum, B. cenepequi, B. hereditatis* e *B. semiaridum,* as quais foram isoladas de nódulos radiculares de leguminosas da Austrália e da África do Sul. As estirpes estudadas foram diferenciadas das demais espécies do gênero por uma abordagem polifásica, incluindo aspectos filogenéticos, genômicos e fenotípicos. Além disso, foi possível identificar a existência de três novos simbiovares no gênero*,* grupos de estirpes que podem se assemelhar por filogenia de genes simbióticos ou planta hospedeira, os quais foram nomeados como sv. cenepequi, sv. glycinis, e sv. cajani, de acordo com o nome da espécie da estirpe que ocupava a posição central no ramo da filogenia dos genes de nodulação *nodC* e de fixação de nitrogênio *nifH*. O primeiro capítulo revela a importância da taxonomia bacteriana como uma ciência colaborativa e dinâmica, que vem sendo alterada com o passar do tempo. Além disso, a reunião de informações acerca da taxonomia bacteriana e descrição de novas espécies podem servir como um guia para microbiologistas e taxonomistas. Os resultados obtidos nos capítulos II, III e IV, contribuem com informações sobre a diversidade do gênero *Bradyrhizobium*, mais especificamente, sobre aspectos metabólicos, evolutivos e genômicos. Destaca-se a importância de tais estudos na microbiologia do solo para a compreensão de quem são os habitantes do solo, a sua variabilidade genética, seus aspectos evolutivos e quais interações realizam, de modo a revelar estirpes que possam ser exploradas biotecnologicamente.

Palavras-chave: taxonomia bacteriana; sequenciamento de genomas; novas espécies; rizóbios; fixação biológica de nitrogênio.

KLEPA, Milena Serenato. **Taxonomy of bacteria in the genomic era and description of ten new species of *Bradyrhizobium***. 2022. 131 p. Thesis (Doctorate degree in Microbiology) – State University of Londrina, Londrina, 2022.

ABSTRACT

Rhizobia are globally widespread bacteria in soils and contribute significantly to the nitrogen input into the biosphere via symbiosis with leguminous plants. However, the magnitude of the rhizobial diversity and their metabolic and evolutionary properties are still far from being fully known. Taxonomy is the science responsible for identifying, classifying and naming organisms following specific rules; it has been carried out since the 1870s and has been revolutionized by technological progress. This thesis aimed to study how the taxonomy of bacteria evolved and the implications on the taxonomy of rhizobia; in addition, ten new species of *Bradyhrizobium* were described, a genus widely known for its successful interaction with the soybean crop and several other legumes, with an emphasis on the tropics. The first chapter is a review where we detail the evolution of the taxonomy of bacteria until the genomic era. We also address requirements and techniques commonly used for the description of new species, and the rules established by bacterial taxonomy committees. Finally, the impact on the number of bacterial species and on the diversity and evolution of rhizobia were analyzed. Chapters II, III and IV involved the description of ten new species of *Bradyrhizobium* named as *B. archetypum, B. australiense, B. murdochi, B. agreste, B. diversitatis, B. glycinis, B. australafricanum, B. cenepequi, B. hereditatis* and *B. semiaridum*, which were isolated from root nodules of leguminous plants from Australia and South Africa. The strains under study were separated from the other species of the genus using a polyphasic approach based on phylogenetic, genomic and phenotypic aspects. In addition, three new symbiovars were identified in the genus, representing groups of strains that may resemble each other by the phylogeny of symbiotic genes or host range. The new symbiovars were named as sv. cenepequi, sv. glycinis, and sv. cajani, according to the name of the species with a central position in the branch of the nodulation *nodC* and nitrogen fixation *nifH* genes phylogenies. The first chapter points out the importance of the taxonomy of bacteria as a collaborative and dynamic science, which has changed over time. In addition, the information about taxonomy and description of new species can be used as a guide for microbiologists and taxonomists. Chapters II, III and IV provide information about the diversity of the genus *Bradyrhizobium*; more specifically, on metabolic, evolutionary and genomic aspects. Such studies are important in soil microbiology to show the soil bacterial population, their genetic variability, evolutionary aspects and interactions, in order to reveal strains that can be explored for biotechnological purposes.

Key words: taxonomy of bacteria; sequencing of genomes; new species; rhizobia; biological nitrogen fixation.

SUMÁRIO

1. INTRODUÇÃO

A biomassa e a atividade de microrganismos no solo é um dos fatores mais influentes na disponibilidade de nutrientes e na produtividade de plantas em ecossistemas terrestres. Apesar de o solo predominantemente apresentar condições desafiadoras para a sobrevivência dos microrganismos, estima-se que um grama de solo pode conter 10^9 células microbianas, distribuídas em diversos filos e, portanto, o solo é considerado um grande reservatório de biodiversidade genética e metabólica (FIERER, 2017; ZHAO et al., 2017; CUSTÓDIO et al., 2022).

Além de estabelecer interações entre si, alguns microrganismos do solo também podem interagir simbioticamente com plantas, como é o caso dos rizóbios, bactérias capazes de induzir a formação de nódulos nas raízes ou caules de plantas da família Fabaceae, as leguminosas. Essas estruturas especializadas permitem a ação da enzima nitrogenase, responsável por reduzir o nitrogênio atmosférico N_2 a uma forma assimilável aos hospedeiros vegetais, num processo denominado fixação biológica de nitrogênio (FBN) (WANG et al., 2019; KUMAR et al., 2020).

A FBN é essencial para o fornecimento de nitrogênio a plantas em ambientes naturais e agrícolas. Nesse último caso, a adição de estirpes elite de rizóbios para cada leguminosa pode reduzir significativamente ou até mesmo suprimir o uso de fertilizantes nitrogenados. A síntese e uso desses fertilizantes resultam na emissão de gases do efeito estufa, sem contar os possíveis impactos negativos ao solo, lençóis freáticos e, consequentemente, na biodiversidade local (KOPTTIKE et al., 2019). Além disso, a redução ou substituição de fertilizantes nitrogenados por inoculantes, produtos biológicos certificados por órgãos competentes e contendo uma alta concentração de células de estirpes de rizóbios selecionadas, é viável economicamente, gerando uma economia incomparável para os países que fazem uso dessa tecnologia (HUNGRIA e MENDES, 2015). Dada a importância da FBN, conhecer os microrganismos presentes no solo representa o primeiro passo para a identificação e preservação dos microrganismos adaptados às plantas hospedeiras específicas e às condições de cada solo, os quais podem apresentar um potencial biotecnológico relevante para a agricultura ou regeneração de áreas degradadas.

A taxonomia bacteriana é a ciência destinada à classificação de

bactérias de acordo com a similaridade e seguindo um sistema hierárquico, o qual permite interpretar as relações evolutivas existentes entre eles. Esse sistema hierárquico é dado por domínio, filo, classe, ordem, família, gênero e espécie, sendo a categoria espécie portadora de maior diversidade (KÄMPFER e GLAESER, 2012). Além disso, a taxonomia bacteriana é responsável por nomear as bactérias seguindo regras específicas e identificar se organismos recém-isolados pertencem a uma categoria taxonômica já descrita (ROSSELLÓ-MÓRA e WHITMAN, 2018; CARRO et al., 2021). Com o avanço tecnológico, o aprimoramento de técnicas voltadas ao estudo de microrganismos permitiu o estabelecimento do conceito de taxonomia polifásica, a qual impulsionou a descrição de milhares de espécies bacterianas durante um longo período (HELENE et al., 2022). Entende-se por taxonomia polifásica o delineamento de espécies baseado em critérios fenotípicos, genotípicos e filogenéticos (RAINA et al., 2019).

O primeiro genoma bacteriano sequenciado foi da espécie *Haemophilus influenzae*, em 1995, usando a técnica de sequenciamento de Sanger (HELENE et al., 2022). Hoje, é possível sequenciar um genoma bacteriano completo em até 48 horas, com um preço relativamente acessível, utilizando aparelhos portáteis até mesmo fora de um laboratório. Os caminhos que a ciência percorreu nos permitem afirmar que estamos vivendo uma era genômica na classificação bacteriana (HELENE et al., 2022). Com isso, a descrição de novas espécies bacterianas seguindo uma perspectiva genômica é altamente recomendada (CHUN et al., 2018), aumentando significativamente o número de sequências genômicas disponíveis em bancos de dados. Em julho de 2022, cerca de 1.213.548 genomas bacterianos estavam disponíveis no *National Center for Biotechnological Information* (NCBI) (NCBI, 2022).

A acessibilidade aos dados genômicos permitiu a expansão de técnicas de comparação da similaridade entre genomas *in silico*, como *Average Nucleotide Identity* (ANI) e hibridização DNA-DNA digital (HDDd) que são, atualmente, consideradas padrão-ouro na taxonomia bacteriana, em que valores de corte de 96% e 70% de similaridade, respectivamente, foram estabelecidos para o delineamento de espécies (MEIER-KOLTHOFF et al., 2013; CHUN et al., 2018). Além disso, os avanços na anotação de genomas bacterianos têm facilitado a realização de análises filogenéticas do genoma *core*, que inclui centenas de genes presentes em todas as estirpes em estudo, os quais são conservados e participantes

do metabolismo basal, permitindo uma filogenia mais fidedigna (CARRO et al., 2021; HUGENHOLTZ et al., 2021).

Tratando-se da taxonomia de rizóbios, esses organismos são pertencentes ao filo Proteobacteria e distribuídos tanto na classe Alfaproteobactéria, a qual abrange maior número de taxa, quanto na classe Betaproteobactéria, a qual há cerca de duas décadas foi relatada como contendo espécies capazes de fixar nitrogênio em simbiose com leguminosas. A classe Alfaproteobactéria possui a ordem Hyphomicrobiales (=Rhizobiales), na qual os rizóbios são encontrados em sete famílias e 16 gêneros, enquanto a classe Betaproteobactéria apresenta rizóbios na ordem Burkholderiales, em uma família e três gêneros. Ambos os grupos, Alfa e Betaproteobactéria, totalizam 226 espécies de rizóbios até o momento (HELENE et al., 2022). Também é possível perceber um aumento no número de genomas de rizóbios disponíveis em bancos de dados. *Bradyrhizobium*, um gênero amplamente conhecido pela associação simbiótica com soja e com espécies de leguminosas tropicais, apresentava 187 genomas de estirpes depositados no NCBI em 2018 (ORMEÑO-ORRILLO e MARTÍNEZ-ROMERO, 2019); em julho de 2022, o gênero conta com 587 genomas disponíveis (NCBI, 2022).

Outro interesse crescente reside em diferentes análises genômicas que podem ser aplicadas ao estudo dos genes relacionados à nodulação e à FBN, sua organização no genoma e transferência horizontal. Diante disso, pode-se afirmar que a taxonomia de rizóbios avançou conjuntamente com a evolução da taxonomia bacteriana, gerando uma melhor compreensão da origem e evolução desses organismos, além da caracterização de estirpes que apresentam um papel fundamental na produtividade agrícola (HELENE et al., 2022).

Bradyrhizobium é um dos gêneros mais importantes quando se fala em rizóbios. Como exemplo, estirpes selecionadas por apresentar uma alta competitividade em relação às bactérias estabelecidas no solo e com alta capacidade de fixar nitrogênio em associação com a soja têm sido usadas como inoculantes para essa cultura no Brasil, gerando a economia estimada, em 2015, em 15 bilhões de dólares para o país (HUNGRIA e MENDES, 2015). No entanto, este gênero é cosmopolita e apresenta uma larga faixa de hospedeiros; 24 das 33 tribos de Fabaceae são capazes de estabelecer relações simbióticas com estirpes deste gênero (WEISBURG et al., 2022). Além do estilo de vida simbiótico, *Bradyrhizobium* apresenta uma grande comunidade de estirpes habitando o solo livremente (TAO et

al., 2021), ou promovendo o crescimento de plantas em associações endofíticas (de CASTILHO et al., 2021). Outras peculiaridades do gênero incluem estirpes capazes de nodular plantas não-leguminosas do gênero *Parasponia* (DUPIN et al., 2020), e estirpes que preservaram a capacidade fotossintética, as quais foram relatadas como capazes de nodular leguminosas utilizando mecanismos não tradicionais (GIRAUD et al., 2007).

Embora a *List of Prokaryotic Names with Standing in Nomenclature* (LPSN) aponte que o gênero *Bradyrhizobium* possuía, em agosto de 2022, 82 espécies descritas (LPSN, 2022), dentre as quais algumas necessitam de uma validação formal por regras de nomenclatura, alguns estudos genômicos e estatísticos estimam que o gênero apresente aproximadamente 800 espécies na natureza (ORMEÑO-ORILLO e MARTÍNEZ-ROMERO, 2019). *Bradyrhizobium* é conhecido por apresentar genomas relativamente grandes, com um tamanho médio de 8.6 Mpb, no entanto, o maior genoma descrito no gênero atingiu 11.7 Mpb (ORMEÑO-ORRILLO e MARTÍNEZ-ROMERO, 2019), tamanho próximo ao maior genoma bacteriano já relatado na literatura, de 14.7 Mpb (HAN et al., 2013). Além disso, os genes responsáveis pela nodulação e FBN, resumidamente representados pelos genes *nod* e *nif*, são agrupados em ilhas genômicas incorporadas no cromossomo, apresentando grande diversidade e número de tais genes (WEISBURG et al., 2022). Diante de tamanha variabilidade genética, o gênero *Bradyrhizobium* tornou-se um intrigante e importante objeto de estudo na área de microbiologia do solo.

A presente tese foi dedicada ao estudo da taxonomia de rizóbios na era genômica, resultando em uma revisão bibliográfica que discutiu detalhadamente a evolução das técnicas utilizadas na taxonomia bacteriana e como isso impactou o estudo da diversidade de rizóbios; além disso, dez novas espécies de *Bradyrhizobium* foram descritas, as quais foram chamadas de *B. archetypum, B. australiense, B. murdochi, B. agreste, B. diversitatis, B. glycinis, B. australafricanum, B. cenepequi, B. hereditatis* e *B. semiaridum*.

REFERÊNCIAS BIBLIOGRÁFICAS

CARRO, L.; PEIX, Á.; VELÁZQUEZ, E. The Taxonomy of bacteria in the genomic era. In: VILLA, T. G.; de MIGUEL BOUZAS, T. (eds.). **Developmental biology in prokaryotes and lower eukaryotes**, 2021. p. 289–309. https://doi.org/10.1007/978-3-030-77595-7_12

CHUN, J. et al. Proposed minimal standards for the use of genome data for the taxonomy of prokaryotes. **International Journal of Systematic and Evolutionary Microbiology**, v. 68, n. 1, p. 461–466, 2018. https://doi.org/10.1099/ijsem.0.002516

CUSTÓDIO, V. et al. Sculpting the soil microbiota. **The Plant Journal**, v. 109, p. 508-522, 2022. https://doi.org/10.1111/tpj.15568

de CASTILHO, C. L. et al. Growth-promoting effects of *Bradyrhizobium* soybeans symbionts in black oats, white oats, and ryegrass. **Brazilian Journal of Microbiology**, v. 52, p. 1451-1460, 2021. https://doi.org/10.1007/s42770-021-00523-1

DUPIN, S. E.; GEURTS, R.; KIERS, T. E. The non-legume *Parasponia andersonii* mediates the fitness of nitrogen-fixing rhizobial symbionts under high nitrogen conditions. **Frontiers in Plant Science**, v. 10, p. 1779, 2019. https://doi.org/10.3389/fpls.2019.01779

GIRAUD, E. et al. Legumes symbioses: absence of nod genes in photosynthetic *Bradyrhizobia*. **Science**, v. 316, n. 5829, p. 1307–1312, 2007. https://doi.org/10.1126/science.1139548

HAN, K. et al. Extraordinary expansion of a *Sorangium cellulosum* genome from an alkaline milieu. **Scientific reports**, v. 3, n. 1, p. 1-7, 2013. https://doi.org/10.1038/srep02101

HELENE, L. C. F.; KLEPA, M. S.; HUNGRIA, M. New insights into the taxonomy of bacteria in the genomic era and a case study with rhizobia. **International Journal of Microbiology**, v. 2022, p. 4623713, 2022. https://doi.org/10.1155/2022/4623713

HUGENHOLTZ, P. et al. Prokaryotic taxonomy and nomenclature in the age of big sequence data. **Multidisciplinary journal of Microbial Ecology**, v. 15, p. 1879-1892, 2021. https://doi.org/10.1038/s41396-021-00941-x

HUNGRIA, M. e MENDES, I. C. Nitrogen fixation with soybean: the perfect symbiosis? In: Frans J. de Bruijn (eds.) **Biological nitrogen fixation**. 2015. p. 1009-1024. http://dx.doi.org/10.1002/9781119053095.ch99

KÄMPFER, P. e GLAESER, S. P. Prokaryotic taxonomy in the sequencing era - the polyphasic approach revisited. **Environmental Microbiology**, v. 14, n. 2, p. 291–317, 2012. https://doi.org/10.1111/j.1462-2920.2011.02615.x

KOPTTIKE, P. M. et al. Soil and the intensification of agriculture for global food security. **Environment International**, v. 132, p. 105078, 2019. https://doi.org/10.1016/j.envint.2019.105078

KUMAR, N. et al. The rhizobium-plant symbiosis: state of the art. In: VARMA, A.; Tripathi, S.; PRASAD, R. (eds.). **Plant microbe symbiosis**. 2020, p. 1-21. https://doi.org/10.1007/978-3-030-36248-5

LPSN – List of Prokaryotic Names with Standing in Nomenclature. Disponível em: https://lpsn.dsmz.de/ Acesso em: 4 jul. 2022.

MEIER-KOLTHOFF, J. P. et al. Genome sequence-based species delimitation with confidence intervals and improved distance functions. **BMC Bioinformatics**, v. 14, n. 60, p. 1–14, 2013. http://www.biomedcentral.com/1471-2105/14/60

NCBI – National Center *for* Biotechnological Information. Disponível em: https://www.ncbi.nlm.nih.gov/ Acesso em: 7 jul. 2022.

ORMEÑO-ORRILLO, E. e MARTÍNEZ-ROMERO, E. A genomotaxonomy view of the *Bradyrhizobium* genus. **Frontiers in Microbiology**, v. 10, p. 1334, 2019. https://doi.org/10.3389/fmicb.2019.01334

RAINA, V. et al. A polyphasic taxonomic approach for designation and description of novel microbial species. In: DAS, S. e DASH, H. R. (eds.). **Microbial diversity in the**

genomic era. 2019. p. 137-152. https://doi.org/10.1016/B978-0-12-814849-5.00009-5

ROSSELLÓ-MÓRA, R. e WHITMAN, W. B. Dialogue on the nomenclature and classification of prokaryotes. **Systematic and Applied Microbiology**, v. 42, n. 1, p. 5-15, 2018. https://doi.org/10.1016/j.syapm.2018.07.002

TAO, J. et al. Evolutionary origin and ecological implication of a unique *nif* island in free-living *Bradyrhizobium* lineages. **Multidisciplinary Journal of Microbial Ecology**, v. 15, p. 3195-3206, 2021. https://doi.org/10.1038/s41396-021-01002-z

WANG, E. T. et al. Symbiosis between rhizobia and legumes. In: WANG, E. T. et al. (eds.). **Ecology and evolution of rhizobia:** principles and applications. 2019. p. 3-15. https://doi.org/10.1007/978-981-32-9555-1

WEISBERG, A. et al. Pangenome evolution reconciles robustness and instability of rhizobial symbiosis. **mBio**, v. 13, n. 3, p. e00074-22, 2022. https://doi.org/10.1128/mbio.00074-22

ZHANG, Z. et al. Soil bacterial quantification approaches coupling with relative abundances reflecting the changes of taxa. **Scientific Reports**, v. 7, p. 4837, 2017. https://doi.org/10.1038/s41598-017-05260-w

CAPÍTULO I

NOVOS INSIGHTS NA TAXONOMIA BACTERIANA NA ERA GENÔMICA E UM ESTUDO DE CASO COM RIZÓBIOS

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RESUMO

Desde o princípio, a história da taxonomia de procariotos tem sido alterada com o desenvolvimento de novas e mais robustas tecnologias. Como resultado, o número de descrições de novos taxa tem aumentando exponencialmente e um número cada vez maior de outros taxa tem sido alvo de reclassificações, exigindo esforços para manter um sistema hierárquico organizado. No entanto, com a era genômica, espera-se que a taxonomia de procariotos atinja um status mais estável. Embora algumas análises de caracterização dos microrganismos ainda sejam necessárias e muito utilizadas, o uso de dados genômicos pode ser suficiente para o delineamento de taxa, facilitando a classificação e identificação correta dos organismos. Aqui nós descrevemos a evolução da taxonomia de procariotos, com ênfase no domínio Bacteria, até a era genômica. A história da taxonomia de rizóbios foi utilizada como exemplo devido a importância da simbiose entre rizóbios e leguminosas para o fornecimento de nitrogênio em ambientes naturais e agrícolas pela fixação biológica de nitrogênio. O presente estudo mostra os avanços tecnológicos e metodologias utilizadas para classificar e identificar espécies bacterianas, assim como, as regras requeridas para uma descrição adequada de novos taxa.

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Review Article

New Insights into the Taxonomy of Bacteria in the Genomic Era and a Case Study with Rhizobia

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Since early studies, the history of prokaryotes taxonomy has dealt with many changes driven by the development of new and more robust technologies. As a result, the number of new taxa descriptions is exponentially increasing, while an increasing number of others has been subject of reclassification, demanding from the taxonomists more effort to maintain an organized hierarchical system. However, expectations are that the taxonomy of prokaryotes will acquire a more stable status with the genomic era. Other analyses may continue to be necessary to determine microbial features, but the use of genomic data might be sufficient to provide reliable taxa delineation, helping taxonomy to reach the goal of correct classification and identification. Here we describe the evolution of prokaryotes' taxonomy until the genomic era, emphasizing bacteria and taking as an example the history of rhizobia taxonomy. This example was chosen because of the importance of the symbiotic nitrogen fixation of legumes with rhizobia to the nitrogen input to both natural ecosystems and agricultural crops. This case study reports the technological advances and the methodologies used to classify and identify bacterial species and indicates the actual rules required for an accurate description of new taxa.

1. Introduction

The taxonomy terminology has been broadly discussed. Some researchers consider the taxonomy as systematics, while others define taxonomy as the classification of organisms and part of the systematics, which would have a broader scope, including studies with evolutionary and phylogenetic components [1]. Taxonomy is the science responsible for the orderly arrangement of living organisms respecting a hierarchical system that can presume the evolutionary relationships; it also offers relevant information about the origin of life and how it evolved on Earth [2, 3]. The objective of general taxonomy is to establish a classification system based on genealogical relationships, aiming to reach a natural system that mirrors the "order in nature" [4–6].

Prokaryotes include living organisms belonging to both domains, Archaea and Bacteria, known as archaebacteria or archaea and eubacteria, respectively. Those microorganisms do not have a distinct nucleus or other organelles due to the lack of internal membranes, main characteristics distinguishing them from the eukaryotes. The prokaryotic taxonomy is traditionally split into three correlated areas: classification, nomenclature, and identification [7, 8]. The orderly arrangement of organisms into taxonomic ranks designates the classification. The nomenclature objective is

to name the organisms following the International Code of Nomenclature of Prokaryotes (ICNP) rules. The identification involves allocating the strains into the described taxonomic groups [7, 9, 10]. However, if the taxonomy of a strain remains undefined, the characterization and phylogenetic analysis should be carefully carried out to describe and name the new taxon.

The basic unit of taxonomy is the species. Bergey's Manual of Systematic Bacteriology defines a bacterial species as a group of strains with certain distinctive features that generally resemble each other in essential features of an organization [11]. Estimates are of about 1012 bacterial species on Earth [12]. In November 2021, there were about 17,845 valid species names (without synonyms) [13]. Considering this number, we can conclude that bacterial diversity is not sufficiently explored and that most might still be composed of still uncultivable species.

Besides arranging the organisms, the taxonomic tools are used to study microbial diversity and establish phylogenetic relationships. Biodiversity represents the basis of the stability of the ecosystems, providing environmental resilience [14]. Assessing microbial biodiversity also provides various biotechnological resources for food production and regenerative agriculture, environmentally-friendly technologies such as bioremediation and bioprospection of new enzymes, antibiotics, and biological processes. Studies on microbial diversity and taxonomy are crucial to guarantee the environmental and socioeconomic benefits be still unknown to the microbial world. In one attempt to put a monetary value on goods and services provided by ecosystems, the worth of biodiversity was estimated at the outstanding value of US$ 33 trillion per year, close to the GDP (growth domestic product) of the United States and China combined [15]. Other estimates point out to lower or similar values, but undoubtedly, all of them are important [16].

Although many techniques, rules, and concepts are used for prokaryotes in general, in this review we describe how bacterial taxonomy evolved until genomic era and important tools developed to assess bacterial diversity and guide to proper classification. We also present a case study with rhizobia to clarify how the evolution of the taxonomic science impacted this group of bacteria, probably amongst the most important for ecosystems and agricultural sustainability, and improved our knowledge about them.

2. The Bacteria Species Concept and Description of New Taxa

The species concept is considered a universal theory limiting the category "species" for all living organisms. Concerning the prokaryotes, several incongruences are discussed, since they do not fit into the most common eukaryotic species perceptions, such as the morphological, biological, or evolutionary concepts [17]. For years, the bacterial species were classified based on phenotypic features, followed by polyphasic analyses involving phenotypic, genotypic, and phylogenetic properties, but with the methods differing among bacterial groups. As a result, each described taxon is represented by a lower taxon, and a type species represents each genus. Nowadays, the advancement of next-generation sequencing (NGS) has encouraged the description of novel species based mainly on genomic sequences [18], and this practice is revolutionizing the bacterial species concept and the taxonomic groups [19–23].

For the description of novel bacterial taxa, the taxonomists follow guidelines from the International Committee on Systematic of Prokaryotes (ICSP), split into several subcommittees, according to the knowledge areas. Regarding the rhizobial species, the Subcommittee on Taxonomy of Rhizobia and Agrobacteria is responsible for the guidelines. For many years, the only guideline for rhizobia taxa description available was published by Graham and collaborators in 1991 [24]. More recently, subcommittee members proposed an improvement to the minimal standards for the species and genera description [25], provided on the website https://sites.google.com/view/taxonomyagrorhizo/home. The genomic profile comparison of strains studied and related type strains is required among the updates, and the genome sequence of the type strain representing the new species must be deposited in databases. This requirement will increase the number of genomes available for further studies.

Another critical step in taxonomy concerns the proper nomenclature, which the ICNP regulates. The scientific name of a novel bacterial taxon needs to be in Latin, referring to the history of the taxon, and be published in the "Approved List" of prokaryotes to become a valid name. In the International Journal of Systematic and Evolutionary Microbiology (IJSEM), the official journal of the ICSP, a clear statement of the name (i.e., fam. nov., gen. nov., sp. nov., etc.) and its etymology, as well as the characterization data of the taxon and the type strain designation, must be provided [26]. The valid names outside the IJSEM must follow these same guidelines and request the official journal validation [27].

The List of Prokaryotic names with Standing in Nomenclature (LPSN) is an online tool constantly updated by the Leibniz Institute DSMZ—German Collection of Microorganisms and Cell Cultures GmbH (https://lpsn.dsmz.de/). It includes a broad range of taxonomic information for each described taxon, such as etymology, nomenclatural and taxonomic status, type strain designations, and the link to the description publication [13]. However, it is worth mentioning that some prokaryotes, such as those still uncultivable, or genetic material retrieved from the environment, although might have sequences indicating that could represent new species, do not have sufficient information required to be described as a novel taxon [28]. Therefore, these organisms are classified as a "candidate" of a new species or a new genus, receiving the taxonomic status *Candidatus* [29]. Nowadays, with the development of metagenomics, which involves acquiring genome sequences from sampled materials of a community of organisms inhabiting a common environment, millions of 16S rRNA sequences have been deposited in databases [30], and a large number of them share less than the suggested threshold value of similarity for species circumscription. Therefore, even though the uncultivable prokaryotes have not been isolated yet, the analyses of those sequences imply that they might represent different species. Given this evidence, many

efforts have been carried out for gene sequences of uncultivable taxa to be considered by ICSP and to change the status of *Candidatus* to valid names [31].

3. A Brief View of Bacteria Taxonomy

Ferdinand Cohn developed the first studies of bacterial classification in the 1870s. He used morphologic traits as criteria to define six different genera [1]. From this, different methods of classification based on phenotypic traits were published, making the studies of taxonomists difficult because of the lack of minimal standards and common agreement on classification [17]. From the first edition of the Bergey's Manual of Determinative Bacteriology (1984–1989), this status was changed, with the definition of a reference guideline for bacterial classification, providing conditions for the microbiologists to merge the criteria adopted among them [32].

Even though the clustering methods emerged before the advent of computers, they were applied in bacteriology just after using computers, around 1957, by Sneath and Ludwig et al. [33, 34]. This era of bacterial taxonomy was marked by numerical studies where several phenotypic properties were electronically tabulated and used as a relatedness measurement. However, although the numerical taxonomy improved the identification of bacteria, it did not consider phylogenetic analyses [35]. Together with the evolution of numerical taxonomy, new techniques considering the physiologic and biochemical traits started to be developed, such as chromatographic and electrophoretic methods to define chemotaxonomic markers, aiming to reach the goal of a universal classification system for bacteria [17].

In the early 1960s, the development of molecular techniques supported the inclusion of methodologies such as the DNA guanine and cytosine content (GC mol%) and DNA-DNA hybridization (DDH) [36, 37] for taxonomic studies. Those techniques allowed the comparison of genomes, improving the classification of bacteria. Since the first experiments based on single-stranded DNA reassociation conducted by Schildkraut et al. in 1961 [38], and for about 50 years, the DDH was used as a standard technique for bacterial species circumscription [8, 9, 17].

A remarkable breakthrough in the attempts to determine relationships between distantly related organisms came around the 1970s when the Taq polymerase enzyme was discovered and used for DNA amplification through the polymerase chain reaction (PCR) techniques [39], and the dideoxy sequencing technology was described by Sanger et al. [40]. Almost simultaneously, the small ribosomal subunits 16S rRNA for prokaryotes and 18S rRNA for eukaryotes were described and started to be broadly used as molecular markers to organize all living organisms into three superior taxa, Archaea, Bacteria, and Eukarya, named as domains [41]. This new hierarchical taxonomic system based on these molecular markers allowed the incorporation of phylogenetic information in the prokaryotic classification [2, 42–45].

The phylogeny studies the evolutionary relationships among organisms, and using conserved molecular data became commonly accepted in taxonomy. After the ribosomal sequences, other conserved genes started to be used [46]. Although less sequences can be used, the Multilocus Sequence Typing (MLST) generally evaluates 8 to 12 concatenated housekeeping genes or other protein-coding genes to identify genotypes and differentiate closely related strains, becoming broadly applied in molecular epidemiology. In the MLST, each gene (locus) contains different alleles; the allelic differences are converted in values resulting in the "sequence type" for the bacteria, which are available in a specific database for comparisons (as https://pubmlst.org/) of several pathogenic bacteria. Even though the MLST is not commonly used to infer phylogeny in epidemiology studies, it was applied for this purpose in taxonomic studies, contributing to the development of the Multilocus Sequence Analysis (MLSA) [47–52].

The MLSA accesses the evolutionary information of concatenated housekeeping genes to build phylogenetic trees with more robust data than the analysis with single sequences [53, 54]. It is an important tool for studying prokaryotic diversity and classifying taxonomic groups due to its high-resolution power on species delineation. The sequences are aligned, and informative sites are compared. As in the 16S rRNA analysis, the nucleotide identity (NI), which is a percentage of sequence similarity, must respect the threshold values suggested for each respective group of study [46, 55, 56]. The phylogenetic reconstructions can be achieved using different classes of inference methods. Each one has its particular strengths and weaknesses, requiring careful considerations to choose the best method that fits the analysis [25, 57].

Today, advanced sequencing technologies allow the taxonomy to use genomic data in silico to compare microorganisms, helping to allocate them in their respective taxa or describe new taxa to accommodate the new group. With sequenced genomes, the taxonomists can calculate the overall genome-related indices (OGRIs) and estimate the relatedness among microorganisms; however, suggested threshold values must also be considered. The OGRIs came to replace the DDH due to its low cost, reproducibility, and quality of genomic information. Furthermore, the genome sequences can be deposited in databases so that other scientists can use the data without cultivating the respective bacteria [51, 58]. The OGRIs include average nucleotide identity (ANI) and digital DNA-DNA hybridization (dDDH), the most broadly used to calculate the relatedness between orthological sequences of two genomes. Those sequences are descended from the same ancestral sequence, which kept conserved across the evolution. Studies showing that ANI is a robust measure of evolutionary distance that correlates with DDH values have been published since 2005 [59, 60].

In conclusion, until the genomic era, the polyphasic taxonomy was used to identify, classify, and name prokaryotes according to phenotypic, genotypic, and phylogenetic characteristics. It enabled considerable progress and stability in microbial taxonomy. However, with advancements in genome sequencing, there are today better tools to delineate species, study phylogeny, and ordinate microbial

diversity. The history of microbial taxonomy incorporates the most advanced technologies and adheres to standards and rules, representing a scientific field where the progress goes alongside conservatism [61, 62].

4. Bioinformatics, Evolutionary Markers, and Threshold Values

The evolutionary molecular markers are constitutive genes that reflect the phylogeny of the organisms because the bases' substitution on DNA sequences (given by mutations and recombination) is proportional to the evolution that each species underwent from its ancestors, allowing estimates of the differentiation level of the species [63]. In bacterial taxonomy, those molecular markers started to be amplified, sequenced, and used to phylogenetically ordinate bacteria with the development of the PCR and sequencing techniques. The sequences from amplicons of molecular markers of strains under study and related strains need to be aligned using specific algorithms, such as MUSCLE [64] and ClustalW [65], to identify significant sites for the analysis.

In the next step, it is recommended to choose the best substitution model for the multiple sequence alignment, which depends on the phylogenetic method used to understand the phylogeny of the group under study. Models of substitution are algorithms responsible for evaluating the frequency of each nucleotide and its frequency of substitution, differentiating between transitions (exchange for nitrogen bases of the same biochemical class) and transversions (exchange for nitrogen bases of a different biochemical class). With this, the models can infer the evolutive history for the alignment [66, 67]. Finally, the phylogenetic methods are used to construct the phylogenies based on the alignment and best evolutive model. The result is a graphic representation of the evolution of the strains given by a phylogenetic tree.

In a phylogenetic tree, the extremities are represented by the investigated lineages (sequences, strains). The horizontal lines are called branches, and the nodes that connect the branches represent the most recent common ancestry among the strains [67]. However, a reliable phylogeny reconstruction needs to consider the number of sequences, the size of the alignment, the statistical support, and the outgroup. The bootstrap is commonly used in phylogenies to provide statistical support for the tree nodes [68]. This confidence test consists of multiple resampling of a dataset with the same size as the original alignment. For each resampling, the algorithm randomly chooses the sites, that is, the columns of the alignment, which may be repeated or excluded. Different resamplings result in phylogenetic trees that are compared with the tree obtained in the original alignment. The number of resamplings is variable and may be chosen by the researcher to ensure a reliable phylogeny [69]. The bootstrap value corresponds to the percentage of each group found among all trees obtained in the resampling. The outgroup represents a related taxonomic group, and it is used to show the ancestral strains, helping to determine the tree's root [70].

It is also possible to calculate the NI, a mathematic parameter used to measure the percentage of similarity among the nucleotide sequences from the alignment, but it does not include evolutionary analyses. Specific software is available to calculate the NI, such as BioEdit [71]. Several studies compare NI of particular sequences and genomic analysis, such as dDDH or ANI, of a large group of strains to suggest standard values of NI that reflect whole-genome features of prokaryotes, saving time and costs. These values are commonly known as threshold or cutoff values and are used for taxon circumscription [56, 72].

The 16S rRNA gene, a critical molecular marker in bacterial taxonomy, contains approximately 1,500 base pairs (bp) and plays a role in synthesizing essential proteins to the functioning of every prokaryote. It is originated from a common ancestor among all prokaryotes, being homologous and keeping conserved throughout the evolution process, but having variable sites with evolutionary information [42, 73]. The degree of divergence in the 16S rRNA sequences allows to estimate the phylogenetic distance among strains and, for this reason, is considered a molecular chronometer in bacterial taxonomy [1, 67].

Some numerical values of 16S rRNA NI have been suggested to delimit species boundaries. For example, Stackebrandt and Goebel, in 1994 [74] suggested that strains sharing less than 97% NI on the 16S rRNA sequences should not belong to the same species because the homology would not give a DNA-DNA reassociation above 60–70%. After that, a threshold ranging from 98.7 to 99% was recognized as corresponding to the DDH reassociation value for species delineation [1]. More recently, Kim et al. [72] carried out a large comparative study between 16S rRNA sequence similarities and ANI and proposed the threshold of 98.65% similarity in the 16S rRNA sequence for differentiating two species.

The increase in the number of novel taxa described using the 16S rRNA sequence data has revolutionized our knowledge of the microbial taxonomy, especially at the species level [51]. However, the resolution power of the 16S rRNA analysis is limited due to the predominance of conserved sites in these sequences, not giving enough evolutionary information for the analysis and restricting the identification at the genus level [6, 75–77], with the suggested threshold of 95% for genus delimitation [78]. In addition, studies have reported that those sequences can have multiple heterogeneous copies and, in some groups of microorganisms, the horizontal gene transference (HGT) may happen [79, 80]. These events may lead to misinterpretation of the evolutionary data [81]. Concerning these issues involving the ribosomal sequences, the information from the 16S rRNA phylogeny must corroborate with the suggested values of molecular analyses with a better resolution power and should not replace other methodologies.

Many taxonomists analyzed the internal transcribed spacer (ITS) as an alternative molecular marker to increase the knowledge about the ribosomal region [75, 82–84]. Those sequences are located between the small-subunit (16S rRNA) and the large subunit (23S rRNA) of the ribosomal RNA and contain highly variable sequence regions, allowing

the differentiation of bacteria even at the strain level, showing higher phylogenetic diversity [85–87]. For example, a study with the *Bradyrhizobium* genus demonstrated that strains sharing 95.5% NI on the ITS sequences would correspond to 60% of reassociation on the DDH, belonging to the same species [88]. However, as the 16S rRNA, these sequences do not have a high-resolution power to infer taxonomic groups at the species level. Despite being not often used, the 23S rRNA may also be applied as a molecular marker. The 23S rRNA is located in the large subunit of the prokaryotic ribosome, has a conserved function, universal distribution, and presents sequences with different levels of variation. However, it is commonly characterized by showing insertions and deletions with large sizes, resulting in more information to improve phylogenetic resolution as an additional analysis [66, 89].

Even though the 16S rRNA sequences have been broadly used as an effective tool for basic evolutionary analyses of cultivable and uncultivable bacteria, for closely related groups, they are unable to determine their nearest neighbors since different species can share identical or nearly identical 16S rRNA sequences [83, 90–93]. An approach that can be used to fill this gap is the MLSA. As mentioned before, the MLSA puts together evolutionary information from concatenated housekeeping genes to better assure the correct taxa identification of strains under study. The housekeeping genes are responsible for fundamental roles in the basal microbial metabolism, encoding for essential proteins of the cell functions. Therefore, they have high conservation levels but faster evolutionary rates when compared with the ribosomal sequences, carrying more phylogenetic information [94].

The MLSA allows the analysis of genes together as a larger phylogenetic dataset [35, 46, 52, 95]. Therefore, the methodology aggregates sufficient phylogenetic signals, offering a buffer effect to the noncongruent signals that influence smaller datasets, being more reliable [77, 94, 96]. However, it is worth mentioning that the single housekeeping genes analysis does not reflect the entire evolutionary pattern of the strain; it is recommended to proceed to the comparison between the 16S rRNA and each housekeeping gene phylogeny to detect and avoid potential HGT and recombination events, since each housekeeping gene may have a different history of evolution [75, 92].

The main requirements of MLSA involve the selection of housekeeping genes that should be present in the genome of all organisms object of study as a single copy and spread in the genome. They must also have a consistent size to allow phylogenetic reconstructions and sequencing. Consequently, different genera may vary in the set of genes used in the analysis [46, 52]. In addition, the resulting data from MLSA must corroborate with the 16S rRNA, dDDH, and ANI analyses. It is recommended to use at least five housekeeping genes, considering that the number of genes reflects directly in the discriminatory power of the technique [52]. Some of the most common housekeeping genes applied for the classification of new taxa are ATP synthase β-subunit (*atpD*), chaperone protein (*dnaK*), glutamine synthase II (*glnII*), glutamate synthase (*gltB*), DNA gyrase β-subunit (*gyrB*), recombinase A (*recA*), RNA polymerase β-subunit (*rpoB*), and tryptophan synthase β-subunit (*trpB*) [10, 75, 97–99]. In addition, new genes can be studied, aiming to determine if they provide relevant and congruent phylogenetic information as those genes cited above.

After the housekeeping genes selection, each set of single-gene sequences should be aligned and trimmed to keep the same region of comparison and the same size for the alignment. Subsequently, the phylogeny of each single gene is individually built and compared to each other, and if they are congruent, the alignments must be concatenated to proceed with the MLSA. The concatenation process can be carried out manually using software or any text editor program. Some of the most common software for alignment of prokaryotic sequences are MEGA (Molecular Evolutionary Genetics Analysis) [100] which provides alignment tools as MUSCLE and ClustalW, and BioEdit [71] which also provides the alignment tool ClustalW. To concatenate the sequences, the SeaView [101] is commonly used. In addition, it is important to search for updated sequences in databases to construct reliable phylogenies, verifying the quality of the sequences and the availability of the sequences of the closely related strains.

In 2002, the ad hoc committee of the ICSP recommended the analysis of the concatenated housekeeping genes as a promising method to replace the DDH association in bacterial taxonomy [102]. Following this, Konstantinidis et al. [56] studied extensive whole-genome comparisons of four important bacterial groups. They concluded that the phylogeny of six to eight genes reflects the threshold of 70% DDH and 96% ANI for species circumscription. The authors did not suggest any specific set of genes and affirmed that even if the genes are randomly combined, they provide a robust phylogeny for the group studied [56, 103]. Since then, the NI of concatenated housekeeping genes has been accepted to predict the whole-genome information and differentiate bacterial groups [98, 104–106]. However, no universal threshold for MLSA has been determined for species circumscription yet [18]. Nevertheless, the MLSA has been used as a great strategy to differentiate species in bacterial taxonomic studies, and it is considered as a critical step of bacterial taxonomy since it is more phylogenetic sensible than 16S rRNA analysis and involves conserved regions able to infer phylogeny, unlike OGRIs, which are mathematic parameters [51].

5. Evaluating Genomic Traits

As the DNA molecules represent the identity of the species, studying the genomic profiles allows obtaining relevant information for taxonomic purposes. Prokaryotic genomes contain repetitive sequences distributed throughout the chromosome; however, the sites, length, and the number of times they are repeated are characteristic of each strain, representing a fingerprint of each genome. To evaluate those genomic profiles, the taxonomists use DNA amplification by PCR with specific primers for those regions or restriction enzymes to cut the chromosome in the restriction sites. As a result, in both procedures, there is a mixture of different

fragments of DNA that can be separated by electrophoresis revealing the respective genetic profile [9, 107, 108]. For taxonomic studies, there are three main sets of repetitive elements: repetitive extragenic palindromic (REP) [109], enterobacterial repetitive intergenic consensus (ERIC) [110], and BOX elements [111–113]. Those analyses are limited for species circumscription, but if properly used, they can be applied to identify variability from strains of the same species, to find clones, and to authenticate strains in a study or culture collection [8, 108, 114].

The DDH evaluates the extension and stability of DNA hybrid strands after the dissociation and consecutive reassociation of a two-genomes mixture incubated under controlled conditions [115]. The cutoff is based on the percentage of reassociation using the difference in melting temperature of the hybrid DNA strand (heterologous) compared to 100% of the original strand (homologous). If this association is 70% or higher, or the melting temperature difference (ΔTm) is below 5°C, the genomes compared belong to the same species [7, 18, 35]. The DDH started to be applied when a putative novel group shared more than 97% NI in the 16S rRNA sequence with the related species, aiming to assure enough difference among the genomes of the strains to support the description of a new group. Since the introduction of the DDH in microbiology [116], it became the "gold standard" for species delineation of Bacteria and Archaea and a required technique in every prokaryote species description [8]. However, despite the cited advantages, the DDH has limitations, especially for being time-consuming, requiring intensive labor, and not allowing comparison of results obtained in different experiments, not enabling establishing a global database [44, 51, 117, 118].

The first prokaryote genome sequenced was of the bacteria *Haemophilus influenzae* in 1995, using the conventional Sanger sequencing technique [119]. Although a new genomic era was starting, the high cost and time-consuming process of genome sequencing did not allow significant progress for one decade. In 2005, with the arrival of the NGS technologies, also known as high-throughput sequencing methods, the easy access and low cost of the genome sequencing launched the report of sequenced genomes of prokaryotes [51, 120]. Today, many sequencing platforms can be used, and they are split into two main groups, depending on the type of template used for the sequencing reactions, the high-end instruments, and the bench-top instruments. The high-end platforms demand more technology infrastructure for data tracking, analysis, and storage, while the bench-top ones have more modest requirements. Nowadays, the most popular platforms are the MiSeq and HiSeq (Illumina, USA), Ion Torrent (Thermo Fisher Scientific, USA), and Pacific Biosciences (USA) [60].

The statistical parameters used to report the quality of the genome assembly recommended for taxonomic purposes are (i) the genome size, defined as the length of all contigs sequenced; (ii) the N50, defined as the length of the shortest contig that accumulatively shows 50% or more of the genome size when the lengths of the contigs are summed from the largest to shortest; and (iii) the depth of coverage from the sequencing, indicating how many sequencing reads are generated; this value is usually given as folds and is recommended a minimum of 50X (50-fold) for the platforms cited before [60, 121]. Another critical issue to consider in taxonomy is the genome authenticity, which can be achieved by comparing complete conserved sequences, as the 16S rRNA or housekeeping genes extracted from the genome, with the same sequence obtained through the conventional Sanger sequencing. When describing a new species, this comparison should be performed at least with the type strain [51].

Presently, using genome sequencing, the taxonomists can use other analyses to study the relatedness among the DNAs of bacteria. The threshold values suggested for these analyses relate to the 70% from the DDH technique. As mentioned above, these values are known as OGRIs and effectively calculate genomes' similarities *in silico* [51, 58]. The ANI and the dDDH are now extensively used to replace the original DDH, adopting the threshold values of 95–96% and 70%, respectively, for species circumscription. The OGRIs can be calculated using software tools for taxonomic purposes. Today there are many readily available web services, such as the Genome-to-Genome Distance Calculator and the ANI calculator, and standalone tools, such as JSpecies and OrthoANI with USEARCH. Besides being fast and of low cost, the high quality of the sequences allows the improvement of genomic databases that researchers can use worldwide [60, 117, 122, 123].

With the availability of genome sequences, another parameter that can be calculated *in silico* is the DNA guanine and cytosine (GC) content, which is also used as a genotypic marker in taxonomy [124]. In the beginning, the bases' composition variation from strains of the same species should not exceed 3 mol%, and for strains from the same genus, 10 mol% [7]. Later, a survey comparing the GC content with the genomic data and dDDH indicated that the variation should not exceed 1 mol% among strains of the same species [58]. The GC content can be calculated using tools to assemble the genomes, such as the SEED viewer provided by the RAST server [125], QUAST [121], and BioEdit [71]. Although the GC content is important to distinguish nonrelated bacteria, similar DNA base composition does not necessarily imply that the two strains are closely related [35].

6. Phenotypic Traits

In contrast to the taxonomy of eukaryotes, where the phenotypic characteristics can be used to differentiate some organisms, these traits are questionable in prokaryotes since different bacterial species can present identical phenotypes [17]. Nevertheless, starting the prokaryotic classification using the phenotypic evaluation may help delimit further analyses; for example, most pathogenic bacterial groups present well-established phenotypes in the literature and are critical to quick diagnostics.

The classical phenotypic tests in microbiology include morphological, physiologic, and biochemical analysis. Morphological characteristics describe the cellular and colony features, such as the cell shape, endospore formation,

presence of flagella, Gram stain, color colony, diameter, opacity, mucus production, and their consistency. On the other hand, the physiological and biochemical characteristics include data about the culture under different growth conditions, such as range of temperature, pH (4–12), salinity tolerance (1–10%), O_2 or CO_2 requirements, tolerance to different antibiotics, enzymatic activity (i.e., urease), and metabolism of compounds (i.e., carbon source and nitrogen source) [7, 9, 28, 126]. Therefore, it is recommended to compare the data obtained with reference or type strains and to include negative and positive controls [8]. In addition, the use of commercial tests with minimal standards such as API (bioMérieux) or Biolog (Biolog, Inc.) that evaluate the carbon source utilization by bacteria is suggested to avoid incongruences among laboratories.

Another common phenotypic test is the chemical characterization of cells, which evaluates extracellular elements (peptidoglycan, teichoic acids, and mycolic acids), cell membrane composition (fatty acids, polar lipids, respiratory lipoquinones, and pigments), or cytoplasm compounds (polyamines) [8]. These features are also known as chemotaxonomic markers because they are usually stable within a bacterial group [127]. The resulting data are available in specific databases. One of the most common is the database of fatty acids profiles implemented by Sherlock Microbial Identification System (MIDI, Inc.) [8, 105, 128]. Even though the chemical characterization provides important information about the cells, it has become unusual in taxonomic studies because the tests are laborious, require specific equipment and methodologies, and usually allow the classification only at the genus level identification. Lately, the use of Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) for bacterial identification has become a rapid, precise, and cost-effective method, especially compared to traditional phenotypic and molecular techniques. It is based on the analyses of bacteria components profiles, as proteins directly extracted from intact bacteria, and compared with a reference database for identification [129, 130]. This methodology is commonly used on clinical isolates' identification, but as the reference databases are being updated, it is becoming practicable for other groups of bacteria, such as the rhizobia [131–133].

It is worth mentioning that there are many genes coding proteins without known function. Therefore, the phenotypic tests could help the search for proteins with biotechnological interest, improving the knowledge about the interactions of microorganisms with the environment [18]. However, the phenotype results from the combination of genotype and environmental conditions. Consequently, it is impossible to know the whole phenotype of a prokaryote based only on observable characteristics or on the information obtained from the genome. In taxonomy, it is common to find some incongruent data in phenotypic evaluations, probably because the accessory genome codes many phenotypic traits; for example, the plasmids easily exchanged among the organisms. In addition, as there are no laboratory conditions able to mimic the environment entirely, the phenotypic characterization can exclude some microorganisms, such as the uncultivable. Therefore, these data need to be validated by genomic analysis [134].

7. Rhizobia Study Case

7.1. History of Rhizobia Taxonomy. More than 2,000 years ago, ancient Chinese literature reported that crop rotation of legumes with cereals was traditionally used to enhance grain production. Improvement in soil fertility by cultivating legumes was thus already noticed at that time, although the mechanisms involved were not known yet [28]. However, it was just by the end of the nineteenth century that the assimilation of atmospheric nitrogen was related to the root nodules in legumes, and in 1888 the first root-nodule bacterium was isolated from nodules of *Pisum sativum* plants (pea) and reported by the Dutch microbiologist Martinus Willem Beijerinck to be responsible for the nitrogen fixation process. The isolated bacterium was first named *Bacillus radicicola* but later reclassified as *Rhizobium*, comprising the *Rhizobium leguminosarum* species [28, 135, 136]. Since then, the nodulating nitrogen-fixing bacteria have been generally called rhizobia [137].

In the early twentieth century, nodulation tests using a broad range of host plants and different bacteria were conducted, and the specificity between the host plants and the symbiotic bacteria was reported. Based on this, Baldwin and Fred [138] proposed the cross-nodulation concept, indicating specificity between rhizobia and host plants. For about 80 years, taxonomists used this concept for species definition, and six main species were described: *R. leguminosarum* [136], *R. phaseoli*, *R. trifolii*, and *R. meliloti* [139], which produced acid reaction on yeast-extract mannitol agar (YMA) medium, and *R. lupini* [140] and *R. japonicum* [141], which produced alkaline reaction on the same medium. Besides those six species, some other strains isolated from cowpea were defined as *Rhizobium* spp. [135, 142].

The taxonomy dropped the cross-nodulation concept after several studies reporting both exceptions and strains sharing high similarity and belonging to different groups. Additionally, the rhizobia classification needed to include more information to adjust to the general bacterial taxonomy [143–145]. However, even after the cross-inoculation concept was dropped in rhizobia taxonomy, it continued to be studied, representing an important feature regarding the inoculation and efficiency of the symbiosis [28].

The next step of rhizobia taxonomy was based on the numerical taxonomy using computers to compare bacteria properties. Around the 1960s, many analyses were included in taxonomic studies involving phenotypic traits, growth conditions, nutrient resources, metabolic features, and resistance to antibiotics and other chemicals, among others. Also, the DNA molecule started to be investigated, and the base composition (GC mol%) was added to bacterial classification [145–148]. Using the numerical taxonomy combined with DNA analyses, three other rhizobial genera were described. In 1982, Jordan [149] proposed the description of the genus *Bradyrhizobium* to allocate the slow-growing

species *B. japonicum* and *B. lupini*. Six years later, the genus *Azorhizobium* [150] was described, encompassing strains that can effectively nodulate roots and stems of *Sesbania rostrata* and fix nitrogen under free-living aerobic conditions. In the same year, the *Sinorhizobium* genus was proposed for the fast-growing soybean species *Rhizobium fredii* [151].

Around the 1990s, many other analyses were included in taxonomic studies. The polyphasic taxonomy confirmed some of the taxa proposed with the numerical taxonomy but also pointed out that the numerical taxonomy lacked information about the evolutionary relationships among rhizobia. Consistent DNA studies allowed the taxonomists to assess the diversity and phylogenetic relationship among bacteria at a molecular level [28]. The 16S rRNA sequence analysis was included in rhizobia species descriptions and reclassifications in 1991 when Graham and collaborators [24] published the minimal standards for species description of new rhizobia and *Agrobacterium* [152]. Considering the phylogeny and similarity of 16S rRNA, rhizobia were classified in the phylum Proteobacteria, subdivision α-Proteobacteria [153]. In the same decade, two more genera were described. First, Jarvis et al. [154] proposed the genus *Mesorhizobium* to allocate five *Rhizobium* species with an intermediate growth rate than fast-growing *Rhizobium* and *Sinorhizobium* and slow-growing *Bradyrhizobium*. Following, de Lajudie et al. [155] proposed the description of the genus *Allorhizobium* of symbiotic bacteria associated with the aquatic legume *Neptunia natans*.

The six rhizobia genera were allocated in four distinct families: *Bradyrhizobium* [149], *Mesorhizobium* [154], and *Azorhizobium* [150] belonging to families Bradyrhizobiaceae (today Nitrobacteraceae), Phyllobacteriaceae, and Xanthobacteraceae, respectively, and *Rhizobium* [136], *Sinorhizobium* (reclassified as *Ensifer*) [151, 156], and *Allorhizobium* [155] belonging to the family Rhizobiaceae [157]. Additionally, a study with members of the family Rhizobiaceae reported similarities of the 16S rRNA gene higher than 92% among the genera, suggesting that this value could be a threshold for family delineation [158].

The new century started a revolution on the rhizobia taxonomy. A first milestone occurred in January of 2001, with the first report of a non-rhizobia nitrogen-fixing legume-symbiotic bacterium isolated from the nodules of *Crotalaria*, classified as *Methylobacterium* [159], with the species *M. nodulans* [160]. In the same month, based on the 16S rRNA analysis [161], Young et al. [162] suggested the inclusion of all species of *Agrobacterium* [152] and *Allorhizobium undicola* [155] in the genus *Rhizobium*. In 2002, a new species of the genus *Devosia* was reported to induce nitrogen-fixing root-nodules in *Neptunia natans* [163]. In 2005, the first rhizobia in the genus *Phyllobacterium* were described as *P. trifolii*, isolated from the nodules of *Trifolium pratense* [164]. From 2005 to 2007, the genus *Ochrobactrum* (today, *Brucella*) [165, 166] allocated two nodulating species, *O. lupini* and *O. cytisi* [167, 168], isolated from nodules of *Lupinus albus* and *Cytisus scoparius*, respectively. In 2008, Lin and collaborators [169] described in the *Shinella* genus [170] the symbiotic bacterium *S. kummerowiae* isolated from root nodules of *Kummerowia stipulacea*. In 2009, the first rhizobial isolate (BA135) belonging to the species *Aminobacter aminovorans* was reported, isolated from nodules of *Lotus tenuis* [171]. In the following decade, from 2012 to 2014, the genus *Microvirga* [172] allocated four nodulating and nitrogen-fixing new species, *M. lupini* isolated from *Lupinus texensis*, *M. lotononidis*, and *M. zambiensis* isolated from *Listia angolensis*, and *M. vignae* isolated from *Vigna unguiculata* [173, 174].

Besides all those changes in the α-Proteobacteria class, 2001 was outstanding by the report of a nodulating β-Proteobacteria [175], belonging to the genus *Burkholderia*, described by Yabuuchi and collaborators in 1992 [176]. It was the first time that the essential nodulation genes (*nod*) and the nodulation capacity were reported in symbiotic bacteria not belonging to α-Proteobacteria [175]. After that, several nodulating bacteria of β-Proteobacteria genera were described, including the *Ralstonia* genus [177], reclassified in 2004 as *Cupriavidus* [178–180], in addition to several species of the *Burkholderia* genus, later reclassified as the new genus *Paraburkholderia* [181, 182]. In the following years, the introduction of single and concatenated housekeeping genes in phylogenetic studies culminated in the reclassification of many species and the proposal of the new genera *Neorhizobium* and *Pararhizobium*, and also the revision of the genera *Agrobacterium* and *Allorhizobium* [183, 184]. More recently, using whole-genome analyses, Santos and collaborators [21] suggested the description of two new genera, *Mycetohabitans* and *Trinickia*, this last one containing the nodulating nitrogen-fixing species *T. symbiotica*.

With the evolution of taxonomic analyses, we may conclude that many descriptions of nodulating bacteria, isolated from nodules of different hosts and belonging to nonrhizobial genera have been published, and many taxonomic groups were reclassified. Most of those bacteria have a diverse set of nodulation (*nod*) and nitrogen fixation (*nif*) genes, some of which are related to genes of different members of classical rhizobial genera. All those findings show that the ability to establish symbiosis with legumes is more widespread in bacteria than anticipated before [137, 175].

Today, rhizobia are distributed in eight families, seven belonging to the α-Proteobacteria class and one in the β-Proteobacteria. From both subclasses, the α-rhizobia are reported as broadly distributed, both in geography and host-plant range, and although the β-rhizobia are well established, their distribution seems more restricted [28]. The α-Proteobacteria families are the Rhizobiaceae that allocate seven genera, the Phyllobacteriaceae with three genera, the Methylobacteriaceae with two genera, and the other four families allocating only one genus, Nitrobacteriaceae (old Bradyrhizobiaceae), Brucellaceae, Hyphomicrobiaceae, and Xanthobacteiaceae. The β-Proteobacteria family is Burkholderiaceae with three genera. The list of genera and the number of species with valid names standing in nomenclature (without synonyms) according to the LPSN in October of 2021 are listed in Table 1.

In 2020, a study performed phenotypic, genomic, and phylogenetic analyses of the genus *Ensifer* and suggested that

Table 1: List of genera comprising rhizobial species with valid names standing in nomenclature (LPSN) in October 2021. The number of valid names does not include synonyms.

α-Proteobacteria		
Hyphomicrobiales (=Rhizobiales)		
Rhizobiaceae		
Agrobacterium	11 valid names	2 rhizobia
Allorhizobium	8 valid names	2 rhizobia
Ensifer (=*Sinorhizobium*)	20 valid names	20 rhizobia
Neorhizobium	5 valid names	4 rhizobia
Pararhizobium	6 valid names	2 rhizobia
Rhizobium	92 valid names	49 rhizobia
Shinella	8 valid names	1 rhizobia
Phyllobacteriaceae		
Aminobacter	7 valid names	1 rhizobia
Phyllobacterium	12 valid names	4 rhizobia
Mesorhizobium	59 valid names	48 rhizobia
Nitrobacteraceae (=Bradyrhizobiaceae)		
Bradyrhizobium	63 valid names	58 rhizobia
Methylobacteriaceae		
Microvirga	18 valid names	4 rhizobia
Methylobacterium	47 valid names	1 rhizobia
Brucellaceae		
Brucella (=*Ochrobactrum*)	25 valid names	2 rhizobia
Hyphomicrobiaceae		
Devosia	26 valid names	1 rhizobia
Xanthobacteraceae		
Azorhizobium	3 valid names	2 rhizobia
β-Proteobacteria		
Burkholderiales		
Burkholderiaceae		
Cupriavidus	18 valid names	2 rhizobia
Paraburkholderia	79 valid names	22 rhizobia
Trinickia	7 valid names	1 rhizobia

the genus should be separated into two genera, one for the symbiotic clade and the other for the nonsymbiotic clade [185]. More recently, a publication on the IJSEM suggested the revision of family Rhizobiaceae [186]. The extensive study suggests a threshold for core-proteome average amino acid identity (cpAAI) of approximately 86% as a new framework for genus delimitation. It is noteworthy to mention that cpAAI must not be used as sole information for genus delimitation, and the authors specify "approximately 86%" to provide some flexibility regarding the evolution of each genus. Although not yet updated in the LPSN website as validated names, among the reclassifications based on the study of Kuzmanović and collaborators [186], there are arguments that the unification of the genera *Ensifer* and *Sinorhizobium* is no longer justified, and eight new combinations were suggested, but not all involving rhizobial strains [186].

From Table 1 based on the LPSN, we may conclude that today over 200 known rhizobial species are split into 19 genera of the *α*- and *β*-Proteobacteria subclasses, and the number increases every year. However, less than half of these valid names from the 19 genera have species comprising strains already reported for their symbiotic properties, including nodulation and nitrogen fixation abilities. Furthermore, many species are reported as endophytes, or were isolated from environmental samples, or from nodules but unable to reestablish symbiotic associations. Therefore, the symbiotic capacity remains largely unknown for many species.

Additionally, in 2004, Benhizia and collaborators [187] published for the first time the isolation of *γ*-Proteobacteria species from legume nodules. In this study, 52 isolates belonging to the *Pseudomonas*, *Escherichia*, *Leclercia*, *Pantoea*, and *Enterobacter* genera were isolated from three *Hedysarum* species, and rhizobia-like bacteria were found occupying the nodules. However, Koch's postulates and the symbiotic parameters from the isolates were not investigated. Shiraishi and collaborators [188] also reported in 2010 the existence of rhizobial strains in the *γ*-Proteobacteria subdivision. The authors described *nod* and *nif* genes in the *Pseudomonas* sp. strain Ch10048, sharing high similarity with the symbiotic sequences of *Agrobacterium* sp., suggesting the acquisition of these genes through HGT from rhizobial species in the soil. Despite the report of *nod* and *nif* genes in strain Ch10048, and the confirmation of the ability to nodulate the host legume *Robinia pseudoacacia*, the existence of *γ*-rhizobia remains controversial until additional evidence confirms that the genes were not provided by other bacteria coexisting in the nodules and that the nitrogen fixation ability of the strain is tested [28].

Undoubtedly, rhizobia taxonomy advanced together with prokaryotes' taxonomy, and improvements regarding the origin and evolution of these bacteria were obtained. However, there is a need to increase the studies relating taxonomy and phylogeny with the phylogeny of nitrogen fixation and biotechnological properties of rhizobia.

8. Rhizobial Symbiotic Parameters and Genome Architecture

As commented before, the members of the Subcommittee on Taxonomy of Rhizobia and Agrobacteria of the ICSP reviewed the taxonomic developments for this group of bacteria and updated the minimal standards for taxonomic studies, including additional considerations specific to rhizobia and agrobacteria. According to them, taxonomic definitions should not include symbiotic or pathogenic characters because the interactions with plants are determined by accessory genes that may be present in several bacterial species, and be gained or lost, imposing taxonomic limits [25]. Instead, if a strain has a phenotype regarding plant-interaction, this should be described in the taxonomic proposal but considered as a property of the strain, not of the whole taxon. It is worth mentioning that symbiovars are also studied in rhizobial surveys, although not accepted as a formal taxonomic category. The term symbiovar was proposed in 2011 to name a group of strains able to nodulate and fix nitrogen with a range of legumes or a specific legume, and today this definition is based mainly on symbiotic genes' phylogeny [25, 189].

Concerning the description of new rhizobial species, it is especially recommended to evaluate the symbiotic ability of

the strains based on Koch's postulates using the original host and/or other legume species. This last alternative may be used to expand the information about the host range of the strains and to define symbiovar groups or when the seeds of the original host plant are not available. The species *Phaseolus vulgaris*, *Macroptilium atropurpureum*, *Vigna unguiculata*, and *Mimosa pudica* are promiscuous legumes commonly used in taxonomic studies of rhizobia. The symbiotic ability may be evaluated compared to negative controls by the presence/absence of root nodules, plant biomass, N content, or the acetylene reduction assay. In addition, the strains must be reisolated from the nodules, keeping the original phenotypic, phylogenetic, or genotypic features, obeying Koch's postulates [25, 190, 191].

Based on the meta-analysis of 1,708 completed bacterial genomes performed in 2017 by diCenzo and Finan [192], the average and median of bacterial genomes found were 3.65 Mb and 3.46 Mb, respectively. In a review study of 2020, Geddes and collaborators [193] compared the genomes of representative strains of α-rhizobia and β-rhizobia and showed that rhizobial genomes range from 3.42 Mb in *Cupriavidus taiwanensis* LMG 19424 to 9.36 Mb in *Microvirga lupini* Lut6. However, the authors highlighted that some strains of *Bradyrhizobium, Mesorhizobium*, and *Azorhizobium* genera might have higher genomes, which means that the rhizobial genomes can be twice or more times higher than the average size of bacterial genomes reported in the two studies [192, 193]. In contrast, another study reported that *Ensifer* strains from the symbiotic clade carried an average of 325 fewer genes and appeared to have fewer rRNA operons when compared to strains belonging to the nonsymbiotic clade [185]. Large genomes may be related to adaptations to the soil and rhizosphere conditions [194].

In general, the rhizobial genes responsible for plant infection, nodulation, and nitrogen fixation are clustered together in symbiotic plasmids or symbiotic islands in the chromosome, or even in both genomic regions [195–200]. Those clusters are frequently part of mobile genetic elements (MGE) that have independent evolutionary pathways [201]. An exception was first reported in 2007 revealing a group of Bradyrhizobium strains with photosynthetic ability that does not possess nodulation genes and can induce nodulation without nodulation (Nod) factors [202]. It is known that Nod factors play a crucial role in host specificity in the rhizobia-legume interactions; those molecules differ on the symbiosis specific backbone length and other structures, determining the set of plants that the rhizobia can nodulate [203, 204]. In a review recently published, Patra and Mandal [205] pointed out other studies reporting that even in absence of Nod factors, other bradyrhizobia strains, not belonging to the photosynthetic group, are also able to establish successful nodulation. Among the hypotheses discussed, there is the possibility that Nod factors independent nodulation start with the host infection through crack invasion process, instead of the formation of the common infection thread. After the infection, the nodulation might take place using similar signals and mechanisms present in Nod-dependent nodulation [205, 206].

Besides the main chromosome, some bacteria have a "second chromosome" or "megaplasmid," for which the term "chromid" was proposed. These elements have some core genes and nucleotide composition similar to the associated chromosomes, but most of their genes are accessory. Some rhizobia and agrobacteria also have genus-specific chromids, similar within a genus but with different sets of conserved genes among genera. An example is some *Agrobacterium* species with linear chromids carrying a unique replication system and conserved genes [207].

9. Rhizobial Origin Hypothesis and Evolution

As biological nitrogen fixation is considered one of the most important biological processes for life on Earth, there is great biotechnological interest in diazotrophic bacteria [208]. Studying the nodulation ability and nitrogen fixation efficiency, together with the phylogenetic comparison of core and symbiotic genes, gives insights about the origin of the diazotrophic bacteria and the evolution of the biological fixation ability on prokaryotes [106, 209, 210].

Remigi and collaborators [201] reviewed that the nitrogen fixation ability is older than the nodulation process and broadly spread among Bacteria and Archaea. The nodulation genes might have clustered early in the symbiosis evolution path by duplication and specialization of other functional genes. Nowadays, the high diversity of nodulation genes makes it difficult to suggest which bacterial lineage was ancestral. The *nif* and *nod* genes have different phylogenies, implying that rhizobia inherited the nitrogen fixation ability of their free-living relatives [211]. Besides, as the proximity of those genes is not essential for function, it suggests a relatively recent HGT event as a symbiosis set.

Evidence indicates that the *Bradyrhizobium* genus might be the rhizobia's ancestor [212]. Using the genes from the glutamine synthetase enzymes (GSI and GSII), essential for nitrogen assimilation, the estimates are that *Bradyrhizobium* originated 553 million years ago (MYA). Other rhizobia evolved 400-324 MYA, originating the *Mesorhizobium*, *Rhizobium,* and *Sinorhizobium* (=*Ensifer*) genera. Interestingly, the first legumes ascended on Earth long after, around 70 MYA [63, 213]. Another piece of evidence of *Bradyrhizobium* ancestry is that some strains were detected with nitrogen fixation ability as free-living bacteria, as observed in some *Azorhizobium*, and both lineages are very distant from the other rhizobial genera [28].

It is well known that bacteria have different mechanisms to exchange genetic material. This event is more recurrent among organisms sharing the same ecological environment, reinforcing that some rhizobia evolved by acquiring symbiosis genes from other species by HGT [197, 214, 215]. Furthermore, a study with *A. caulinodans* reported an increase in horizontal transference frequency of its symbiosis island in the legume rhizosphere or in the presence of plant flavonoids, suggesting a host-dependent evolution [216]. Over evolutionary time, the horizontal transference of symbiotic functional genes among symbiotic and nonsymbiotic bacteria is hypothetically responsible for the growing number of studies reporting great rhizobia diversity

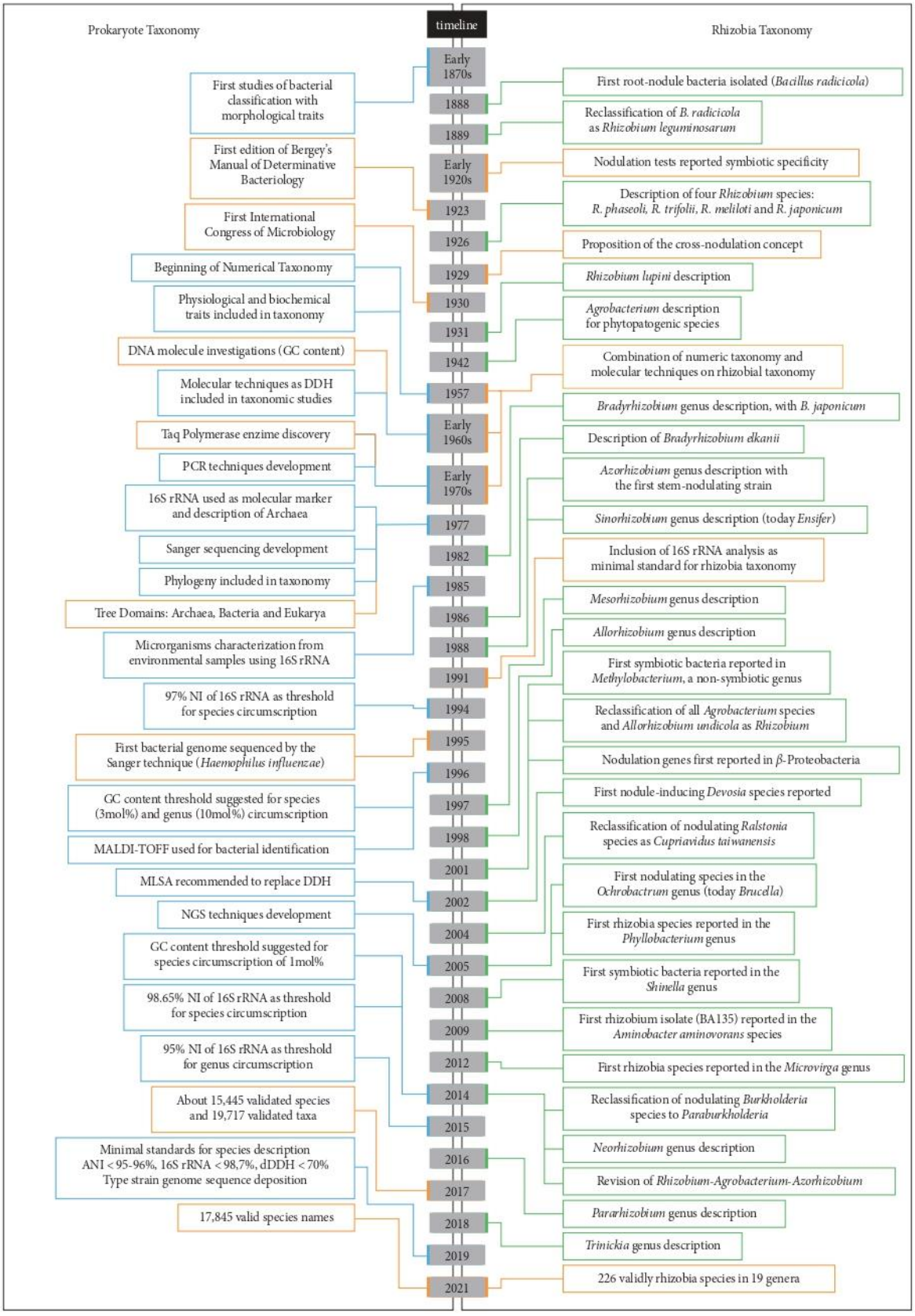

FIGURE 1: Timeline showing the evolution of methods and achievements in prokaryote and rhizobia taxonomy.

[201, 217]. Given the vast population of rhizospheric bacteria, it might seem paradoxical that the symbiosis is restricted to only nineteen genera. However, an increasing number of studies report the coexistence of nonrhizobial bacteria inside the nodules, which deserve further studies and might help to explain that additional partners help the symbiosis [153, 218–222].

10. Conclusions

As presented in this review, the main goal of taxonomy is to ordinate living organisms in a stable and hierarchical system. As shown in Figure 1, remarkable progress has been achieved in both prokaryote and rhizobia taxonomy and phylogeny. However, profound changes may arise with the genomic era. Nevertheless, robust taxonomic methodologies are becoming gradually available in an increasing number of laboratories, allowing researchers to conduct surveys of great interest. These studies have contributed to new insights about the origin, evolution, and diversity of bacteria on Earth and the description of almost 18,000 valid species, of which more than 220 are rhizobia. However, more studies are needed to correlate taxonomy with biotechnological properties of nitrogen-fixing rhizobia to improve their contribution to agricultural and environmental sustainability.

Abbreviations

ANI: Average nucleotide identity
DDH: DNA-DNA hybridization
dDDH: Digital DNA-DNA hybridization
ERIC: Enterobacterial repetitive intergenic consensus
GDP: Growth domestic product
HGT: Horizontal gene transference
ICNP: International Code of Nomenclature of Prokaryotes
ICSP: International Committee on Systematic of Prokaryotes
IJSEM: International Journal of Systematic and Evolutionary Microbiology
ITS: Internal transcribed spacer
LPSN: List of prokaryotic names with standing in nomenclature
MEGA: Molecular evolutionary genetics analysis
MGE: Mobile genetic elements
MLSA: Multilocus sequence analysis
MLST: Multilocus sequence typing
MYA: Million years ago
NGS: Next-generation sequencing
NI: Nucleotide identity
OGRI: Overall genome-related indices
PCR: Polymerase chain reaction
REP: Repetitive extragenic palindromic.

Conflicts of Interest

The authors declare that they have no conflicts of interest regarding the publishing of this paper.

Authors' Contributions

Luisa Caroline Ferraz Helene and Milena Serenato Klepa contributed equally to this work. The authors declare that they have consented to participate in the manuscript and publish it.

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References

[1] E. Stackebrandt and J. Ebers, "Taxonomic parameters revisited: tarnished gold standards," *Microbiology Today*, vol. 33, pp. 152–155, 2006.
[2] P. Kämpfer, "Systematics of prokaryotes: the state of the art," *Antonie Van Leeuwenhoek*, vol. 101, 2012.
[3] I. C. Sutcliffe, M. E. Trujillo, and M. Goodfellow, "A call to arms for systematists: revitalising the purpose and practises underpinning the description of novel microbial taxa," *Antonie Van Leeuwenhoek*, vol. 101, no. 1, pp. 13–20, 2012.
[4] H. G. Schlegel and W. Köhler, "Bacteriology paved the way to cell biology: a historical account," *Biology of the Prokaryotes*, Wiley, Stuttgart, Germany, 1999.
[5] G. Drews, "The roots of microbiology and the influence of Ferdinand Cohn on microbiology of the 19th century," *FEMS Microbiology Reviews*, vol. 24, no. 3, pp. 225–249, 2000.
[6] P. Kämpfer and S. P. Glaeser, "Prokaryotic taxonomy in the sequencing era and the role of MLSA in classification," *Microbiology Australia*, vol. 32, no. 2, p. 66, 2011.
[7] P. Vandamme, B. Pot, M. Gillis, P. De Vos, K. Kersters, and J. Swings, "Polyphasic taxonomy, a consensus approach to bacterial systematics," *Microbiological Reviews*, vol. 60, no. 2, pp. 407–438, 1996.
[8] B. J. Tindall, R. Rosselló-Móra, H. J. Busse, W. Ludwig, and P. Kämpfer, "Notes on the characterization of prokaryote strains for taxonomic purposes," *International Journal of Systematic and Evolutionary Microbiology*, vol. 60, no. 1, pp. 249–266, 2010.
[9] E. R. B. Moore, S. A. Mihaylova, P. Vandamme, M. I. Krichevsky, and L. Dijkshoorn, "Microbial systematics and taxonomy: relevance for a microbial commons," *Research in Microbiology*, vol. 161, no. 6, pp. 430–438, 2010.
[10] P. Kämpfer and S. P. Glaeser, "Prokaryotic taxonomy in the sequencing era—the polyphasic approach revisited," *Environmental Microbiology*, vol. 14, no. 2, pp. 291–317, 2012.
[11] D. J. Brenner, J. T. Staley, and N. R. Krieg, "Classification of prokaryotic organisms and the concept of bacterial speciation," *Bergey's Manual of Systematic Bacteriology*, Wiley, Boston, MA, USA, 2005.
[12] K. J. Locey and J. T. Lennon, "Scaling laws predict global microbial diversity," *Proceedings of the National Academy of*

Sciences of the United States of America, vol. 113, no. 21, pp. 5970–5975, 2016.

[13] A. C. Parte, J. Sardà Carbasse, J. P. Meier-Kolthoff, L. C. Reimer, and M. Göker, "List of prokaryotic names with standing in nomenclature (LPSN) moves to the DSMZ," *International Journal of Systematic and Evolutionary Microbiology*, vol. 70, no. 11, pp. 5607–5612, 2020.

[14] M. Loreau and C. de Mazancourt, "Biodiversity and ecosystem stability: a synthesis of underlying mechanisms," *Ecology Letters*, vol. 16, pp. 106–115, 2013.

[15] G. Bos, "Tomorrow's production systems will be closer to nature," 2017, https://www.iucn.org/news/business-and-biodiversity/201701/tomorrows-production-systems-will-be-closer-nature.

[16] World Economic Forum, "The global wisks report 2020, insight report," 2020, https://www3.weforum.org/docs/WEF_Global_Risk_Report_2020.pdf.

[17] R. Rosselló-Móra and R. Amann, "The species concept for prokaryotes," *FEMS Microbiology Reviews*, vol. 25, no. 1, pp. 39–67, 2001.

[18] R. Rosselló-Móra and R. Amann, "Past and future species definitions for Bacteria and Archaea," *Systematic & Applied Microbiology*, vol. 38, no. 4, pp. 209–216, 2015.

[19] L. C. Ferraz Helene, R. F. Dall'Agnol, J. R. M. Delamuta, and M. Hungria, "*Mesorhizobium atlanticum* sp. nov., a new nitrogen-fixing species from soils of the Brazilian Atlantic Forest biome," *International Journal of Systematic and Evolutionary Microbiology*, vol. 69, no. 6, pp. 1800–1806, 2019.

[20] E. S. P. Bromfield, S. Cloutier, and H. D. T. Nguyen, "Description and complete genome sequence of *Bradyrhizobium amphicarpaeae* sp. nov., harbouring photosystem and nitrogen- fixation genes," *International Journal of Systematic and Evolutionary Microbiology*, vol. 69, no. 9, pp. 2841–2848, 2019.

[21] E. L. P. Santos, M. Palmer, B. Chávez-Ramírez et al., "Whole genome analyses suggests that burkholderia sensu lato contains two additional novel genera (mycetohabitans gen. Nov., and trinickia gen. Nov.): implications for the evolution of diazotrophy and nodulation in the burkholderiaceae," *Genes*, vol. 9, 2018.

[22] J. D. Flores-Félix, E. Menéndez, A. Peix, P. García-Fraile, and E. Velázquez, "History and current taxonomic status of genus Agrobacterium," *Systematic & Applied Microbiology*, vol. 43, no. 1, Article ID 126046, 2020.

[23] J. R. Avontuur, M. Palmer, C. W. Beukes et al., "Genome-informed *Bradyrhizobium* taxonomy: where to from here?" *Systematic & Applied Microbiology*, vol. 42, no. 4, pp. 427–439, 2019.

[24] P. H. Graham, M. J. Sadowsky, H. H. Keyser et al., "Proposed minimal standards for the description of new genera and species of root- and stem-nodulating bacteria," *International Journal of Systematic Bacteriology*, vol. 41, no. 4, pp. 582–587, 1991.

[25] P. M. de Lajudie, M. Andrews, J. Ardley et al., "Minimal standards for the description of new genera and species of rhizobia and agrobacteria," *International Journal of Systematic and Evolutionary Microbiology*, vol. 69, no. 7, pp. 1852–1863, 2019.

[26] C. T. Parker, B. J. Tindall, and G. M. Garrity, "Prokaryotic code (2008 revision) International code of nomenclature of prokaryotes," *International Journal of Systematic and Evolutionary Microbiology*, vol. 69, 2019.

[27] M. E. Trujillo, "Publication of new taxa," 2021, https://www.microbiologyresearch.org/journal/ijsem/scope.

[28] E. T. Wang, C. F. Tian, W. F. Chen, J. P. W. Young, and W. X. Chen, *Ecology and Evolution of Rhizobia: Principles and Applications*, Springer, Berlin, Germany, 2019.

[29] R. G. Murray and E. Stackenbrandt, "Taxonomic Note: Implementation of the provisional status Candidatus for incompletely described procaryotes," *International Journal of Systematic Bacteriology*, vol. 45, no. 1, pp. 186-187, 1995.

[30] C. Quast, E. Pruesse, P. Yilmaz et al., "The SILVA ribosomal RNA gene database project: improved data processing and web-based tools," *Nucleic Acids Research*, vol. 41, no. D1, pp. D590–D596, 2012.

[31] W. B. Whitman, I. C. Sutcliffe, and R. Rossello-Mora, "Proposal for changes in the International code of nomenclature of prokaryotes: granting priority to *Candidatus* names," *International Journal of Systematic and Evolutionary Microbiology*, vol. 69, no. 7, pp. 2174-2175, 2019.

[32] D. J. Brenner, J. T. Staley, and N. R. Krieg, *Classification of Procaryotic Organisms: an Overview*, Wiley, New York, NY, USA, 1989.

[33] P. H. A. Sneath, "The application of computers to taxonomy," *Microbiology*, vol. 17, no. 1, pp. 201–226, 1957.

[34] W. Ludwig, K. Schleifer, and E. Stackebrandt, "Databases," *The Prokaryotes*, Springer, Berlin, Germany, 2006.

[35] K. H. Schleifer, "Classification of Bacteria and Archaea: past, present and future," *Systematic & Applied Microbiology*, vol. 32, no. 8, pp. 533–542, 2009.

[36] L. G. Wayne, W. E. C. Moore, E. Stackebrandt et al., "Report of the Ad Hoc Committee on reconciliation of approaches to bacterial systematics," *International Journal of Systematic and Evolutionary Microbiology*, vol. 37, no. 4, pp. 463-464, 1987.

[37] D. J. Brenner, G. R. Fanning, A. V. Rake, and K. E. Johnson, "Batch procedure for thermal elution of DNA from hydroxyapatite," *Analytical Biochemistry*, vol. 28, pp. 447–459, 1969.

[38] C. L. Schildkraut, J. Marmur, and P. Doty, "The formation of hybrid DNA molecules and their use in studies of DNA homologies," *Journal of Molecular Biology*, vol. 3, no. 5, pp. 595–IN16, 1961.

[39] R. K. Saiki, D. H. Gelfand, S. Stoffel et al., "Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase," *Science*, vol. 239, no. 4839, pp. 487–491, 1988.

[40] F. Sanger, S. Nicklen, and A. R. Coulson, "DNA sequencing with chain-terminating inhibitors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 74, no. 12, pp. 5463–5467, 1977.

[41] C. R. Woese and G. E. Fox, "Phylogenetic structure of the prokaryotic domain: the primary kingdoms," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 74, no. 11, pp. 5088–5090, 1977.

[42] C. R. Woese, O. Kandler, and M. L. Wheelis, "Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 87, no. 12, pp. 4576–4579, 1990.

[43] J. T. Staley, "The bacterial species dilemma and the genomic-phylogenetic species concept," *Philosophical Transactions of the Royal Society B: Biological Sciences*, vol. 361, 2006.

[44] A. Oren and G. M. Garrity, "Then and now: a systematic review of the systematics of prokaryotes in the last 80 years," *Antonie Van Leeuwenhoek*, vol. 106, no. 1, pp. 43–56, 2014.

[45] E. Stackebrandt, W. Ludwig, and G. E. Fox, "3 16 S ribosomal RNA oligonucleotide cataloguing," *Methods in Microbiology*, vol. 18, pp. 75–107, 1985.

[46] D. Gevers, F. M. Cohan, J. G. Lawrence et al., "Re-evaluating prokaryotic species," *Nature Reviews Microbiology*, vol. 3, no. 9, pp. 733–739, 2005.

[47] J. Feng, E. L. Schuenzel, J. Li, and N. W. Schaad, "Multilocus sequence typing reveals two evolutionary lineages of acidovorax avenae subsp. citrulli," *Phytopathology*, vol. 99, 2009.

[48] S. Yan, H. Liu, T. J. Mohr et al., "Role of recombination in the evolution of the model plant pathogen *Pseudomonas syringae* pv. tomato DC3000, a very atypical tomato strain," *Applied and Environmental Microbiology*, vol. 74, no. 10, pp. 3171–3181, 2008.

[49] M. C. J. Maiden, J. A. Bygraves, E. Feil et al., "Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 6, pp. 3140–3145, 1998.

[50] C. B. Sullivan, M. A. Diggle, and S. C. Clarke, "Multilocus sequence typing: data analysis in clinical microbiology and public health," *Molecular Biotechnology*, vol. 29, pp. 245–254, 2005.

[51] J. Chun and F. A. Rainey, "Integrating genomics into the taxonomy and systematics of the Bacteria and Archaea," *International Journal of Systematic and Evolutionary Microbiology*, vol. 64, no. Pt_2, pp. 316–324, 2014.

[52] S. P. Glaeser and P. Kämpfer, "Multilocus sequence analysis (MLSA) in prokaryotic taxonomy," *Systematic & Applied Microbiology*, vol. 38, no. 4, pp. 237–245, 2015.

[53] P. De Vos, "Multilocus sequence determination and analysis," *Methods in Microbiology*, vol. 38, pp. 385–407, 2012.

[54] X. Rong and Y. Huang, "Taxonomic evaluation of the *Streptomyces hygroscopicus* clade using multilocus sequence analysis and DNA-DNA hybridization, validating the MLSA scheme for systematics of the whole genus," *Systematic & Applied Microbiology*, vol. 35, no. 1, pp. 7–18, 2012.

[55] D. R. Zeigler, "Gene sequences useful for predicting relatedness of whole genomes in bacteria," *International Journal of Systematic and Evolutionary Microbiology*, vol. 53, no. 6, pp. 1893–1900, 2003.

[56] K. T. Konstantinidis, A. Ramette, and J. M. Tiedje, "Toward a more robust assessment of intraspecies diversity, using fewer genetic markers," *Applied and Environmental Microbiology*, vol. 72, no. 11, pp. 7286–7293, 2006.

[57] Z. Yang and B. Rannala, "Molecular phylogenetics: principles and practice," *Nature Reviews Genetics*, vol. 13, no. 5, pp. 303–314, 2012.

[58] J. P. Meier-Kolthoff, H. P. Klenk, and M. Göker, "Taxonomic use of DNA G+C content and DNA-DNA hybridization in the genomic age," *International Journal of Systematic and Evolutionary Microbiology*, vol. 64, no. Pt_2, pp. 352–356, 2014.

[59] K. T. Konstantinidis and J. M. Tiedje, "Genomic insights that advance the species definition for prokaryotes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 7, pp. 2567–2572, 2005.

[60] J. Chun, A. Oren, A. Ventosa et al., "Proposed minimal standards for the use of genome data for the taxonomy of prokaryotes," *International Journal of Systematic and Evolutionary Microbiology*, vol. 68, no. 1, pp. 461–466, 2018.

[61] P. Vandamme and C. Peeters, "Time to revisit polyphasic taxonomy," *Antonie Van Leeuwenhoek*, vol. 106, no. 1, pp. 57–65, 2014.

[62] C. C. Thompson, G. R. Amaral, M. Campeão et al., "Microbial taxonomy in the post-genomic era: rebuilding from scratch?" *Archives of Microbiology*, vol. 197, no. 3, pp. 359–370, 2015.

[63] L. Lloret and E. Martínez-Romero, "Evolución y filogenia de *Rhizobium*," *Revista Latinoamericana de Microbiología*, vol. 47, no. 1-2, pp. 43–60, 2005.

[64] R. C. Edgar, "MUSCLE: multiple sequence alignment with high accuracy and high throughput," *Nucleic Acids Research*, vol. 32, no. 5, pp. 1792–1797, 2004.

[65] J. D. Thompson, D. G. Higgins, and T. J. Gibson, "Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice," *Nucleic Acids Research*, vol. 22, no. 22, pp. 4673–4680, 1994.

[66] W. Ludwig and K. H. Schleifer, "Bacterial phylogeny based on 16S and 23S rRNA sequence analysis," *FEMS Microbiology Reviews*, vol. 15, no. 2-3, pp. 155–173, 1994.

[67] W. Ludwig and H. P. Klenk, "Overview: a phylogenetic backbone and taxonomic framework for prokaryotic systematics," *Bergey's Manual of Systematic Bacteriology*, Springer, Berlin, Germany, 2001.

[68] J. Felsenstein, "Confidence limits on phylogenies: an approach using the bootstrap," *Evolution*, vol. 39, 1985.

[69] S. B. Hedges, "The number of replications needed for accurate estimation of the bootstrap P value in phylogenetic studies," *Molecular Biology and Evolution*, vol. 9, pp. 366–369, 1992.

[70] J. D. Retief, "Phylogenetic analysis using PHYLIP," *Bioinformatics Methods and Protocols*, Human Press, Totowa, New Jersey, USA, 2000.

[71] T. Hall, "BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT," *Nucleic Acids Symposium Series*, vol. 41, pp. 95–98, 1999.

[72] M. Kim, H. S. Oh, S. C. Park, and J. Chun, "Towards a taxonomic coherence between average nucleotide identity and 16S rRNA gene sequence similarity for species demarcation of prokaryotes," *International Journal of Systematic and Evolutionary Microbiology*, vol. 64, no. Pt_2, pp. 346–351, 2014.

[73] W. Ludwig, R. Amann, E. Martinez-Romero et al., "rRNA based identification and detection systems for rhizobia and other bacteria," *Plant and Soil*, vol. 204, pp. 1–19, 1998.

[74] E. Stackebrandt and B. M. Goebel, "Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology," *International Journal of Systematic and Evolutionary Microbiology*, vol. 44, no. 4, pp. 846–849, 1994.

[75] P. Menna, F. G. Barcellos, and M. Hungria, "Phylogeny and taxonomy of a diverse collection of *Bradyrhizobium* strains based on multilocus sequence analysis of the 16S rRNA gene, ITS region and *glnII, recA, atpD* and *dnaK* genes," *International Journal of Systematic and Evolutionary Microbiology*, vol. 59, no. 12, pp. 2934–2950, 2009.

[76] D. Durán, L. Rey, J. Mayo et al., "*Bradyrhizobium paxllaeri* sp. nov. and *Bradyrhizobium icense* sp. nov., nitrogen-fixing rhizobial symbionts of Lima bean (*Phaseolus lunatus* L.) in Peru," *International Journal of Systematic and Evolutionary Microbiology*, vol. 64, no. Pt_6, pp. 2072–2078, 2014.

[77] L. C. Ferraz Helene, G. O'Hara, and M. Hungria, "Characterization of *Bradyrhizobium* strains indigenous to Western Australia and South Africa indicates remarkable genetic diversity and reveals putative new species,"

Systematic & Applied Microbiology, vol. 43, no. 2, Article ID 126053, 2020.

[78] M. Rossi-Tamisier, S. Benamar, D. Raoult, and P. E. Fournier, "Cautionary tale of using 16S rRNA gene sequence similarity values in identification of human-associated bacterial species," *International Journal of Systematic and Evolutionary Microbiology*, vol. 65, no. Pt_6, pp. 1929–1934, 2015.

[79] L. M. Schouls, C. S. Schot, and J. A. Jacobs, "Horizontal transfer of segments of the 16S rRNA genes between species of the *Streptococcus anginosus* group," *Journal of Bacteriology*, vol. 185, no. 24, pp. 7241–7246, 2003.

[80] A. Y. Pei, W. E. Oberdorf, C. W. Nossa et al., "Diversity of 16S rRNA genes within individual prokaryotic genomes," *Applied and Environmental Microbiology*, vol. 76, no. 12, pp. 3886–3897, 2010.

[81] X. Y. Zhi, W. Zhao, W. J. Li, and G. P. Zhao, "Prokaryotic systematics in the genomics era," *Antonie Van Leeuwenhoek*, vol. 101, no. 1, pp. 21–34, 2011.

[82] J. García-Martínez, S. G. Acinas, A. I. Antón, and F. Rodríguez-Valera, "Use of the 16S–23S ribosomal genes spacer region in studies of prokaryotic diversity," *Journal of Microbiological Methods*, vol. 36, no. 1-2, pp. 55–64, 1999.

[83] M. S. Klepa, M. C. d O. Urquiaga, P. Somasegaran, J. R. M. Delamuta, R. A. Ribeiro, and M. Hungria, "*Bradyrhizobium niftali* sp. nov., an effective nitrogen-fixing symbiont of partridge pea [*Chamaecrista fasciculata* (Michx.) Greene], a native caesalpinioid legume broadly distributed in the USA," *International Journal of Systematic and Evolutionary Microbiology*, vol. 69, no. 11, pp. 3448–3459, 2019.

[84] M. C. d O. Urquiaga, M. S. Klepa, P. Somasegaran, R. A. Ribeiro, J. R. M. Delamuta, and M. Hungria, "*Bradyrhizobium frederickii* sp. nov., a nitrogen-fixing lineage isolated from nodules of the caesalpinioid species *Chamaecrista fasciculata* and characterized by tolerance to high temperature in vitro," *International Journal of Systematic and Evolutionary Microbiology*, vol. 69, no. 12, pp. 3863–3877, 2019.

[85] P. van Berkum and J. J. Fuhrmann, "Evolutionary relationships among the soybean bradyrhizobia reconstructed from 16S rRNA gene and internally transcribed spacer region sequence divergence," *International Journal of Systematic and Evolutionary Microbiology*, vol. 50, no. 6, pp. 2165–2172, 2000.

[86] A. Willems, R. Coopman, and M. Gillis, "Comparison of sequence analysis of 16S–23S rDNA spacer regions, AFLP analysis and DNA–DNA hybridizations in *Bradyrhizobium*," *International Journal of Systematic and Evolutionary Microbiology*, vol. 51, no. 2, pp. 623–632, 2001.

[87] L. Carro, Á. Peix, and E. Velázquez, "The taxonomy of bacteria in the genomic era," *Developmental Biology in Prokaryotes and Lower Eukaryotes*, Springer, Berlin, Germany, 2021.

[88] A. Willems, A. Munive, P. de Lajudie, and M. Gillis, "In most *Bradyrhizobium* groups sequence comparison of 16S-23S rDNA internal transcribed spacer regions corroborates DNA-DNA hybridizations," *Systematic & Applied Microbiology*, vol. 26, no. 2, pp. 203–210, 2003.

[89] A. Pei, Cw Nossa, P. Chokshi et al., "Diversity of 23S rRNA genes within individual prokaryotic genomes," *PLoS One*, vol. 4, no. 5, Article ID e5437, 2009.

[90] A. J. Probst, C. Hertel, L. Richter, L. Wassil, W. Ludwig, and W. P. Hammes, "*Staphylococcus condimenti* sp. nov., from soy sauce mash, and *Staphylococcus carnosus* (Schleifer and Fischer 1982) subsp. *utilis* subsp. nov," *International Journal of Systematic Bacteriology*, vol. 48, no. 3, pp. 651–658, 1998.

[91] J. Yan, H. Yan, L. X. Liu et al., "*Rhizobium hidalgonense* sp. nov., a nodule endophytic bacterium of *Phaseolus vulgaris* in acid soil," *Archives of Microbiology*, vol. 199, no. 1, pp. 97–104, 2017.

[92] M. S. Klepa, L. C. Ferraz Helene, G. O'Hara, and M. Hungria, "*Bradyrhizobium agreste* sp. nov., *Bradyrhizobium glycinis* sp. nov. and *Bradyrhizobium diversitatis* sp. nov., isolated from a biodiversity hotspot of the genus *Glycine* in Western Australia," *International Journal of Systematic and Evolutionary Microbiology*, vol. 71, no. 3, 2019.

[93] L. C. F. Helene, M. S. Klepa, G. O'Hara, and M. Hungria, "*Bradyrhizobium archetypum* sp. nov., *Bradyrhizobium australiense* sp. nov. and *Bradyrhizobium murdochi* sp. nov., isolated from nodules of legumes indigenous to Western Australia," *International Journal of Systematic and Evolutionary Microbiology*, vol. 70, no. 8, pp. 4623–4636, 2020.

[94] J. M. Lang, A. E. Darling, and J. A. Eisen, "Phylogeny of Bacterial and Archaeal genomes using conserved genes: supertrees and supermatrices," *PLoS One*, vol. 8, no. 4, Article ID e62510, 2013.

[95] H. Berrada and K. Fikri-Benbrahim, "Taxonomy of the rhizobia: current perspectives," *British Microbiology Research Journal*, vol. 4, no. 6, pp. 616–639, 2014.

[96] J. Z. M. Chan, M. R. Halachev, N. J. Loman, C. Constantinidou, and M. J. Pallen, "Defining bacterial species in the genomic era: insights from the genus *Acinetobacter*," *BMC Microbiology*, vol. 12, no. 1, p. 302, 2012.

[97] F. L. Thompson, D. Gevers, C. C. Thompson et al., "Phylogeny and molecular identification of vibrios on the basis of Multilocus Sequence Analysis," *Applied and Environmental Microbiology*, vol. 71, pp. 5107–5115, 2005.

[98] M. Martens, P. Dawyndt, R. Coopman, M. Gillis, P. De Vos, and A. Willems, "Advantages of multilocus sequence analysis for taxonomic studies: a case study using 10 housekeeping genes in the genus *Ensifer* (including former *Sinorhizobium*)," *International Journal of Systematic and Evolutionary Microbiology*, vol. 58, no. 1, pp. 200–214, 2008.

[99] L. Mavima, C. W. Beukes, M. Palmer et al., "*Paraburkholderia youngii* sp. nov. and "*Paraburkholderia atlantica*" Brazilian and Mexican *Mimosa*-associated rhizobia that were previously known as *Paraburkholderia tuberum* sv. mimosae," *Systematic & Applied Microbiology*, vol. 44, no. 1, Article ID 126152, 2021.

[100] K. Tamura, G. Stecher, D. Peterson, A. Filipski, and S. Kumar, "MEGA6: molecular evolutionary genetics analysis version 6.0," *Molecular Biology and Evolution*, vol. 30, no. 12, pp. 2725–2729, 2013.

[101] M. Gouy, S. Guindon, and O. Gascuel, "SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building," *Molecular Biology and Evolution*, vol. 27, no. 2, pp. 221–224, 2010.

[102] E. Stackebrandt, W. Frederiksen, G. M. Garrity et al., "Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology," *International Journal of Systematic and Evolutionary Microbiology*, vol. 52, pp. 1043–1047, 2002.

[103] K. T. Konstantinidis and J. M. Tiedje, "Prokaryotic taxonomy and phylogeny in the genomic era: advancements and challenges ahead," *Current Opinion in Microbiology*, vol. 10, no. 5, pp. 504–509, 2007.

[104] M. Mulet, J. Lalucat, and E. Garcia-Valdes, "DNA sequence-based analysis of the Pseudomonas species," *Environmental Microbiology*, vol. 12, 2010.

[105] J. R. M. Delamuta, R. A. Ribeiro, E. Ormeño-Orrillo, I. S. Melo, E. Martínez-Romero, and M. Hungria, "Polyphasic evidence supporting the reclassification of *Bradyrhizobium japonicum* group Ia strains as *Bradyrhizobium diazoefficiens* sp. nov," *International Journal of Systematic and Evolutionary Microbiology*, vol. 63, no. Pt_9, pp. 3342–3351, 2013.

[106] F. Paulitsch, R. F. Dall'Agnol, J. R. M. Delamuta, R. A. Ribeiro, J. S. da Silva Batista, and M. Hungria, "*Paraburkholderia atlantica* sp. nov. and *Paraburkholderia franconis* sp. nov., two new nitrogen-fixing nodulating species isolated from Atlantic forest soils in Brazil," *Archives of Microbiology*, vol. 202, no. 6, pp. 1369–1380, 2020.

[107] J. Versalovic, M. Schneider, F. J. Bruijn, and J. R. Lupski, "Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction," *Methods in Molecular and Cellular Biology*, vol. 5, pp. 25–40, 1994.

[108] K. S. B. Mannan, R. M. Mazumdar, and A. Chowdhury, "Identification and characterization of microorganisms: DNA fingerprinting methods," *Songklanakarin Journal of Science and Technology*, vol. 35, no. 4, pp. 397–404, 2013.

[109] M. J. Stern, G. F. L. Ames, N. H. Smith, E. Clare Robinson, and C. F. Higgins, "Repetitive extragenic palindromic sequences: a major component of the bacterial genome," *Cell*, vol. 37, no. 3, pp. 1015–1026, 1984.

[110] C. S. J. Hulton, C. F. Higgins, and P. M. Sharp, "ERIC sequences: a novel family of repetitive elements in the genomes of *Escherichia coli, Salmonella typhimurium* and other enterobacteria," *Molecular Microbiology*, vol. 5, no. 4, pp. 825–834, 1991.

[111] B. Martin, O. Humbert, M. Camara et al., "A highly conserved repeated DNA element located in the chromosome of *Streptococcus pneumoniae*," *Nucleic Acids Research*, vol. 20, no. 13, pp. 3479–3483, 1992.

[112] F. J. de Bruijn, "Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergeneric consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria," *Applied and Environmental Microbiology*, vol. 58, no. 7, pp. 2180–2187, 1992.

[113] G. Laguerre, P. Mavingui, M. R. Allard et al., "Typing of rhizobia by PCR DNA fingerprinting and PCR-restriction fragment length polymorphism analysis of chromosomal and symbiotic gene regions: application to *Rhizobium leguminosarum* and its different biovars," *Applied and Environmental Microbiology*, vol. 62, no. 6, pp. 2029–2036, 1996.

[114] P. Menna, A. A. Pereira, E. V. Bangel, and M. Hungria, "rep-PCR of tropical rhizobia for strain fingerprinting, biodiversity appraisal and as a taxonomic and phylogenetic tool," *Symbiosis*, vol. 48, pp. 120–130, 2009.

[115] R. Rosselló-Móra, M. Urdiain, and A. López-López, "DNA-DNA hybridization," *Taxonomy of Prokaryotes*, Academic Press, Cambridge, MA, USA, 2012.

[116] J. L. Johnson and E. J. Ordal, "Deoxyribonucleic acid homology in bacterial taxonomy: effect of incubation temperature on reaction specificity," *Journal of Bacteriology*, vol. 95, no. 3, pp. 893–900, 1968.

[117] J. Goris, K. T. Konstantinidis, J. A. Klappenbach, T. Coenye, P. Vandamme, and J. M. Tiedje, "DNA-DNA hybridization values and their relationship to whole-genome sequence similarities," *International Journal of Systematic and Evolutionary Microbiology*, vol. 57, no. 1, pp. 81–91, 2007.

[118] N. K. Mahato, V. Gupta, P. Singh et al., "Microbial taxonomy in the era of OMICS: application of DNA sequences, computational tools and techniques," *Antonie van Leeuwenhoek*, vol. 110, 2017.

[119] R. D. Fleischmann, M. D. Adams, O. White et al., "Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd," *Science*, vol. 269, no. 5223, pp. 496–512, 1995.

[120] E. Sentausa and P. E. Fournier, "Advantages and limitations of genomics in prokaryotic taxonomy," *Clinical Microbiology and Infection*, vol. 19, no. 9, pp. 790–795, 2013.

[121] A. Gurevich, V. Saveliev, N. Vyahhi, and G. Tesler, "QUAST: quality assessment tool for genome assemblies," *Bioinformatics*, vol. 29, no. 8, pp. 1072–1075, 2013.

[122] J. P. Meier-Kolthoff, A. F. Auch, H. P. Klenk, and M. Göker, "Genome sequence based species delimitation with confidence intervals and improved distance functions," *BMC Bioinformatics*, vol. 14, no. 1, p. 60, 2013.

[123] M. Richter and R. Rosselló-Mora, "Shifting the genomic gold standard for the prokaryotic species definition," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 45, pp. 19126–19131, 2009.

[124] N. M. Mesbah, W. B. Whitman, and M. Mesbah, "Determination of the G+C content of prokaryotes," *Taxonomy of Prokaryotes*, Elsevier, Amsterdam, Netherlands, 1st edition, 2012.

[125] R. Overbeek, R. Olson, G. D. Pusch et al., "The seed and the rapid annotation of microbial genomes using subsystems technology (RAST)," *Nucleic Acids Research*, vol. 42, no. D1, pp. D206–D214, 2014.

[126] M. Hungria, L. M. D O. Chueire, R. G. Coca, and M. Megías, "Preliminary characterization of fast growing rhizobial strains isolated from soyabean nodules in Brazil," *Soil Biology and Biochemistry*, vol. 33, no. 10, pp. 1349–1361, 2001.

[127] P. Kämpfer, "Continuing importance of the "phenotype" in the genomic era," *Methods in Microbiology*, vol. 41, pp. 307–320, 2014.

[128] M. Sasser, "Identification of bacteria by gas chromatography of cellular fatty acids," 1990, https://www.researchgate.net/publication/284025789_Identification_of_bacteria_by_gas_chromatography_of_cellular_fatty_acids.

[129] E. Carbonnelle, C. Mesquita, E. Bille et al., "MALDI-TOF mass spectrometry tools for bacterial identification in clinical microbiology laboratory," *Clinical Biochemistry*, vol. 44, no. 1, pp. 104–109, 2011.

[130] R. D. Holland, J. G. Wilkes, F. Rafii et al., "Rapid identification of intact whole bacteria based on spectral patterns using matrix-assisted laser desorption/ionization with time-of-flight mass spectrometry," *Rapid Communications in Mass Spectrometry*, vol. 10, pp. 1227–1232, 1996.

[131] D. Ziegler, J. F. Pothier, J. Ardley et al., "Ribosomal protein biomarkers provide root nodule bacterial identification by MALDI-TOF MS," *Applied Microbiology and Biotechnology*, vol. 99, no. 13, pp. 5547–5562, 2015.

[132] L. Rolim, T. R. Santiago, F. B. dos Reis Junior, I. de Carvalho Mendes, M. Hungria, and L. P. Silva, "Identification of soybean *Bradyrhizobium* strains used in commercial inoculants in Brazil by MALDI-TOF mass spectrometry," *Brazilian Journal of Microbiology*, vol. 50, no. 4, pp. 1147-1148, 2019.

[133] P. Rahi, O. Prakash, and Y. S. Shouche, "Matrix-assisted laser desorption/ionization time-of-flight mass-spectrometry (MALDI-TOF MS) based microbial identifications:

challenges and scopes for microbial ecologists," *Frontiers in Microbiology*, vol. 7, 2016.

[134] E. Ormeño-Orrillo and E. Martínez-Romero, "Phenotypic tests in *Rhizobium* species description: an opinion and (a sympatric speciation) hypothesis," *Systematic & Applied Microbiology*, vol. 36, no. 3, pp. 145–147, 2013.

[135] A. Willems, "The taxonomy of rhizobia: an overview," *Plant and Soil*, vol. 287, no. 1-2, pp. 3–14, 2006.

[136] B. Frank, "Ueber die Pilzsymbiose der Leguminosen," *Berichte der Deutschen Botanischen Gesellschaft*, vol. 7, pp. 332–346, 1889.

[137] E. Velázquez, P. García-Fraile, M. H. Ramírez-Bahena, R. Rivas, and E. Martínez-Molina, "Current status of the taxonomy of bacteria able to establish nitrogen-fixing legume symbiosis," *Microbes for Legume Improvement*, Springer, Berlin, Germany, 2nd edition, 2017.

[138] I. L. Baldwin and E. B. Fred, "Nomenclature of the root nodule bacteria of the Leguminosae," *Journal of Bacteriology*, vol. 17, no. 2, pp. 141–150, 1929.

[139] P. A. Dangeard, "Recherches sur les tubercules radicaux des Légumineuses," *Botaniste*, vol. 16, pp. 1–275, 1926.

[140] M. M. Eckhardt, I. L. Baldwin, and E. B. Fred, "Studies on the root-nodule organism of *Lupinus*," *Journal of Bacteriology*, vol. 21, no. 4, pp. 273–285, 1931.

[141] R. E. Buchanan, "What names should be used for the organisms producing nodules on the roots of leguminous plants?" *Proceedings of the Iowa Academy of Science*, vol. 33, 1926.

[142] E. B. Fred, I. L. Baldwin, and E. Mc Coy, *Root Nodule Bacteria and Leguminous Plants*, Madison, Madison, Wisconsin, 1932.

[143] J. K. Wilson, "Over five hundred reasons for abandoning the cross-inoculation groups of legumes," *Soil Science*, vol. 58, no. 1, pp. 61–70, 1944.

[144] F. Zakhia and P. De Lajudie, "Taxonomy of rhizobia," *Agronomie*, vol. 21, 2001.

[145] P. H. Graham, "The application of computer techniques to the taxonomy of the root-nodule bacteria of legumes," *Journal of General Microbiology*, vol. 35, no. 3, pp. 511–517, 1964.

[146] P. H. Graham, "Antigenic affinities of the root-nodule bacteria of legumes," *Antonie van Leeuwenhoek*, vol. 29, no. 1, pp. 281–291, 1963.

[147] J. De Ley and A. Rassel, "DNA base composition, flagellation and taxonomy of the genus *Rhizobium*," *Journal of General Microbiology*, vol. 41, no. 1, pp. 85–92, 1965.

[148] M. L. Moffett and R. R. Colwell, "Adansonian analysis of the Rhizobiaceae," *Journal of General Microbiology*, vol. 51, no. 2, pp. 245–266, 1968.

[149] D. C. Jordan, "Transfer of *Rhizobium japonicum* Buchanan 1980 to *Bradyrhizobium* gen. nov., a genus of slow-growing, root nodule bacteria from leguminous plants," *International Journal of Systematic Bacteriology*, vol. 32, no. 1, pp. 136–139, 1982.

[150] B. Dreyfus, J. L. Garcia, and M. Gillis, "Characterization of *Azorhizobium cauhodans* gen. nov., sp. nov., a stem-nodulating nitrogen-fixing bacterium isolated from *Sesbania rostrata*," *International Journal of Systematic Bacteriology*, vol. 38, no. 1, pp. 89–98, 1988.

[151] W. X. Chen, G. H. Yan, and J. L. Li, "Numerical taxonomic study of fast-growing soybean rhizobia and proposal that *Rhizobium fredii* be assigned to *Sinorhizobium* gen. nov," *International Journal of Systematic Bacteriology*, vol. 38, no. 4, pp. 392–397, 1988.

[152] H. J. Conn, "Validity of the genus *Alcaligenes*," *Journal of Bacteriology*, vol. 44, no. 3, pp. 353–360, 1942.

[153] A. Peix, M. H. Ramírez-Bahena, E. Velázquez, and E. J. Bedmar, "Bacterial associations with legumes," *Critical Reviews in Plant Sciences*, vol. 34, no. 1–3, pp. 17–42, 2015.

[154] B. D. W. Jarvis, P. van Berkum, W. X. Chen et al., "Transfer of *Rhizobium loti, Rhizobium huakuii, Rhizobium ciceri, Rhizobium mediterraneum*, and *Rhizobium tianshanense* to *Mesorhizobium* gen. nov," *International Journal of Systematic and Evolutionary Microbiology*, vol. 47, no. 3, pp. 895–898, 1997.

[155] P. de Lajudie, E. Laurent-Fulele, A. Willems et al., "*Allorhizobium undicola* gen. nov., sp. nov., nitrogen-fixing bacteria that efficiently nodulate *Neptunia natans* in Senegal," *International Journal of Systematic Bacteriology*, vol. 48, no. 4, pp. 1277–1290, 1998.

[156] L. E. J. Casida, "*Ensifer adhaerens* gen nov. sp. nov.: a bacterial predator of bacteria in soil," *International Journal of Systematic Bacteriology*, vol. 32, no. 3, pp. 339–345, 1982.

[157] P. van Berkum and B. D. Eardly, "Molecular evolutionary systematics of the Rhizobiaceae," *The Rhizobiaceae*, Elsevier, Dordrecht, Netherlands, 1998.

[158] M. Yanagi and K. Yamasato, "Phylogenetic analysis of the family Rhizobiaceae and related bacteria by sequencing of 16S rRNA gene using PCR and DNA sequencer," *FEMS Microbiology Letters*, vol. 107, no. 1, pp. 115–120, 1993.

[159] T. E. Patt, G. C. Cole, and R. S. Hanson, "*Methylobacterium*, a new genus of facultatively methylotrophic bacteria," *International Journal of Systematic Bacteriology*, vol. 26, no. 2, pp. 226–229, 1976.

[160] A. Sy, E. Giraud, P. Jourand et al., "Methylotrophic *Methylobacterium* bacteria nodulate and fix nitrogen in symbiosis with legumes," *Journal of Bacteriology*, vol. 183, no. 1, pp. 214–220, 2001.

[161] H. Sawada, H. Ieki, H. Oyaizu, and S. Matsumoto, "Proposal for rejection of *Agrobacterium tumefaciens* and revised descriptions for the genus *Agrobacterium* and for *Agrobacterium radiobacter* and *Agrobacterium rhizogenes*," *International Journal of Systematic Bacteriology*, vol. 43, no. 4, pp. 694–702, 1993.

[162] J. M. Young, L. D. Kuykendall, E. Martínez-Romero, A. Kerr, and H. Sawada, "A revision of *Rhizobium* Frank 1889, with an emended description of the genus, and the inclusion of all species of *Agrobacterium* Conn 1942 and *Allorhizobium undicola* de Lajudie et al. 1998 as new combinations: *Rhizobium radiobacter, R. rhizogenes, R. rubi, R. undicola* and *R. vitis*," *International Journal of Systematic and Evolutionary Microbiology*, vol. 51, no. 1, pp. 89–103, 2001.

[163] R. Rivas, E. Velázquez, A. Willems et al., "A new species of *Devosia* that forms a unique nitrogen-fixing root-nodule symbiosis with the aquatic legume *Neptunia natans* (L.f.) Druce," *Applied and Environmental Microbiology*, vol. 68, no. 11, pp. 5217–5222, 2002.

[164] A. Valverde, E. Velázquez, F. Fernández-Santos et al., "*Phyllobacterium trifolii* sp. nov., nodulating *Trifolium* and *Lupinus* in Spanish soils," *International Journal of Systematic and Evolutionary Microbiology*, vol. 55, no. 5, pp. 1985–1989, 2005.

[165] B. Holmes, M. Popoff, M. Kiredjian, and K. Kersters, "*Ochrobactrum anthropi* gen. nov., sp. nov. from human clinical specimens and previously known as group Vd," *International Journal of Systematic Bacteriology*, vol. 38, no. 4, pp. 406–416, 1988.

[166] K. F. Meyer and E. B. Shaw, "A comparison of the morphologic, cultural and biochemical characteristics of *B. abortus* and *B. melitensis* from cattle. Studies on the genus *Brucella* nov," *Journal of Infectious Diseases*, vol. 27, no. 3, pp. 173–184, 1920.

[167] M. E. Trujillo, A. Willems, A. Abril et al., "Nodulation of *Lupinus albus* by strains of *Ochrobactrum lupini* sp. nov," *Applied and Environmental Microbiology*, vol. 71, pp. 1318–1327, 2005.

[168] J. L. Zurdo-Piñeiro, R. Rivas, M. E. Trujillo et al., "*Ochrobactrum cytisi* sp. nov., isolated from nodules of *Cytisus scoparius* in Spain," *International Journal of Systematic and Evolutionary Microbiology*, vol. 57, no. 4, pp. 784–788, 2007.

[169] D. X. Lin, E. T. Wang, H. Tang et al., "*Shinella kummerowiae* sp. nov., a symbiotic bacterium isolated from root nodules of the herbal legume *Kummerowia stipulacea*," *International Journal of Systematic and Evolutionary Microbiology*, vol. 58, no. 6, pp. 1409–1413, 2008.

[170] D. S. An, W. T. Im, H. C. Yang, and S. T. Lee, "*Shinella granuli* gen. nov., sp. nov., and proposal of the reclassification of *Zoogloea ramigera* ATCC 19623 as *Shinella zoogloeoides* sp. nov," *International Journal of Systematic and Evolutionary Microbiology*, vol. 56, no. 2, pp. 443–448, 2006.

[171] M. J. Estrella, S. Muñoz, M. J. Soto, O. Ruiz, and J. Sanjuán, "Genetic diversity and host range of rhizobia nodulating *Lotus tenuis* in typical soils of the Salado River Basin (Argentina)," *Applied and Environmental Microbiology*, vol. 75, no. 4, pp. 1088–1098, 2009.

[172] S. Kanso and B. K. C. Patel, "*Microvirga subterranea* gen. nov., sp. nov., a moderate thermophile from a deep subsurface Australian thermal aquifer," *International Journal of Systematic and Evolutionary Microbiology*, vol. 53, no. 2, pp. 401–406, 2003.

[173] J. K. Ardley, M. A. Parker, S. E. De Meyer et al., "*Microvirga lupini* sp. nov., *Microvirga lotononidis* sp. nov. and *Microvirga zambiensis* sp. nov. are alphaproteobacterial root-nodule bacteria that specifically nodulate and fix nitrogen with geographically and taxonomically separate legume hosts," *International Journal of Systematic and Evolutionary Microbiology*, vol. 62, no. Pt_11, pp. 2579–2588, 2012.

[174] V. Radl, J. L. Simões-Araújo, J. Leite et al., "*Microvirga vignae* sp. nov., a root nodule symbiotic bacterium isolated from cowpea grown in semi-arid Brazil," *International Journal of Systematic and Evolutionary Microbiology*, vol. 64, no. Pt_3, pp. 725–730, 2014.

[175] L. Moulin, A. Munive, B. Dreyfus, and C. Boivin-Masson, "Nodulation of legumes by members of the beta-subclass of Proteobacteria," *Nature*, vol. 411, no. 6840, pp. 948–950, 2001.

[176] E. Yabuuchi, Y. Kosako, H. Oyaizu et al., "Proposal of burkholderia gen. Nov. and transfer of seven species of the genus pseudomonas homology group II to the new genus, with the type species Burkholderia cepacia (palleroni and holmes 1981) comb. Nov," *Microbiology and Immunology*, vol. 36, 1992.

[177] E. Yabuuchi, Y. Kosako, I. Yano, H. Hotta, and Y. Nishiuchi, "Transfer of two burkholderia and an alcaligenes species to ralstonia gen. nov.:proposal of Ralstonia pickettii (ralston, palleroni and doudoroff 1973) comb. nov., Ralstonia solanacearum (Smith 1896) comb. nov. and Ralstonia eutropha (Davis 1969) comb. nov," *Microbiology and Immunology*, vol. 39, 1995.

[178] N. S. Makkar and L. E. Casida Jr, "*Cupriavidus necator* gen. nov., sp. nov.: a nonobligate bacterial predator of bacteria in soil," *International Journal of Systematic Bacteriology*, vol. 37, no. 4, pp. 323–326, 1987.

[179] W. M. Chen, E. K. James, A. R. Prescott, M. Kierans, and J. I. Sprent, "Nodulation of *Mimosa* spp. by the beta-proteobacterium *Ralstonia taiwanensis*," *Molecular Plant-Microbe Interactions®*, vol. 16, no. 12, pp. 1051–1061, 2003.

[180] P. Vandamme and T. Coenye, "Taxonomy of the genus *Cupriavidus*: a tale of lost and found," *International Journal of Systematic and Evolutionary Microbiology*, vol. 54, no. 6, pp. 2285–2289, 2004.

[181] A. Sawana, M. Adeolu, and R. S. Gupta, "Molecular signatures and phylogenomic analysis of the genus *Burkholderia*: proposal for division of this genus into the emended genus *Burkholderia* containing pathogenic organisms and a new genus *Paraburkholderia* gen. nov. harboring environmental species," *Frontiers in Genetics*, vol. 5, 2014.

[182] A. P. Dobritsa and M. Samadpour, "Transfer of eleven *Burkholderia* species to the genus *Paraburkholderia* and proposal of *Caballeronia* gen. nov., a new genus to accommodate twelve species of *Burkholderia* and *Paraburkholderia*," *International Journal of Systematic and Evolutionary Microbiology*, vol. 66, no. 8, pp. 2836–2846, 2016.

[183] S. A. Mousavi, J. Österman, N. Wahlberg et al., "Phylogeny of the Rhizobium–Allorhizobium–Agrobacterium clade supports the delineation of Neorhizobium gen. nov," *Systematic and Applied Microbiology*, vol. 37, 2014.

[184] S. A. Mousavi, A. Willems, X. Nesme, P. de Lajudie, and K. Lindström, "Revised phylogeny of Rhizobiaceae: proposal of the delineation of *Pararhizobium* gen. nov., and 13 new species combinations," *Systematic & Applied Microbiology*, vol. 38, no. 2, pp. 84–90, 2015.

[185] C. Fagorzi, A. Ilie, F. Decorosi et al., "Symbiotic and non-symbiotic members of the genus Ensifer (syn. Sinorhizobium) are separated into two clades based on comparative genomics and high-throughput phenotyping," *Genome Biology and Evolution*, vol. 12, no. 12, pp. 2521–2534, 2020.

[186] N. Kuzmanović, C. Fagorzi, A. Mengoni, F. Lassalle, and G. C. DiCenzo, "Taxonomy of Rhizobiaceae revisited: proposal of a new framework for genus delimitation," *International Journal of Systematic and Evolutionary Microbiology*, vol. 72, no. 3, 2022.

[187] Y. Benhiziia, H. Benhizia, A. Benguedouar, R. Muresu, A. Giacomini, and A. Squartini, "Gamma Proteobacteria can nodulate legumes of the genus Hedysarum," *Systematic & Applied Microbiology*, vol. 27, no. 4, pp. 462–468, 2004.

[188] A. Shiraishi, N. Matsushita, and T. Hougetsu, "Nodulation in black locust by the gammaproteobacteria *Pseudomonas* sp. and the Betaproteobacteria *Burkholderia* sp," *Systematic & Applied Microbiology*, vol. 33, no. 5, pp. 269–274, 2010.

[189] M. A. Rogel, E. Ormeño-Orrillo, and E. Martinez Romero, "Symbiovars in rhizobia reflect bacterial adaptation to legumes," *Systematic & Applied Microbiology*, vol. 34, no. 2, pp. 96–104, 2011.

[190] J. R. M. Delamuta, R. A. Ribeiro, E. Ormeño-Orrillo et al., "*Bradyrhizobium tropiciagri* sp. nov. and *Bradyrhizobium embrapense* sp. nov nitrogen-fixing symbionts of tropical forage legumes," *International Journal of Systematic and Evolutionary Microbiology*, vol. 65, no. Pt_12, pp. 4424–4433, 2015.

[191] M. Hungria, G. W. O'Hara, J. E. Zilli, R. S. Araujo, R. Deaker, and J. G. I. Howieson, "Isolation and growth of rhizobia,"

Working with Rhizobia, ACIAR, Canberra, ACT, Australia, 2016.

[192] G. C. diCenzo and T. M. Finan, "The divided bacterial genome: structure, function, and evolution," *Microbiology and Molecular Biology Reviews*, vol. 81, no. 3, 2017.

[193] B. A. Geddes, J. Kearsley, R. Morton, G. C. diCenzo, and T. M. finan, "The genomes of rhizobia," *Regulation of Nitrogen-Fixing Symbioses in Legumes*, Elsevier, Amsterdam, Netherlands, 2020.

[194] G. C. diCenzo, A. Checcucci, M. Bazzicalupo et al., "Metabolic modelling reveals the specialization of secondary replicons for niche adaptation in *Sinorhizobium meliloti*," *Nature Communications*, vol. 7, no. 1, Article ID 12219, 2016.

[195] G. Dávila and R. Palacios, "Origins of genomics in nitrogen-fixation research," *Genomes and Genomics of Nitrogen-Fixing Organisms*, Springer, Berlin, Germany, 2005.

[196] F. Galibert, T. M. Finan, S. R. Long et al., "The composite genome of the legume symbiont *Sinorhizobium meliloti*," *Science*, vol. 293, no. 5530, pp. 668–672, 2001.

[197] J. T. Sullivan and C. W. Ronson, "Evolution of rhizobia by acquisition of a 500-kb symbiosis island that integrates into a phe-tRNA gene," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 9, pp. 5145–5149, 1998.

[198] S. Wang, B. Hao, J. Li et al., "Whole-genome sequencing of *Mesorhi zobium huakuii* 7653R provides molecular insights into host specificity and symbiosis island dynamics," *BMC Genomics*, vol. 15, no. 1, p. 440, 2014.

[199] M. Black, P. Moolhuijzen, B. Chapman et al., "The genetics of symbiotic nitrogen fixation: comparative genomics of 14 rhizobia strains by resolution of protein clusters," *Genes*, vol. 3, no. 1, pp. 138–166, 2012.

[200] S. E. de Meyer, L. Briscoe, P. Martínez-Hidalgo et al., "Symbiotic *Burkholderia* species show diverse arrangements of *nif/fix* and nod genes and lack typical high-affinity cytochrome *cbb3* oxidase genes," *Molecular Plant-Microbe Interactions*, vol. 29, no. 8, pp. 609–619, 2016.

[201] P. Remigi, J. Zhu, J. P. W. Young, and C. Masson-Boivin, "Symbiosis within symbiosis: Evolving nitrogen-fixing legume symbionts," *Trends in Microbiology*, vol. 24, no. 1, pp. 63–75, 2016.

[202] E. Giraud, L. Moulin, D. Vallenet et al., "Legumes symbioses: absence of *nod* genes in photosynthetic bradyrhizobia," *Science*, vol. 316, no. 5829, pp. 1307–1312, 2007.

[203] X. Perret, C. Staehelin, and W. J. Broughton, "Molecular basis of symbiotic promiscuity," *Microbiology and Molecular Biology Reviews*, vol. 64, pp. 180–201, 2000.

[204] G. E. D. Oldroyd, J. D. Murray, P. S. Poole, and J. A. Downie, "The rules of engagement in the legume-rhizobial symbiosis," *Annual Review of Genetics*, vol. 45, no. 1, pp. 119–144, 2011.

[205] D. Patra and S. Mandal, "Nod–factors are dispensable for nodulation: a twist in bradyrhizobia-legume symbiosis," *Symbiosis*, vol. 86, no. 1, pp. 1–15, 2022.

[206] L. H. Madsen, L. Tirichine, A. Jurkiewicz et al., "The molecular network governing nodule organogenesis and infection in the model legume *Lotus japonicus*," *Nature Communications*, vol. 1, no. 1, p. 10, 2010.

[207] P. W. Harrison, R. P. Lower, N. K. Kim, and J. P. W. Young, "Introducing the bacterial 'chromid': not a chromosome, not a plasmid," *Trends in Microbiology*, vol. 18, no. 4, pp. 141–148, 2010.

[208] J. S. Silva Batista, M. Hungria, F. G. Barcellos, M. C. Ferreira, and I. C. Mendes, "Variability in *Bradyrhizobium japonicum* and *B. elkanii* seven years after introduction of both the exotic microsymbiont and the soybean host in a Cerrados soil," *Microbial Ecology*, vol. 53, no. 2, pp. 270–284, 2007.

[209] L. C. F. Helene, J. R. M. Delamuta, R. A. Ribeiro, and M. Hungria, "*Bradyrhizobium mercantei* sp. nov., a nitrogen-fixing symbiont isolated from nodules of *Deguelia costata* (syn. *Lonchocarpus costatus*)," *International Journal of Systematic and Evolutionary Microbiology*, vol. 67, no. 6, pp. 1827–1834, 2017.

[210] L. C. Ferraz Helene, J. R. Marçon Delamuta, R. Augusto Ribeiro et al., "*Bradyrhizobium viridifuturi* sp. nov., encompassing nitrogen-fixing symbionts of legumes used for green manure and environmental services," *International Journal of Systematic and Evolutionary Microbiology*, vol. 65, no. Pt_12, pp. 4441–4448, 2015.

[211] J. P. W. Young, "The phylogeny and evolution of nitrogenases," *Genomes and Genomics of Nitrogen-Fixing Organisms*, Springer, Berlin, Germany, 2005.

[212] M. Hungria, P. Menna, and J. R. M. Delamuta, "Bradyrhizobium, the ancestor of all rhizobia: phylogeny of housekeeping and nitrogen-fixation genes," *Biological Nitrogen Fixation*, John Wiley & Son, Hoboken, NJ, USA, 2015.

[213] S. L. Turner and J. P. W. Young, "The glutamine synthetases of rhizobia: phylogenetics and evolutionary implications," *Molecular Biology and Evolution*, vol. 17, no. 2, pp. 309–319, 2000.

[214] P. Menna and M. Hungria, "Phylogeny of nodulation and nitrogen-fixation genes in *Bradyrhizobium*: supporting evidence for the theory of monophyletic origin, and spread and maintenance by both horizontal and vertical transfer," *International Journal of Systematic and Evolutionary Microbiology*, vol. 61, no. 12, pp. 3052–3067, 2011.

[215] C. S. Smillie, M. B. Smith, J. Friedman, O. X. Cordero, L. A. David, and E. J. Alm, "Ecology drives a global network of gene exchange connecting the human microbiome," *Nature*, vol. 480, no. 7376, pp. 241–244, 2011.

[216] J. Ling, H. Wang, P. Wu et al., "Plant nodulation inducers enhance horizontal gene transfer of *Azorhizobium caulinodans* symbiosis island," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 113, no. 48, pp. 13875–13880, 2016.

[217] C. Masson-Boivin, E. Giraud, X. Perret, and J. Batut, "Establishing nitrogen-fixing symbiosis with legumes: how many rhizobium recipes?" *Trends in Microbiology*, vol. 17, no. 10, pp. 458–466, 2009.

[218] D. Bulgarelli, M. Rott, K. Schlaeppi et al., "Revealing structure and assembly cues for *Arabidopsis* root-inhabiting bacterial microbiota," *Nature*, vol. 488, no. 7409, pp. 91–95, 2012.

[219] J. Edwards, C. Johnson, C. Santos-Medellín et al., "Structure, variation, and assembly of the root-associated microbiomes of rice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 112, no. 8, 2015.

[220] F. Ibanez, J. Angelini, T. Taurian, M. L. Tonelli, and A. Fabra, "Endophytic occupation of peanut root nodules by opportunistic gammaproteobacteria," *Systematic & Applied Microbiology*, vol. 32, no. 1, pp. 49–55, 2009.

[221] A. M. Chibeba, C. S. Pereira, J. E. L. Antunes et al., "Polyphasic characterization of nitrogen-fixing and co-resident bacteria in nodules of *Phaseolus lunatus* inoculated with soils from Piauí State, northeast Brazil," *Symbiosis*, vol. 80, no. 3, pp. 279–292, 2020.

[222] S. M. B. Rocha, L. W. Mendes, L. M. D S. Oliveira et al., "Nodule microbiome from cowpea and lima bean grown in composted tannery sludge-treated soil," *Applied Soil Ecology*, vol. 151, Article ID 103542, 2020.

CAPÍTULO II

Bradyrhizobium archetypum sp. nov., *Bradyrhizobium australiense* sp. nov. E *Bradyrhizobium murdochi* sp. nov., ISOLADAS DE NÓDULOS DE LEGUMINOSAS INDÍGENAS DA AUSTRÁLIA OCIDENTAL.

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RESUMO

O gênero *Bradyrhizobium* é considerado como uma possível linhagem ancestral de todos os rizóbios, o qual é amplamente distribuído em uma variedade de ecossistemas e com uma diversidade notável. O presente estudo utilizou uma abordagem polifásica com o objetivo de caracterizar a posição taxonômica de oito estirpes de *Bradyrhizobium* isoladas de leguminosas indígenas da Austrália Ocidental. Como esperado para o gênero, as sequências do gene 16S RNAr foram altamente conservadas, no entanto, os resultados de *multilocus sequence analysis* com quatro genes *housekeeping* (*dnaK, gnlII, gyrB* e *recA*) confirmaram a existência de três novos clados que incluíram as estirpes (1) WSM 1744^{T}, WSM 1736 e WSM 1737; (2) WSM 1791^{T} e WSM 1742; e (3) WSM 1741^{T}, WSM 1735 e WSM 1790. Os valores mais altos de *average nucleotide identity* dos três grupos em relação às estirpes tipo mais próximas foram 92.4, 92.3 e 93.3%, respectivamente; valores que estão abaixo do valor de corte para delineamento de espécies. A hibridação DNA-DNA digital também confirmou a descrição de novas espécies, com menos de 52% de parentesco em relação às estirpes tipo mais próximas. A filogenia do gene simbiótico *nodC* agrupou as oito estirpes em estudo no simbiovar retamae, junto com sete estirpes tipo de *Bradyrhizobium*, compartilhando 94.2-98.1% de identidade nucleotídica (IN), e menos de 88.7% de similaridade com outras estirpes relacionadas e simbiovares. As características morfofisiológicas, filogenéticas, genômicas e simbióticas foram determinadas para as novas espécies e os dados são congruentes com a descrição de três novas espécies, *Bradyrhizobium archetypum* sp. nov., *Bradyrhizobium australiense* sp. nov. e *Bradyrhizobium murdochi* sp. nov., com WSM 1744^{T} (=CNPSo 4013^{T}=LMG 31646^{T}), WSM 1791^{T}

(=CNPSo 4014^{T}=LMG 31647^{T}) e WSM 1741^{T} (=CNPSo 4020^{T}=LMG 31651^{T}) designadas como estirpes tipo, respectivamente.

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TAXONOMIC DESCRIPTION

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Bradyrhizobium archetypum sp. nov., *Bradyrhizobium australiense* sp. nov. and *Bradyrhizobium murdochi* sp. nov., isolated from nodules of legumes indigenous to Western Australia

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Abstract

The genus *Bradyrhizobium* is considered as the probable ancestor lineage of all rhizobia, broadly spread in a variety of ecosystems and with remarkable diversity. A polyphasic study was performed to characterize and clarify the taxonomic position of eight bradyrhizobial strains isolated from indigenous legumes to Western Australia. As expected for the genus, the 16S rRNA gene sequences were highly conserved, but the results of multilocus sequence analysis with four housekeeping genes (*dnaK*, *glnII*, *gyrB* and *recA*) confirmed three new distinct clades including the following strains: (1) WSM 1744^T, WSM 1736 and WSM 1737; (2) WSM 1791^T and WSM 1742; and (3) WSM 1741^T, WSM 1735 and WSM 1790. The highest ANI values of the three groups in relation to the closest type strains were 92.4, 92.3 and 93.3%, respectively, below the threshold of species circumscription. The digital DNA–DNA hybridization analysis also confirmed new species descriptions, with less than 52% relatedness with the closest type strains. The phylogeny of the symbiotic gene *nodC* clustered the eight strains into the symbiovar retamae, together with seven *Bradyrhizobium* type strains, sharing from 94.2–98.1% nucleotide identity (NI), and less than 88.7% NI with other related strains and symbiovars. Morpho-physiological, phylogenetics, genomic and symbiotic traits were determined for the new groups and our data support the description of three new species, *Bradyrhizobium archetypum* sp. nov., *Bradyrhizobium australiense* sp. nov. and *Bradyrhizobium murdochi sp. nov.*, with WSM 1744^T (=CNPSo 4013^T=LMG 31646^T), WSM 1791^T (=CNPSo 4014^T=LMG 31647^T) and WSM 1741^T (=CNPSo 4020^T=LMG 31651^T) designated as type strains, respectively.

The process of biological nitrogen fixation (BNF) greatly impacts agriculture, supplying nitrogen (N) not just for important grain crops such as soybean (*Glycine max* (L.) Merr), but also for other plants used as green manure, pastures and wood production [1, 2]. The efficiency of the BNF process depends on the level of interaction between the plants and the microsymbionts, but the most relevant contribution occurs with the symbiotic association of bacteria collectively known as 'rhizobia', and a broad range of legumes (Fabaceae=Leguminosae). The main feature of the symbiosis is the presence of specific organs, the nodules, mainly in roots, structures where the BNF process takes place. The resulting nitrogenous compounds are assimilated by the hosts, improving plant nutrition, biomass production, yield and soil fertility. Therefore, BNF helps to decrease the expensive costs and to mitigate the environmental effects of chemical N-fertilizers, helping to reach the goal of sustainable agriculture [3, 4].

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Keywords: *Bradyrhizobium*; phylogeny of prokaryotes; biological nitrogen fixation; MLSA; ANI; dDDH; NI.

Abbreviations: ANI, average nucleotide identity; BNF, biological nitrogen fixation; dDDH, digital DNA–DNA hybridization; DDH, DNA–DNA hybridization; LB, Luria–Bertani; ML, maximum-likelihood; MLSA, multilocus sequence analysis; NI, nucleotide identity; OGRI, overall genome related index; YMA, yeast–mannitol agar.

The following new sequences have been deposited at the GenBank/EMBL/DDBJ database: *atpD* of *B. archetypum* WSM 1744^T (MT070745), WSM 1736 (MT070741), WSM 1737 (MT070742); *atpD* of *B. australiense* WSM 1791^T (MT070746), WSM 1742 (MT070744); *atpD* of *B. murdochi* WSM 1741^T (MT070743), WSM 1735 (MT070740). Accession numbers of the 16S rRNA gene sequences retrieved from the GenBank database: *B. archetypum* WSM 1744^T (MK676065), WSM 1736 (MK676059), WSM 1737 (MK676060); *B. australiense* WSM 1791^T (MK676067), WSM 1742 (MK676063); and *B. murdochi* WSM 1741^T (MK676062), WSM 1790 (MK676066), WSM 1735 (MK676058). Genome accession numbers of *B. archetypum* WSM 1744^T (JAAVLW000000000), *B. australiense* WSM 1791^T (JAAVLX000000000), *B. murdochi* WSM 1741^T (AXAU00000000).

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One supplementary table and seven supplementary figures are available with the online version of this article.

Table 1. Strains from this study (bold) and most related type strains used as comparison

Strain	Synonomy	Original host	Geographical origin	Reference
***Bradyrhizobium archetypum* WSM 1744^T**	CNPSo 4013^T, LMG 31646^T	*Muelleranthus trifoliolatus*	Wooramel, WA	[12, 24]
***Bradyrhizobium archetypum* WSM 1736**	CNPSo 4012	*Indigofera* species	Minilya, WA	[12, 24]
***Bradyrhizobium archetypum* WSM 1737**	CNPSo 4024	*Indigofera* species	Minilya, WA	[12, 24]
***Bradyrhizobium australiense* WSM 1791^T**	CNPSo 4014^T, LMG 31647^T	*Indigofera* species	Carnarvon, WA	[12, 24]
***Bradyrhizobium australiense* WSM 1742**	CNPSo 4021	*Indigofera* species	Carnarvon, WA	[12, 24]
***Bradyrhizobium murdochi* WSM 1741^T**	CNPSo 4020^T, LMG 31651^T	*Rhynchosia minima*	Cape Range National Park, WA	[12, 24]
***Bradyrhizobium murdochi* WSM 1735**	CNPSo 4023	*Rhynchosia minima*	Cape Range National Park, WA	[12, 24]
***Bradyrhizobium murdochi* WSM 1790**	CNPSo 4018	*Indigofera colueta*	North West Cape, WA	[12, 24]
Bradyrhizobium icense LMTR 13^T	HAMBI 3584^T, CECT 8509^T, CNPSo 2583^T	*Phaseolus lunatus*	Peru	[22]
Bradyrhizobium jicamae PAC68^T	LMG 24556^T, CECT 7395^T	*Pachyrhizus erosus*	Honduras	[55]
Bradyrhizobium lablabi CCBAU 23086^T	LMG 25572^T, HAMBI 3052^T	*Lablab purpureus* and *Arachis hypogaea*	China	[56]
Bradyrhizobium namibiense 5-10^T	LMG 28789^T, DSM 100300^T, NTCCM0017^T	*Lablab purpureus*	Namibia	[57]
Bradyrhizobium paxllaeri LMTR 21^T	DSM 18454^T, HAMBI 2911^T	*Phaseolus lunatus*	Peru	[22]
Bradyrhizobium retamae Ro19^T	LMG 27393^T, CECT 8261^T	*Retama sphaerocarpa* and *Retama monosperma*	Morocco and Spain	[42]

In tropical soils, the genus *Bradyrhizobium* [5] is broadly spread and predominant in rhizobial populations [6]. *Bradyrhizobium* is considered as the probable ancestor lineage of all rhizobia and, in the last decade, several studies reported remarkable genetic diversity within the genus, as well as symbiotic associations with an increasing number of legumes [e.g. [1, 2, 6–13]. The environmental and economic applications of *Bradyrhizobium* and its high diversity reassure the importance of characterization and seeking for strains with biotechnological potential.

Although the revolutionary importance of the 16S rRNA gene in establishing the backbone of modern phylogeny [14], the gene is limited in discriminating diversity [15]. The 16S rRNA gene is still used to support taxonomic studies, with the value of nucleotide identity (NI) of 98.7%, proposed in the last decade [16], representing the threshold for species delimitation [17]. Yet, this value needs to corroborate with more robust molecular analyses, including the evaluation of others molecular markers that enhance evolutionary information [18]. Until few years ago, the value of 70% of DNA–DNA hybridization (DDH) between the genome of two strains was also used to discriminate species [19, 20]; however, with the development of bioinformatics tools, genomic parameters, including multilocus sequence analysis (MLSA) [21, 22], average nucleotide identity (ANI) [18], and digital DNA–DNA hybridization (dDDH) [23], have replaced the traditional methods.

Here, we describe a polyphasic study that included genomic parameters aiming at the classification of eight *Bradyrhizobium* strains isolated from nodules of indigenous legumes to Western Australia (WA), that resulted in the description of three new species.

ISOLATION AND ECOLOGY

The eight *Bradyrhizobium* strains used in this study are listed in Table 1. All strains were isolated in the north-west of WA from root nodules of indigenous legumes collected and isolated in a previous study [24] and preliminary characterized [12].

All strains from this study were deposited at the Diazotrophic and Plant Growth Promoting Bacteria Culture Collection of Embrapa Soja (WFCC Collection No. 1213; WDCM Collection No. 1054), located at Londrina, State of Paraná, Brazil; at the Western Australian Soil Microbiology Gene Bank (WSM Culture Collection); at the Belgian Co-ordinated Collection of Micro-organisms (BCCM/LMG); and at the Culture Collection of the Biology Department of the Universidad de Sevilla, Spain.

The strains were grown on modified yeast extract–mannitol agar (YMA) medium [25] at 28 °C, unless otherwise indicated. The stock cultures were maintained on modified YMA medium in a cold room at 4 °C, while long-term storage was by cryopreservation in liquid modified YM medium containing 30% glycerol (v/v) at −80 °C and at −150 °C, and also by lyophilization.

PHYLOGENY

For the molecular analysis, total DNA of the strains was extracted using the DNeasy blood and tissue kit (Qiagen), according to the manufacturer's procedure. The amplification of partial sequences of the *atpD* housekeeping gene, that codes for the enzyme ATP synthase (beta subunit), was obtained using the pair of primers TSatpDf (5′-TCTGGTCCGYGGCCAGGAAG-3′) and TSatpDr (5′-CGACACTTCCGARCCS GCCTG-3′), with the conditions described by Stepkowski *et al.* [26]. The other gene sequences (housekeeping and symbiotic genes) were obtained from our previous study [12]. The PCR products were purified using PureLinkTMQuick PCR Purification Kit (Invitrogen), according to the manufacturer's procedure. Genes sequencing was performed in an ABI3500xL analyzer (Applied Biosystems), as described before [10], and the sequences were deposited at the GenBank database. The remaining sequences were retrieved from GenBank and all the accession numbers are shown in the phylograms and in Table S1 (available in the online version of this article). Maximum-likelihood (ML) phylogenies [27] were obtained with the Molecular Evolutionary Genetics Analysis (MEGA) software (version 7) [28]. Multiple sequence alignment was constructed with MUSCLE [29] and the tree node support was evaluated with bootstrap analysis [30] using 1000 replicates. *Xanthobacter autotrophicus* Py2 was used as outgroup. To build the phylograms, the software eliminated all positions containing gaps and missing data, and the best model of evolution was determined considering the lowest Bayesian information criterion (BIC) score [31]. The sequence lengths in the final dataset and the model of evolution are indicated in each figure legend. For the MLSA, each housekeeping gene sequence was aligned and trimmed to the same length and then manually concatenated. The NIs of single and concatenated sequences were calculated using Bioedit software (version 7.0.4.1) [32], and the values of the groups from our study and the most related type strains in the MLSA are shown in Table 2.

The 16S rRNA gene phylogeny (Fig. 1) split the *Bradyrhizobium* type strains into the two well-known superclades of *Bradyrhizobium japonicum* (GI) and *Bradyrhizobium elkanii* (GII) [2, 8–10, 33]. All strains from our study were clustered in the *B. elkanii* superclade, showing higher similarity with six type strains: *Bradyrhizobium retamae*, *Bradyrhizobium valentinum*, *Bradyrhizobium lablabi*, *Bradyrhizobium icense*, *Bradyrhizobium paxllaeri* and *Bradyrhizobium namibiense* (Fig. 1). The NI values among the strains from our study and related type strains ranged from 99.4 to 99.9% (Table 2). The results agree with several reports that the 16S rRNA sequences are highly conserved in the genus *Bradyrhizobium*, with many species sharing more than 99% NI, not allowing proper evolutionary information [7, 12, 13, 34].

Phylogenetic analyses of each single housekeeping gene were performed (Figs S1–S5), but to improve the information MLSA was performed with two sets of concatenated genes. The first set included four genes (*dnaK+glnII+gyrB+recA*) and all eight strains from our study (Fig. 2). The second set included five genes (*atpD+dnaK+glnII+gyrB+*recA), but without *B. murdochi* WSM 1790, whose *atpD* gene we were unable to amplify with the primers used (Fig. S6). The MLSA phylogenies were congruent in both analysis of concatenated genes, confirming the two superclades (GI and GII) and, as expected, defining more distinct groups when compared with the 16S rRNA gene phylogeny (Figs 2 and S6). In addition, similar NI values were found in both analyses of concatenated genes (Table 2); therefore, we will discuss the data considering the analysis with four genes. All eight strains from our study clustered into the *B. elkanii* superclade with consistent statistic support, but were clearly split into three different clusters (Fig. 2). Group GII.I included *B. archetypum* strains WSM 1744^T, WSM 1736 and WSM 1737 with 100% bootstrap support; these two last strains shared 100% NI with each other and 99.3% NI with WSM 1744^T. The most related species from this group is *B. namibiense* 5-10^T, sharing 94.1% NI, followed by *B. paxllaeri* LMTR 21^T, *B. jicamae* PAC68^T and *B. icense* LMTR 13^T, with NIs 93.2, 93.0 and 92.6%, respectively. Group GII.II clustered *B. australiense* strains WSM 1791^T and WSM 1742 with 100% bootstrap support and sharing 100% NI. *B. icense* LMTR 13^T, *B. murdochi* WSM 1741^T, *B. retamae* Ro19^T and *B. jicamae* PAC68^T are the most related species from this group, sharing 94.8, 94.7, 94.6 and 93.7% NI, respectively. Finally, group GII.III included *B. murdochi* strains WSM 1741^T, WSM 1735 and WSM 1790, also with 100% bootstrap support. The first two strains shared 100% NI with each other and 97.6% with WSM 1790. The most-related species of this group are *B. retamae* Ro19^T, *B. icense* LMTR 13^T, *B. australiense* WSM 1791^T and *B. lablabi* CCBAU 23086^T, with 97.3, 95.3, 94.7 and 93.7% NI, respectively (Fig. 2, Table 2).

Nowadays, the suggested threshold for species delineation for the genus *Bradyrhizobium* using five concatenated genes is 97% NI [22]. Several phylogenetic studies have suggested a minimum of four [6, 12], or even three [8] concatenated housekeeping genes in the MLSA, and a considerable number of *Bradyrhizobium* species have been described using four genes and an NI value of 97% as the threshold for species delineation [2, 13, 33, 35, 36]. In our study, both sets of genes on the

Table 2. Percentage of nucleotide identity among strains from this study and the most related type strains in the MLSA

	Nucleotide Identity (%)							
Strains	**16S rRNA gene**	***atpD***	***dnaK***	***glnII***	***gyrB***	***recA***	**MLSA (four genes)**	**MLSA (five genes)**
				***Bradyrhizobiumarchetypum* WSM 1744^T, WSM 1736 and WSM 1737**				
Among strains	100	99.4–100	99.5–100	99.8–100	99.2–100	98.6–100	99.3–100	99.3–100
Bradyrhizobium namibiense 5-10^T	99.5	94.9–95.4	96.3–96.8	93–93.2	93.4	94.4–94.9	94–94.1	94.2
Bradyrhizobium paxllaeri LMTR 21^T	99.5	93.1–93.6	95.4–95.8	90.8–91	93.2–93.6	93.8	92.9–93.2	93–93.3
Bradyrhizobium jicamae PAC68^T	99.1	94.9–95.4	93.2–93.6	90.8–91	94.2–94.5	93.3	92.8–93	93.2–93.5
Bradyrhizobium icense LMTR 13^T	99.5	94.1	95–95.4	90–90.2	0.94	92–92.2	92.5–92.6	92.8–92.9
Bradyrhizobium lablabi CCBAU 23086^T	0.994	93.6–94.1	95–95.4	89.6–89.8	92.9–93.2	93.6–93.6	92.4–92.5	92.7–92.8
Bradyrhizobium murdochi WSM 1741^T	99.7	93.4	93.2–93.6	90–90.2	93.6–93.8	93–93.3	92.4–92.5	92.6–92.7
Bradyrhizobium australiense WSM 1791^T	99.5	92.9–93.4	92.3–92.7	89.6–89.8	94	93–93.3	92.3	92.4–92.5
Bradyrhizobium retamae Ro19^T	99.9	92.4–92.9	91.4–91.8	90.4	93.2	93–93.3	92.1	92.2–92.3
				***Bradyrhizobium australiense* WSM 1791^T and WSM 1742**				
Among strains	100	100	100	100	100	100	100	100
Bradyrhizobium icense LMTR 13^T	99.8	93.9	92.3	97.8	93.4	94.4	94.8	94.6
Bradyrhizobium murdochi WSM 1741^T	99.8	93.4	95.4	96.6	93.1	94.1	94.7	94.4
Bradyrhizobium retamae Ro19^T	99.6	92.9	93.6	96.4	93.4	94.6	94.6	94.3
Bradyrhizobium jicamae PAC68^T	99.4	94.4	93.6	94.2	93.4	93.6	93.7	93.8
Bradyrhizobium lablabi CCBAU 23086^T	99.7	93.1	93.6	93.4	92.3	94.9	93.4	93.4
Bradyrhizobium paxllaeri LMTR 21^T	99.8	93.4	92.3	0.94	92.3	94.1	93.2	93.3
B. archetypum WSM 1744^T	99.5	92.9	92.3	89.8	94	93.3	92.3	92.4
Bradyrhizobium namibiense 5-10^T	99.8	94.4	91.8	89.8	92.5	0.93	91.7	92.2
				***Bradyrhizobium murdochi* WSM 1741^T, WSM 1735 and WSM 1790**				
Among strains	99.7–100	100	99.5–100	98.4–100	96.3–100	97.6–100	97.6–100	100
Bradyrhizobium retamae Ro19^T	99.6–99.9	96.2	97.7–98.1	97.6–98	96.1–96.9	96.8–97	97–97.3	97.1
Bradyrhizobium icense LMTR 13^T	99.5–99.8	96.4	94.1–94.5	96.4–96.8	93.4–94.9	94.9–95.7	95–95.3	95.6
Bradyrhizobium australiense WSM 1791^T	99.7–100	93.4	95.4–95.9	96.2–96.6	92.5–93.1	94.1–94.4	94.5–94.7	94.4

Continued

Table 2. Continued

	Nucleotide Identity (%)							
Bradyrhizobium lablabi CCBAU 23086^T	99.4–99.7	93.9	95.9–96.3	92.6–93	91.4–92.2	95.4–95.7	93.4–93.7	93.7
Bradyrhizobium jicamae PAC68^T	99.1–99.4	92.9	94.1–94.5	93.6–94	92–92.3	92.2	92.9–93	93
Bradyrhizobium paxllaeri LMTR 21^T	99.5–99.8	93.4	93.6–94.1	93.2–93.8	91.3–91.8	92.2–93	92.6–92.7	92.9
Bradyrhizobium archetypum WSM 1744^T	99.5–99.8	93.4	93.2–93.6	89.2–90.2	92.7–93.8	93.3–93.6	92–92.5	92.7
Bradyrhizobium namibiense 5-10^T	99.5–99.8	93.9	92.7–93.2	89.8	90.3–92.2	93.8–94.4	91.5–91.9	92.3

MLSA presented NI for the new groups compared with the most related species far below 97%; the only exception was of group GII.III, which shared from 97 to 97.3% NI with *B. retamae* Ro19^T. This value is slightly above the 97% suggested cut-off for species delineation [22], but as the NI is not a phylogenetic but a mathematic parameter, we decided to keep the distinction of this group from *B. retamae* species; moreover, the following analyses corroborated this decision.

Another important parameter related to symbiotic bacteria is the phylogeny of symbiotic genes. The term symbiovar (sv.) refers to a group of strains capable of associating with a specific legume [37]. As the symbiotic gene *nodC* encodes an enzyme (*N*-acetylglucosaminyl transferase) involved in the synthesis of Nod factors, molecules responsible for the legume-rhizobia association, this gene has become a molecular marker required for the description of rhizobial symbiovars [10, 37, 38].

The *nodC* phylogeny (Fig. 3) clustered all strains from this study within the sv. retamae, one out of the 11 symbiovars described so far for the genus *Bradyrhizobium* [10, 39–44]. When described, the sv. retamae contained mainly nodulating strains isolated from *Retama* species in different continents [42], and includes today seven type strains [12]. A recent phylogenetic study of *Bradyrhizobium* symbiotic genes proposed that the similarity of *nodC* genes from the same symbiovar might be of 92.5% [10], and in our study the identity with sv. retamae ranged from 94.2–98.1% NI. The phylogram also shows that the two most-related symbiovars to our strains were sv. lupini sharing from 86.8–88.7% NI and sv. sierranevadense, with 85.0–86.3% NI. The other two isolated strains related from sv. retamae are *B. jicamae* PAC68^T and *B. erythrophlei* CCBAU 53325^T, sharing from 84.2–85.0% and 83.7–84.7% NI respectively. Considering those values, our results support the suggested threshold of 92.5% NI for *nodC* sequences belonging to same symbiovar [10], and confirm the position of all three groups from our study as belonging to sv. retamae.

GENOME FEATURES

Genome analyses provide reliable and high informative means to study phylogenetic relationships among prokaryotes. Several genomic analyses and bioinformatics tools are available today, and the values obtained are known as overall genome related indices (OGRIs) [45].

The genome of *B. murdochi* WSM 1741^T has been sequenced before by the DOE Joint Genome Institute and was retrieved from the GenBank database (accession no. AXAU00000000). Total DNAs of strains WSM 1744^T and WSM 1791^T were used to build the library with the Nextera XT kit, according to the manufacturer's procedure. The library was processed on the MiSeq platform (Illumina) at Embrapa Soja, and the drafts of the whole genomes were assembled with the A5-miseq pipeline (*de novo*) version 20140604. The genomes were deposited in the GenBank database and received the accession numbers of *B. archetypum* WSM 1744^T (accession no. JAAVLW000000000) and *B. australiense* WSM 1791^T (accession no. JAAVLX000000000). The genome assembly of strain WSM 1744^T allowed a coverage of 139-fold assembled in 35 contigs, with an *N50* of 804703; for strain WSM 1791^T, a coverage of 82-fold was obtained and assembled in 46 contigs, with an *N50* of 720641. The genomes were estimated at 7017218 bp and 7436256 bp for WSM 1744^T and WSM 1791^T, respectively, and RAST annotation [46] identified 7256 coding DNA sequences (CDSs) for WSM 1744^T, and 7,949 CDSs for WSM 1791^T.

The genome sequences of strains WSM 1741^T, WSM 1744^T and WSM 1791^T were used to calculate the ANI and dDDH values in comparison to the five closest-related type strains according to the MLSA of *B. jicamae* PAC68^T (LLXZ01000000), *B. paxllaeri* LMTR 21^T (MAXB00000000), *B. lablabi* CCBAU 23086^T (LLYB01000000), *B. icense* LMRT13^T (CP016428) and *B. retamae* Ro19^T (LLYA01000000). The only type strain close from the group which was not included in the analyses was *B. namibiense* 5-10^T, as there is no genome available for this strain. ANI values were calculated using the ANI calculator [47] and the dDDH values were calculated using the Genome-to-Genome Distance Calculator version 2.1 [23] and the recommended 'formula 2' (identities/HSP length), considering the values of 95~96% and 70% as threshold for species delineation, respectively, as proposed by Chun *et al.* [18]. The three groups from our study shared less than 93.3%

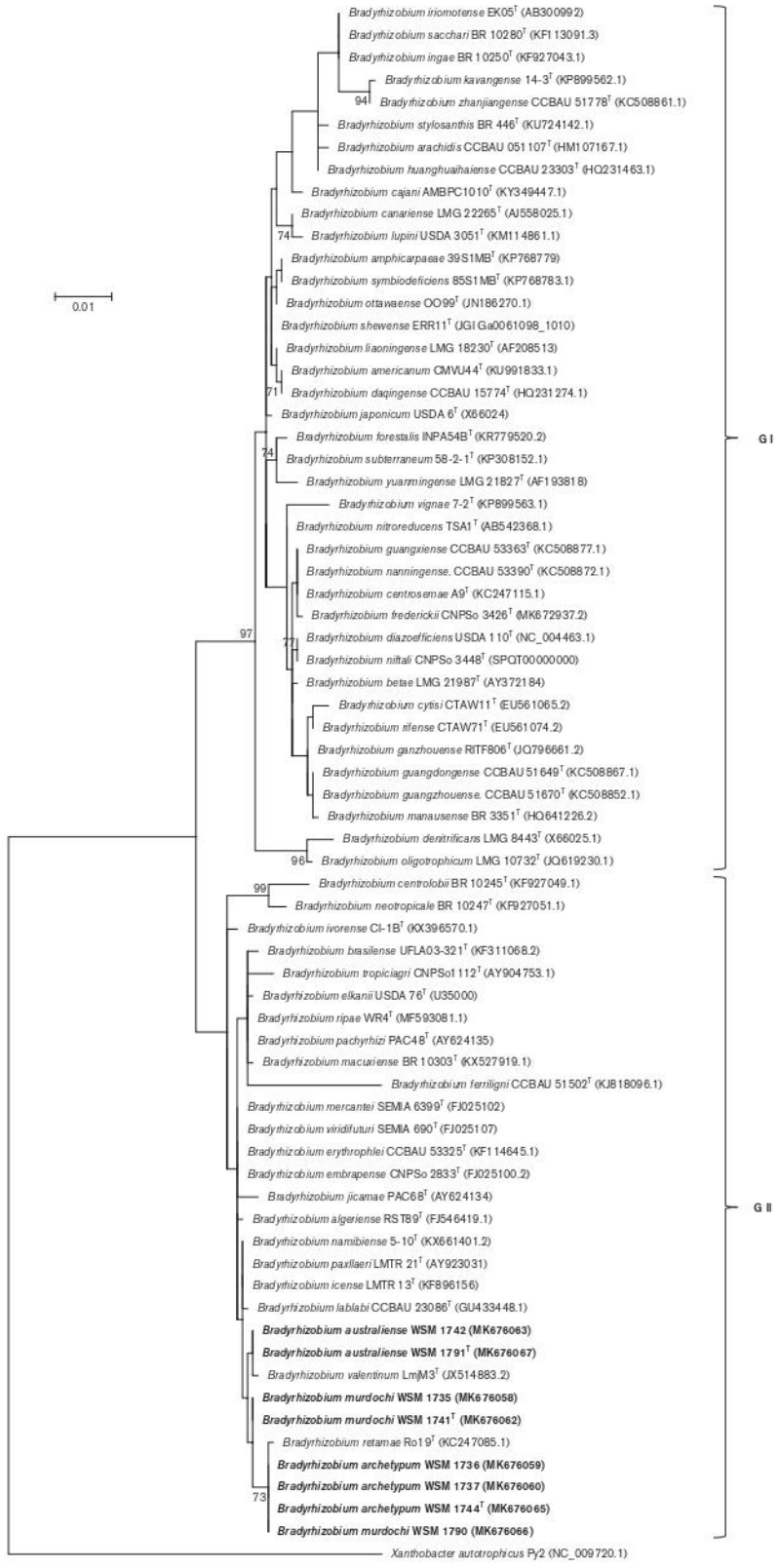

Fig. 1. Maximum-likelihood (ML) phylogeny based on the 16S rRNA gene sequences (1167 bp), using the Tamura three-parameter+G+I model. Accession numbers are indicated within parenthesis, and strains from this study are shown in bold. Bootstrap values >70% are indicated at the nodes. *Xanthobacter autotrophicus* Py2 was used as an outgroup. Bar, one substitution per 100 nucleotide positions.

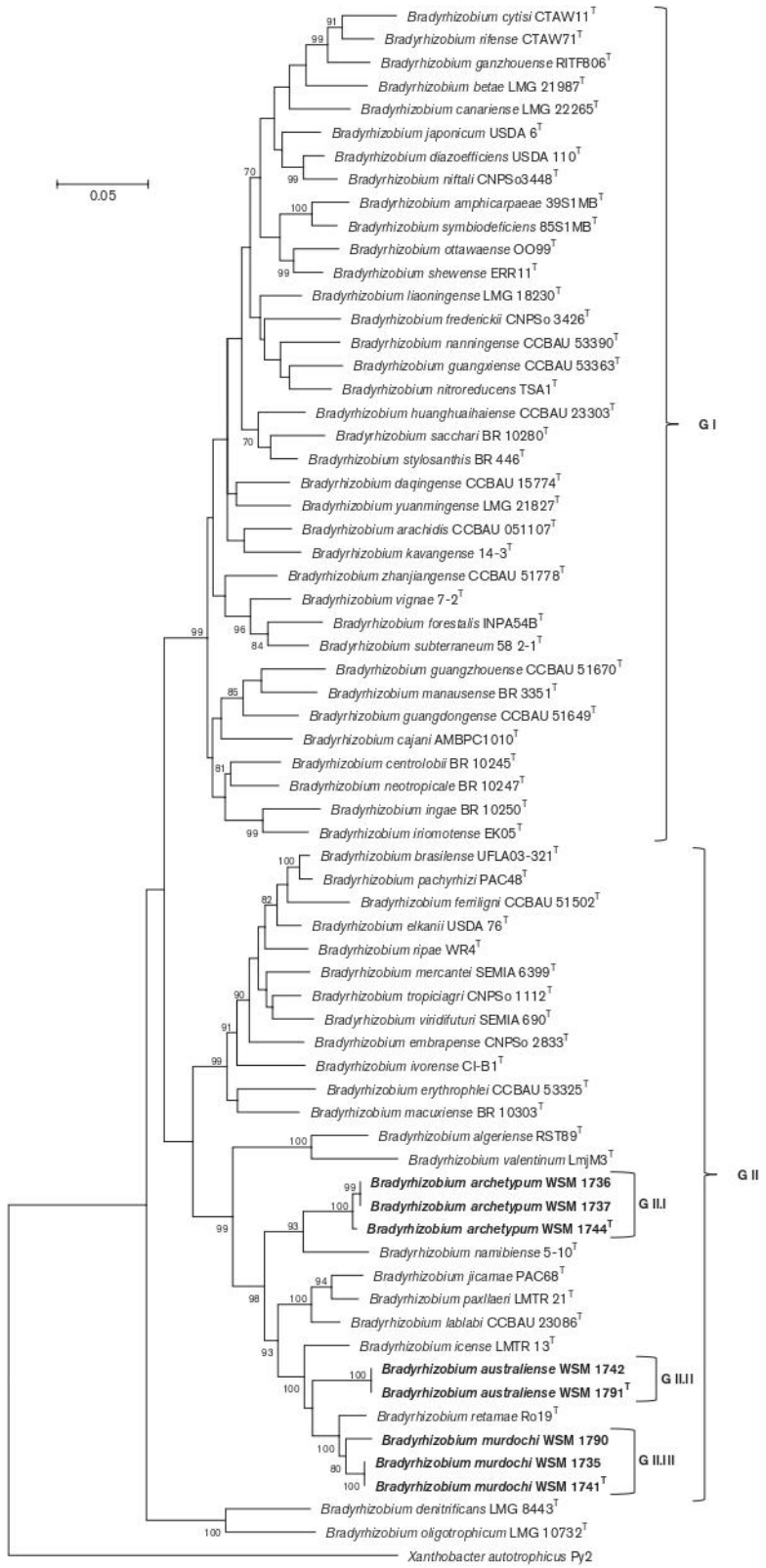

Fig. 2. Maximum-likelihood phylogeny based on the *dnaK+glnII+gyrB+recA* concatenated gene sequences (1649 bp), using General Time Reversible+G+I model. Strains from this study are shown in bold. Bootstrap values >70% are indicated at the nodes. *Xanthobacter autotrophicus* Py2 was used as an outgroup. Bar, five substitutions per 100 nucleotide positions.

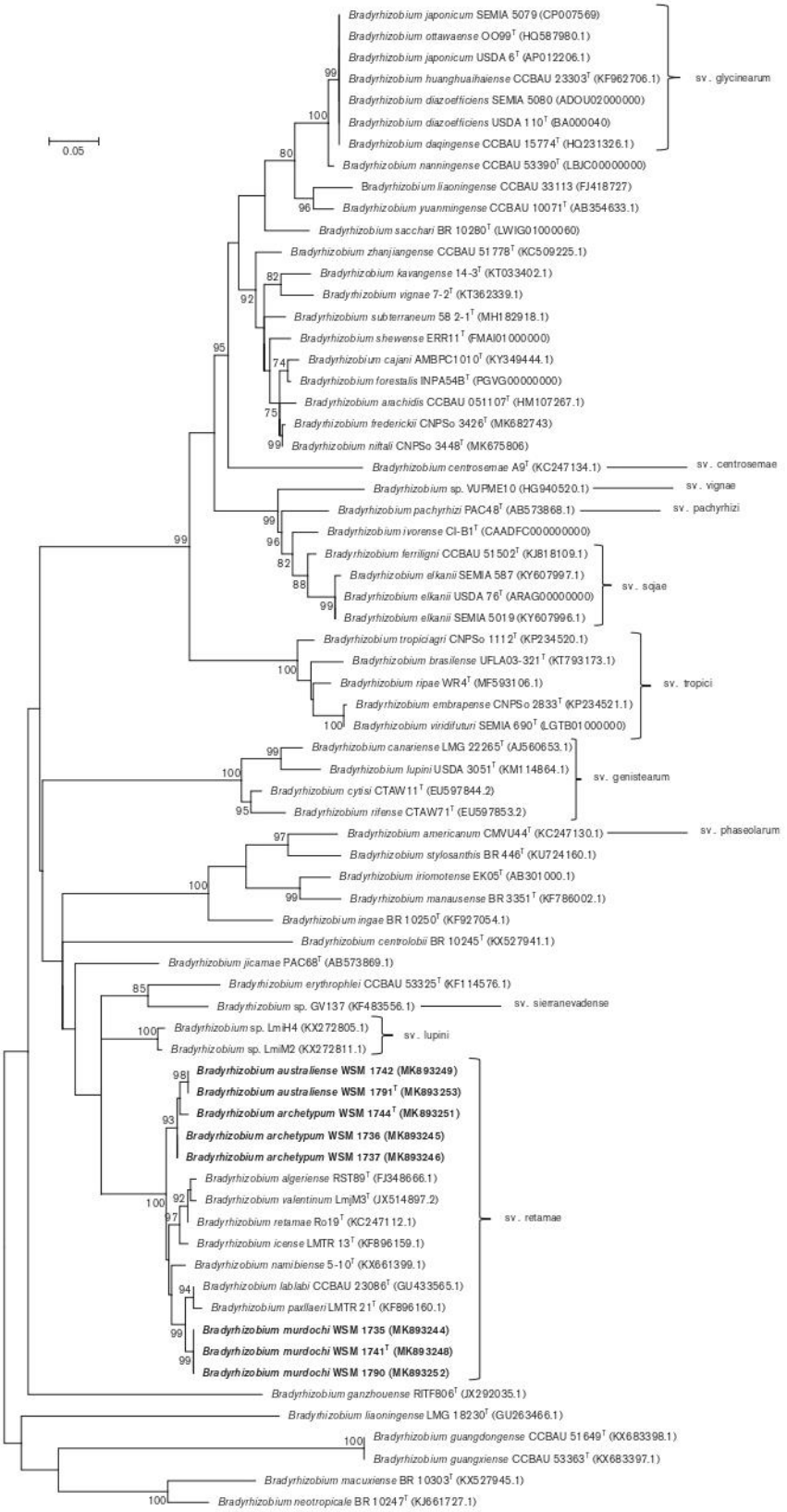

Fig. 3. Maximum-likelihood phylogeny based on *nodC* gene sequences (380 bp), using Tamura's three-parameter+G+I model. Strains from this study are shown in bold. Bootstrap values >70% are indicated at the nodes. Bar, five substitutions per 100 nucleotide positions.

Table 3. Overall genome related indices between the type strains from this study and closely related species

	Bradyrhizobium archetypum WSM 1744^T		*Bradyrhizobium australiense* WSM 1791^T		*Bradyrhizobium murdochi* WSM 1741^T	
Strain	**ANI (%)**	**dDDH (%)**	**ANI (%)**	**dDDH (%)**	**ANI (%)**	**dDDH (%)**
Bradyrhizobium icense LMTR^T	87.6	34.3	89.8	39.9	90.9	43.2
Bradyrhizobium jicamae PAC68^T	86.6	32.4	86.7	32.8	86.9	33.2
Bradyrhizobium lablabi CCBAU 23086^T	87.1	33.4	87.3	33.8	87.5	34.2
Bradyrhizobium paxllaeri LMTR 21^T	92.4	48.4	92.3	47.9	92.4	47.3
Bradyrhizobium retamae Ro19^T	87.4	33.8	89.7	39.6	93.3	52.0
Bradyrhizobium archetypum WSM 1744^T	–	–	88.0	34.9	87.9	35.0
Bradyrhizobium australiense WSM 1791^T	88.0	34.9	–	–	90.0	40.2
Bradyrhizobium murdochi WSM 1741^T	87.9	35.0	90.0	40.2	–	–

ANI and 52% dDDH values with most related type strains (Table 3), values far below the threshold suggested for species discrimination. The OGRI values were in agreement in both analyses, corroborating the description of three new groups.

The genome G+C content was calculated using SEED [48]. The G+C content of strain WSM 1744^T was estimated at 62.3 mol%, strain WSM 1791^T at 61.9 mol%, and strain WSM 1741^T at 62.1 mol%. Those values are within the range of 61–63%, which are usual for genus *Bradyrhizobium* [49].

To evaluate the genetic profile of the strains from this study BOX-PCR fingerprinting was performed using the primer BOX-A1R and the cycles described before [50]. *B. icense* LMTR 13^T and *B. retamae* Ro19^T were also included in the analysis for being phylogenetically closely related to the groups. The dendrogram was generated with Bionumerics software (version 7.6, Applied Mathematics) using the unweighted pair group method with arithmetic mean algorithm (UPGMA) [51], and the Jaccard coefficient [52], with 2% tolerance. Using the parameters cited above, the BOX dendrogram (Fig. S7) revealed high similarity (above 80%, but below 88%, indicating that they are not clones) between strains WSM 1736 and WSM 1737; between strains WSM 1741^T and WSM 1735; and between strains WSM 1791^T and WSM 1742, and unique profiles for the remaining strains, including WSM 1744^T. In addition, the dendrogram also revealed genomic distinction among the strains from our study and the two type strains of *B. icense* and *B. retamae*.

PHYSIOLOGY

Phenotypic analyses including morphologic and physiologic characterization were performed with the strains from our study and related type strains. Phenotypic tests were performed at 28 °C (unless indicated), and included colony morphology, ability to grow in modified YMA medium [25] with 1% NaCl, acid/alkaline reaction on modified YMA medium with bromothymol blue, ability to grow in modified YMA medium with pH 4.0 and pH 8.0, and in solid Luria–Bertani (LB) medium. Enzymatic degradation of urea was determined in modified YMA medium containing 2% urea and phenol red as indicator. The ability of strains to grow at 37 °C was also tested and all analyses were conducted as previously described [53], in triplicate, and evaluated over the course of 10 days of incubation.

The API 50CH kit (BioMérieux) was used following the manufacturer's recommendations to evaluate the carbon (C) source utilization capacity of the strains. Modified YM without mannitol was used as basal medium and bromothymol blue as indicator of acid or alkaline reaction when the use of each C source was evaluated. It is worth mentioning that we have often verified that API evaluation has limitations, e.g. one strain can show weak growth in the API with a C source, but grow well in a culture medium containing the same C source. Antibiotic tolerance was evaluated using the disc-diffusion method on modified YMA plates with the ten antibiotics (concentration per disc): ampicillin (10 µg), bacitracin (10 U), cefuroxime (30 µg), chloramphenicol (30 µg), nalidixic acid (30 µg), neomycin (30 µg), penicillin (10 U), streptomycin (10 µg), tetracycline (30 µg) and erythromycin (15 µg). All tests were performed in duplicate. The eight strains from our study could tolerate ampicillin, bacitracin, chloramphenicol, nalidixic acid, penicillin and erythromycin. Different results among the strains from this study and type strains are shown in Table 4, and the main properties are included in each species description.

All strains confirmed the ability of nodulating and fixing nitrogen with the original host legume, specified in Table 1. The nodulation ability and efficiency of nitrogen fixation (pink or red internal color of the nodules) were also evaluated in soybean (*Glycine max*) and in the promiscuous papilionoid siratro (*Macroptilium atropurpureum*). Evaluation was performed in modified Leonard jars containing sand and vermiculite and receiving sterile N-free nutrient solution of Broughton and Dilworth [54]. The eight strains from our study were able to produce effective nitrogen-fixing nodules on *Macroptilium atropurpureum*, but none of the strains nodulated *Glycine max*.

Table 4. Distinctive morphophysiological features of the type strains from this study and the most related type strains

Strains: 1, *Bradyrhizobium archetypum* WSM 1744^T; 2, *Bradyrhizobium australiense* WSM 1791^T; 3, *Bradyrhizobium murdochi* WSM 1741^T; 4, *Bradyrhizobium icense* LMTR 13^T; 5, *Bradyrhizobium retamae* Ro19^T. +, Positive growth; –, no growth; w, weak growth.

Characteristic	1	2	3	4	5*
Carbon source assimilation:					
Erythritol	w	w	–	–	–
D-Arabinose	+	w	+	+	+
L-Arabinose	w	w	+	+	+
D-Ribose	w	w	+	+	+
D-Xylose	w	w	w	+	w
L-Xylose	+	w	+	+	+
D-Adonitol	+	w	–	w	–
Methyl β-D-xylopiranoside	+	–	–	+	–
D-Galactose	w	w	+	+	w
D-Glucose	w	w	w	+	w
D-Fructose	w	w	w	+	w
D-Mannose	w	w	w	+	–
L-Sorbose	+	w	w	w	–
Dulcitol	w	w	–	–	–
Inositol	w	w	–	–	–
D-Mannitol	w	w	w	–	–
D-Sorbitol	w	w	w	–	–
Methyl α-D-mannopyranoside	w	w	–	–	–
Methyl α-D-glucopyranoside	w	w	–	–	–
N-Acetylglucosamine	w	w	–	–	–
Amygdalin	w	w	–	–	–
Arbutin	w	w	–	–	–
Aesculin ferric citrate	+	+	+	+	w
Salicin	w	w	–	–	–
Cellobiose	w	w	–	–	–
Maltose	w	w	–	–	–
Lactose	w	w	w	–	–
Melibiose	+	w	w	+	–
Sucrose	w	w	–	–	–
Trehalose	w	w	–	–	–
Insulin	w	w	–	–	–
Melezitose	w	w	–	–	–
Raffinose	w	w	–	–	–
Glycogen	+	w	+	+	–
Xylitol	+	w	–	–	–

Continued

Table 4. Continued

Characteristic	1	2	3	4	5*
Gentiobiose	+	w	w	–	–
Turanose	w	w	–	–	–
D-Tagatose	w	w	–	–	–
D-Fucose	w	w	w	+	w
L-Fucose	+	w	+	+	+
D-Arabitol	+	w	–	–	–
L-Arabitol	+	w	w	–	–
Potassium gluconate	+	+	+	+	–
Potassium 2-ketogluconate	+	+	+	w	–
Potassium 5-ketogluconate	+	+	+	–	–
Enzymatic activity:					
Urease	+	w	+	w	–
Grow at/in:					
37 °C	+ (10 days)	w (10 days)	w (7 days)	–	–
pH 4	w	w	–	–	–
pH 8	+	w	+	+	w
Resistance to (μg):					
Ampicillin (10)	+	+	+	–	+
Neomycin (30)	+	+	+	+	w
Penicillin G (10 U)	+	+	+	–	+
Tetracycline (30)	+	–	+	+	–
Cefuroxima (30)	+	+	–	–	–

In conclusion, this extensive polyphasic study encompassing phenotypic, genotypic, phylogenetic and genomic analyses indicated that strains isolated from nodules of indigenous legumes to WA comprise three novel species of the genus *Bradyrhizobium*, for which we propose the names *Bradyrhizobium archetypum* sp. nov., *Bradyrhizobium australiense* sp. nov. and *Bradyrhizobium murdochi* sp. nov., represented by type strains WSM 1744^T, WSM 1791^T and WSM 1741^T, respectively.

DESCRIPTION OF *BRADYRHIZOBIUM ARCHETYPUM* SP. NOV.

Bradyrhizobium archetypum (ar.che.ty'pum. Gr. masc. adj. *archetypos* first-molded; N.L. neut. adj. *archetypum*, indicating a first origin, referring to the aboriginal history of Australia).

Cells are Gram-stain-negative, aerobic and non-spore-forming. Colonies on modified YMA medium with Congo red are circular, translucent, with low production of mucus, and less than 1 mm in diameter with light pink colour after 7 days of growth at 28 °C. They alkalinize modified YMA medium with bromothymol blue after 7 days, and optimum growth occurs at pH 6.8 and 28 °C. Strains do not grow in LB medium or in the presence of 1% NaCl, but with 10 days of incubation strain WSM 1744^T is able to grow at 37 °C and at pH 8.0, with weak growth at pH 4.0. Strains are positive for urease activity and are tolerant to the antibiotics (per disc) ampicillin (10 μg), chloramphenicol (30 μg), erythromycin (15 μg), nalidixic acid (30 μg), neomycin (30 μg), penicillin (10 U), tetracycline (30 μg), bacitracin (10 U) and cefuroxime (30 μg), and sensitive to streptomycin (10 μg). With respect to carbon sources in the API test, strain WSM 1744^T is able to use: D-arabinose, L-xylose, D-adonitol, methyl β-D-xylopyranoside, L-sorbose, aesculin ferric citrate, melibiose, starch, glycogen, xylitol, gentiobiose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium 2-ketogluconate and potassium 5-ketogluconate; able to grow weakly in glycerol, erythritol, L-arabinose, D-ribose, D-xylose, 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, amygdalin, arbutin, salicin, cellobiose, maltose, lactose,

sucrose, trehalose, inulin, melezitose, raffinose, turanose, D-lyxose, D-tagatose and D-fucose. Strains of *B. archetypum* form effective nitrogen-fixing nodules on their original hosts and on *Macroptilium atropurpureum*, but do not nodulate *Glycine max*.

The type strain is WSM 1744^T (=CNPSo 4013^T=LMG 31646^T), isolated from nodules of *Muelleranthus trifoliolatus* in Australia. The DNA G+C content of strain WSM 1744^T is 62.3 mol%. Type strain accession numbers: 16S (MK676065), *atpD* (MT070745), *dnaK* (MK674820), *glnII* (MK860847) and *gyrB* (MK860868), *recA* (MK863448).

DESCRIPTION OF *BRADYRHIZOBIUM AUSTRALIENSE* SP. NOV.

Bradyrhizobium australiense (aus.tra.li.en'se. N.L. neut. adj. *australiense* Australian, referring to Australia, the country where the strains were isolated).

Cells are Gram-stain-negative, aerobic and non-spore-forming. Colonies on modified YMA medium with Congo red are circular, translucent, with low production of mucus, and less than 1 mm in diameter with light pink colour after 7 days of incubation at 28 °C. Strains alkalinize modified YMA medium [25] with bromothymol blue after 7 days, and optimum growth occurs at pH 6.8 and 28 °C. Strains do not grow in LB medium and in presence of 1% NaCl, and grow weakly at 37 °C, at pH 4.0 and pH 8.0. Strains are positive for urease activity, and are tolerant to the antibiotics (per disc) ampicillin (10 µg), chloramphenicol (30 µg), erythromycin (15 µg), nalidixic acid (30 µg), neomycin (30 µg), penicillin (10 U) and bacitracin (10 U), cefuroxime (30 µg), and sensitive to tetracycline (30 µg) and streptomycin (10 µg). With respect to carbon sources in the API test, strain WSM 1791^T is able to use: aesculin ferric citrate, starch, D-lyxose, potassium gluconate, potassium 2-ketogluconate and potassium 5-ketogluconate; able to weakly grow in glycerol, erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, D-galactose, D-glucose, D-fructose, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, *N*-acetylglucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose, glycogen, xylitol, gentiobiose, turanose, D-tagatose, D-fucose, L-fucose, D-arabitol and L-arabitol; and unable to grow in methyl β-D-xylopyranoside. Strains of *B. australiense* form effective nitrogen-fixing nodules on *Indigofera* sp. and on *Macroptilium atropurpureum*, but do not nodulate *Glycine max*.

The type strain is WSM 1791^T (=CNPSo 4014^T=LMG 31647^T), isolated from nodules of *Indigofera* species in Australia. The DNA G+C content of strain WSM 1791^T is 61.9 mol%. Type strain accession numbers: 16S (MK676067), *atpD* (MT070746), *dnaK* (MK674822), *glnII* (MK860849), *gyrB* (MK860870) and *recA* (MK863450).

DESCRIPTION OF *BRADYRHIZOBIUM MURDOCHI* SP. NOV.

Bradyrhizobium murdochi (mur'do.chi. N.L. gen. n. *murdochi* of Murdoch University, referring to Murdoch University, which has a long tradition of studies of rhizobia).

Cells are Gram-stain-negative, aerobic and non-spore-forming. Colonies on modified YMA medium with Congo red are circular, translucent, with low production of mucus, and less than 1 mm in diameter with light pink colour after 7 days of incubation at 28 °C. Strains alkalinize modified YMA medium with bromothymol blue after 7 days, and optimum growth occurs at pH 6.8 and 28 °C. Strains do not grow in LB medium, in the presence of 1% NaCl, or in pH 4.0, and grow weakly at 37 °C. Strains are able to grow at pH 8.0, are positive for urease activity, are tolerant to the antibiotics (per disc) ampicillin (10 µg), chloramphenicol (30 µg), erythromycin (15 µg), nalidixic acid (30 µg), neomycin (30 µg), penicillin (10 U), tetracycline (30 µg) and bacitracin (10 U), and sensitive to streptomycin (10 µg) and cefuroxime (30 µg). With respect to carbon sources in the API test, strain WSM 1741^T is able to use D-arabinose, L-arabinose, D-ribose, L-xylose, D-galactose, aesculin ferric citrate, starch, glycogen, D-lyxose, L-fucose, potassium gluconate, potassium 2-ketogluconate and potassium 5-ketogluconate; able to weakly grow in glycerol, D-glucose, D-fructose, D-mannose, L-sorbose, L-rhamnose, D-mannitol, D-sorbitol, lactose, melibiose, gentiobiose, D-fucose and L-arabitol; and unable to grow in erythritol, D-xylose, D-adonitol, methyl β-D-xylopyranoside, dulcitol, inositol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, *N*-acetylglucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, sucrose, trehalose, inulin, melezitose, raffinose, xylitol, turanose, D-tagatose and D-arabitol. Strains of *B. murdochi* form effective nitrogen-fixing nodules on their original hosts and on *Macroptilium atropurpureum*, but do not nodulate *Glycine max*.

The type strain is WSM 1741^T (=CNPSo 4020^T=LMG 31651^T), isolated from nodules of *Rhynchosia minima* in Australia. The DNA G+C content of strain WSM 1741^T is 62.1 mol%. Type strain accession numbers: 16S (MK676062), *atpD* (MT070743), *dnaK* (MK674817), *glnII* (MK860844), *gyrB* (MK860865) and *recA* (MK863445).

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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SUPPLEMENTARY MATERIAL

***Bradyrhizobium archetypum* sp. nov., *Bradyrhizobium australiense* sp. nov., and *Bradyrhizobium murdochi* sp. nov. isolated from nodules of legumes indigenous to Western Australia**

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Table S1. Sequences and genomes GenBank accession numbers. Strains from this study are shown in bold.

Strain	Genome	16S	*atpD*	*dnaK*	*glnII*	*gyrB*	*recA*
***B. archetypum* WSM 1744^T**	JAAVLW000000000	**MK676065**	**MT070745**	**MK674820**	**MK860847**	**MK860868**	**MK863448**
***B. archetypum* WSM 1736**		**MK676059**	**MT070741**	**MK674814**	**MK860841**	**MK860862**	**MK863442**
***B. archetypum* WSM 1737**		**MK676060**	**MT070742**	**MK674815**	**MK860842**	**MK860863**	**MK863443**
***B. australiense* WSM 1791^T**	JAAVLX000000000	**MK676067**	**MT070746**	**MK674822**	**MK860849**	**MK860870**	**MK863450**
***B. australiense* WSM 1742**		**MK676063**	**MT070744**	**MK674818**	**MK860845**	**MK860866**	**MK863446**
***B. murdochi* WSM 1741^T**	AXAU00000000	**MK676062**	**MT070743**	**MK674817**	**MK860844**	**MK860865**	**MK863445**
***B. murdochi* WSM 1790**		**MK676066**	NA	**MK674821**	**MK860848**	**MK860869**	**MK863449**
***B. murdochi* WSM 1735**		**MK676058**	**MT070740**	**MK674813**	**MK860840**	**MK860861**	**MK863441**
B. algeriense RST89^T	PYCM00000000	FJ546419.1	KF956544.1	FJ546419.1	FJ264924.1		FJ264927.1
B. americanum CMVU44^T		KU991833.1	KC247125.1	NA	KX012942.1	NA	KC247141.1
B. amphicarpaeae 39S1MB^T		KP768779	KP768547.1	CP029426	KP768605.1	KP768721.1	KF615002.1
B. arachidis CCBAU 051107^T		HM107167.1	HM107217.1	KJ560556	HM107251	JX437675	HM107233
B. betae LMG 21987^T		AY372184	FM253129.1	FM253303	AB353733	AB353735	AB353734
B. brasilense UFLA03-321^T	MPVQ00000000	KF311068.2	KF452730.1	KF452791.1		KF452827.1	KT793142.1
B. cajani AMBPC1010^T	WQNE01000000	KY349447.1		NA	KY349442.1	NA	KY349440.1
B. canariense LMG 22265^T		AJ558025.1	FM253135.1	AY923047	AY386765	FM253220	FM253177
B. centrolobii BR 10245^T	LUUB01000000	KF927049.1		KX527928.1	KX527991.1	KX528004.1	KX527954.1
B. centrosemae A9^T		KC247115.1	KC247129.1	NA	KX012940.1	NA	KC247145.1
B. cytisi CTAW11^T		EU561065.2	GU001613.1	KF532219	GU001594	KF532653	KF532947
B. daqingense CCBAU 15774^T		HQ231274.1	HQ231289.1	KF962684	HQ231301	JX437669	HQ231270
B. denitrificans LMG 8443^T		X66025.1	FM253153.1	KF962685	HM047121	FM253239	EU665419

B. diazoefficiens USDA 110^T	NC_004463.1						
B. elkanii USDA 76^T	ARAG00000000	U35000	AY386758.1		AY599117	AM418800	AY591568
B. embrapense CNPSo 2833^T		FJ025100.2	HQ634875.1	KP234519.2	GQ160500	HQ634891	HQ634899
B. erythrophlei CCBAU 53325^T		KF114645.1		KX690620	KF114693.1	KF114717.1	KF114669.1
B. ferriligni CCBAU 51502^T		KJ818096.1		KX690619	KJ818099.1	KJ818102.1	KJ818112.1
B. forestalis INPA54B^T	PGVG00000000	KR779520.2	KF452722.1	KF452796.1		KF452831.1	KF452867.1
B. frederickii CNPSo 3426T	SPQS00000000	MK672937.2			MK682688	MK682721	MK682710
B. ganzhouense RITF806^T		JQ796661.2	JX277182.1	KP420023	JX277110	KP420022	JX277144
B. guangdongense CCBAU 51649^T		KC508867.1	KC508916.1	KC508964.1	KC509023.1	KC509072.1	KC509269.1
B. guangxiense CCBAU 53363^T		KC508877.1	KC508926.1	KC508974.1	KC509033.1	KC509082.1	KC509279.1
B. guangzhouense CCBAU 51670^T		KC508852.1	KC508902.1	KC508950.1	KC509008.1	KC509057.1	KC509254.1
B. huanghuaihaiense CCBAU 23303^T		HQ231463.1	HQ231682.1	KF962686	HQ231639	JX437672	HQ231595
B. icense LMTR 13^T		KF896156	KF896192	KF896182	KF896175	KF896201	JX943615
B. ingae BR 10250^T		KF927043.1	KY753593.1	KF927055	KF927067	KF927079	KF927061
B. iriomotense EK05^T		AB300992	AB300994.1	JF308944	AB300995	AB300997	AB300996
B. ivorense CI-B1T	CAADFC000000000	KX396570.1		MK376326	MH756157	MH756161	MK376330
B. japonicum USDA 6^T		X66024	AP012206.1	AM182120	HQ587875	AB070586	AM182158
B. jicamae PAC68^T		AY624134	FJ428211.1	JF308945	FJ428204	HQ873309	HM047133
B. kavangense 14-3^T		KP899562.1	KY753592.1	KR259949.1	KM378446.1	KX661397.1	KM378399.1
B. lablabi CCBAU 23086^T		GU433448.1	GU433473.1	KF962687	GU433498	JX437670	GU433522
B. liaoningense LMG 18230^T		AF208513	AY386752.1	AY923041	AY386775	FM253223	FM253180
B. lupini USDA 3051^T		KM114861.1	KU738808.1		KM114862.1	KM114862.1	KM114866.1
B. macuxiense BR 10303^T	LNCU01000000	KX527919.1		KX527932.1	KX527995.1	KX528008.1	KX527958.1

B. manausense BR 3351^T	LJYG01000000	HQ641226.2		KF786001	KF785986	KF786000	KF785992
B. mercantei SEMIA 6399^T	MKFI01000000	FJ025102		KX690617	KX690621	KX690623	KX690615
B. namibiense 5-10^T		KX661401.2	KX661387.1	KP402058.1	KM378440.1	KX661393.1	KM378377.1
B. nanningense CCBAU 53390^T		KC508872.1	KC508921.1	KC508969.1	KC509028.1	KC509077.1	KC509274.1
B. neotropicale BR 10247^T	LSEF01000000	KF927051.1		KJ661693	KJ661700	KJ661707	KJ661714
B. niftali CNPSo 3448T	SPQT00000000				MK675791	MK675794	MK675797
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Xanthobacter autotrophycus Py2	NC_009720						

*Sequence retrieved from Joint Genome Portal (JGI).

Legends of Supplementary Figures

Figure S1. Maximum likelihood phylogeny based on *atpD* gene sequences (395 bp), using General Time Reversible + G model. Accession numbers are indicated within parenthesis. Strains from this study are shown in bold. Bootstrap values > 70 % are indicated at the nodes. *Xanthobacter autotrophicus* Py2 was used as outgroup. Bar indicates two substitutions per 100 nucleotide positions.

Figure S2. Maximum likelihood phylogeny based on *dnaK* gene sequences (220 bp), using Tamura-Nei + G + I model. Accession numbers are indicated within parenthesis. Strains from this study are shown in bold. Bootstrap values > 70 % are indicated at the nodes. *Xanthobacter autotrophicus* Py2 was used as outgroup. Bar indicates five substitutions per 100 nucleotide positions.

Figure S3. Maximum likelihood phylogeny based on *glnII* gene sequences (504 bp), using General Time Reversible + G model. Accession numbers are indicated within parenthesis. Strains from this study are shown in bold. Bootstrap values > 70 % are indicated at the nodes. *Xanthobacter autotrophicus* Py2 was used as outgroup. Bar indicates two substitutions per 100 nucleotide positions.

Figure S4. Maximum likelihood phylogeny based on *gyrB* gene sequences (550 bp), using General Time Reversible + G + I model. Accession numbers are indicated within parenthesis. Strains from this study are shown in bold. Bootstrap values > 70 % are indicated at the nodes. *Xanthobacter autotrophicus* Py2 was used as outgroup. Bar indicates five substitutions per 100 nucleotide positions.

Figure S5. Maximum likelihood phylogeny based on *recA* gene sequences (375 bp), using Tamura-Nei + G + I model. Accession numbers are indicated within parenthesis. Strains from this study are shown in bold. Bootstrap values > 70 % are indicated at the nodes. *Xanthobacter autotrophicus* Py2 was used as outgroup. Bar indicates five substitutions per 100 nucleotide positions.

Figure S6. Maximum likelihood phylogeny based on the *atpD+dnaK+glnII+gyrB+recA* concatenated gene sequences (2,044 bp), using General Time Reversible + G + I model. Strains from this study are shown in bold. Bootstrap values > 70 % are indicated at the nodes. *Xanthobacter autotrophicus* Py2 was used as outgroup. Bar indicates five substitutions per 100 nucleotide positions.

Figure S7. Cluster analysis of products obtained by BOX-A1R-PCR of strains from this study (shown in bold) and closely related *Bradyrhizobium* species, analyzed with 2 % of tolerance. Analysis performed with the program Bionumerics (Applied Mathematics, Kortrijk, Belgium, v.7.6) using the UPGMA algorithm (Unweighted Pair-Group Method with Arithmetic mean) and the Jaccard coefficient.

Fig. S1

atpD

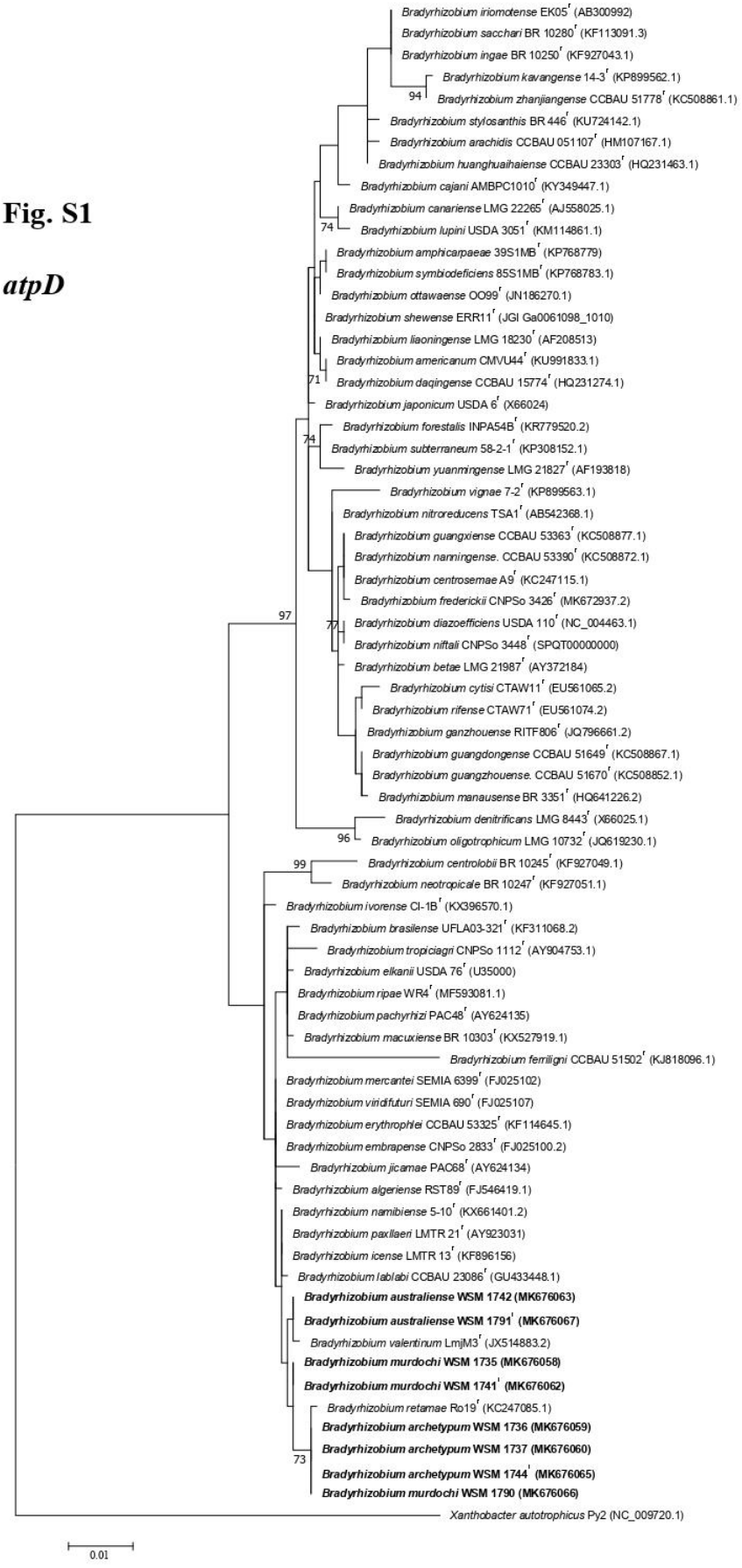
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Fig. S2

dnaK

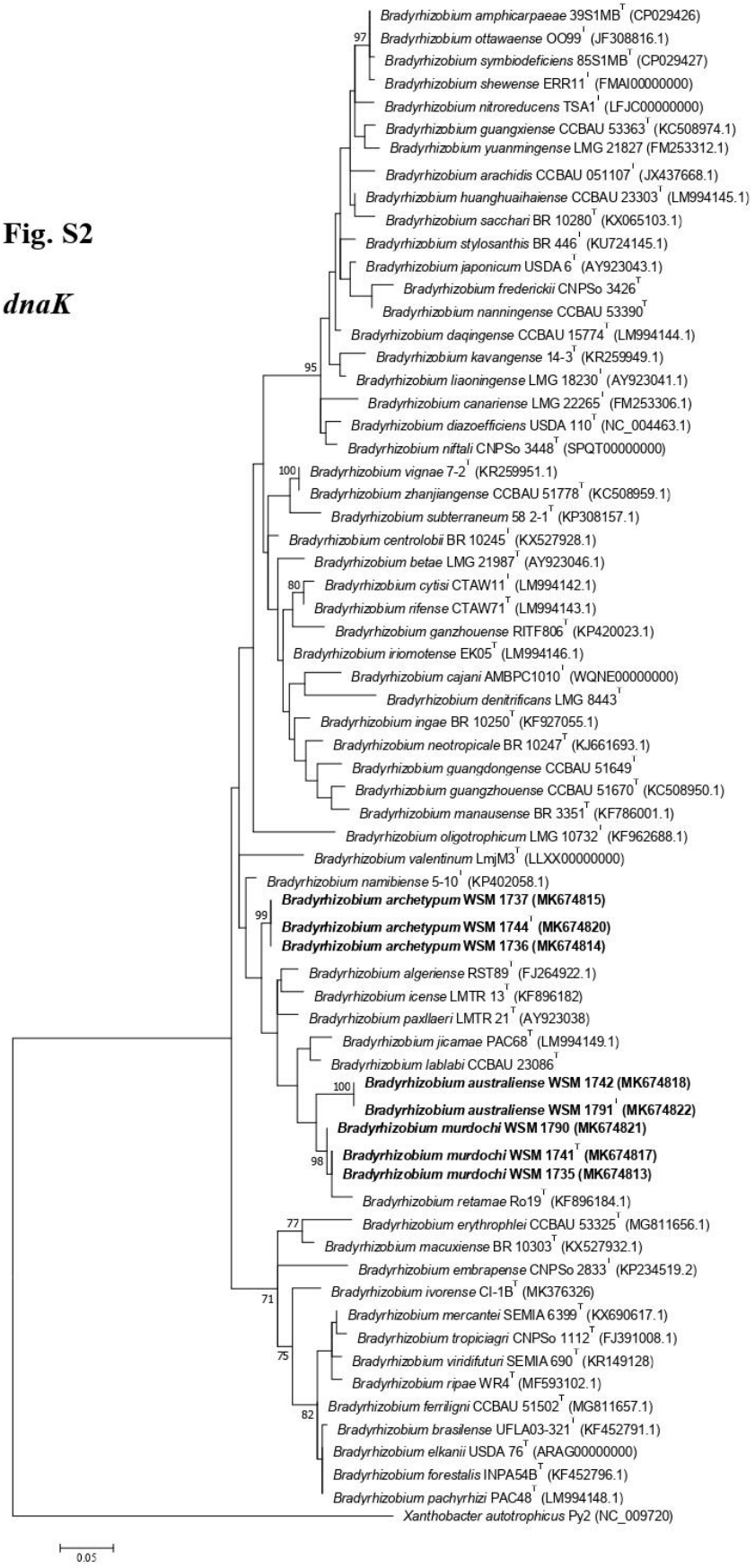
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Fig. S3

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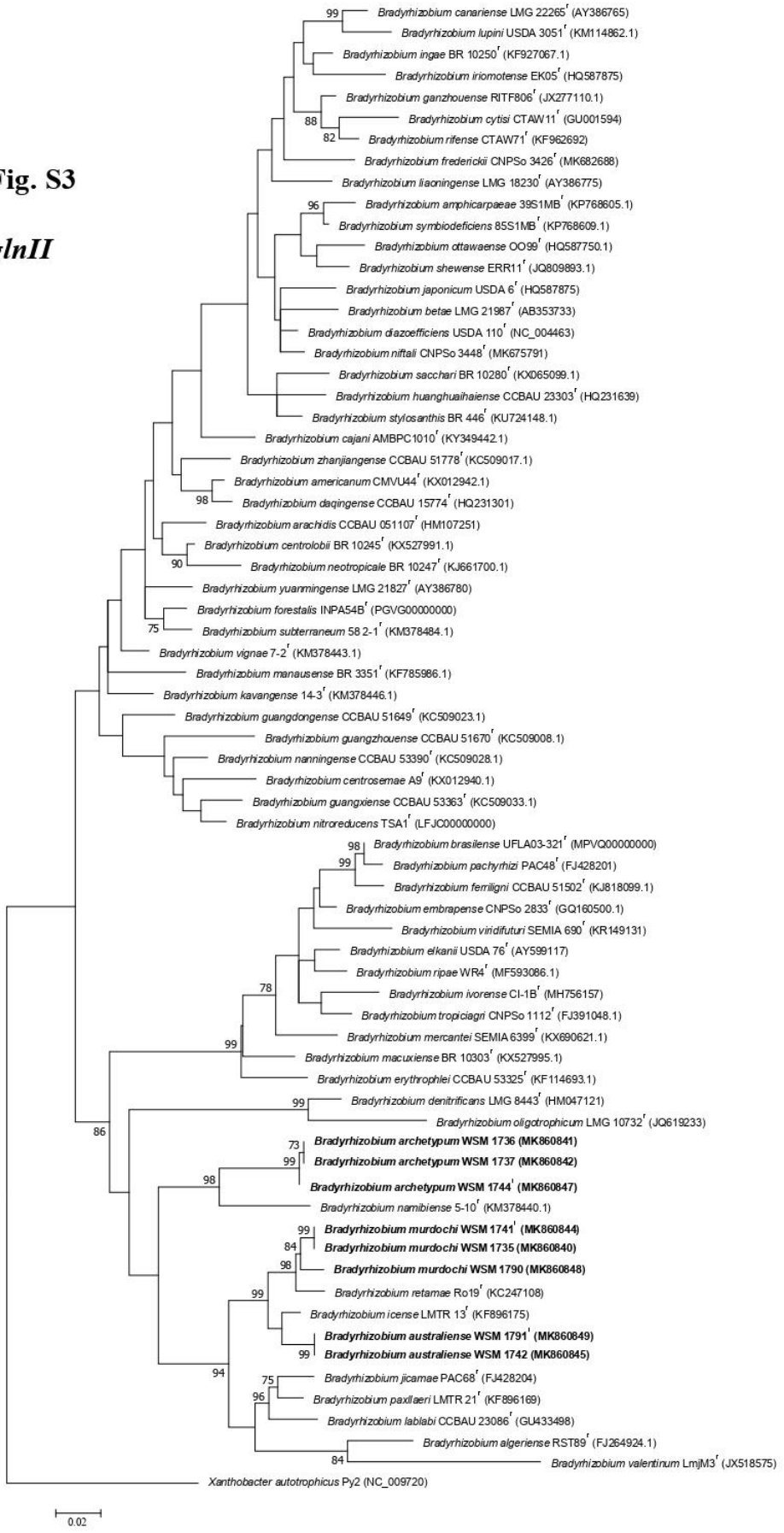
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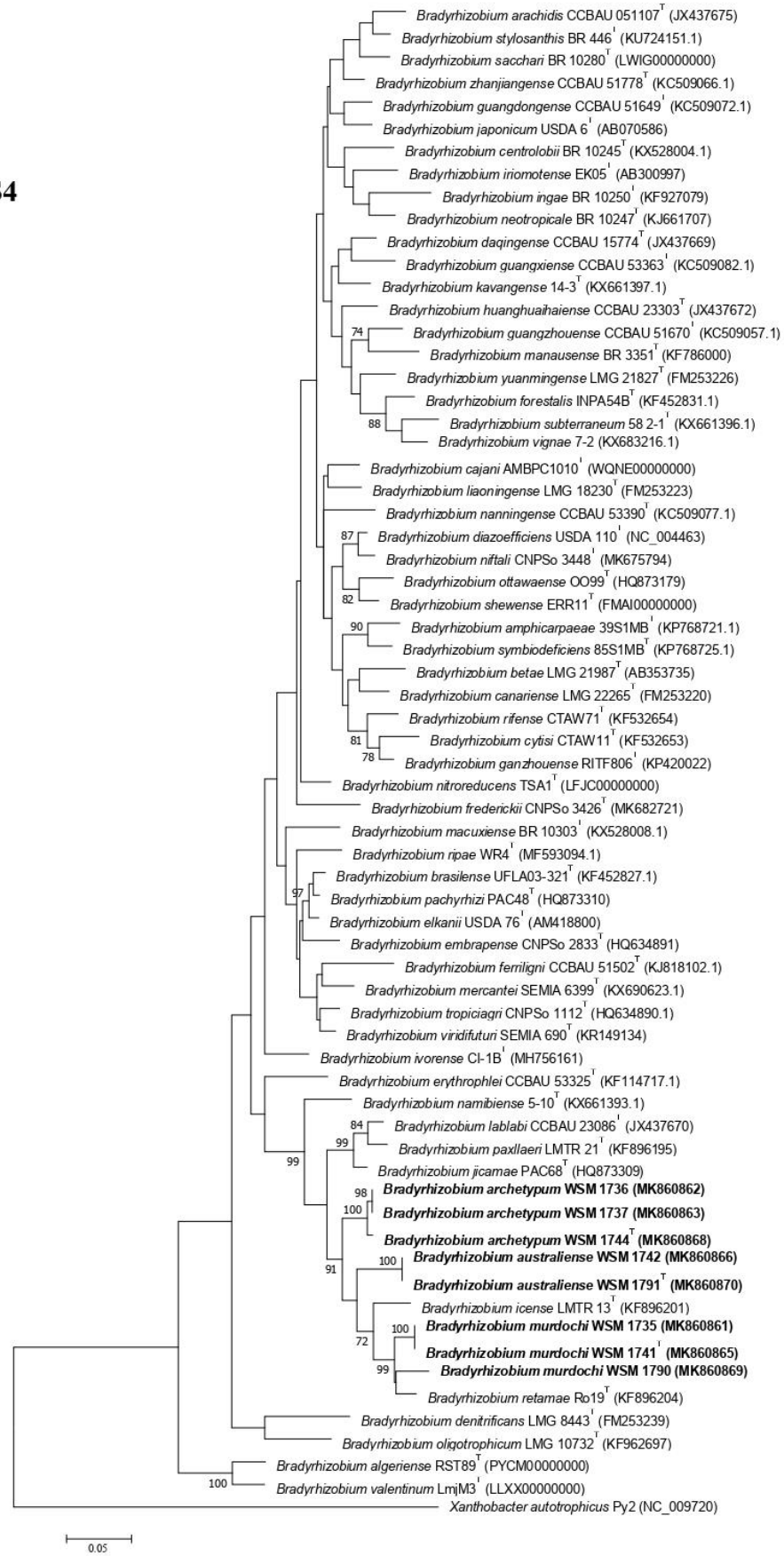
Fig. S4

gyrB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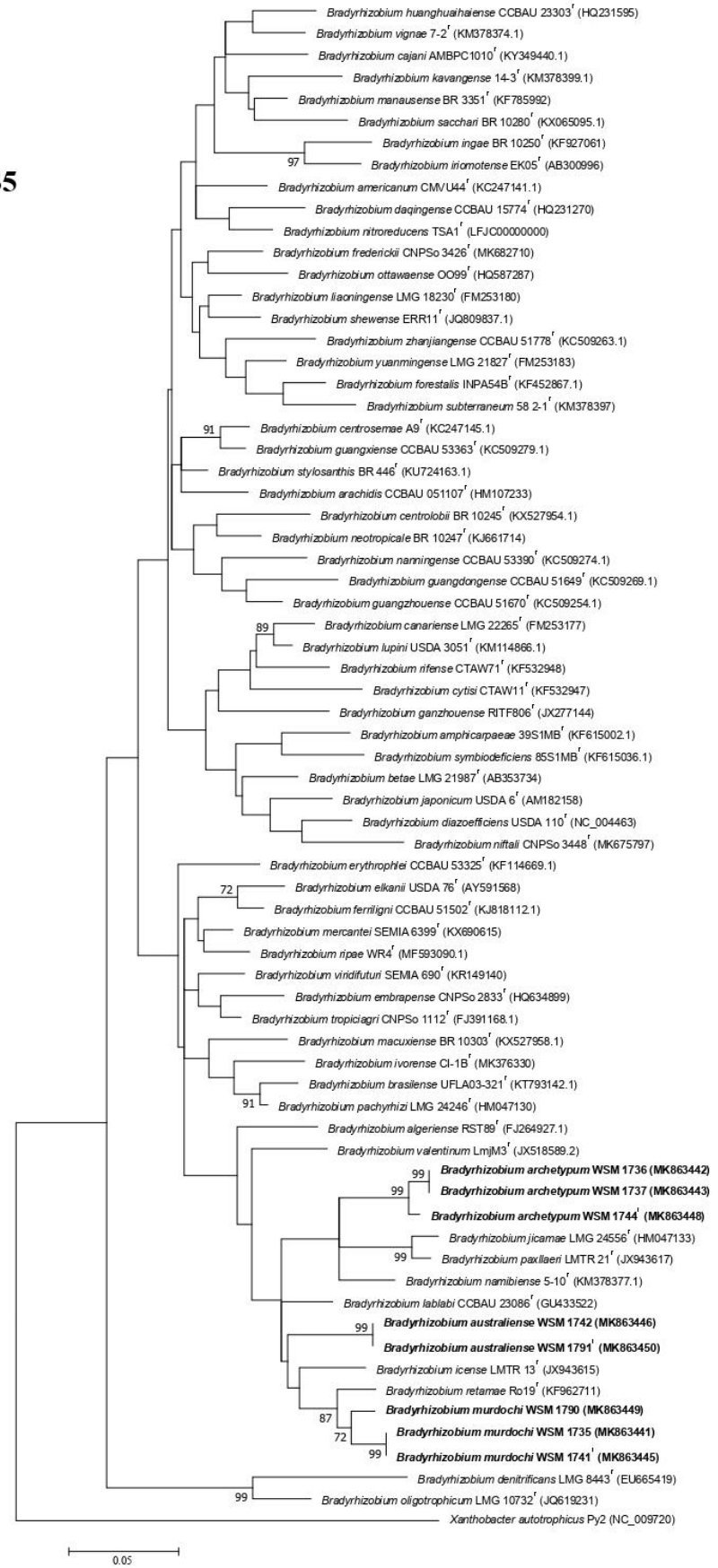
Bradyrhizobium tropiciagri CNPSo 1112T (HQ634890.1)
Bradyrhizobium viridifuturi SEMIA 690T (KR149134)
Bradyrhizobium ivorense CI-1BT (MH756161)
Bradyrhizobium erythrophlei CCBAU 53325T (KF114717.1)
Bradyrhizobium namibiense 5-10T (KX661393.1)
Bradyrhizobium lablabi CCBAU 23086T (JX437670)
Bradyrhizobium paxllaeri LMTR 21T (KF896195)
Bradyrhizobium jicamae PAC68T (HQ873309)
Bradyrhizobium archetypum WSM 1736 (MK860862)
Bradyrhizobium archetypum WSM 1737 (MK860863)
Bradyrhizobium archetypum WSM 1744T (MK860868)
Bradyrhizobium australiense WSM 1742 (MK860866)
Bradyrhizobium australiense WSM 1791T (MK860870)
Bradyrhizobium icense LMTR 13T (KF896201)
Bradyrhizobium murdochi WSM 1735 (MK860861)
Bradyrhizobium murdochi WSM 1741T (MK860865)
Bradyrhizobium murdochi WSM 1790 (MK860869)
Bradyrhizobium retamae Ro19T (KF896204)
Bradyrhizobium denitrificans LMG 8443T (FM253239)
Bradyrhizobium oligotrophicum LMG 10732T (KF962697)
Bradyrhizobium algeriense RST89T (PYCM00000000)
Bradyrhizobium valentinum LmjM3T (LLXX00000000)
Xanthobacter autotrophicus Py2 (NC_009720)
74
88
87
82
90
81
78
97
84
99
99
98
100
100
91
72
99
100
100
0.05

Fig. S5

recA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Bradyrhizobium vignae 7-2ᵀ (KM378374.1)
Bradyrhizobium cajani AMBPC1010ᵀ (KY349440.1)
Bradyrhizobium kavangense 14-3ᵀ (KM378399.1)
Bradyrhizobium manausense BR 3351ᵀ (KF785992)
Bradyrhizobium sacchari BR 10280ᵀ (KX065095.1)
Bradyrhizobium ingae BR 10250ᵀ (KF927061)
Bradyrhizobium iriomotense EK05ᵀ (AB300996)
Bradyrhizobium americanum CMVU44ᵀ (KC247141.1)
Bradyrhizobium daqingense CCBAU 15774ᵀ (HQ231270)
Bradyrhizobium nitroreducens TSA1ᵀ (LFJC00000000)
Bradyrhizobium frederickii CNPSo 3426ᵀ (MK682710)
Bradyrhizobium ottawaense OO99ᵀ (HQ587287)
Bradyrhizobium liaoningense LMG 18230ᵀ (FM253180)
Bradyrhizobium shewense ERR11ᵀ (JQ809837.1)
Bradyrhizobium zhanjiangense CCBAU 51778ᵀ (KC509263.1)
Bradyrhizobium yuanmingense LMG 21827ᵀ (FM253183)
Bradyrhizobium forestalis INPA54Bᵀ (KF452867.1)
Bradyrhizobium subterraneum 58 2-1ᵀ (KM378397)
Bradyrhizobium centrosemae A9ᵀ (KC247145.1)
Bradyrhizobium guangxiense CCBAU 53363ᵀ (KC509279.1)
Bradyrhizobium stylosanthis BR 446ᵀ (KU724163.1)
Bradyrhizobium arachidis CCBAU 051107ᵀ (HM107233)
Bradyrhizobium centrolobii BR 10245ᵀ (KX527954.1)
Bradyrhizobium neotropicale BR 10247ᵀ (KJ661714)
Bradyrhizobium nanningense CCBAU 53390ᵀ (KC509274.1)
Bradyrhizobium guangdongense CCBAU 51649ᵀ (KC509269.1)
Bradyrhizobium guangzhouense CCBAU 51670ᵀ (KC509254.1)
Bradyrhizobium canariense LMG 22265ᵀ (FM253177)
Bradyrhizobium lupini USDA 3051ᵀ (KM114866.1)
Bradyrhizobium rifense CTAW71ᵀ (KF532948)
Bradyrhizobium cytisi CTAW11ᵀ (KF532947)
Bradyrhizobium ganzhouense RITF806ᵀ (JX277144)
Bradyrhizobium amphicarpaeae 39S1MBᵀ (KF615002.1)
Bradyrhizobium symbiodeficiens 85S1MBᵀ (KF615036.1)
Bradyrhizobium betae LMG 21987ᵀ (AB353734)
Bradyrhizobium japonicum USDA 6ᵀ (AM182158)
Bradyrhizobium diazoefficiens USDA 110ᵀ (NC_004463)
Bradyrhizobium niftali CNPSo 3448ᵀ (MK675797)
Bradyrhizobium erythrophlei CCBAU 53325ᵀ (KF114669.1)
Bradyrhizobium elkanii USDA 76ᵀ (AY591568)
Bradyrhizobium ferriligni CCBAU 51502ᵀ (KJ818112.1)
Bradyrhizobium mercantei SEMIA 6399ᵀ (KX690615)
Bradyrhizobium ripae WR4ᵀ (MF593090.1)
Bradyrhizobium viridifuturi SEMIA 690ᵀ (KR149140)
Bradyrhizobium embrapense CNPSo 2833ᵀ (HQ634899)
Bradyrhizobium tropiciagri CNPSo 1112ᵀ (FJ391168.1)
Bradyrhizobium macuxiense BR 10303ᵀ (KX527958.1)
Bradyrhizobium ivorense CI-1Bᵀ (MK376330)
Bradyrhizobium brasilense UFLA03-321ᵀ (KT793142.1)
Bradyrhizobium pachyrhizi LMG 24246ᵀ (HM047130)
Bradyrhizobium algeriense RST89ᵀ (FJ264927.1)
Bradyrhizobium valentinum LmjM3ᵀ (JX518589.2)
Bradyrhizobium archetypum WSM 1736 (MK863442)
Bradyrhizobium archetypum WSM 1737 (MK863443)
Bradyrhizobium archetypum WSM 1744ᵀ (MK863448)
Bradyrhizobium jicamae LMG 24556ᵀ (HM047133)
Bradyrhizobium paxllaeri LMTR 21ᵀ (JX943617)
Bradyrhizobium namibiense 5-10ᵀ (KM378377.1)
Bradyrhizobium lablabi CCBAU 23086ᵀ (GU433522)
Bradyrhizobium australiense WSM 1742 (MK863446)
Bradyrhizobium australiense WSM 1791ᵀ (MK863450)
Bradyrhizobium icense LMTR 13ᵀ (JX943615)
Bradyrhizobium retamae Ro19ᵀ (KF962711)
Bradyrhizobium murdochi WSM 1790 (MK863449)
Bradyrhizobium murdochi WSM 1735 (MK863441)
Bradyrhizobium murdochi WSM 1741ᵀ (MK863445)
Bradyrhizobium denitrificans LMG 8443ᵀ (EU665419)
Bradyrhizobium oligotrophicum LMG 10732ᵀ (JQ619231)
Xanthobacter autotrophicus Py2 (NC_009720)
97
91
89
72
99
87
0.05

Fig. S6

MLSA
5 genes

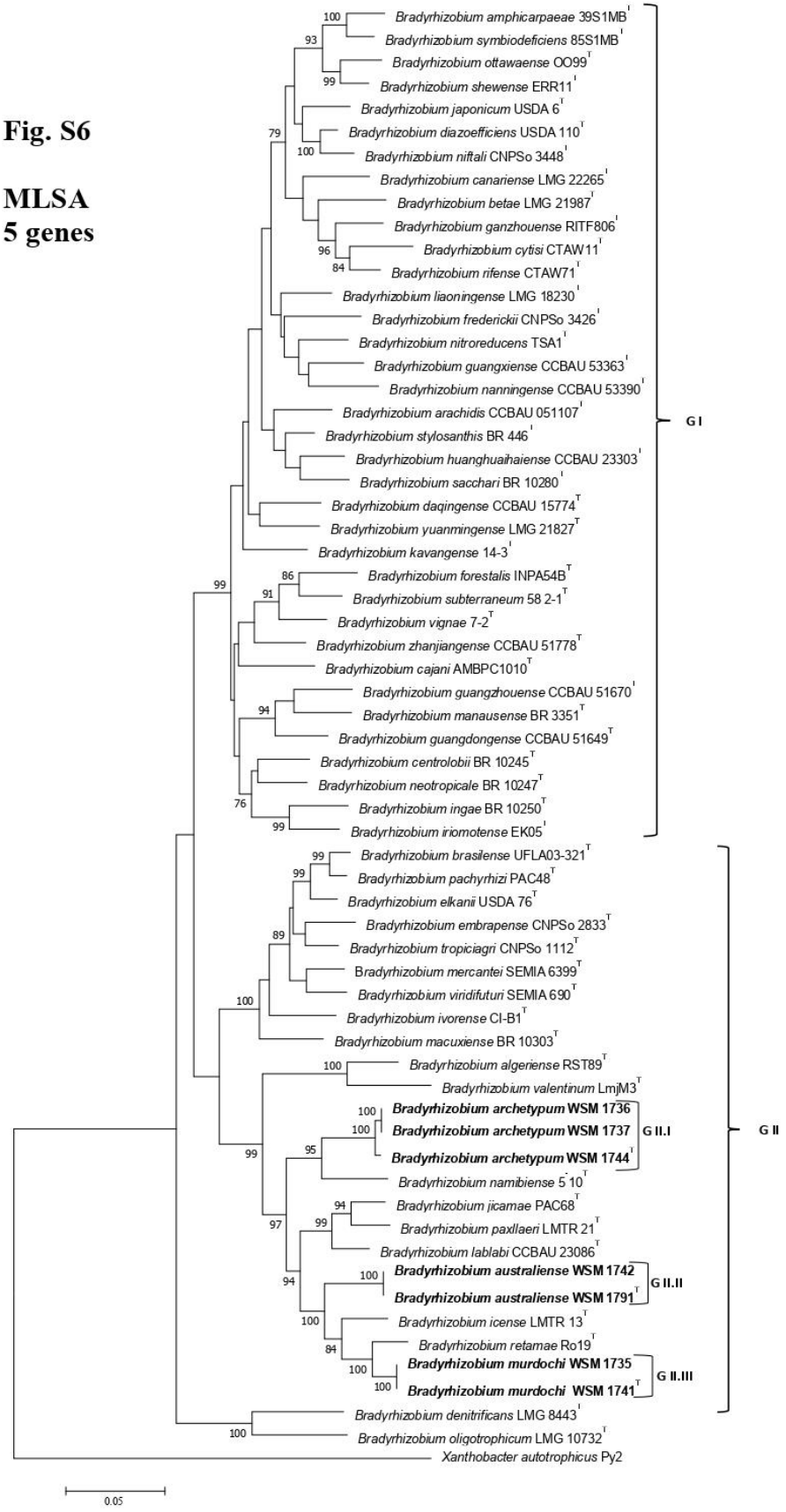
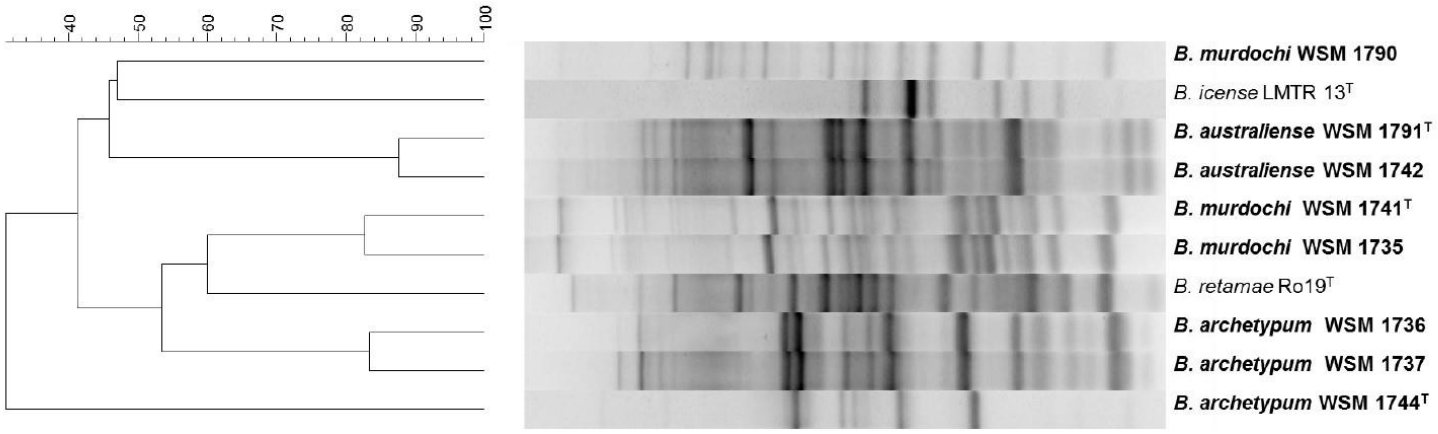


Fig. S7

BOX-PCR



CAPÍTULO III

Bradyrhizobium agreste sp. nov., *Bradyrhizobium glycinis* sp. nov. E *Bradyrhizobium diversitatis* sp. nov., ISOLADAS DE UM HOTSPOT DE BIODIVERSIDADE DO GÊNERO *Glycine* NA AUSTRÁLIA OCIDENTAL.

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RESUMO

Estirpes do gênero *Bradyrhizobium* associadas com leguminosas de importância agronômica como a soja (*Glycine max*) têm sido cada vez mais estudadas; no entanto, informações sobre os simbiontes de espécies *Glycine* selvagem ainda são escassos. Austrália é um centro genético de espécies de *Glycine* selvagem e, nesse estudo, nós realizamos uma análise polifásica da taxonomia de três estirpes de *Bradyrhizobium* – CNPSo 4010^T, CNPSo 4016^T e CNPSo 4019^T – isoladas de plantas iscas das espécies *Glycine clandestina, Glycine tabacina* e *Glycine max*, respectivamente, na Austrália Ocidental. A árvore filogenética das sequências do gene 16S RNAr agrupou todas as estirpes no super clado *Bradyrhizobium japonicum*; as estirpes CNPSo 4010^T e CNPSo 4016^T tiveram *Bradyrhizobium yuanmingense* CCBAU 10071^T como espécie mais próxima, enquanto a estirpe CNPSo 4019^T foi mais próxima de *Bradyrhizobium liaoningense* LMG 18230^T. O *multilocus sequence analysis* (MLSA) com os cinco genes *housekeeping -dnaK, glnII, gyrB, recA* e *rpoB*- confirmaram os mesmos grupos encontrados na filogenia do gene 16S RNAr, mas indicaram baixa similaridade com as espécies do gênero descritas; as identidades nucleotídicas variaram entre 93.6 e 97.6 % de similaridade. Em relação aos genomas das três espécies aqui descritas, os valores da análise de *average nucleotide identity* e a hibridação DNA-DNA digital foram menores que 94.97 e 59.80%, respectivamente, com as espécies mais próximas. Na filogenia do *nodC,* as estirpes CNPSo 4010^T e CNPSo 4019^T agruparam com *Bradyrhizobium zhanjiangense* e *Bradyrhizobium ganzhouense*, respectivamente, enquanto a estirpe CNPSo 4016^T foi posicionada separadamente de todas as espécies de *Bradyrhizobium* simbióticas. Outras propriedades genômicas (BOX-PCR), fenotípicas e simbióticas foram avaliadas e corroboraram a descrição de três novas

linhagens de *Bradyrhizobium.* Nós propusemos os nomes de *Bradyrhizobium agreste* sp. nov. para CNPSo 4010^{T} (=WSM 4802^{T}=LMG 31645^{T}) isolada de *Glycine clandestina*, *Bradyrhizobium glycinis* sp. nov. para CNPSo 4016^{T} (=WSM 4799^{T}=LMG 31645^{T}) isolada de *G. tabacina* e *Bradyrhizobium diversitatis* sp. nov. para CNPSo 4019^{T} (=WSM 4799^{T}=LMG 31650^{T}) isolada de *G. max.*

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TAXONOMIC DESCRIPTION
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MICROBIOLOGY SOCIETY
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Bradyrhizobium agreste sp. nov., *Bradyrhizobium glycinis* sp. nov. and *Bradyrhizobium diversitatis* sp. nov., isolated from a biodiversity hotspot of the genus *Glycine* in Western Australia

Milena Serenato Klepa[1,2,3]†, Luisa Caroline Ferraz Helene[1,2]†, Graham O'Hara[4] and Mariangela Hungria[1,2,3,*]

Abstract

Strains of the genus *Bradyrhizobium* associated with agronomically important crops such as soybean (*Glycine max*) are increasingly studied; however, information about symbionts of wild *Glycine* species is scarce. Australia is a genetic centre of wild *Glycine* species and we performed a polyphasic analysis of three *Bradyrhizobium* strains—CNPSo 4010^T, CNPSo 4016^T, and CNPSo 4019^T—trapped from Western Australian soils with *Glycine clandestina*, *Glycine tabacina* and *Glycine max*, respectively. The phylogenetic tree of the 16S rRNA gene clustered all strains into the *Bradyrhizobium japonicum* superclade; strains CNPSo 4010^T and CNPSo 4016^T had *Bradyrhizobium yuanmingense* CCBAU 10071^T as the closest species, whereas strain CNPSo 4019^T was closer to *Bradyrhizobium liaoningense* LMG 18230^T. The multilocus sequence analysis (MLSA) with five housekeeping genes—*dnaK*, *glnII*, *gyrB*, *recA* and *rpoB*—confirmed the same clusters as the 16S rRNA phylogeny, but indicated low similarity to described species, with nucleotide identities ranging from 93.6 to 97.6% of similarity. Considering the genomes of the three strains, the average nucleotide identity and digital DNA–DNA hybridization values were lower than 94.97 and 59.80%, respectively, with the closest species. In the *nodC* phylogeny, strains CNPSo 4010^T and CNPSo 4019^T grouped with *Bradyrhizobium zhanjiangense* and *Bradyrhizobium ganzhouense*, respectively, while strain CNPSo 4016^T was positioned separately from the all symbiotic *Bradyrhizobium* species. Other genomic (BOX-PCR), phenotypic and symbiotic properties were evaluated and corroborated with the description of three new lineages of *Bradyrhizobium*. We propose the names of *Bradyrhizobium agreste* sp. nov. for CNPSo 4010^T (=WSM 4802^T=LMG 31645^T) isolated from *Glycine clandestina*, *Bradyrhizobium glycinis* sp. nov. for CNPSo 4016^T (=WSM 4801^T=LMG 31649^T) isolated from *Glycine tabacina* and *Bradyrhizobium diversitatis* sp. nov. for CNPSo 4019^T (=WSM 4799^T=LMG 31650^T) isolated from *G. max*.

INTRODUCTION

Balanced levels of N in soil are required to obtain high productivities and healthy plants. Biological nitrogen fixation (BNF) is carried out by a restricted group of prokaryotes able to reduce the atmospheric dinitrogen (N_2) to ammonium (NH_3), the assimilable form by plants [1, 2]. The highest level of evolution of BNF occurs with the symbiotic interaction between diazotrophic bacteria collectively known as rhizobia and members of the family Fabaceae (=Leguminosae). The symbiosis involves a mutual exchange of molecular signals, culminating in the development of specialized structures on roots, and occasionally on stems, called nodules, in which the BNF process takes place [3, 4]. A variety of elite rhizobial strains have been introduced in agricultural systems for over a century as a sustainable way to improve soil fertility and crop yield, in addition to lowering costs, due to the replacement of

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***Correspondence:** Mariangela Hungria, mariangela.hungria@embrapa.br;biotecnologia.solo@hotmail.com
Keywords: *Bradyrhizobium*; wild soybean; *Glycine*; nodulation; MLSA; ANI; dDDH.
Abbreviations: ANI, average nucleotide identity; BNF, biological nitrogen fixation; dDDH, digital DNA–DNA hybridization; DDH, DNA–DNA hybridization; GGDC, Genome-to-Genome Distance Calculator; LB, Luria–Bertani; ML, maximum-likelihood; MLSA, multilocus sequence analysis; NI, nucleotide identity; UPGMA, unweighted pair group method with arithmetic mean algorithm; YMA, yeast–mannitol agar.
The following new sequences have been deposited at the GenBank/EMBL/DDBJ database: *atpD* of *B. agreste* CNPSo 4010^T (MT683853); *atpD* of *B. glycinis* CNPSo 4016^T (MT683854); *atpD* of *B. diversitatis* CNPSo 4019^T (MT683855). Genome accession numbers of *B. agreste* CNPSo 4010^T (JACCHP000000000); *B. glycinis* CNPSo 4016^T (JACCHQ000000000); *B. diversitatis* CNPSo 4019^T (JACEGD000000000).
†These authors contributed equally to this work
One supplementary table and nine supplementary figures are available with the online version of this article.

Table 1. Strains used in this study

Species/strain name	Other nomenclatures	Original host species	Geographical origin	Reference
Bradyrhizobium agreste CNPSo 4010^T	WSM 4802^T=LMG 31645	*Glycine clandestina*	Kununurra, Australia	Helene *et al.* [16]
Bradyrhizobium glycinis CNPSo 4016^T	WSM 4801^T=LMG 31649^T	*Glycine tabacina*	Kununurra, Australia	Helene *et al.* [16]
Bradyrhizobium diversitatis CNPSo 4019^T	WSM 4799^T=LMG 31650^T	*Glycine max*	Nambung, Australia	Helene *et al.* [16]
Bradyrhizobium fredereickii CNPSo 3426^T	USDA 10052^T=U686^T=CL 20^T	*Chamaecrista fasciculata*	Missouri, USA	Urquiaga *et al.* [38]
Bradyrhizobium liaoningense LMG 18230^T	DSM 24092^T=CECT 4845^T=USDA 3622^T	*Glycine max*	China	Xu *et al.* [9]
Bradyrhizobium yuanmingense CCBAU 10071^T	CFNEB 101^T=CIP 108027^T=NBRC 100594^T	*Lespedeza* species	China	Yao *et al.* [59]

chemical N-fertilizers [5, 6]. One remarkable example relies on the symbiosis of *Bradyrhizobium* with the soybean (*Glycine max*) crop in Brazil, saving billions of dollars every year [7, 8].

Several *Bradyrhizobium* species have been isolated from nodules of soybean, mostly grown in China, the main genetic centre of this legume species [9–11]. However, *Bradyrhizobium* is a geographically widespread genus that nodulates several legume tribes, from herbaceous to trees, mainly in tropical regions [12–15]. Recently, Helene *et al.* [16] reported a high diversity of *Bradyrhizobium* strains isolated from Western Australian soils using species of *Glycine* species as trapping hosts. It is worth mentioning that the genus *Glycine* is split into two subgenera: *Soja*, with *Glycine max* and *Glycine soja* species, and *Glycine*, which includes 25 species of wild soybean, most indigenous to Australia [17–20]; for this reason, although not often mentioned, Australia is a great hotspot of diversity of the genus *Glycine* [21].

Populations of Australian indigenous *Glycine* species are distributed all over the country and are the wild relatives of the economically important soybean crop [17, 21, 22]. Due to the inhospitable conditions of most areas growing indigenous *Glycine* in Australia, bioprospecting their genomes may help to understand and improve the adaptation of soybean to climate change [21]. In addition, to investigate the diversity of rhizobia symbionts of indigenous *Glycine* species in soils of Western Australia should provide valuable information for biotechnological applications. Here, we report a polyphasic study with three lineages of *Bradyrhizobium* isolated from *Glycine clandestina, G. tabacina* and *G. max* that resulted in the description of three new species.

ISOLATION AND ECOLOGY

Strains CNPSo 4010^T, CNPSo 4016^T and CNPSo 4019^T were isolated from root nodules of three different species, *G. clandestina* JC Wendl [23], *G. tabacina* (Labill.) Benth [24] and *G. max*, respectively, used as trap plants in Western Australian soil, as previously reported by our group [16]. Information about the strains used in this study as well as the sampling soil points are shown in Table 1.

The strains are deposited at the Diazotrophic and Plant Growth Promoting Bacteria Culture Collection of Embrapa Soja (WFCC Collection No. 1213, WDCM Collection No. 1054), in Londrina, State of Parana, Brazil; at the Western Australian Soil Microbiology Gene Bank (WSM Culture Collection); at the Belgian Coordinated Collections of Microorganisms (BCCM/LMG); and in other culture collections.

The strains were short-term maintained on modified yeast extract–mannitol agar (YMA) medium [25] at 4 °C in a cold room and periodically cultured, while for long-term preservation the cultures were stored in modified-YM with 30% glycerol (v/v) at −80 and −150 °C by cryopreservation, and lyophilized as previously described [26].

PHYLOGENY

Sequences of the 16S rRNA and of four housekeeping genes (*dnaK*, *glnII*, *gyrB* and *recA*), as well as of the symbiotic gene *nodC* were obtained from a previous study [16], and their accession numbers are shown in Table S1. Amplicons for the housekeeping gene *atpD* were obtained using the pair of primers TSatpDf (5′-TCTGGTCCGYGGCCAGGAAG-3′) and TSatpDr (5′-CGACACTTCCGARCCSGCCTG-3′), with the conditions described by Stepkowski *et al.* [27]. The PureLink kit (Invitrogen) was used following the manufacturer's instructions for the purification of the PCR products, which were sequenced in an ABI 3500XL (Applied Biosystems) capillary sequencer analyzer, as described by Delamuta *et al.* [28]. All sequences were deposited at the GenBank database (NCBI) and the accession numbers are listed in parentheses in the phylogenetic trees and in Table S1. The complete sequences of housekeeping genes *atpD*, *dnaK*, *glnII*, *gyrB*, *recA* and *rpoB* were also retrieved from the genome of the type strains CNPSo 4010^T, CNPSo 4016^T and CNPSo 4019^T in order to build a robust multilocus sequence analysis (MLSA) phylogeny. Multiple sequence alignments were obtained

Table 2. Nucleotide identity among new lineages of *Bradyrhizobium* and closely related species, based on the sequences of single and concatenated housekeeping genes (*atpD*, *dnaK*, *glnII*, *gyrB*, *recA* and *rpoB*) and the 16S rRNA

	Nucleotide identity (%)								
Strains	**MLSA (five genes)**	**MLSA (six genes)**	**16S rRNA**	***atpD***	***dnaK***	***glnII***	***gyrB***	***recA***	***rpoB***
			***Bradyrhizobium agreste* CNPSo 4010^T**						
B. glycinis CNPSo 4016^T	97.2	97.5	100	97.7	98.1	97.4	96.8	95.5	98.6
B. yuanmingense CCBAU 10071^T	96.4	96.9	99.6	94.9	99	95.8	95.3	95.8	98
B. liaoningense LMG 18230^T	95.3	95.3	99.6	94.1	95.9	94.6	94.4	94.7	97.8
B. diversitatis CNPSo 4019^T	95.1	95.3	99.6	94.1	95.9	94.4	94.8	93.8	97.5
B. frederickii CNPSo 4026^T	93.6	94.7	99.5	94.6	95.4	93.8	91.3	93.8	95.6
			***Bradyrhizobium glycinis* CNPSo 4016^T**						
B. agreste CNPSo 4010^T	97.2	97.5	100	97.7	98.1	97.4	96.8	95.5	98.6
B. yuanmingense CCBAU 10071^T	96.1	97.1	99.6	94.1	98.1	95.8	94.1	96.3	98.3
B. diversitatis CNPSo 4019^T	95.2	95.1	99.6	93.9	96.8	94.8	93.9	94.7	97.2
B. liaoningense LMG 18230^T	94.9	95.1	99.6	94.1	96.8	94.2	93.9	94.4	97
B. frederickii CNPSo 4026^T	93.6	94.5	99.5	95.1	95	94.2	90.7	93.8	95.9
			***Bradyrhizobium diversitatis* CNPSo 4019^T**						
B. liaoningense LMG 18230^T	97.6	97.8	100	96.9	98.6	94.8	98.1	98.6	99.1
B. yuanmingense CCBAU 10071^T	95.4	95.3	99.5	93.4	95.9	94.4	94.4	95.5	97.8
B. glycinis CNPSo 4016^T	95.2	95.1	99.6	94.1	95.9	94.4	94.8	93.8	97.5
B. agreste CNPSo 4010^T	95.1	95.3	99.6	93.9	96.8	94.8	93.9	94.7	97.2
B. frederickii CNPSo 4026^T	94.8	95.7	99.7	95.4	96.3	93.8	93.9	94.7	97

with MUSCLE [29] and the best evolutionary distance model was inferred by the lowest Bayesian information criterion scores [30] for maximum-likelihood (ML) reconstructions in Molecular Evolutionary Genetics Analysis (MEGA) software version 7 [31]. The evolutionary models are described in the figure captions. The statistical support of the trees was estimated by bootstrap analysis [32] with 1000 re-samplings [33]. *Xanthobacter autotrophicus* Py2 was used as an outgroup for all phylogenies, except for the *nodC* tree. The sequences of housekeeping genes were concatenated with SeaView software version 4.7 [34] for the MLSA. Nucleotide identity was calculated with BioEdit software version 7.0.4.1 [35] for single and concatenated genes and the values are indicated in Table 2.

The 16S rRNA phylogeny traditionally splits the genus *Bradyrhizobium* into two well-supported superclades, *B. japonicum* and *B. elkanii* [36–38]. We included sequences of all described *Bradyrhizobium* species described at the time of writing and the three strains from our study fit into the *B. japonicum* superclade; strains CNPSo 4010^T and CNPSo 4016^T showed higher relatedness with *B. yuanmingense* CCBAU 10071^T (99.6% of similarity), whereas the CNPSo 4019^T showed 100% similarity to *B. liaoningense* LMG 18230^T and 99.8% to *B. daqingense* CGMCC 1.10947^T (Fig. 1). The nucleotide identity values of the 16S rRNA genes among strains CNPSo 4010^T, CNPSo 4016^T and CNPSo 4019^T ranged from 99.6 to 100% (Table 2). Considering the threshold of 98.65% for species boundary suggested by Kim *et al.* [39], these results corroborate that the 16S rRNA gene provides limited taxonomic information in the genus *Bradyrhizobium* [15, 40–42].

In the analysis of housekeeping genes, it is important to verify each single phylogeny to detect congruence with the 16S rRNA phylogeny and possible events of horizontal gene transfer and recombination [36, 43]. Therefore, single phylogenies of the genes *atpD* (398 bp), *dnaK* (221 bp), *glnII* (504 bp), *gyrB* (553 bp), *recA* (360 bp) and *rpoB* (371 bp) were built and are shown in Figs S1–S6 (available in the online version of this article). All phylogenies of single housekeeping genes clustered the strains from this study in clades separated from all described *Bradyrhizobium* species. However, some differences in the topology of the trees were verified on *atpD* (Fig. S1) and *glnII* (Fig. S3) phylogenies. The well-supported *B. japonicum* and *B. elkanii* superclades were not detected in the *atpD* phylogeny, as previously reported by Menna *et al.* [36]. Therefore, in order to improve the phylogenetic information of strains CNPSo 4010^T, CNPSo 4016^T and CNPSo 4019^T, two alignments of concatenated

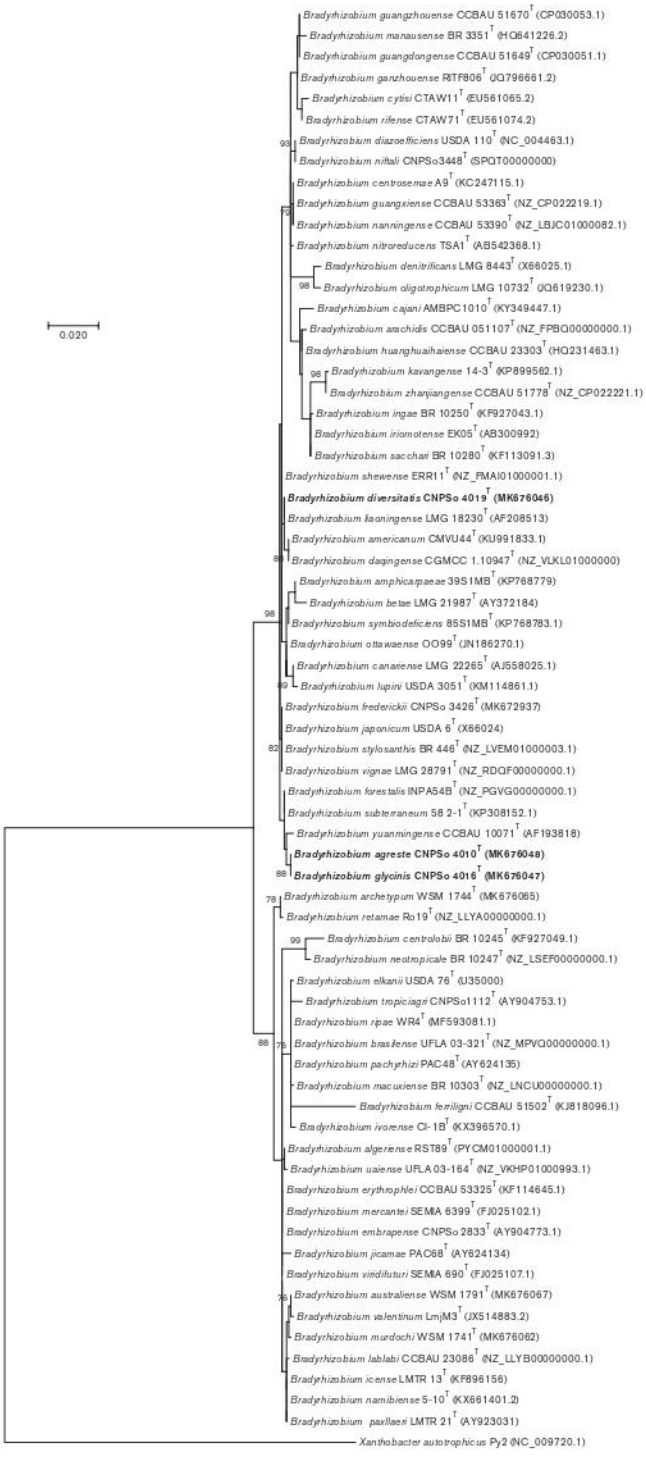

Fig. 1. Maximum-likelihood phylogeny based on 16S rRNA alignment (1312 bp), using the T92 (Tamura three-parameter+G+I) model by MEGA version 7. Accession numbers are indicated in parentheses and in Table S1. The novel species are shown in bold. Bootstrap values >70% are indicated at the nodes. *Xanthobacter autotrophicus* Py2 was used as an outgroup. Bar indicates two substitutions per 100 nucleotide positions.

housekeeping genes were performed for the MLSA; the first including *dnaK+glnII+gyrB+recA+rpoB* genes (2009 bp), and the second with complete sequences of *atpD+dnaK+glnII+gyrB+recA+rpoB* genes (11704 bp), and they are shown in Figs 2 and S7, respectively. The resulting concatenated phylograms maintained the main groups of strains observed in the 16S rRNA phylogeny, but improved the species delineation. Also, the similar topology of both MLSA trees demonstrated that this approach represents a reliable buffer against possible events of recombination at a single locus [44].

In the MLSA with five housekeeping genes (Fig. 2), strains CNPSo 4010^T and CNPSo 4016^T remained in a consistent cluster with 99% bootstrap support, with *B. yuanmingense* CCBAU 10071^T as the closest species with 96.4 and 96.1% nucleotide identity, respectively (Fig. 2, Table 2). Concerning strain CNPSo 4019^T, *B. liaoningense* LMG 18230^T was the closest species with 99% bootstrap support and 97.6% similarity; another close species was *B. frederickii* CNPSo 3426^T, sharing 94.8% nucleotide identity. In addition, the nucleotide identity values among strains CNPSo 4010^T, CNPSo 4016^T and CNPSo 4019^T ranged from 95.1 to 97.2%.

Based on an MLSA with five housekeeping genes, Durán *et al.* [45] proposed the threshold of 97% similarity for *Bradyrhizobium* species delineation. Even though the strains of this study showed nucleotide identity values slightly above this threshold in the MLSA, other species also shared nucleotide identity values higher than 97%; for example, *B. amphicarpaeae* 39S1MB^T and *B. symbiodeficiens* 85S1MB^T (97.1%), *B. elkanii* USDA 76^T and *B. pachyrhizi* PAC 48^T (97.5%), *B. elkanii* USDA 76^T and *B. brasilense* UFLA03-321^T (97.6%) (data not shown). Recently, also in studies with *Bradyrhizobium*, Klepa *et al.* [15], Helene *et al.* [46] and Fossou *et al.* [47] reported values higher than 97% when considering five concatenated housekeeping genes. Therefore, as the nucleotide identity is a mathematic parameter and does not take into account specific mutations on gene sequences, we suggest that the threshold of *Bradyrhizobium* species delineation should be revised.

The symbiotic process between rhizobia and legumes relies on a complex molecular signal exchange that activates several genes responsible for nodule development [48–50]. Phylogeny of nodulation genes does not elucidate the taxonomic status; however, it is important to reveal the evolutionary history of symbiotic relationships, host-range specificity and symbiovar definitions [3, 28, 46, 51, 52]. In this study, the *nodC* gene analysis revealed that CNPSo 4010^T from *G. clandestina* clustered in a well-supported clade with *B. zhanjiangense* CCBAU 51778^T isolated from nodules of *Arachis hypogaea* in southeast China [53], CNPSo 4019^T from *G. max* clustered with 100% bootstrap support with *B. ganzhouense* RITF806^T isolated from nodules of *Acacia melanoxylon* grown in China [54]; whereas CNPSo 4016^T, isolated from *G. tabacina*, occupied an isolated position (Fig. 3). Note that the phylogenetic position of each of the three strains in the *nodC* tree was different. Comparing the core 16S rRNA and housekeeping genes with the *nodC* gene phylogenies, a different evolutionary pattern

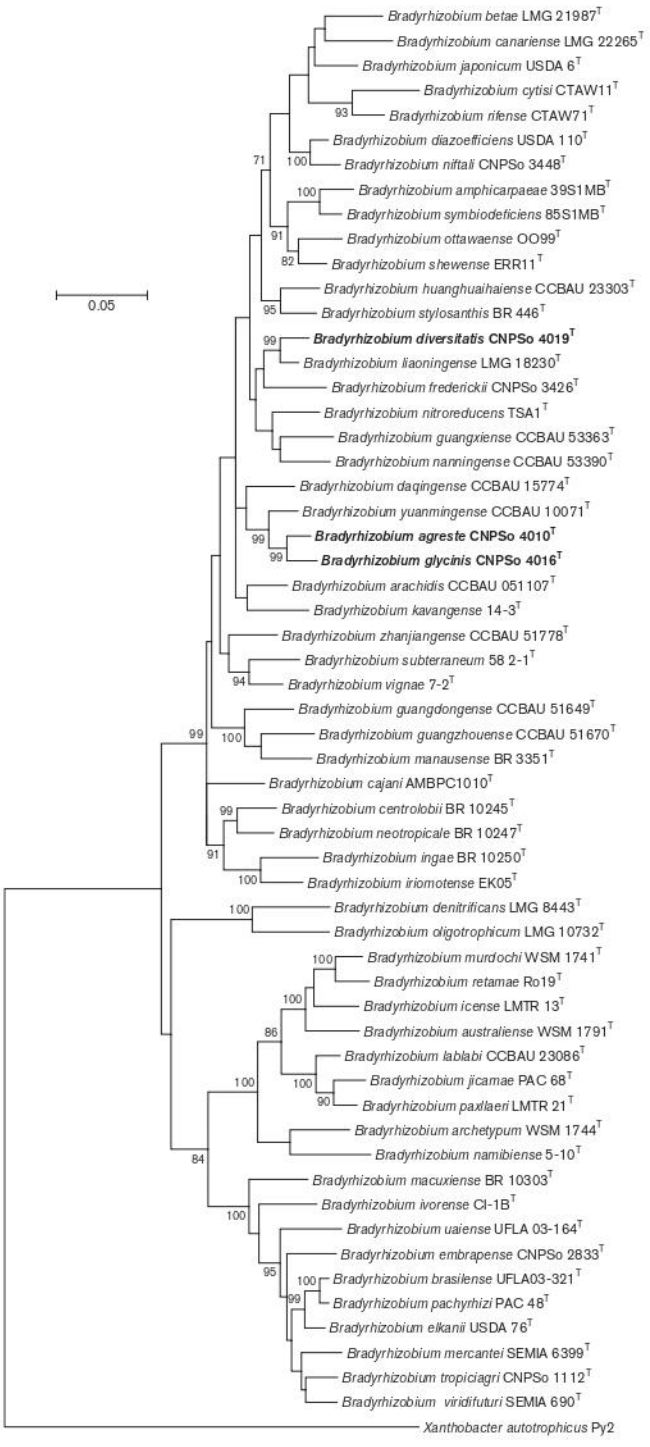

Fig. 2. Maximum-likelihood phylogeny based on alignment of *dnaK+glnII+gyrB+recA+rpoB* concatenated genes (2009 bp), using the GTR (general time reversible)+G+I model by MEGA version 7. Accession numbers are indicated in Table S1. The novel species are shown in bold. Bootstrap values >70% are indicated at the nodes. *Xanthobacter autotrophicus* Py2 was used as an outgroup. Bar indicates five substitution per 100 nucleotide positions.

was detected in symbiotic phylogeny, suggesting different evolutionary histories, confirming previous reports of other species within the genus *Bradyrhizobium* [16, 28, 51].

GENOME FEATURES

Total DNA of strains CNPSo 4010^T, CNPSo 4016^T and CNPSo 4019^T was extracted and used to reconstruct the sequence libraries according to the manufacturer's protocol of Nextera XT kit. The genome sequencing was performed using the MiSeq platform (Illumina) at Embrapa Soja. *De novo* sequence assemblies were carried out by A5-MiSeq pipeline version 20140604. The genomes were annotated with RAST version 2.0 [55], using default parameters. The draft genome of CNPSo 4010^T (JACCHP000000000) presented about 7877331 bp with 75 contigs and an N50 value of 251874 bp. The genome size of CNPSo 4016^T (JACCHQ000000000) was estimated at 7794411 bp, containing 83 contigs and an N50 value of 202190 bp. The genome of CNPSo 4019^T (JACEGD000000000) was estimated at 8450368 bp, with 133 contigs and an N50 value of 198871 bp. The sequence coverages of genomes CNPSo 4010^T, CNPSo 4016^T and CNPSo 4019^T were estimated at 95-, 91-, and 94-fold, respectively. Coding DNA sequences were identified as 7883 for CNPSo 4010^T, as 7987 for CNPSo 4016^T and 8603 for CNPSo 4019^T.

Genomic-based measurements of similarity simplify and ensure robust data for microbial taxonomy as a replacement for the traditional DNA–DNA hybridization (DDH) technique [56, 57]. We used average nucleotide identity (ANI) and the digital DNA–DNA hybridization (dDDH) analyses for species delineation considering the suggested cut-off values of 95–96 and 70%, respectively [56, 58, 59]. The genomes of strains CNPSo 4010^T, CNPSo 4016^T and CNPSo 4019^T were compared with the closest related strains according to the phylogenetic analyses: *B. frederickii* CNPSo 3426^T (SPQS00000000), *B. liaoningense* LMG 18230^T (project ID: 1052895 - JGI) and *B. yuanmingense* CCBAU 10071^T (FMAE01000000). ANI values were calculated using the ANI calculator [60] and the dDDH values were estimated with the Genome-to-Genome Distance Calculator (GGDC) version 2.1 [61], using the recommended 'formula 2' (identities/HSP length). Strains CNPSo 4010^T and CNPSo 4016^T shared 94.18% of ANI and 55.50% of dDDH to each other and both strains showed values lower than 93.32% for ANI and 51.80% for dDDH when compared with *B. yuanmingense* CCBAU 10071^T isolated from nodules of *Lespedeza* species plants in China [62] (Table 3). The ANI and dDDH values between CNPSo 4019^T and *B. liaoningense* LMG 18230^T isolated from nodules of *G. max* grown in China [9] were of 94.97 and 59.80 %, respectively. The values below the threshold for species delineation recorded for the three Australian strains isolated from *Glycine* species confirm that they belong to new species.

The genome G+C contents were calculated using the SEED platform [55] and estimated to be 63.8, 63.7 and 63.8 mol% for CNPSo 4010^T, CNPSo 4016^T and CNPSo 4019^T, respectively.

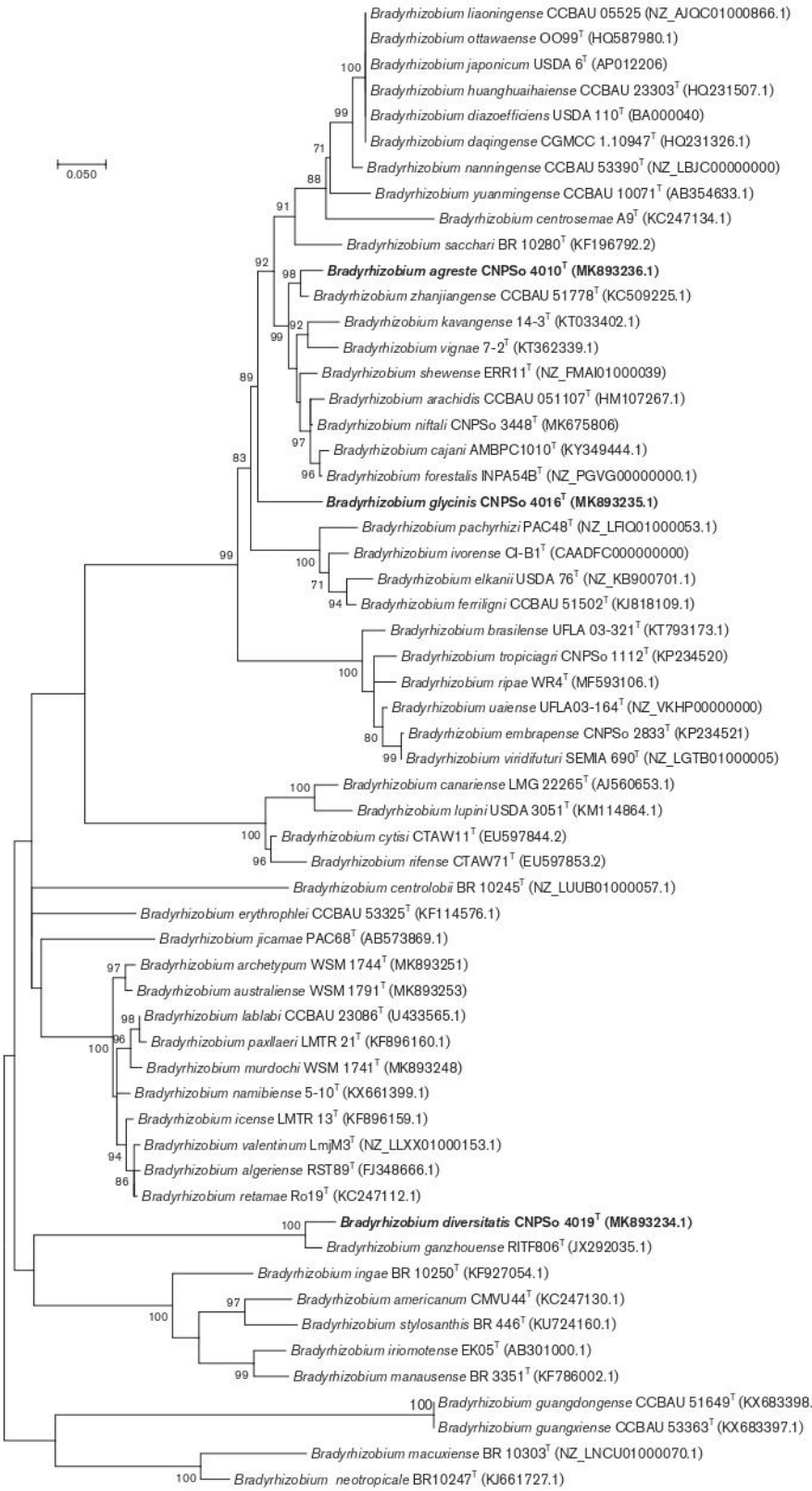

Fig. 3. Maximum-likelihood phylogeny based on *nodC* alignment (426 bp), using the T92 (Tamura three-parameter+G+I) model by MEGA version 7. Accession numbers are indicated in parentheses. The novel species are shown in bold. Bootstrap values >70% are indicated at the nodes. Bar indicates five substitutions per 100 nucleotide positions.

Table 3. ANI and dDDH values among new lineages of *Bradyrhizobium* and closely related *Bradyrhizobium* species

	Bradyrhizobium agreste CNPSo 4010^T		*Bradyrhizobium glycinis* CNPSo 4016^T		*Bradyrhizobium diversitatis* CNPSo 4019^T	
Strains	**ANI %**	**dDDH %**	**ANI %**	**dDDH %**	**ANI %**	**dDDH %**
B. fredereickii CNPSo 3426^T (SPQS00000000)	88.20	35.80	88.13	35.60	88.66	36.90
B. liaoningense LMG 18230^T (JGI Project Id: 1052895)	89.20	38.40	89.19	38.60	94.97	59.80
B. yuanmingense CCBAU 10071^T (FMAE01000000)	92.62	48.60	93.32	51.80	89.21	38.40
B. agreste CNPSo 4010^T (JACCHP000000000)	–	–	94.18	55.50	89.25	38.30
B. glycinis CNPSo 4016^T (JACCHQ000000000)	94.18	55.50	–	–	89.28	38.50
B. diversitatis CNPSo 4019^T (JACEGD000000000)	89.26	38.40	89.28	38.40	–	–

Genomic diversity at strain level was determined by BOX-PCR, using BOX-A1R primer (5′-CTACGGCAAGGCGACG CTGACG-3′) [63], with the conditions described by Chibeba *et al.* [64]. The BOX-PCR profiles of strains CNPSo 4010^T, CNPSo 4016^T and CNPSo 4019^T and the most related species based on the MLSA, *B. liaoningense* LMG 18230^T and *B. yuanmingense* CCBAU 10071^T, were analysed and compared with the Bionumerics software version 7.6 (Applied Mathematics) using the UPGMA (unweighted pair-group method with arithmetic mean) [65] and the Jaccard coefficient [66], with 2% tolerance. The profiles shared less than 50% similarity among the strains of this study, confirming high diversity, and also varied in relation to the closest *Bradyrhizobium* species (Fig. S8).

Therefore, this is the first study describing novel species isolated from wild *Glycine* (*G. clandestina* and *G. tabacina*) indigenous to Australia, which are usually neglected compared to the economically important soybean crop. Even though these wild species are not cultivated, they are considered as a secondary genetic source for desirable agronomic traits such as drought tolerance and disease resistance, due the closeness to soybean [17, 20, 22]. Considering the symbiosis with compatible and well-adapted *Bradyrhizobium*, the nodulated wild *Glycine* may represent an interesting option to restore degraded habitats in Australia.

The phylogenetic relationships of the genus *Glycine* have not yet been fully explained. Whereas *G. max* was domesticated in China around 5000 years ago, through the crossing of wild soybean species [67–69], Australia harbours most of the wild species of the genus *Glycine* [17–21]. Some events during the Earth's evolution have been hypothesized to explain the diversification of these legumes. After the Pangea breakup, the Gondwana supercontinent was formed, connecting Australia and Antarctica; these landmasses separated and Australia moved northwards close to Asia. This moment possibly allowed the successfully entry of Asian legumes in Australia and, consequently, their symbionts [14, 70–72].

Taking into account that China is the original centre of soybean, the region might also be a diversification centre for compatible rhizobia [73–75]. The high similarity of CNPSo 4010^T and CNPSo 4016^T to *B. yuanmingense* CCBAU 10071^T and CNPSo 4019^T to *B. liaoningense* LMG 18230^T may be related to dispersal events. Although *B. yuamingense* CCBAU 10071^T was the original symbiont of *Lespezeda* species in China [62], many strains of this species have been isolated from soybean in Asian soils [74–76]. Also, *B. liaoningense* is a widespread soybean symbiont in India and China [9, 77, 78]. Therefore, our findings support the hypothesis of legume and symbionts exchange between Asian and Australian continents.

PHENOTYPIC CHARACTERIZATION

Several morphophysiological evaluations were performed and compared with strains CNPSo 4010^T, CNPSo 4016^T and CNPSo 4019^T and closest species *B. liaoningense* LMG 18230^T and *B. yuanmingense* CCBAU 10071^T. Except where indicated, the tests were assessed on modified-YMA medium at 28 °C [25]. Colony morphology was analysed using Congo red and acid and alkaline reaction using bromothymol blue after 7–10 days of growth. In order to evaluate growth under different conditions, the strains were cultured with 1% NaCl, at 37 °C, on Luria–Bertani (LB) medium, at pH 4.0 and 8.0, adapted from Hungria *et al.* [79]. The urease activity was tested using 2% urea and phenol red as indicator. Carbohydrate metabolism was detected by the API 50CH kit platform (bioMérieux), according to the manufacturer's instructions and using modified-YM-minus-mannitol with bromothymol blue. Tolerance of antibiotics was determined by the disc-diffusion technique proposed by Bauer *et al.* [80] using ampicillin (10 µg), bacitracin (10 U), cefuroxime (30 µg), chloramphenicol (30 µg), nalidixic acid (30 µg), neomycin (30 µg), penicillin (10 U), streptomycin (10 µg), tetracycline (30 µg) and erythromycin (15 µg). All tests were conducted in duplicate.

In general, the phenotypic results are in agreement with those commonly found in the genus *Bradyrhizobium*. However, it is worth mentioning some unusual features detected in this study: CNPSo 4010^T showed a neutral reaction on modified-YMA with bromothymol blue as indicator and the three strains from this study presented optimal growth on modified-YMA at 37 °C in 3–4 days, indicating a possible mechanism of high temperature tolerance. CNPSo 4019^T was able to grow in a large pH range, pH 4.0–8.0, while strain CNPSo 4016^T grew well at pH 8.0. Carbohydrate metabolism by API 50CH was heterogeneous among the strains and revealed slightly incongruences with the universal culture medium used to grow rhizobia (YMA), since the CNPSo strains weakly used D-mannitol and also glycerol, another C source commonly used for this culture media. Interestingly, CNPSo 4016^T was positive or weak for metabolism of all carbohydrate sources available on the kit. Although our findings revealed different metabolic features, the phenotypic characteristics are generally encoded by the accessory genome, being unstable over time. Also, they may present different results according to the laboratory conditions [81, 82]. The differential phenotypical features among the strains from this study and closest species are reported in Table 4.

Nodulation and nitrogen fixation assays were carried out in Leonard jars with soybean commercial cultivar BRASMAX Potência RR and with the promiscuous papilionoid siratro (*Macroptilium atropurpureum*). Each jar was sterilized with sand, vermiculite (2:1, v:v) and the N-free nutrient solution described by Broughton and Dilworth [83]. The strains were cultured in modified-YM medium [25] and inoculated on the seeds (1 ml) after planting. The plants grew under controlled glasshouse conditions for 30 days. All strains were able to form effective red colour nodules with siratro but only strain CNPSo 4019^T was able to effectively nodulate soybean, its original host (Fig. S9).

Therefore, the phylogenetic, genomic and phenotypic data accomplished in this study support the proposed descriptions of the novel species *Bradyrhizobium agreste* sp. nov., *Bradyrhizobium glycinis* sp. nov. and *Bradyrhizobium diversitatis* sp. nov., isolated from three different *Glycine* species, for which the type strains are CNPSo 4010^T, CNPSo 4016^T and CNPSo 4019^T, respectively.

DESCRIPTION OF *BRADYRHIZOBIUM AGRESTE* SP. NOV.

Bradyrhizobium agreste (a.gres'te. L. neut. adj. *agreste*, wild, referring to the importance of isolation from wild species, such as the *Glycine clandestina* from this study).

Cells are Gram-stain-negative, aerobic and non-spore-forming. Colonies on modified-YMA medium at pH 6.8–7.0 and Congo red are slightly pink, less than 1 mm in diameter, circular, opaque, with low mucus production and a gummy consistency after 7 days of growth at 28 °C. The strain shows a neutral reaction on modified-YMA with bromothymol blue as indicator and weak urease activity. CNPSo 4010^T shows weak growth at pH 4.0 and 8.0. The strain is able to grow at 37 °C for 4 days, but unable to grow on solid LB medium and modified-YMA containing 1% NaCl. With respect to carbon sources in the API test, the strain is able to use starch, glycogen, D-se, L-fucose and potassium 2-ketogluconate; weakly uses glycerol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, methyl β-D-xylopyranoside, D-galactose, D-glucose, D-fructose, D-mannose, L-sorbose, D-mannitol, *N*-acetylglucosamine, aesculin ferric citrate, D-fucose, D-arabitol and potassium 5-ketogluconate; does not use erythritol, D-adonitol, L-rhamnose, dulcitol, inositol, D-sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, amygdalin, arbutin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose, xylitol, gentiobiose, turanose, D-tagatose, L-arabitol and potassium gluconate. The strain is tolerant to the antibiotics ampicillin (10 µg), bacitracin (10 U), chloramphenicol (30 µg), nalidixic acid (30 µg) and penicillin G (10 U), moderately sensitive to neomycin (30 µg) and tetracycline (30 µg) and sensitive to cefuroxime (30 µg), erythromycin (15 µg) and streptomycin (10 µg). The strain is able to form effective nitrogen-fixing nodules with *Macroptilium atropurpureum*, but does not nodulate *Glycine max*.

The type strain, CNPSo 4010^T (=WSM 4802^T=LMG 31645^T), was isolated from a nodule of *Glycine clandestina* in Kununurra, Australia. The DNA G+C content of strain CNPSo 4010^T is 63.8 mol%.

DESCRIPTION OF *BRADYRHIZOBIUM GLYCINIS* SP. NOV.

Bradyrhizobium glycinis (gly.ci'nis. N.L. gen. n. *glycinis*, of the genus *Glycine*, a genus that encompasses host plants of several *Bradyrhizobium* species, including this new species, isolated from *G. tabacina*).

Cells are Gram-stain-negative, aerobic and non-spore-forming. Colonies on modified-YMA medium at pH 6.8–7.0 and Congo red are slightly pink, less than 1 mm in diameter, circular, opaque and exhibit low mucus production with a gummy consistency after 7 days of growth at 28 °C. The strain shows an alkaline reaction on modified-YMA with bromothymol blue and weak urease activity. CNPSo 4016^T shows weak growth at pH 4.0 but grows well at pH 8.0 and at 37 °C after 4 days. The strain is unable to grow on solid LB medium and modified-YMA containing 1% NaCl. With respect to carbon sources in the API test, strain CNPSo 4016^T is able to use D-arabinose, amygdalin, aesculin ferric citrate, starch, turanose, D-ose, D-fucose, L-fucose, L-arabitol, potassium gluconate, potassium 2-ketogluconate and potassium 5-ketogluconate; weakly uses glycerol, erythritol, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, methyl β-D-xylopyranoside, D-galactose, D-glucose, D-fructose, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl

Table 4. Distinctive phenotypical properties of new lineages of *Bradyrhizobium* and closely related strains

Strains: 1, *Bradyrhizobium agreste* CNPSo 4010^T; 2, *Bradyrhizobium glycinis* CNPSo 4016^T; 3, *Bradyrhizobium diversitatis* CNPSo 4019^T; 4, *Bradyrhizobium liaoningense* LMG 18230^T; 5, *Bradyrhizobium yuanmingense* CCBAU 10071^T. Data are evaluated as: +, growth; w, weakly positive; –, no growth. NA, No data available.

Characteristic	1	2	3	4*	5†
Carbon source utilization:					
Erythritol	–	w	–	–	–
D-Arabinose	w	+	w	+	w
L-Arabinose	w	w	w	+	+
D-Ribose	w	w	w	+	+
D-Xylose	w	+	w	+	+
L-Xylose	w	+	+	+	+
D-Adonitol	–	w	–	–	–
Methyl β-D-xylopyranoside	w	w	w	–	w
D-Galactose	w	w	–	w	w
D-Fructose	w	w	–	–	w
L-Sorbose	w	w	w	–	–
L-Rhamnose	–	w	w	w	w
Dulcitol	–	–	w	–	–
Inositol	–	w	–	–	–
D-Mannitol	w	w	w	–	w
D-Sorbitol	–	w	w	–	–
Methyl α-D-mannopyranoside	–	–	w	–	–
Methyl α-D-glusopyranoside	–	w	w	–	–
N-Acetylglucosamine	w	–	w	–	–
Amygdalin	–	+	w	–	–
Arbutin	–	w	w	–	–
Aesculin ferric citrate	w	+	+	+	+
Salicin	–	w	w	–	–
Cellobiose	–	w	w	–	–
Maltose	–	w	w	–	w
Lactose	–	w	w	–	–
Melibiose	–	w	w	–	–
Sucrose	–	w	w	–	–
Trehalose	–	w	w	w	–
Inulin	–	w	w	–	–
Melezitose	–	w	w	–	–
Raffinose	–	w	w	–	–
Glycogen	+	w	+	+	–
Xylitol	–	w	–	–	–

Continued

Table 4. Continued

Characteristic	1	2	3	4*	5†
Gentiobiose	–	w	w	–	–
Turanose	–	+	w	–	–
D-Lyxose	+	+	w	+	+
D-Tagatose	–	w	w	–	–
D-Fucose	w	+	w	+	+
D-Arabitol	w	w	w	w	–
L-Arabitol	–	+	–	–	–
Potassium gluconate	–	+	+	–	+
Potassium 2-ketogluconate	+	+	+	–	+
Potassium 5-ketogluconate	w	+	+	–	+
Enzymatic activity:					
Urease	w	w	+	+	NA
Growth at/in:					
pH 4	w	w	+	+	–
pH 8	w	+	+	+	+
Alkaline reaction	w	+	+	+	+
37 °C	+ (4 days)	+ (4 days)	+ (3 days)	–	+
Tolerance to antibiotics:					
Ampicillin (10 µg)	+	–	+	–	w
Bacitracin (10 U)	+	+	+	+	NA
Erythromycin (15 µg)	–	–	+	+	+
Neomycin (30 µg)	w	w	+	–	–
Streptomycin (10 µg)	–	–	–	–	+
Tetracycline (30 µg)	w	–	+	+	+

*Data obtained from Urquiaga *et al.* [42].
†Data obtained from Delamuta *et al.* [37]; Yao *et al.* [62] and Grönemeyer *et al.* [84].

α-D-mannopyranoside, methyl α-D-glucopyranoside, *N*-acetylglucosamine, arbutin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose, glycogen, xylitol, gentiobiose, D-tagatose and D-arabitol. The strain is tolerant to bacitracin (10 U), chloramphenicol (30 µg), nalidixic acid (30 µg) and penicillin G (10 U), moderately sensitive to neomycin (30 µg) and sensitive to ampicillin (10 µg), cefuroxime (30 µg), erythromycin (15 µg), streptomycin (10 µg) and tetracycline (30 µg). The strain is able to form effective nitrogen-fixing nodules with *Macroptilium atropurpureum* but does not nodulate *Glycine max*.

The type strain CNPSo 4016^T (=WSM 4801^T=LMG 31649^T) was isolated from a nodule of *Glycine tabacina* in Kununurra, Australia. The DNA G+C content of strain CNPSo 4016^T is 63.7 mol%.

DESCRIPTION OF *BRADYRHIZOBIUM DIVERSITATIS* SP. NOV.

Bradyrhizobium diversitatis (di.ver.si.ta'tis. L. fem. n. *diversitas* diversity, N.L. gen. n. *diversitatis*, of diversity, referring to the importance of studies on microbial diversity revealing, as in the case of this study, genetic richness of *Bradyrhizobium* isolated from *Glycine max*).

Cells are Gram-stain-negative, aerobic and non-spore-forming. Colonies on modified-YMA medium at pH 6.8–7.0 and Congo red are slightly pink, less than 1 mm in diameter,

circular, opaque and exhibit low mucus production with a gummy consistency after 7 days of growth at 28 °C. The strain shows alkaline reaction on modified-YMA with bromothymol blue, is urease-positive and able to grow at pH 4.0 and 8.0. CNPSo 4019^T is able to grow at 37 °C after 3 days. The strain is unable to grow on solid LB medium and modified-YMA containing 1% NaCl. With respect to carbon sources in the API test, strain CNPSo 4019^T is able to use L-xylose, aesculin ferric citrate, starch, glycogen, L-fucose, potassium gluconate, potassium 2-ketogluconate, and potassium 5-ketogluconate; weakly uses glycerol, D-arabinose, L-arabinose, D-ribose, D-xylose, D-adonitol, methyl β-D-xylopyranoside, D-glucose, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, *N*-acetylglucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose, gentiobiose, turanose, D-lyxose, D-tagatose, D-fucose and D-arabitol; does not use erythritol, D-galactose, D-fructose, xylitol and L-arabitol. The strain is tolerant to the antibiotics ampicillin (10 µg), bacitracin (10 U), chloramphenicol (30 µg), erythromycin (15 µg), nalidixic acid (30 µg), neomycin (30 µg), penicillin G (10 U) and tetracycline (30 µg); and is sensitive to cefuroxime (30 µg) and streptomycin (10 µg). The strain is able to form nitrogen-fixing nodules with *Macroptilium atropurpureum* and *Glycine max*.

The type strain, CNPSo 4019^T (=WSM 4799^T=LMG 31650^T), was isolated from a nodule of *Glycine max* in Nambung, Australia. The DNA G+C content of strain CNPSo 4019^T is 63.8 mol%.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

References

1. **Dixon R, Kahn D**. Genetic regulation of biological nitrogen fixation. *Nat Rev Microbiol* 2004;2:621–631.
2. **De Bruijn FJ**. Biological nitrogen fixation. In: Lugtenberg B (editor). *Principles of Plant-Microbe Interactions: Microbes for Sustainable Agriculture*, 1st ed. Switzerland: Springer International Publishing; 2015. pp. 1–448.
3. **Peix A, Ramírez-Bahena MH, Velázquez E, Bedmar EJ**. Bacterial associations with legumes. *CRC Crit Rev Plant Sci* 2015;34:17–42.
4. **Mus F, Crook MB, Garcia K, Garcia Costas A, Geddes BA *et al.***. Symbiotic nitrogen fixation and the challenges to its extension to nonlegumes. *Appl Environ Microbiol* 2016;82:3698–3710.
5. **Ormeño-Orrillo E, Hungria M, Martinez-Romero E**. Dinitrogen-fixing prokaryotes. In: Rosenberg E, DeLong E, Stackebrandt E, Lory S, Thompson F (editors). *The Prokaryotes: Prokaryotic Physiology and Biochemistry*, 4th ed. Berlin Heidelberg: Springer-Verlag; 2013. pp. 427–451.
6. **Bashan Y, de-Bashan LE, Prabhu SR, Hernandez JP**. Advances in plant growth-promoting bacterial inoculant technology: formulations and practical perspectives (1998-2013). *Plant Soil* 2014;378:1–33.
7. **Hungria M, Loureiro MF, Mendes IC, Campo RJ, Graham PH**. Inoculant preparation, production and application. In: Werner D, Newton WE (editors). *Nitrogen Fixation in Agriculture, Forestry, Ecology, and the Environment*, 4th ed. Dordrecht, The Netherlands: Springer; 2005. pp. 223–246.
8. **Hungria M, Mendes IC**. Nitrogen fixation with soybean: the perfect symbiosis? In: de Bruijn FJ (editor). *Biological Nitrogen Fixation*. John Wiley & Sons, Inc; 2015. pp. 1009–1023.
9. **Xu LM, Ge C, Cui Z, Li J, Fan H**. *Bradyrhizobium liaoningense* sp. nov., isolated from the root nodules of soybeans. *Int J Syst Bacteriol* 1995;45:706–711.
10. **Wang JY, Wang R, Zhang YM, Liu HC, Chen WF *et al.***. *Bradyrhizobium daqingense* sp. nov., isolated from soybean nodules. *Int J Syst Evol Microbiol* 2013;63:616–624.
11. **Yu X, Cloutier S, Tambong JT, Bromfield ESP**. *Bradyrhizobium ottawaense* sp. nov., a symbiotic nitrogen fixing bacterium from root nodules of soybeans in Canada. *Int J Syst Evol Microbiol* 2014;64:3202–3207.
12. **Menna P, Hungria M, Barcellos FG, Bangel EV, Hess PN *et al.***. Molecular phylogeny based on the 16S rRNA gene of elite rhizobial strains used in Brazilian commercial inoculants. *Syst Appl Microbiol* 2006;29:315–332.
13. **Hungria M, Menna P, Marçon Delamuta JR**. Bradyrhizobium, the ancestor of all rhizobia: phylogeny of housekeeping and nitrogen-fixation genes. In: Bruijn FJ (editor). *Biological Nitrogen Fixation*. New Jersey: Wiley, Sons, Inc; 2015. pp. 191–202.
14. **Sprent JI, Ardley J, James EK**. Biogeography of nodulated legumes and their nitrogen-fixing symbionts. *New Phytol* 2017;215:40–56.
15. **Klepa MS, Urquiaga MCdeO, Somasegaran P, Delamuta JRM, Ribeiro RA *et al.***. *Bradyrhizobium niftali* sp. nov., an effective nitrogen-fixing symbiont of partridge pea [*Chamaecrista fasciculata* (Michx.) Greene], a native caesalpinioid legume broadly distributed in the USA. *Int J Syst Evol Microbiol* 2019;69:3448–3459.
16. **Ferraz Helene LC, O'Hara G, Hungria M, Helene LCF, O'Hara G**. Characterization of *Bradyrhizobium* strains indigenous to Western Australia and South Africa indicates remarkable genetic diversity and reveals putative new species. *Syst Appl Microbiol* 2020;43:126053.
17. **Pueppke SG**. Nodulating associations among rhizobia and legumes of the genus *Glycine* subgenus *Glycine*. *Plant an* 1988;109:189–193.
18. **Hungria M, Franchini JC, Campo RJ, Graham PH**. The importance of nitrogen fixation to soybean cropping in South America. In: Werner D, Newton WE (editors). *Nitrogen Fixation in Agriculture, Forestry, Ecology, and the Environment*, 4th ed. Dordrecht, The Netherlands: Springer; 2005. pp. 25–39.
19. **Pfeil BEA, Craven LAB, Brown A, Murray BGC, Doyle JJA**. Three new species of northern Australian *Glycine* (Fabaceae, Phaseolae), *G. gracei, G. montis-douglas* and *G. syndetika*. *Aust Syst Bot* 2006;19:245–258.
20. **Hermann FJ**. *A Revision of the Genus Glycine and Its Immediate Allies*. Washiton, DC: U.S.D.A. Agricultural Research Service; 1962. p. 79.
21. **González-Orozco CE, Brown AH, Knerr N, Miller JT, Doyle JJ**. Hotspots of diversity of wild Australian soybean relatives and their conservation in situ. *Conserv Genet* 2012;13:1269–1281.
22. **Doyle JJ, Doyle JJ, Doyle JL, Rauscher JT, Brown AHD**. Diploid and polyploid reticulate evolution throughout the history of the perennial soybeans (*Glycine* subgenus *Glycine*). *New Phytol* 2003;161:121–132.

23. **Stajsic V**. Flora of Victoria [Internet]. Glycine clandestina J.C.Wendl. Twining Glycine. 2018 [cited 2020 Oct 27]. Available from: https://vicflora.rbg.vic.gov.au/flora/taxon/4441818f-b0dc-40a4-b2ec-268db551d025.
24. **Herbarium Western Australia**. FloraBase—the Western Australian Flora. [Internet]. Glycine tabacina (Labill.) Benth. Glycine Pea. 1998 [cited 2020 Oct 27]. Available from: https://florabase.dpaw.wa.gov.au/browse/profile/3941.
25. **Hungria M, O'Hara GW, Zilli JE, Araujo RS, Deaker R *et al***. Isolation and growth of rhizobia. In: Howieson JG, Dilworth MJ (editors). *Working with Rhizobia*. Canberra: Australian Centre for International Agriculture Reserch (ACIAR); 2016. pp. 39–60.
26. **Delamuta JRM, Ribeiro RA, Araújo JLS, Rouws LFM, Zilli Jerri Édson, *et al***. *Bradyrhizobium stylosanthis* sp. nov., comprising nitrogen-fixing symbionts isolated from nodules of the tropical forage legume *Stylosanthes* spp. *Int J Syst Evol Microbiol* 2016;66:3078–3087.
27. **Stepkowski T, Moulin L, Krzyzańska A, McInnes A, Law IJ *et al***. European origin of *Bradyrhizobium* populations infecting lupins and serradella in soils of Western Australia and South Africa. *Appl Environ Microbiol* 2005;71:7041–7052.
28. **Delamuta JRM, Menna P, Ribeiro RA, Hungria M**. Phylogenies of symbiotic genes of *Bradyrhizobium* symbionts of legumes of economic and environmental importance in Brazil support the definition of the new symbiovars pachyrhizi and sojae. *Syst Appl Microbiol* 2017;40:254–265.
29. **Edgar RC**. Muscle: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* 2004;32:1792–1797.
30. **Schwarz G**. Estimating the dimension of a model. *Ann Stat* 1978;6:461–464.
31. **Kumar S, Stecher G, Tamura K**. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Mol Evol Genet Anal* 2016;33:1870–1874.
32. **Felsenstein J**. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 1985;39:783–791.
33. **Hedges SB**. The number of replications needed for accurate estimation of the bootstrap P value in phylogenetic studies. *Mol Biol Evol* 1992;9:366–369.
34. **Gouy M, Guindon S, Gascuel O**. SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Mol Biol Evol* 2010;27:221–224.
35. **Hall T**. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* 1999;41:95–98.
36. **Menna P, Barcellos FG, Hungria M**. Phylogeny and taxonomy of a diverse collection of *Bradyrhizobium* strains based on multilocus sequence analysis of the 16S rRNA gene, ITS region and *glnII, recA, atpD* and *dnaK* genes. *Int J Syst Evol Microbiol* 2009;59:2934–2950.
37. **Delamuta JRM, Ribeiro RA, Ormeño-Orrillo E, Melo IS, Martínez-Romero E *et al***. Polyphasic evidence supporting the reclassification of *Bradyrhizobium japonicum* group Ia strains as *Bradyrhizobium diazoefficiens* sp. nov. *Int J Syst Evol Microbiol* 2013;63:3342–3351.
38. **Ferraz Helene LC, Marçon Delamuta JR, Augusto Ribeiro R, Ormeño-Orrillo E, Antonio Rogel M *et al***. *Bradyrhizobium viridifuturi* sp. nov., encompassing nitrogen-fixing symbionts of legumes used for green manure and environmental services. *Int J Syst Evol Microbiol* 2015;65:4441–4448.
39. **Kim M, Oh H-S, Park S-C, Chun J**. Towards a taxonomic coherence between average nucleotide identity and 16S rRNA gene sequence similarity for species demarcation of prokaryotes. *Int J Syst Evol Microbiol* 2014;64:346–351.
40. **Delamuta JRM, Ribeiro RA, Ormeño-Orrillo E, Parma MM, Melo IS *et al***. *Bradyrhizobium tropiciagri* sp. nov. and *Bradyrhizobium embrapense* sp. nov., nitrogen-fixing symbionts of tropical forage legumes. *Int J Syst Evol Microbiol* 2015;65:4424–4433.
41. **Helene LCF, Delamuta JRM, Ribeiro RA, Hungria M**. *Bradyrhizobium mercantei* sp. nov., a nitrogen-fixing symbiont isolated from nodules of *Deguelia costata* (syn. Lonchocarpus costatus). *Int J Syst Evol Microbiol* 2017;67:1827–1834.
42. **Urquiaga MCdeO, Klepa MS, Somasegaran P, Ribeiro RA, Delamuta JRM *et al***. *Bradyrhizobium frederickii* sp. nov., a nitrogen-fixing lineage isolated from nodules of the caesalpinioid species *Chamaecrista fasciculata* and characterized by tolerance to high temperature *in vitro*. *Int J Syst Evol Microbiol* 2019;69:3863–3877.
43. **Tang J, Bromfield ESP, Rodrigue N, Cloutier S, Tambong JT**. Microevolution of symbiotic *Bradyrhizobium* populations associated with soybeans in east North America. *Ecol Evol* 2012;2:2943–2961.
44. **Gevers D, Cohan FM, Lawrence JG, Spratt BG, Coenye T *et al***. Opinion: Re-evaluating prokaryotic species. *Nat Rev Microbiol* 2005;3:733–739.
45. **Durán D, Rey L, Mayo J, Zúñiga-Dávila D, Imperial J *et al***. *Bradyrhizobium paxllaeri* sp. nov. and *Bradyrhizobium icense* sp. nov., nitrogen-fixing rhizobial symbionts of lima bean (*Phaseolus lunatus* L.) in Peru. *Int J Syst Evol Microbiol* 2014;64:2072–2078.
46. **Helene LCF, Klepa MS, O'Hara G, Hungria M**. *Bradyrhizobium archetypum* sp. nov., *Bradyrhizobium australiense* sp. nov. and *Bradyrhizobium murdochi* sp. nov., isolated from nodules of legumes indigenous to Western Australia. *Int J Syst Evol Microbiol* 2020;70:4623–4636.
47. **Fossou RK, Pothier JF, Zézé A, Perret X**. *Bradyrhizobium ivorense* sp. nov. as a potential local bioinoculant for *Cajanus cajan* cultures in Côte d'Ivoire. *Int J Syst Evol Microbiol* 2020;70:1421–1430.
48. **Schultze M, Kondorosi A**. Regulation of symbiotic root nodule development. *Annu Rev Genet* 1998;32:33–57.
49. **Lloret L, Martínez-romero E**. Evolución y filogenia de *Rhizobium*. *Rev Latinoam Microbiol* 2005;47:43–60.
50. **Jones KM, Kobayashi H, Davies BW, Taga ME, Walker GC**. How rhizobial symbionts invade plants: the *Sinorhizobium-Medicago* model. *Nat Rev Microbiol* 2007;5:619–633.
51. **Menna P, Hungria M**. Phylogeny of nodulation and nitrogen-fixation genes in *Bradyrhizobium*: supporting evidence for the theory of monophyletic origin, and spread and maintenance by both horizontal and vertical transfer. *Int J Syst Evol Microbiol* 2011;61:3052–3067.
52. **Rogel MA, Ormeño-Orrillo E, Martinez Romero E, Romero EM**. Symbiovars in rhizobia reflect bacterial adaptation to legumes. *Syst Appl Microbiol* 2011;34:96–104.
53. **Li YH, Wang R, Sui XH, Wang ET, Zhang XX *et al***. Bradyrhizobium nanningense sp. nov., Bradyrhizobium guangzhouense sp. nov. and Bradyrhizobium zhanjiangense sp. nov., isolated from effective nodules of peanut in Southeast China. *Syst Appl Microbiol* 2019;42:126002.
54. **Lu JK, Dou YJ, Zhu YJ, Wang SK, Sui XH *et al***. *Bradyrhizobium ganzhouense* sp. nov., an effective symbiotic bacterium isolated from *Acacia melanoxylon* R. Br. nodules. *Int J Syst Evol Microbiol* 2014;64:1900–1905.
55. **Overbeek R, Olson R, Pusch GD, Olsen GJ, Davis JJ *et al***. The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). *Nucleic Acids Res* 2014;42:206–214.
56. **Chun J, Oren A, Ventosa A, Christensen H, Arahal DR *et al***. Proposed minimal standards for the use of genome data for the taxonomy of prokaryotes. *Int J Syst Evol Microbiol* 2018;68:461–466.
57. **de Lajudie PM, Andrews M, Ardley J, Eardly B, Jumas-Bilak E *et al***. Minimal standards for the description of new genera and species of rhizobia and agrobacteria. *Int J Syst Evol Microbiol* 2019;69:1852–1863.
58. **Goris J, Konstantinidis KT, Klappenbach JA, Coenye T, Vandamme P *et al***. DNA-DNA hybridization values and their relationship to whole-genome sequence similarities. *Int J Syst Evol Microbiol* 2007;57:81–91.
59. **Mahato NK, Gupta V, Singh P, Kumari R, Verma H *et al***. Microbial taxonomy in the era of OMICS: application of DNA sequences, computational tools and techniques. *Antonie van Leeuwenhoek* 2017;110:1357–1371.

60. **Rodriguez-R LM, Konstantinidis KT**. The enveomics collection: a toolbox for specialized analyses of microbial genomes and metagenomes. *Peer J Prepr* 2016;4:e1900v1.
61. **Meier-Kolthoff JP, Auch AF, Klenk H-P, Göker M**. Genome sequence-based species delimitation with confidence intervals and improved distance functions. *BMC Bioinformatics* 2013;14:1–14.
62. **Yao ZY, Kan FL, Wang ET, Wei GH, Chen WX**. Characterization of rhizobia that nodulate legume species of the genus *Lespedeza* and description of *Bradyrhizobium yuanmingense* sp. nov. *Int J Syst Evol Microbiol* 2002;52:2219–2230.
63. **Versalovic J, Schneider M, de BFJ, Lupski JR**. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods Mol Cell Biol* 1994;5:25–40.
64. **Chibeba AM, Kyei-boahen S, Guimarães MF, Nogueira MA, Hungria M**. *Isolation, Characterization and Selection of Indigenous Bradyrhizobium Strains with Outstanding Symbiotic Performance to Increase Soybean Yields in Mozambique*, 246. Agric Ecosyst Environ. Elsevier; 2017. pp. 291–305.
65. **Sneath P, Sokal R**. *Numerical Taxonomy: the Principles and Practice of Numerical Classification*. San Francisco: Freeman; 1973. p. 573.
66. **Jaccard P**. The distribution of flora in the alpine zone. *New Phytol* 1912;11:37–50.
67. **Hymowitz T**. On the domestication of the soybean. *Econ Bot* 1970;24:408–421.
68. **Hymowitx T, Newell CA**. Taxonomy of the genus *Glycine*, domestication and uses of soybeans. *Econ Bot* 1981;35:272–288.
69. **Ruiz Sainz JE, Zhou JC, Rodriguez-Navarro D-N, Vinardell JM, Thomas-Oates JE**. Soybean cultivation and BNF in China. In: Werner D, Newton WE (editors). *Nitrogen Fixation in Agriculture, Forestry, Ecology, and the Environment*. Springer; 2005. pp. 68–85.
70. **Hymowitz T, Singh RJ, Larkin RP**. Long-distance dispersal: the case for the allopolyploid glycine tabacina (Labill.) Benth. and G. tomentella Hayata in the West-Central Pacific. *Micronesica* 1990;23:5–13.
71. **Sniderman JMK, Jordan GJ**. Extent and timing of floristic exchange between Australian and Asian rain forests. *J Biogeogr* 2011;38:1445–1455.
72. **Buerki S, Forest F, Alvarez N**. Proto-South-East Asia as a trigger of early angiosperm diversification. *Bot J Linn Soc* 2014;174:326–333.
73. **Lie TA, Göktan D, Engin M, Pijnenborg J, Anlarsal E**. Co-evolution of the legume-Rhizobium association. *Plant Soil* 1987;100:171–181.
74. **Man CX, Wang H, Chen WF, Sui XH, Wang ET *et al***. Diverse rhizobia associated with soybean grown in the subtropical and tropical regions of China. *Plant Soil* 2008;310:77–87.
75. **Li QQ, Wang ET, Zhang YZ, Zhang YM, Tian CF *et al***. Diversity and biogeography of rhizobia isolated from root nodules of *Glycine max* grown in Hebei Province, China. *Microb Ecol* 2011;61:917–931.
76. **Appunu C, N'Zoue A, Laguerre G**. Genetic diversity of native *Bradyrhizobia* isolated from soybeans (*Glycine max* L.) in different agricultural-ecological-climatic regions of India. *Appl Environ Microbiol* 2008;74:5991–5996.
77. **Appunu C, Sasirekha N, Prabavathy VR, Nair S**. A significant proportion of indigenous rhizobia from India associated with soybean (Glycine max L.) distinctly belong to Bradyrhizobium and Ensifer genera. *Biol Fertil Soils* 2009;46:57–63.
78. **Han LL, Wang ET, Han TX, Liu J, Sui WH *et al***. Unique community structure and biogeography of soybean rhizobia in the saline-alkaline soils of Xinjiang, China. *Plant and* 2009;324:291–305.
79. **Hungria M, Chueire Lígia Maria de O, Coca RG, Megías M**. Preliminary characterization of fast growing rhizobial strains isolated from soyabean nodules in Brazil. *Soil Biol Biochem* 2001;33:1349–1361.
80. **Bauer AW, Kirby WMM, Sherris JC, Turck M**. Antibiotic susceptibility testing by a standardized single disk method. *Am Jounal Clin Pathol* 1966;36:493–496.
81. **Ormeño-Orrillo E, Martínez-Romero E**. Phenotypic tests in *Rhizobium* species description: an opinion and (a sympatric speciation) hypothesis. *Syst Appl Microbiol* 2013;36:145–147.
82. **Rosselló-Móra R, Amann R**. Past and future species definitions for bacteria and archaea. *Syst Appl Microbiol* 2015;38:209–216.
83. **Yates R, Howieson J, Hungria M, Bala A, O'Hara G *et al***. Authentication of rhizobia and assessment of the legume symbiosis in controlled plant growth systems. In: Howieson J, Dilworth M (editors). *Working with Rhizobia*. Canberra: Australian Centre for International Agricultural Research (ACIAR); 2016. pp. 73–108.
84. **Lasse Grönemeyer J, Hurek T, Reinhold-Hurek B**. *Bradyrhizobium kavangense* sp. nov., a symbiotic nitrogen-fixing bacterium from root nodules of traditional Namibian pulses. *Int J Syst Evol Microbiol* 2015;65:4886–4894.

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SUPPLEMENTARY MATERIAL

Bradyrhizobium agreste **sp. nov.,** ***Bradyrhizobium glycinis*** **sp. nov. and** ***Bradyrhizobium diversitatis*** **sp. nov., isolated from a biodiversity hotspot of the genus** ***Glycine*** **in Western Australia**

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Table S1. Accession numbers of 16S rRNA, *atpD*, *dnaK*, *glnII*, *gyrB, recA* and *rpoB* used in this study.

Strains	16S	*atpD*	*dnaK*	*glnII*	*gyrB*	*recA*	*rpoB*
B. agreste CNPSo 4010^T	MK676048	MT683853	MK674804	MK860831	MK860852	MK863432	JACCHP000000000
B. glycinis CNPSo 4016^T	MK676047	MT683854	MK674803	MK860830	MK860851	MK863431	JACCHQ000000000
B. diversitatis CNPSo 4019^T	MK676046	MT683855	MK674802	MK860829	MK860850	MK863430	JACEGD000000000
B. algeriense RST89^T	PYCM01000001.1	KF956544.1	FJ264922.1	FJ264924.1	PYCM01000001.1	FJ264927.1	-
B. americanum CMVU44^T	KU991833.1	KC247125.1	-	KX012942.1	-	KC247141.1	-
B. amphicarpaeae 39S1MB^T	KP768779	KP768547.1	CP029426	KP768605.1	KP768721.1	KF615002.1	KP768663.1
B. arachidis CCBAU 051107^T	NZ_FPBQ00000000.1	HM107217.1	JX437668.1	HM107251.1	JX437675.1	HM107233.1	JX437682.1
B. archetypum WSM 1744^T	MK676065	MT070745	MK674820	MK860847	MK860868	MK863448	JAAVLW000000000
B. australiense WSM 1791^T	MK676067	MT070746	MK674822	MK860849	MK860870	MK863450	JAAVLX000000000
B. betae LMG 21987^T	AY372184	FM253129	AY923046.1	AB353733.1	FM253217	AB353734.1	FM253260
B. brasilense UFLA03-321^T	NZ_MPVQ00000000.1	KF452730.1	KF452791.1	NZ_MPVQ00000000	KF452827.1	KT793142.1	KF452879.1
B. cajani AMBPC1010^T	KY349447.1	NZ_WQNE01000000	NZ_WQNE00000000	KY349442.1	NZ_WQNE00000000	KY349440.1	NZ_WQNE01000005.1
B canariense LMG 22265^T	AJ558025.1	AY386739.1	AY923047.1	AY386765.1	FM253220	FM253177	FM253263
B. centrolobii BR 10245^T	KF927049.1	NZ_LUUB01000000	KX527928.1	KX527991.1	KX528004.1	KX527954.1	KF983827.3
B. centrosemae A9^T	KC247115.1	KC247129.1	-	KX012940.1	-	KC247145.1	-
B. cytisi CTAW11^T	EU561065.2	GU001613.1	JQ945184.1	GU001594.1	JN186292.1	GU001575.1	JN186288.1
B. daqingense CGMCC 1.10947^T	NZ_VLKL01000000	HQ231289.1	KF962684.1	HQ231301.1	JX437669.1	HQ231270.1	JX437676.1
B. denitrificans LMG 8443^T	X66025.1	FM253153.1	KF962685.1	HM047121.1	FM253239.1	FM253196.1	FM253282.1
B. diazoefficiens USDA 110^T	NC_004463.1	NC_004463.1	NC_004463.1	NC_004463.1	NC_004463.1	NC_004463.1	NC_004463.1
B. elkanii USDA 76^T	U35000	AY386758.1	AY328392.1	AY599117.1	AM418800	AY591568.1	AM295348
B. embrapense CNPSo 2833^T	AY904773.1	HQ634875	KP234519.2	GQ160500	HQ634891	HQ634899	HQ634910.1
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B. ferriligni CCBAU 51502^T	KJ818096.1	-	MG811657.1	KJ818099.1	KJ818102.1	KJ818112.1	MG811655.1
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B. guangzhouense CCBAU 51670^T	CP030053.1	KC508902.1	KC508950.1	KC509008.1	KC509057.1	KC509254.1	CP030053.1
B. huanghuaihaiense CCBAU 23303^T	HQ231463.1	HQ231682.1	JX437665.1	HQ231639.1	JX437672.1	HQ231595.1	HQ587647.1
B. icense LMTR 13^T	KF896156	KF896192	KF896182	KF896175	KF896201	JX943615	NZ_CP016428.1
B. ingae BR 10250^T	KF927043.1	KY753593.1	KF927055.1	KF927067.1	KF927079.1	KF927061.1	KF927073.1
B. iriomotense EK05^T	AB300992	AB300994.1	JF308944.1	AB300995	AB300997	AB300996	HQ587646.1
B. ivorense CI-1B^T	KX396570.1	CAADFC000000000	MK376326	MH756157	MH756161	MK376330	KX388393.1
B. japonicum USDA 6^T	X66024	AM168320	AM168362	AF169582	AM418801	AM182158	AM295349
B. jicamae PAC68^T	AY624134	FJ428211	JF308945.1	FJ428204	HQ873309.1	HM047133.1	HQ587647
B. kavangense 14-3^T	KP899562.1	KY753592.1	KR259949.1	KM378446.1	KX661397.1	KM378399.1	KM378311.1
B. lablabi CCBAU 23086^T	NZ_LLYB00000000.1	GU433473.1	KF962687.1	GU433498.1	JX437670.1	GU433522.1	JX437677
B. liaoningense LMG 18230^T	AF208513	AY386752.1	AY923041.1	AY386775.1	FM253223	AY591564.1	FM253266
B. lupini USDA 3051^T	KM114861.1	KU738808.1	-	KM114862.1	-	KM114866.1	-
B. macuxiense BR 10303^T	NZ_LNCU00000000.1	NZ_LNCU01000024.1	KX527932.1	KX527995.1	KX528008.1	KX527958.1	KX527969.1
B. manausense BR 3351^T	HQ641226.2	NZ_LJYG01000000	KF786001.1	KF785986.1	KF786000.1	KF785992.1	KF785998.1
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B. oligotrophicum LMG 10732^T	JQ619230.1	JQ619232.1	KF962688.1	JQ619233.1	KF962697.1	JQ619231.1	KF962713.1
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B. stylosanthis BR 446^T	NZ_LVEM01000003.1	NZ_LVEM00000000	KU724145	KU724148	KU724151	KU724163.1	KU724166
B. subterraneum 58 2-1^T	KP308152.1	KX661391.1	KP308157.1	KM378484.1	KX661396.1	KM378397	KM378349.1
B. symbiodeficiens 85S1MB^T	KP768783.1	KP768551.1	CP029427	KP768609.1	KP768725.1	KF615036.1	KP768667.1
B. tropiciagri CNPSo 1112^T	AY904753.1	FJ390968	FJ391008.1	FJ391048	HQ634890	FJ391168	HQ634909.1
B. uaiense UFLA 03-164^T	NZ_VKHP01000993.1	KF452739.1	KF452780.1	NZ_VKHP00000000	KT793133.1	KT793144.1	NZ_VKHP00000000
B. valentinum LmjM3^T	JX514883.2	JX518561.2	NZ_LLXX01000028.1	JX518575	NZ_LLXX01000044.1	JX518589.2	-
B. vignae 7-2^T	NZ_RDQF00000000.1	KX683215.1	KR259951.1	KM378443.1	KX683216.1	KM378374.1	KM378308.1
B. viridifuturi SEMIA 690^T	FJ025107.1	NZ_LGTB01000039.1	KR149128	KR149131.1	KR149134.1	KR149140	KU724169.1
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Legends of Supplementary Figures

Figure S1. Maximum likelihood phylogeny based from alignment of the *atpD* (398 bp), using the GTR: General Time Reversible +G+I model by MEGA v. 7. Accession numbers are indicated in parentheses and in Table S1. The novel species are shown in bold. Bootstrap values >70 % are indicated at the nodes. *Xanthobacter autotrophicus* Py2 was used as outgroup. Bar indicates five substitution per 100 nucleotide positions.

Figure S2. Maximum likelihood phylogeny based from alignment of the *dnaK* (221 bp), using the T93: Tamura-Nei +G model by MEGA v. 7. Accession numbers are indicated in parentheses and in Table S1. The novel species is shown are bold. Bootstrap values >70 % are indicated at the nodes. *Xanthobacter autotrophicus* Py2 was used as outgroup. Bar indicates five substitutions per 100 nucleotide positions.

Figure S3. Maximum likelihood phylogeny based from alignment of the *glnII* (504 bp), using the GTR: General Time Reversible +G+I model by MEGA v. 7. Accession numbers are indicated in parentheses and in Table S1. The novel species are shown in bold. Bootstrap values >70 % are indicated at the nodes. *Xanthobacter autotrophicus* Py2 was used as outgroup. Bar indicates two substitutions per 100 nucleotide positions.

Figure S4. Maximum likelihood phylogeny based from alignment of the *gyrB* (553 bp), using the GTR: General Time Reversible +G+I model by MEGA v. 7. Accession numbers are indicated in parentheses and in Table S1. The novel species are shown in bold. Bootstrap values >70 % are indicated at the nodes. *Xanthobacter autotrophicus* Py2 was used as outgroup. Bar indicates five substitutions per 100 nucleotide positions.

Figure S5. Maximum likelihood phylogeny based from alignment of the *recA* (360 bp), using the Tamura 3-Parameter +G model by MEGA v. 7. Accession numbers are indicated in parentheses and in Table S1. The novel species are shown in bold. Bootstrap values >70 % are indicated at the nodes. *Xanthobacter autotrophicus* Py2 was used as outgroup. Bar indicates five substitutions per 100 nucleotide positions.

Figure S6. Maximum likelihood phylogeny based from alignment of the *rpoB* (371 bp), using the GTR: General Time Reversible +G model by MEGA v. 7. Accession numbers are indicated in parentheses and in Table S1. The novel species are shown in bold. Bootstrap values >70 % are indicated at the nodes. *Xanthobacter autotrophicus* Py2 was used as outgroup. Bar indicates two substitution per 100 nucleotide positions.

Figure S7. Maximum likelihood phylogeny based from alignment of the *atpD+dnaK+glnII+gyrB+recA+rpoB* concatenated (11,704 bp), using the GTR: General Time Reversible +G+I model by MEGA v. 7. Accession numbers are indicated in parentheses. The novel species are shown in bold. Bootstrap values >70 % are indicated at the nodes. *Xanthobacter*

autotrophicus Py2 was used as outgroup. Bar indicates five substitution per 100 nucleotide positions.

Figure S8. Dendrogram of similarity based on the BOX-PCR profiles of strains in study and closely related *Bradyrhizobium* species, performed with the program Bionumerics (Applied Mathematics, Kortrijk, Belgium, v.7.6) using the UPGMA algorithm (Unweighted Pair-Group Method with Arithmetic mean) and the Jaccard coefficient, with 2 % tolerance.

Figure S9. Siratro with effective red color nodules formed by (a) *B. agreste* CNPSo 4010^{T}, (b) *B. glycinis* CNPSo 4016^{T} and (c) *B. diversitatis* CNPSo 4019^{T}; (d) soybean, the original host plant of *B. diversitatis* CNPSo 4019^{T}, inoculated by the strain; (e) roots of soybean inoculated with *B. diversitatis* CNPSo 4019^{T}. Plants were grown under axenic conditions at the glasshouse.

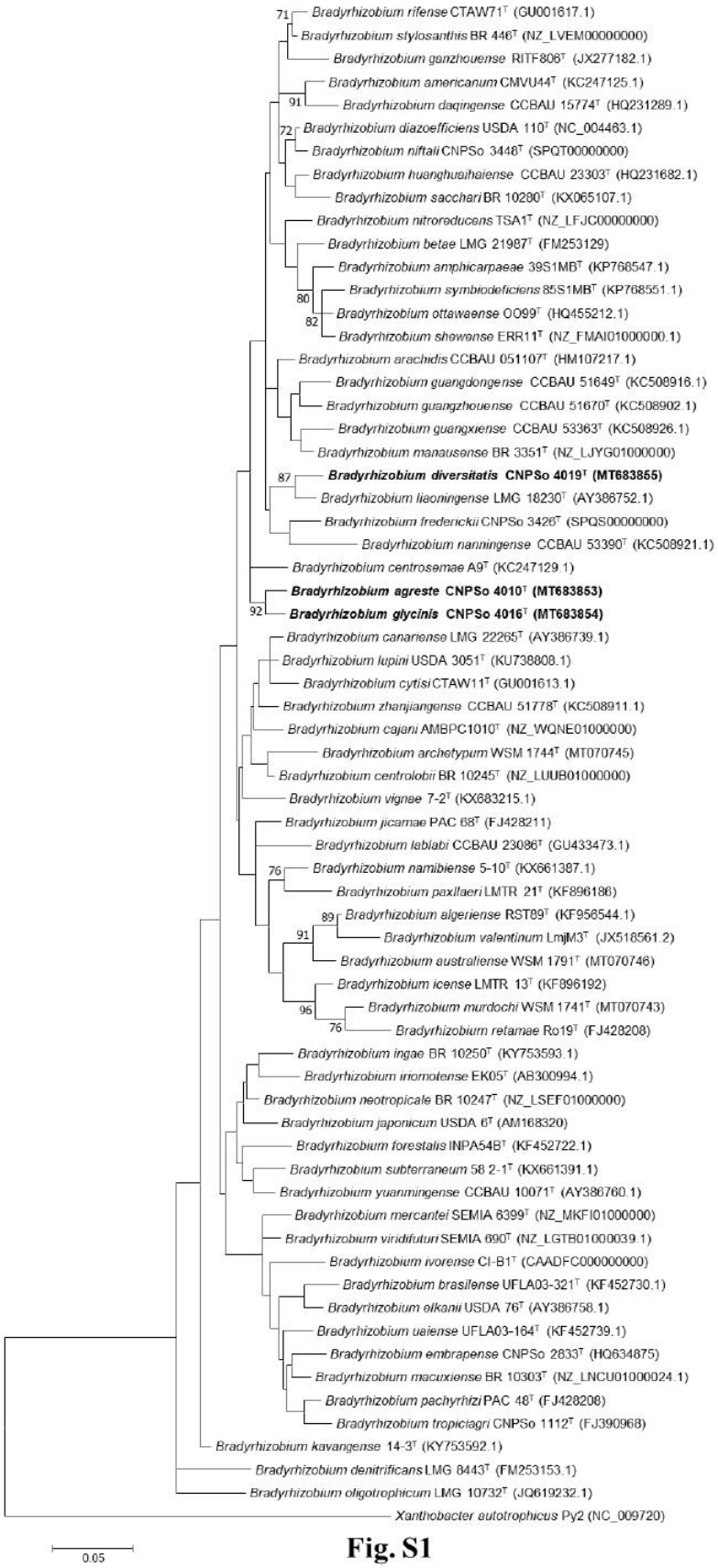
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Fig. S1

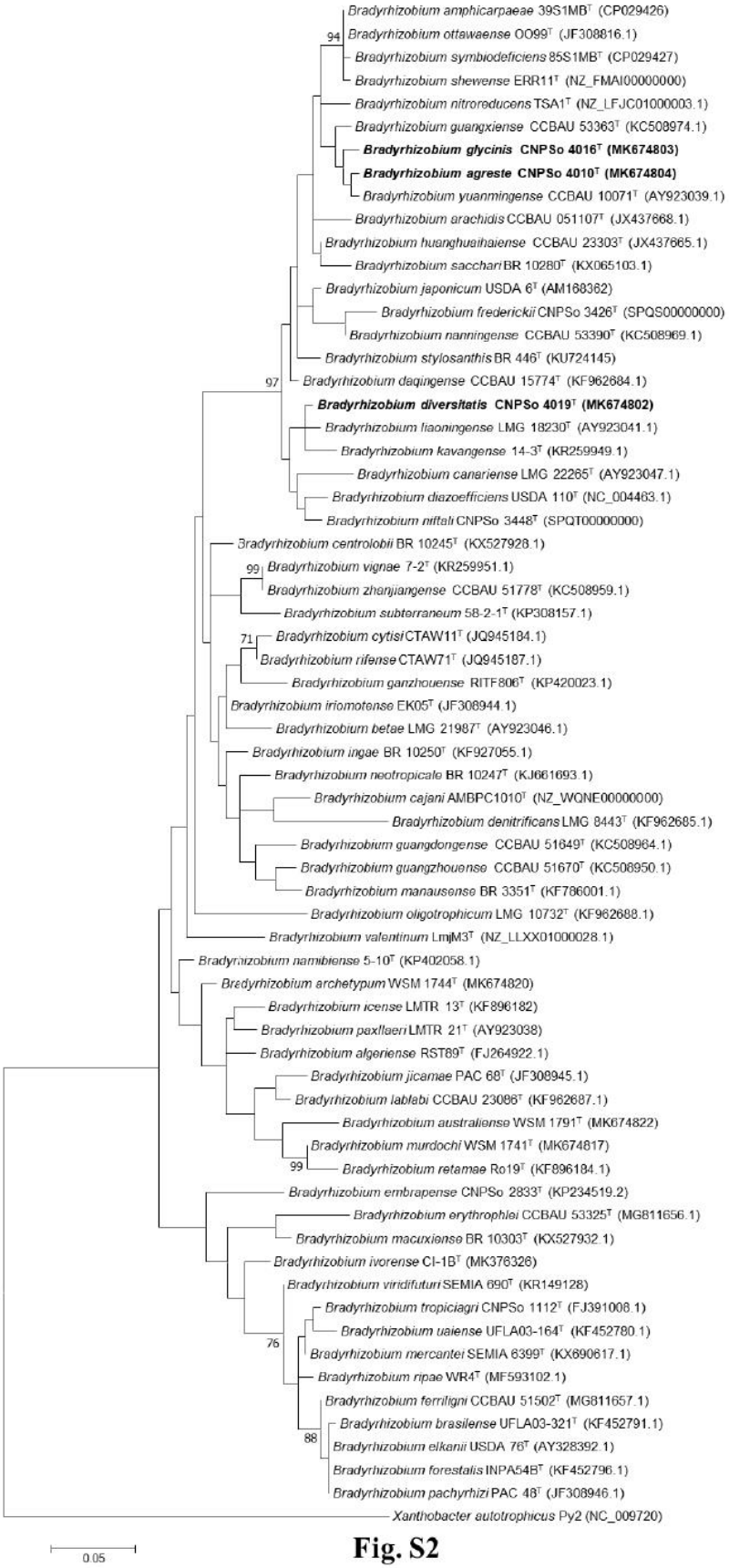
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Fig. S2

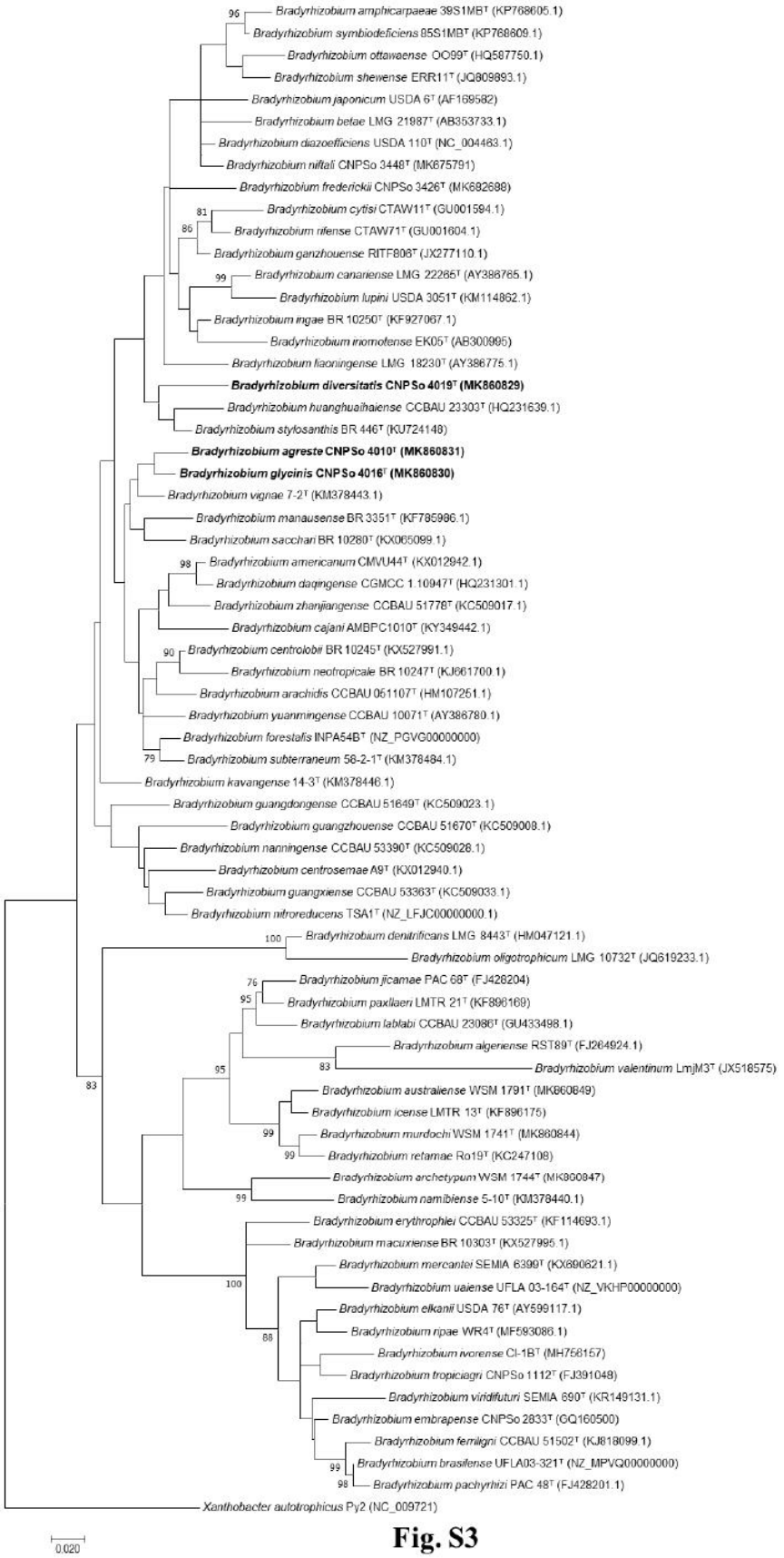
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Fig. S3

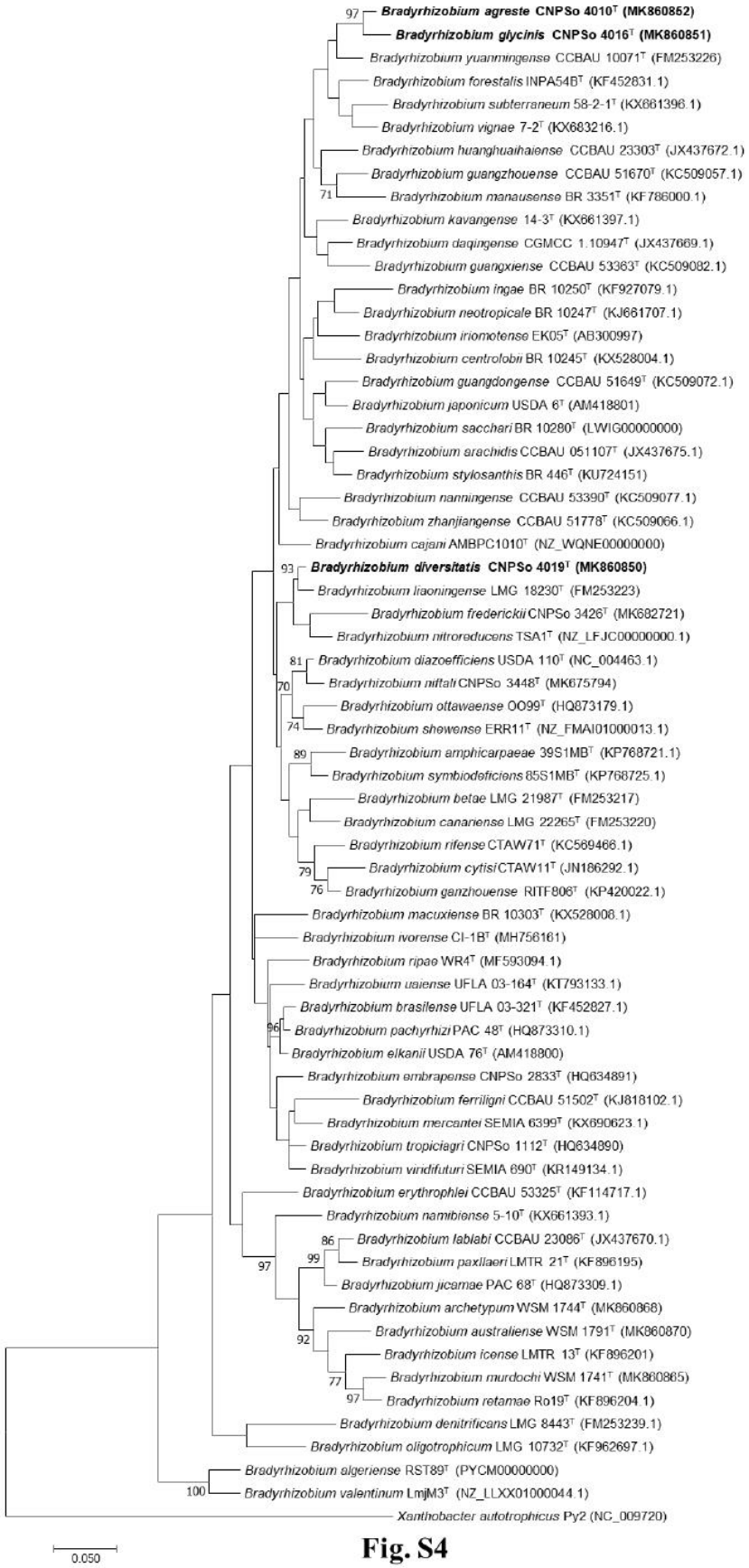
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Fig. S4

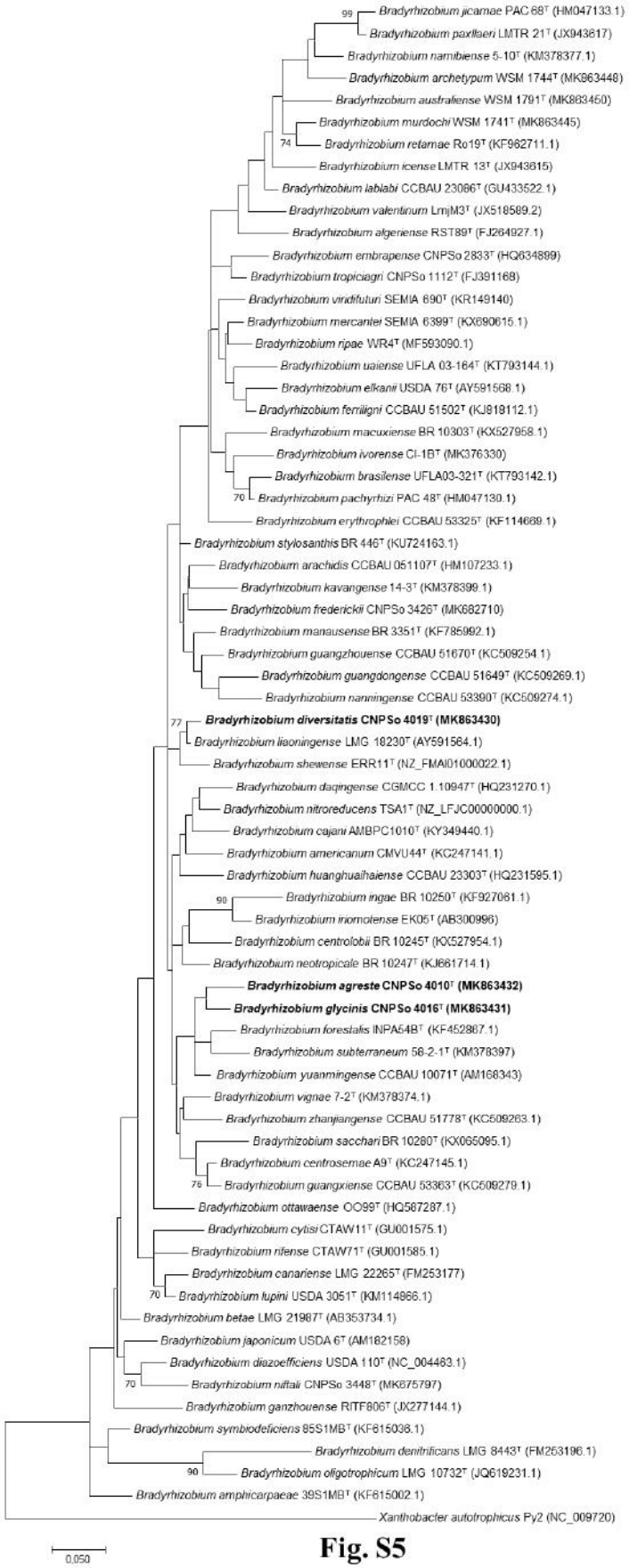
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Fig. S5

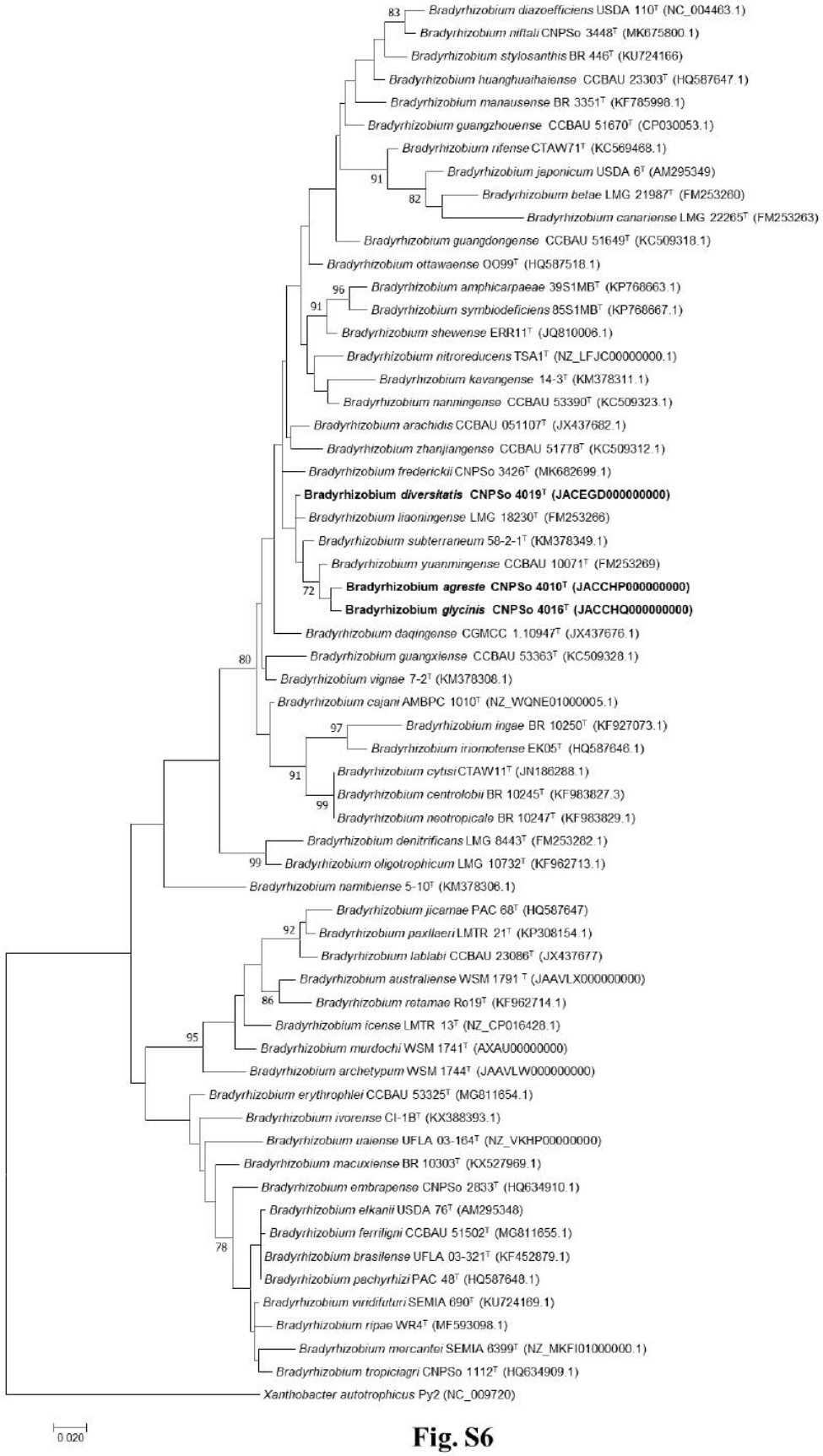
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Fig. S6

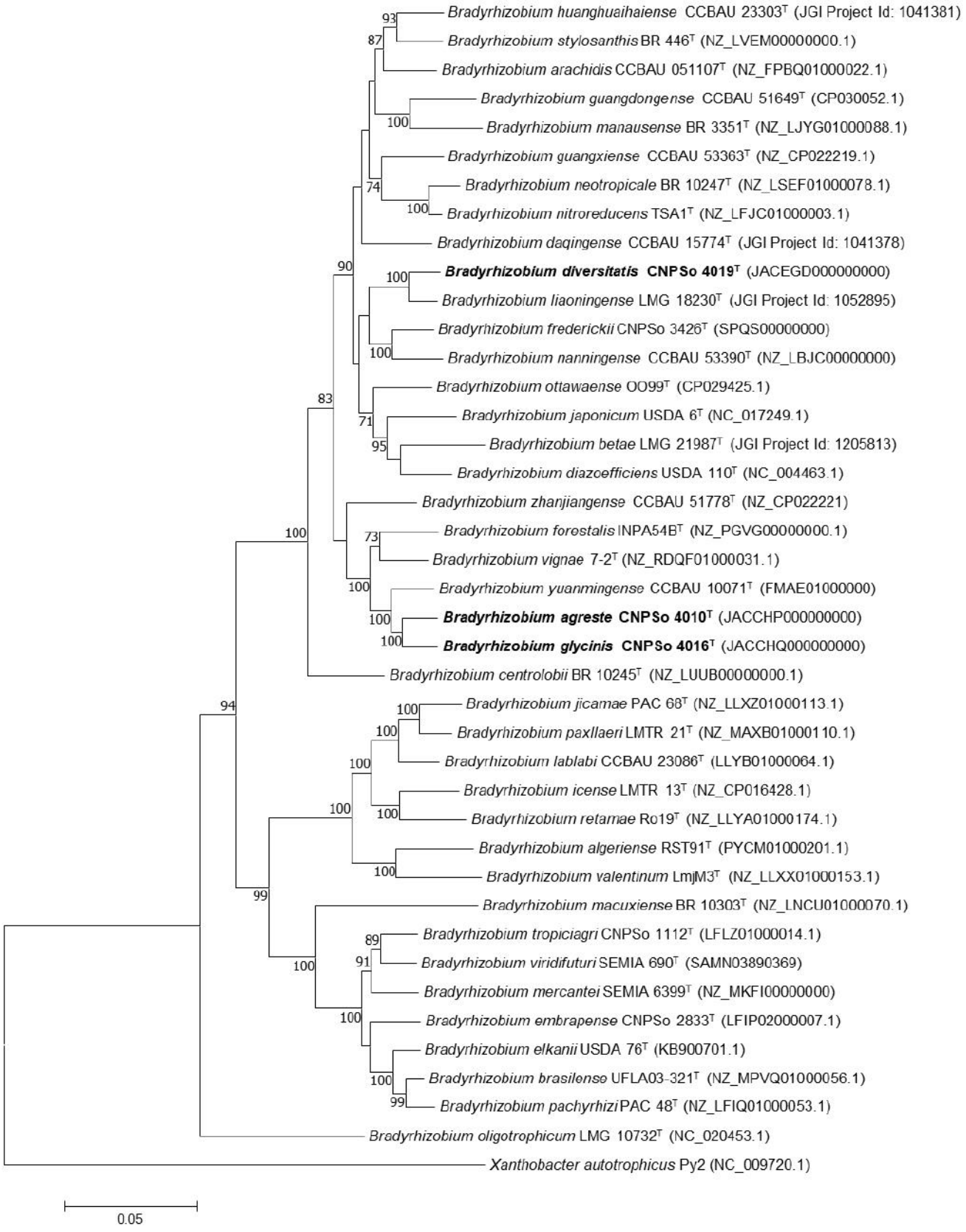
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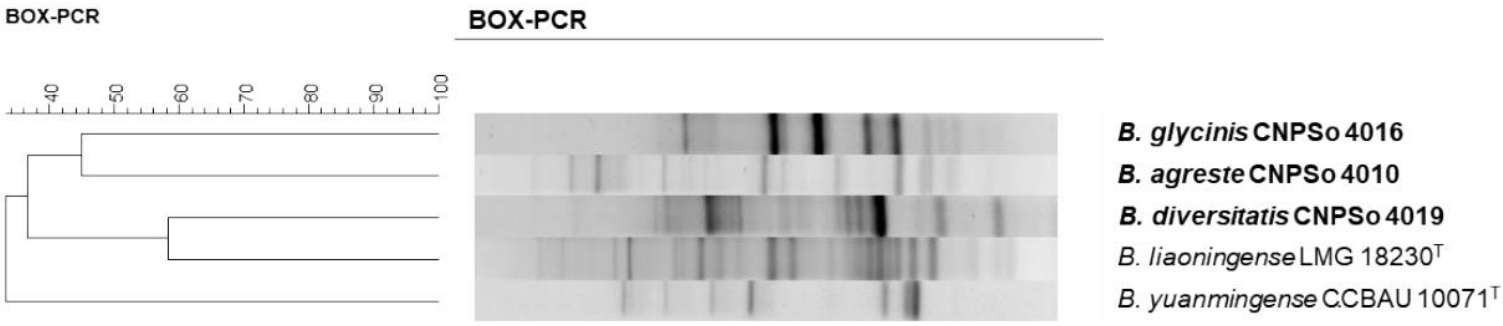
Fig. S7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B. liaoningense LMG 18230T
B. yuanmingense CCBAU 10071T

Fig. S8

Fig. S9

CAPÍTULO IV

Bradyrhizobium cenepequi sp. nov., *Bradyrhizobium semiaridum* sp. nov., *Bradyrhizobium hereditatis* sp. nov. E *Bradyrhizobium australafricanum* sp. nov., SIMBIONTES DE DIFERENTES PLANTAS LEGUMINOSAS DA AUSTRÁLIA OCIDENTAL E ÁFRICA DO SUL E A DEFINIÇÃO DE TRÊS NOVOS SIMBIOVARES.

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RESUMO

Bradyrhizobium é um gênero heterogêneo de bactérias capazes de estabelecer associações simbióticas com uma ampla faixa de leguminosas hospedeiras, incluindo espécies de importância econômica e ambiental. O objetivo do estudo foi a definição taxonômica e de simbiovares de quatro estirpes – CNPSo 4026^T, WSM 1704^T, WSM 1738^T e WSM 4400^T – previamente isoladas de nódulos de leguminosas na Austrália Ocidental e África do Sul. A árvore filogenética do gene 16S RNAr alocou as estirpes no supergrupo *Bradyrhizobium elkanii.* O *multilocus sequence analysis* (MLSA) utilizando sequências parciais de seis genes *housekeeping – atpD, dnaK, glnII, gyrB, recA* e *rpoB* – não agrupou as estirpes em estudo como coespecíficas de nenhuma espécie de *Bradyrhizobium* descrita. Os valores da análise de *average nucleotide identity* e hibridação DNA-DNA digital foram calculados para as quatro estirpes em estudo e para as espécies mais próximas na filogenia do MLSA, sendo que 95.46 e 62.20%, respectivamente, foram os valores mais altos; portanto, ambos os valores são mais baixos que os valores de corte para o delineamento de espécies. As filogenias de *nodC* e *nifH* incluíram as estirpes WSM 1738^T e WSM 4400^T nos simbiovares retamae e vignae, respectivamente. A filogenia dos genes simbióticos também permitiu a definição de três novos simbiovares, sv. cenepequi, sv. glycinis e sv. cajani. A caracterização morfofisiológica reforçou a identificação das quatro novas espécies de *Bradyrhizobium* propostas que foram nomeadas como *Bradyrhizobium cenepequi* sp. nov. (CNPSo 4026^T=WSM 4798^T=LMG 31653^T), isolada de *Vigna unguiculata; Bradyrhizobium semiaridum* sp. nov. (WSM 1704^T=CNPSo 4028^T=LMG 31654^T),

isolada de *Tephrosia gardneri*; *Bradyrhizobium hereditatis* sp. nov. (WSM 1738^{T}=CNPSo 4025^{T}=LMG 31652^{T}), isolada de *Indigofera* sp.; e *Bradyrhizobium australafricanum* sp. nov. (WSM 4400^{T}=CNPSo 4015^{T}=LMG 31648^{T}) isolada de *Glycine* sp.

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Bradyrhizobium cenepequi sp. nov., *Bradyrhizobium semiaridum* sp. nov., *Bradyrhizobium hereditatis* sp. nov. and *Bradyrhizobium australafricanum* sp. nov., symbionts of different leguminous plants of Western Australia and South Africa and definition of three novel symbiovars

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Abstract

Bradyrhizobium is a heterogeneous bacterial genus capable of establishing symbiotic associations with a broad range of legume hosts, including species of economic and environmental importance. This study was focused on the taxonomic and symbiovar definition of four strains – CNPSo 4026^T, WSM 1704^T, WSM 1738^T and WSM 4400^T – previously isolated from nodules of legumes in Western Australia and South Africa. The 16S rRNA gene phylogenetic tree allocated the strains to the *Bradyrhizobium elkanii* supergroup. The multilocus sequence analysis (MLSA) with partial sequences of six housekeeping genes – *atpD*, *dnaK*, *glnII*, *gyrB*, *recA* and *rpoB* – did not cluster the strains under study as conspecific to any described *Bradyrhizobium* species. Average nucleotide identity and digital DNA–DNA hybridization values were calculated for the four strains of this study and the closest species according to the MLSA phylogeny with the highest values being 95.46 and 62.20%, respectively; therefore, both being lower than the species delineation cut-off values. The *nodC* and *nifH* phylogenies included strains WSM 1738^T and WSM 4400^T in the symbiovars retamae and vignae respectively, and also allowed the definition of three new symbiovars, sv. cenepequi, sv. glycinis, and sv. cajani. Analysis of morphophysiological characterization reinforced the identification of four novel proposed *Bradyrhizobium* species that are accordingly named as follows: *Bradyrhizobium cenepequi* sp. nov. (CNPSo 4026^T=WSM 4798^T=LMG 31653^T), isolated from *Vigna unguiculata*; *Bradyrhizobium semiaridum* sp. nov. (WSM 1704^T=CNPSo 4028^T=LMG 31654^T), isolated from *Tephrosia gardneri*; *Bradyrhizobium hereditatis* sp. nov. (WSM 1738^T=CNPSo 4025^T=LMG 31652^T), isolated from *Indigofera* sp.; and *Bradyrhizobium australafricanum* sp. nov. (WSM 4400^T=CNPSo 4015^T=LMG 31648^T) isolated from *Glycine* sp.

INTRODUCTION

Nitrogen is the nutrient most required by plants and is incorporated into a variety of molecules essential for plant growth, especially DNA, RNA and proteins [1]. The main natural input of N into the biosphere occurs via biological nitrogen fixation performed by prokaryotic organisms [2]. A special group of bacteria, collectively called rhizobia, is able to fix atmospheric nitrogen (N_2) in symbiosis with species of the Fabaceae (=Leguminosae) in specialized structures called nodules, mostly formed on roots and, occasionally, on stems [1]. The symbiosis between rhizobia and legumes is reliant upon various genes; the rhizobial *nif* and *fix* genes are key to the synthesis and regulation of nitrogenase, the enzyme responsible for the reduction of N_2, whereas

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Keywords: *Bradyrhizobium*; MLSA; genome of prokaryotes; ANI; dDDH; symbiovars.
Abbreviations: ANI, average nucleotide identity; dDDH, digital DNA–DNA hybridization; HGT, horizontal gene transfer; LB, Luria–Bertani; ML, maximum-likelihood; MLSA, multilocus sequence analysis; NI, nucleotide identity; YMA, yeast–mannitol agar.
Genome and 16S rRNA accession numbers of *B. cenepequi* CNPSo 4026^T (JAGKJI000000000 and MK676055), *B. semiaridum* WSM 1704^T (JAGKJJ000000000 and MK676057); *B. hereditatis* WSM 1738^T (JAGKJK000000000 and MK676061); *B. australafricanum* WSM 4400^T (JAGKJL000000000 and MK676054).
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Ten supplementary figures and three supplementary tables are available with the online version of this article.

the nodulation process depends on the expression of a group of genes referred to as *nod*, *noe* and *nol* genes [3]. The core *nod* genes are generally located in the *nodABC* operon, which is responsible for the synthesis of the main structure of the Nod factor, lipochitooligosaccharide molecules responsible for bacterial infection and nodule organogenesis [4, 5]; *nodD* is a regulatory gene located upstream of the *nod* gene operon responsible for starting Nod factor synthesis [3, 5]. The remaining proteins coded by *nod*, *noe* and *nol* genes are involved in the modification of the Nod factor structure in order to ensure host specificity [4, 5].

Bradyrhizobium is one of the largest and most intriguing genera of rhizobia and can be isolated from nodules of a broad host-range of legumes, including both ancient and more recently evolved species from the Papilionoideae and Caesalpinioideae subfamilies [6, 7]. Many *Bradyrhizobium* strains associate with crops of great agronomic importance, such as soybean (*Glycine max* (L.) Merr.) [6]. In addition to those known to have a symbiotic lifestyle, some non-symbiotic *Bradyrhizobium* ecotypes are found living freely in soils [8], while others have the ability to promote plant growth when in endophytic associations [9, 10]. The genus also has strains which are highly effective nodulators of the non-legume *Parasponia* [11], and some strains have both the ability to photosynthesize and to nodulate legumes without the Nod factor mechanism [12, 13].

In view of the broad host-range of the genus, several symbiovars have been described within the genus *Bradyrhizobium*. The term symbiovar (sv.) was coined by Rogel *et al.* [14] and refers to lineages of different or the same species that are able to establish symbiosis with distinct leguminous species, these entities are differentiated on host range and symbiotic phylogenies. Currently, there are 12 symbiovars described for *Bradyrhizobium*, based mainly on the phylogeny of the *nodC* gene, chosen due to its key role in the synthesis of the Nod factor: sv. glycinearum, sv. genistearum, sv. retamae, sv. vignae, sv. sierranevadense, sv. centrosemae, sv. phaseolarum, sv. tropici, sv. pachyrhizi, sv. sojae, sv. lupini and sv. septentrionale [15–22].

Despite the increasing number of studies reporting great genetic diversity in *Bradyrhizobium* from a great variety of ecosystems [e.g. 6, 15–22], genomic and statistical studies suggest that a far higher number of genotypes estimated at 800 species still remain to be described [23, 24]. Here we delineate and describe four novel *Bradyrhizobium* species based on a polyphasic approach, as well as three novel symbiovars based on *nodC* and *nifH* phylogenies, increasing the current knowledge of *Bradyrhizobium* diversity and the evolutionary history of the rhizobia–legume symbiosis.

ISOLATION AND ECOLOGY

The four novel species described in this study have recently been characterized by Helene *et al.* [25] and emphasize the high diversity of *Bradyrhizobium* strains isolated from indigenous legumes of Western Australia and South Africa. Strain CNPSo 4026^T was isolated from root nodules of *Vigna unguiculata* used as trapping host in Western Australian soils. Strain WSM 4400^T was isolated from nodules of *Glycine* sp., a legume used for cattle forage, grown in Stutterheim, Eastern Cape, in the Amathole District, South Africa. WSM 4400^T is deposited at the WSM Culture Collection and was chosen for the study due to a slower growing property *in vitro*. Strains WSM 1704^T and WSM 1738^T were isolated from *Tephrosia gardneri* and *Indigofera* sp., respectively, in Western Australia by Yates *et al.* [26]. Details of the origin of these strains, as well as the type strains used in this study are shown in Table 1.

All strains are deposited at the 'Diazotrophic and Plant Growth Promoting Bacteria Culture Collection of Embrapa Soja' (WFCC Collection no. 1213, WDCM Collection no. 1054), in Londrina, State of Parana, Brazil, as well as at the Western Australian Soil

Table 1. Strains used in this study

Species/strain name	Other nomenclatures	Original host species	Geographical origin	Reference
***B. cenepequi* CNPSo 4026^T**	WSM 4798^T=LMG 31653^T	*Vigna unguiculata*	Salmon Gums, WA	Helene *et al.* [25]
***B. semiaridum* WSM 1704^T**	CNPSo 4028^T=LMG 31654^T	*Tephrosia gardneri*	Carnarvon, WA	Yates *et al.* [26]
***B. hereditatis* WSM 1738^T**	CNPSo 4025^T=LMG 31652^T	*Indigofera* sp.	Cape Range National Park, WA	Yates *et al.* [26]
***B. australafricanum* WSM 4400^T**	CNPSo 4015^T=LMG 31648^T	*Glycine* sp.	Amathole District, South Africa	Helene *et al.* [25]
B. archetypum WSM 1744^T	CNPSo 4013^T=LMG 31646^T	*Muelleranthus trifoliolatus*	Wooramel, WA	Helene *et al.* [38]
B. brasilense UFLA03-321^T	CBAS645^T=LMG 29353^T	*Vigna unguiculata*	Minas Gerais, Brazil	Costa *et al.* [66]
B. elkanii USDA 76^T	CNPSo 62^T=LMG 6134^T	*Glycine max*	USA	Kuykendall *et al.* [64]
B. ivorense CI-1B^T	CCOS 1862^T=CCMM^T=B1296^T	*Cajanus cajan*	Ivory Coast	Fossou *et al.* [59]
B. pachyrhizi PAC48^T	CECT 7396^T=LMG 24246^T	*Pachyrhizus erosus*	Costa Rica	Ramírez-Bahena *et al.* [67]

Microbiology Gene Bank (WSM Culture Collection), at the Belgian Coordinated Collections of Microorganisms (BCCM/LMG), and also at the Culture Collection of the Department of Microbiology of the University of Seville, Spain.

The strains were maintained on modified-yeast extract–mannitol agar (YMA) medium [27] at 4 °C in a cold room for short-term preservation and were lyophilized and stored in modified-yeast extract–mannitol (YM) broth with 30% (v/v) glycerol at −80 °C and −150 °C by cryopreservation for long-term storage, as previously described [28].

PHYLOGENY

The 16S rRNA gene, and four housekeeping genes (*dnaK*, *glnII*, *gyrB* and *recA*) were previously amplified and sequenced [25]. The accession numbers of the 16S rRNA sequences of the strains are: CNPSo 4026^T (MK676055), WSM 1704^T (MK676057), WSM 1738^T (MK676061) and WSM 4400^T (MK676054). Complete sequences of the housekeeping genes *atpD*, *dnaK*, *glnII*, *gyrB*, *recA* and *rpoB* were also retrieved from the genomes of strains CNPSo 4026^T, WSM 1704^T, WSM 1738^T, WSM 4400^T and other *Bradyrhizobium* type strains with available genomes in the GenBank database of the National Center for Biotechnology Information (NCBI: www.ncbi.nlm.nih.gov). The partial and complete housekeeping gene datasets were used to reconstruct phylogenetic trees of single and concatenated genes.

The symbiotic gene, *nodC*, was amplified and sequenced as previously described [25]. Sequences of *nodC* and *nifH* were also retrieved from the available genomes of the strains used in this study. The symbiovar definition was based upon the phylogeny of the *nodC* and *nifH* symbiotic genes.

The accession numbers of all sequences used in this study are listed in Table S1 and, whenever possible, in parentheses on the phylogenetic trees. MEGA software version 7 [29] was used to obtain the multiple sequence alignments using the MUSCLE algorithm [30], and to reconstruct the maximum-likelihood (ML) phylogenies based on the evolutionary distance models inferred by the lowest Bayesian information criterion scores [31], with 1000 times bootstrap re-sampling [32, 33]. The evolutionary model used for each phylogeny is listed in the corresponding figure caption. In the multilocus sequence analysis (MLSA), the concatenation of the complete and partial housekeeping gene sequences was performed manually. Although nucleotide identity (NI) is a mathematic and not a phylogenetic parameter, the NI values of specific genes can be used for species delimitation. BioEdit version 7.0.4.1 [34] was used to calculate NI, and the values are indicated in the manuscript and in Table 2, Table S2 as well as Table S3. Since we used the same single gene alignment to reconstruct the phylogenies and matrix of identity, the results were discussed together.

Based on previous molecular evidence and on a robust phylogenetic analysis of the ribosomal region of the genus *Bradyrhizobium*, Menna *et al.* [35] highlighted that 16S rRNA analyses were able to divide the genus into two well-supported groups: the *Bradyrhizobium japonicum* and the *Bradyrhizobium elkanii* supergroups. The four strains from our study were located in the *B. elkanii* supergroup in the 16S rRNA phylogeny (1314 bp) (Fig. 1). Strain CNPSo 4026^T clustered with *B. neotropicale* BR 10247^T and *B. centrolobii* BR 10245^T with a 99% bootstrap support and 99.6 and 99% NI, respectively. Strain WSM 1738^T clustered with *B. archetypum* WSM 1744^T and *B. retamae* Ro19^T, sharing 99.8 and 99.7% NI respectively; nevertheless, the cluster had low bootstrap. Strains WSM 1704^T and WSM 4400^T clustered with eight other species with 75% bootstrap support, *B. brasilense* UFLA03-321^T, *B. pachyrhizi* PAC48^T and *B. ripae* WR4^T, all three with 99.9–100% NI, and with *B. elkanii* USDA 76^T (99.8–99.9%), *B. macuxiense* BR 10303^T (99.8–99.9%), *B. ivorense* Cl-1B^T (99.7–99.8%), *B. tropiciagri* CNPSo 1112^T (99.2–99.3%), and *B. ferriligni* CCBAU 51502^T (97.9–98%) (Table 2). High NI values were also found among the strains CNPSo 4026^T, WSM 1704^T, WSM 1738^T and WSM 4400^T, ranging from 98.4 ro 99.9% (Table 2). It is worth mentioning that the majority of the NI values found in the 16S rRNA analysis are above the 98.65% cut-off for species delineation defined by Kim *et al.* [36], confirming that the 16S rRNA gene is very conserved within the genus *Bradyrhizobium*, allowing only limited resolution for species delineation.

Taking into account the high conservation of the 16S rRNA sequences, phylogenetic trees were reconstructed with single and concatenated housekeeping datasets, as they provide more information due to these genes possessing a faster evolutionary rate [37]. The phylogenies of single housekeeping genes *atpD* (398 bp), *dnaK* (221 bp), *glnII* (504 bp), *gyrB* (553 bp), *recA* (360 bp) and *rpoB* (439 bp) were able to differentiate the strains from all described *Bradyrhizobium* species (Figs S1–S6, available in the online version of this article). In general, the phylogeny obtained of each housekeeping gene was congruent to each other (Figs S1–S6). In order to avoid possible discrepancies caused by events of recombination at a single locus, an MLSA was performed with the partial sequences of the housekeeping genes *atpD +dnaK+glnII +gyrB+recA +rpoB* (2475 bp). Based on the MLSA tree (Fig. 2) and on the NI matrix (Table 2), strain CNPSo 4026^T occupied a basal position with 89% bootstrap support; strain WSM 1704^T grouped with *B. ivorense* CI-1B^T with 99% bootstrap support and 95.2% NI; strain WSM 1738^T was clustered with *B. archetypum* WSM 1744^T and *B. namibiense* 5-10^T with 81% bootstrap support and sharing 93.2 and 93.9% NI, respectively; finally, strain WSM 4400^T remained in the same group of the species *B. brasilense* UFLA03-321^T, *B. pachyrhizi* PAC48^T and *B. elkanii* USDA 76^T with 99% bootstrap support, and 98.6, 97.9 and 97.7% NI, respectively (Fig. 2, Table 2). An MLSA based on six genes belonging to the core genome was also performed using the complete sequences of the housekeeping genes *atpD +dnaK+glnII +gyrB+rec A +rpoB* (11,676 bp) (Fig. S7) including the *Bradyrhizobium* type strains with genomes available. The evolutionary pattern was maintained for the strains of this study; however, strains WSM 1738^T and CNPSo 4026^T, presented a basal position with high

Table 2. Nucleotide Identity (NI) among new lineages of *Bradyrhizobium* and closely related species, based on the sequences of single and concatenated housekeeping genes (*atpD*, *dnaK*, *glnII*, *gyrB*, *recA* and *rpoB*) and 16S rRNA

	Nucleotide identity								
Strains	**16S rRNA (1314 bp)**	**MLSA (2475 bp)**	**MLSA (11676 bp)**	***atpD* (398 bp)**	***dnaK* (221 bp)**	***glnII* (504 bp)**	***gyrB* (553 bp)**	***recA* (360 bp)**	***rpoB* (439 bp)**
					***Bradyrhizobium cenepequi* CNPSo 4026^T**				
B. neotropicale BR 10247^T	99.6	89	89.9	90.8	87.3	88.6	88	91.6	87.5
B. centrolobii BR 10245^T	99	89.6	90.5	92.4	90.4	89.6	88.2	90.8	87.5
B. elkanii USDA 76^T	98.7	89.8	91.2	89.8	88.2	88.4	89.8	91.9	90.3
B. ivorense CI-1B^T	98.6	90.4	91.2	90.6	89.5	88.4	89.8	91.1	93.1
B. semiaridum WSM 1704^T	98.8	89.7	91	91.1	89.1	88.2	88.9	89.4	91.9
B. hereditatis WSM 1738^T	98.4	89.9	90.2	93.1	91.4	87.8	87.6	88.6	92.6
B. australafricanum WSM 4400^T	98.7	89.8	91	90.3	88.6	87.6	90	92.2	90.3
					***Bradyrhizobium semiaridum* WSM 1704^T**				
B. brasilense UFLA03-321^T	99.9	94.6	95	93.6	94.5	95.6	94.1	93.8	95.6
B. pachyrhizi PAC48^T	99.9	94.9	95.1	94.6	94.5	94.8	94.1	95.8	95.6
B. ripae WR4^T	99.9	–	–	–	95.4	94.8	93.3	93.6	96.5
B. elkanii USDA 76^T	99.8	94.4	95	94.4	94.5	95.4	94.2	91.9	95.6
B. macuxiense BR 10303^T	99.8	94	92.2	95.1	94.1	93.6	92.4	92.7	96.5
B. ivorense CI-1B^T	99.7	95.2	95.4	95.1	95.4	92.8	96.8	94.1	97
B. tropiciagri CNPSo 1112^T	99.2	94	94.8	94.4	94.5	93.8	93.5	92.7	95.4
B. ferriligni CCBAU 51502^T	97.9	–	–	–	95	94.4	90.4	93	95.4
B. cenepequi CNPSo 4026^T	98.8	89.7	91	91.1	89.1	88.2	88.9	89.4	91.9
B. hereditatis WSM 1738^T	98.4	89.9	90.9	95.4	91.4	88.6	89.1	86.6	89.1
B. australafricanum WSM 4400^T	99.9	94.4	95	94.4	94.5	95.4	94.1	92.7	95.1
					***Bradyrhizobium hereditatis* WSM 1738^T**				
B. archetypum WSM 1744^T	99.8	93.2	93.3	95.1	90	93.8	93.3	91.1	91
B. retamae Ro19^T	99.7	90.6	92.6	93.6	92.3	89.6	90.9	91.6	86.8
Bradyrhizobium namibiense 5-10^T	99.4	93.9	93.3	95.1	90	90	92.2	91.1	90.5

Continued

Table 2. Continued

Strains	16S rRNA (1314 bp)	MLSA (2475 bp)	MLSA (11676 bp)	*atpD* (398 bp)	*dnaK* (221 bp)	*glnII* (504 bp)	*gyrB* (553 bp)	*recA* (360 bp)	*rpoB* (439 bp)
	Nucleotide identity								
B. cenepequi CNPSo 4026^T	98.4	89.9	90.2	93.1	91.4	87.8	87.6	88.6	92.6
B. semiaridum WSM 1704^T	99.1	89.9	90.9	95.4	91.4	88.6	89.1	86.6	89.1
B. australafricanum WSM 4400^T	99	90.7	91	93.4	91.8	90.2	90.2	89.7	89.6
				***Bradyrhizobium australafricanum* WSM 4400^T**					
B. brasilense UFLA03-321^T	100	98.6	99	97.7	100	99.8	99	95.8	99.5
B. pachyrhizi PAC48^T	100	97.9	97.9	95.6	99.5	99	98.3	95.2	99.5
B. ripae WR4^T	100	–	–	–	97.2	96.4	95.2	96.1	97.7
B. elkanii USDA 76^T	99.9	97.7	98	97.4	99.5	96.4	98.5	96.1	99
B. macuxiense BR 10303^T	99.9	94.5	92.2	96.4	94.1	95	94.6	93	96.5
B. ivorense Cl-1B^T	99.8	94.2	94.8	96.2	95	95	94.6	93	95.1
B. tropiciagri CNPSo 1112^T	99.3	95.8	96.6	95.6	96.8	96.4	96.3	94.7	97.9
B. ferriligni CCBAU 51502^T	98	–	–	–	99	98.2	93.5	99.1	99.3
B. cenepequi CNPSo 4026^T	98.7	89.8	91	90.3	88.6	87.6	90	92.2	90.3
B. semiaridum WSM 1704^T	99.9	94.4	95	94.4	94.5	95.4	94.1	92.7	95.1
B. hereditatis WSM 1738^T	99	90.7	91	93.4	91.8	90.2	90.2	89.7	89.6

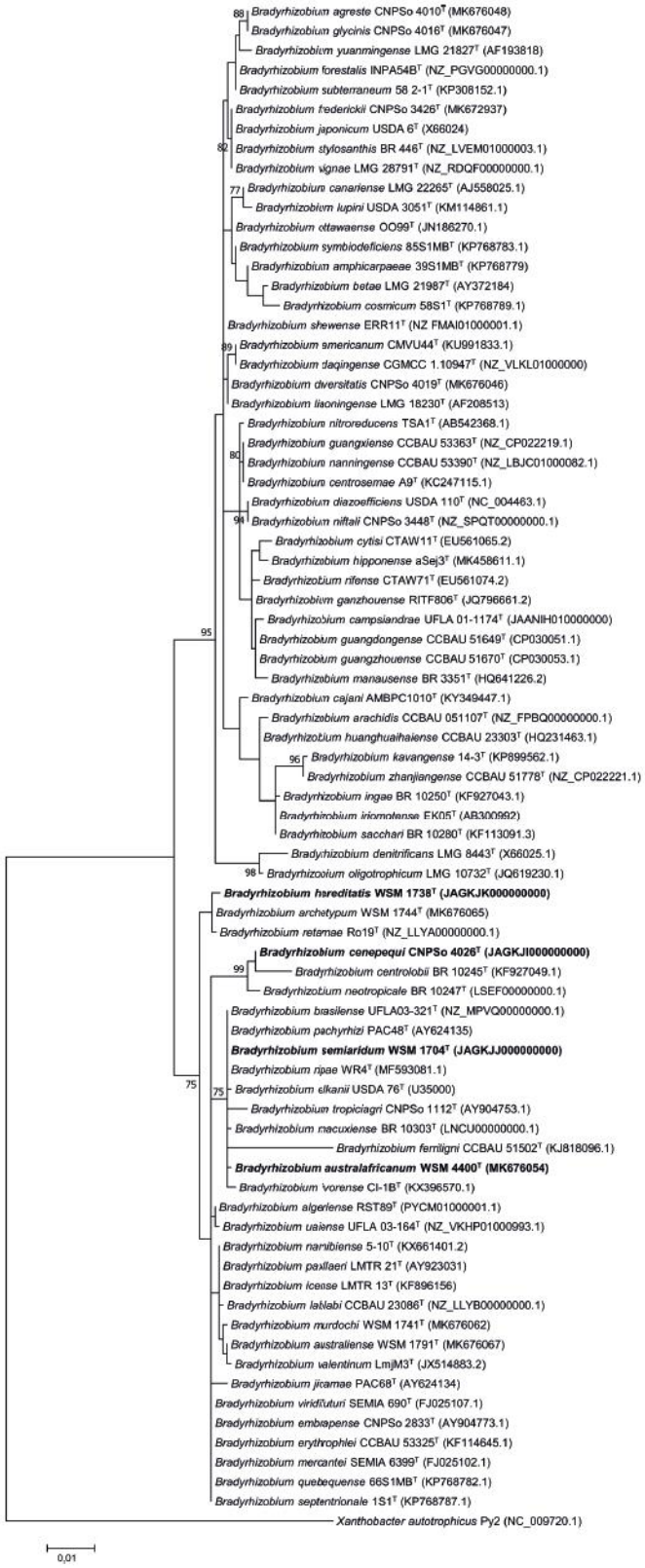

Fig. 1. Maximum-likelihood phylogeny based on the 16S rRNA gene alignment (1,314 bp), using the T92: Tamura three-parameter+G+I model in MEGA version 7. Accession numbers are indicated in parentheses and in Table S1. The novel species are shown in bold. Bootstrap values>70% are indicated at the nodes. *Xanthobacter autotrophicus* Py2 was used as outgroup. Bar indicates one substitution per 100 nucleotide positions.

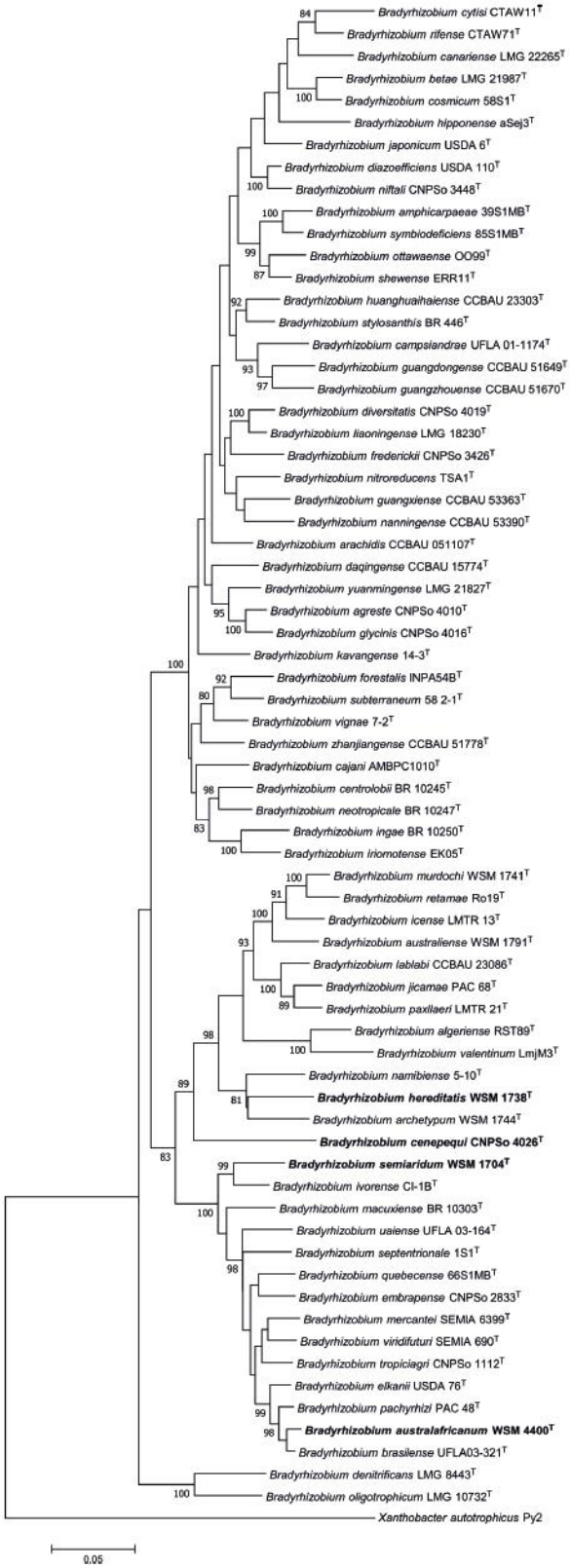

Fig. 2. Maximum-likelihood phylogeny based on concatenated alignment of the partial sequences of *atpD +dnaK+glnII +gyrB+recA +rpoB* genes (2475 bp), using the GTR: general time reversible +G+I model in MEGA version 7. Accession numbers are indicated in Table S1. The novel species are shown in bold. Bootstrap values>70% are indicated at the nodes. *Xanthobacter autotrophicus* Py2 was used as outgroup. Bar indicates five substitutions per 100 nucleotide positions.

bootstrap support to a cluster including *B. jicamae* PAC 68^T, *B. paxllaeri* LMTR 21^T, *B. lablabi* CCBAU 23086^T, *B. valentinum* LmjM3^T, *B. icense* LMTR 13^T, *B. retamae* Ro19^T and *B. murdochi* WSM 1741^T, which were recently described as belonging to the *B. jicamae* supergroup in a phylogenomic study of *Bradyrhizobium* [23]. Since the MLSA using the complete sequences is a larger dataset, the NI values and bootstrap support were slightly higher for the analyses on this dataset versus those based on the alignment with the partial sequences (Table 2), as also observed in other studies [38, 39].

Based on the phylogeny of concatenated sequences of *recA*, *atpD*, *glnII*, *dnaK* and *gyrB*, Durán *et al.* [40] suggested the cut-off of 97% for species delineation in the genus *Bradyrhizobium*. Even though the NI values of WSM 4400^T with *B. brasilense* UFLA03-321^T, *B. pachyrhizi* PAC48^T and *B. elkanii* USDA 76^T were above 97% in the MLSA with partial sequences of six housekeeping genes, that included the *rpoB* gene, strains WSM 4400^T, WSM 1738^T, WSM 1704^T and CNPSo 4026^T were clearly separated from all described *Bradyrhizobium* species, indicating that they are novel lineages.

The analysis of the symbiotic genes may reveal useful information about the evolutionary history of symbiosis in rhizobia [14, 41]. Here two genes, *nodC*, which encodes the main chito-oligosaccharide component of the Nod factor backbone, and *nifH*, encoding the iron subunit of the nitrogenase enzyme [3], were used to infer the diversity of symbiotic genes. Strain WSM 1704^T was not included in the symbiotic analysis, as we were unable to get a successful amplification from this strain with the *nodC* primer used in our study, as well as unable to find both genes in its genome. The NI of *nodC* and *nifH* genes of strains from this study and of close strains are shown in Tables S2 and S3.

The three strains from this study that had an identifiable *nodC* were positioned in three different groups in the *nodC* tree (335 bp) (Fig. 3). Strain CNPSo 4026^T, isolated from *Vigna unguiculata*, occupied a position with 88% bootstrap support and the NI values were equal to or less than 91.6% with all strains used in this analysis. Strain WSM 1738^T, isolated from nodules of *Indigofera* sp., *B. lablab* CCBAU 23086^T isolated from *Lablab purpureus* [42], *B. murdochii* WSM 1741^T from *Rhynchosia minima* [38] and *B. paxllaeri* LMTR 21^T isolated from *Phaseolus lunatus* [40] were grouped with 98% bootstrap support and shared 98.5, 98.5 and 97.6% NI, respectively (Table S2) inside the sv. retamae, which originally included strains isolated mainly from *Retama* species in Africa [16] and today allocates 11 strains. It is interesting to emphasize that all strains of sv. retamae including WSM 1738^T are unable to nodulate soybean [38, 40, 42–44]. Strain WSM 4400^T, originally isolated from nodules of *Glycine* sp. in South Africa, grouped with 99% bootstrap support with *B. pachyrhizi* BR 3262 and *Bradyrhizobium* sp. VULI21 and presented a *nodC* sequence closely related to *Bradyrhizobium* strains isolated from *V. unguiculata* in Spain (VUPME10), Africa (STM3062), Greece (VULI11, VULI21 and VUCR24), and Brazil (BR 3262), composing the sv. vignae [17, 45, 46] with 100% bootstrap support. Even though WSM 4400^T is the first strain isolated from *Glycine* sp. inside the sv. vignae, the nodulation ability of this strain in *V. unguiculata* was confirmed in this study (data not shown). The species *V. unguiculata*, known as cowpea, is indigenous to Africa and represents an important nutritional source around the world; the occurrence of the African strain WSM 4400^T inside the sv. vignae corroborates with the hypothesis that Africa is the centre of origin of this symbiovar, from where the strains and host seeds were dispersed to other continents [17]. The NI values for WSM 4400^T ranged from 95.2–99.1% among the strains of closely related species (Table S2), with the highest NI value found between WSM 4400^T and *B. pachyrhizi* BR 3262, a strain successfully used in commercial inoculants for the cowpea crop in Brazil [45].

In the *nifH* phylogeny (205 bp), the strains of this study confirmed the same clustering as observed for the *nodC* genes, with a basal position of CNPSo 4026^T with 83% bootstrap support, WSM 1738^T in sv. retamae with 94% bootstrap support, and WSM 4400^T in sv. vignae with 99% bootstrap support (Fig. S8). The topology of the *nifH* tree is slightly different from the *nodC* tree, as some *Bradyrhizobium* strains do not have available *nifH* sequences, e.g. strain VUPME10 of sv. vignae. In the *nifH* phylogeny, the other strains from sv. vignae were closer to each other than in the *nodC* phylogeny; in addition, the strains WSM 4400^T, BR 3262, VULI21, VUCR24 and VULI11 shared 99.5% NI (Table S2). Interestingly, strain STM3062 from Africa, which was close to strains of the sv. vignae in the *nodC* phylogeny, presented higher similarity to *B. elkanii* SEMIA 5019 and *B. elkanii* SEMIA 587 from sv. sojae in the *nifH* phylogeny (Fig. S8), sharing 100% NI, whereas the values among STM3062 and strains from sv. vignae ranged from 94.1–94.6% NI (data not shown).

Symbiotic genes, including nodulation and nitrogen-fixation genes, are commonly located in symbiotic plasmids in the genera *Rhizobium*, *Sinorhizobium* and *Paraburkholderia*, while in *Bradyrhizobium* and *Mesorhizobium* they are usually located in the chromosome, in a region called a symbiotic island or an integrative and conjugative element [5, 47–49]. Considering the findings of a large study on the nodulation traits in *Bradyrhizobium*, Menna and Hungria [13] suggested a monophyletic origin for the symbiotic island based upon *nodA*, *nodZ*, *nodY/K* and *nifH* phylogenies, whereafter it is shared among strains ether by vertical inheritance or horizontal transfer. Therefore, the congruence between *nodC* and *nifH* phylogenies found in the strains of the current study, as well as in other studies involving *Bradyrhizobium* [20, 46, 50], support the hypothesis of simultaneous evolution of these genes in the symbiotic island. However, the incongruence presented by STM3062 may indicate a horizontal gene transfer (HGT) event, as has also been demonstrated in other studies [51].

In order to get a better understanding of the evolutionary history of the strains from this study, two novel phylogenetic trees with the 16S rRNA and *glnII* +*recA* housekeeping genes including the available sequences of strains used for symbiovar definition (Fig S9 and Fig S10) were reconstructed and compared with the phylogenies of *nodC* and *nifH* genes. Core and symbiotic genes of

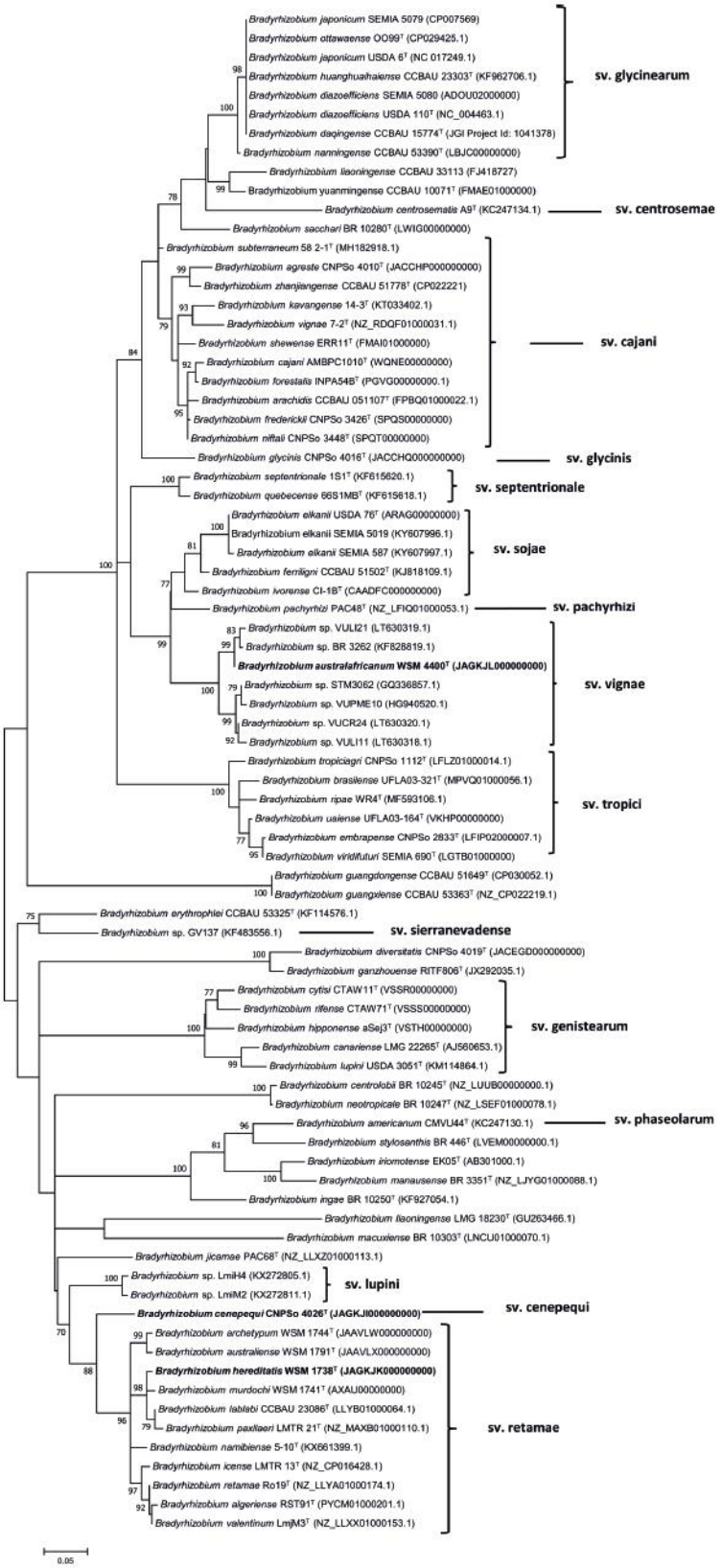

Fig. 3. Maximum-likelihood phylogeny based on *nodC* gene alignment (335 bp), using the T92: Tamura three-parameter+G+I model in MEGA version 7. Accession numbers are indicated in parentheses. The novel species are shown in bold. Bootstrap values >70% are indicated at the nodes. Bar indicates five substitutions per 100 nucleotide positions.

strain WSM 1738^T were not congruent, with *B. archetypum* WSM 1744^T, *B. retamae* Ro19^T and *B. namibiense* 5-10^T representing the closest species in the 16S rRNA and *glnII +recA* phylogenies (Figs S9 and S10), while *B. murdochi* WSM 1741^T was the closest in the symbiotic phylogenies (Fig. 3 and S8), which could indicate HGT of the symbiotic genes, a reasonably common event in rhizobia [52]. Nevertheless, strain WSM 4400^T was close to *B. brasilense* UFLA03-321^T and *B. pachyrhizi* PAC48^T in the 16S rRNA (Fig. 1) and MLSA trees (Fig. 2), but in the novel phylogenies of core genes containing the strains used in symbiovar analysis (Figs S9 and S10), WSM 4400^T was closer to the strains VULI21, VUCR24 and VULI11 from sv. vignae, congruent with *nodC* and *nifH* phylogenies (Fig. 3 and Fig S8). This pattern is generally found in *Mimosa*-nodulating *Paraburkholderia* and it seems to be related to mild HGT events [53]. Therefore, these findings reinforce that both horizontal and vertical transfer may contribute towards the evolution of this symbiosis, resulting in the great diversity of rhizobia found nowadays.

Delamuta *et al.* [20] proposed a cut-off value of approximately 92.5% in *Bradyrhizobium nodC* sequence similarity to define new symbiovars. We will suggest names for the symbiovars according to the strain occupying a central position in the cluster. Based on this cut-off, we confirm that strains WSM 1738^T and WSM 4400^T are included in sv. retamae and sv. vignae, respectively, and we propose the description of three novel symbiovars. More details about the symbiovars described here are shown in Table S3. Strains CNPSo 4026^T described in this study and *B. glycinis* CNPSo 4016^T recently described as a novel species by our research group [39] did not cluster with any *Bradyrhizobium* strain in the *nodC* phylogeny and presented NI values equal or lower than 91.6 and 92.5%, respectively; therefore, we propose two novel symbiovars named cenepequi and glycinis, respectively, named as the first and only species described so far in these symbiovars. The evolutionary history, as well as the host range of these symbiovars should be further investigated as more isolates belonging to these symbiovars become available. We also suggest a novel sv. named 'cajani' for a *nodC* lineage with 79% bootstrap support that contains *B. cajani* and another nine species (Fig. 3). The nucleotide identity of strains *B. agreste* CNPSo 4010^T, *B. arachidis* CCBAU 051107^T, *B. cajani* AMBPC1010^T, *B. forestalis* INPA54B^T, *B. frederickii* CNPSo 3426^T, *B. kavangense* 14-3^T, *B. niftali* CNPSo 3448^T, *B. shewense* ERR11^T, *B. vignae* 7-2^T and *B. zhanjiangense* CCBAU 51778^T ranged from 91.3 to 99.7% similarity among each other (Table S3). Even though strain *B. subterraneum* 58 2-1^T was not included in the branch with 79% bootstrap support, it shared a NI from 93.7–96.4% with the other strains of the sv. cajani, and, therefore, it possibly belongs to the same symbiovar. The sv. cajani contains strains isolated from several hosts, including *Glycine clandestina*, *Arachis hypogaea*, *Cajanus cajan*, *Inga* sp., *Chamaecrista fasciculata*, *Vigna unguiculata* and *Erythrina brucei* isolated in Africa, China, USA, Australia, Brazil and the Dominican Republic (Table S3). Interestingly, all strains from sv. cajani tested for nodulation ability with soybean were unable to nodulate this legume [39, 50, 54–58].

Even though strains *B. nanningense* CCBAU 53390^T and *B. ivorense* CI-1B^T were not described as belonging to any symbiovar, the *nodC* phylogeny (Fig. 3) indicates that these strains belong to pre-existing symbiovars. *Bradyrhizobium nanningense* CCBAU 53390^T was isolated from *A. hypogaea* in China [58] and had 98.8%similarity to strains of sv. glycinearum able to nodulate soybean [15], whereas *B. ivorense* CI-1B^T isolated from *Cajanus cajan* in Africa [59] presented 93.1–95.8% similarity to strains of sv. sojae, commonly associated with *Glycine max* [20].

GENOME FEATURES

The genomes of strains CNPSo 4026^T, WSM 1704^T, WSM 1738^T and WSM 4400^T were sequenced by the MiSeq platform (Illumina) at Embrapa Soja (Londrina, Brazil) using sequence libraries constructed with the Nextera XT kit (Illumina). The reads were assembled *de novo* with the A5-MiSeq pipeline version 20140604 and the genomes were annotated with Rapid Annotation using Subsystems Technology (RAST) version 2.0 [60]. The draft genomes were deposited in the GenBank database (NCBI), and received the accession numbers JAGKJI000000000 for CNPSo 4026^T, JAGKJJ000000000 for WSM 1704^T, JAGKJK000000000 for WSM 1738^T, and JAGKJL000000000 for WSM 4400^T. The final genome assemblies used the recommended statistical parameters for taxonomic purposes [61] and the detailed genomic data are shown in Table 3.

Average nucleotide identity (ANI) and digital DNA–DNA hybridization (dDDH), the state-of-the-art methods for inferring overall genome relatedness, were applied to the genomic sequences of the four strains of this study and the available genomic sequences of the closest species identified in the MLSA, *B. archetypum* WSM 1744^T, *B. brasilense* UFLA03-321^T, *B. elkanii* USDA 76^T, *B. ivorense* CI-1B^T and *B. pachyrhizi* PAC48^T. The genomic comparisons were calculated using an ANI calculator [62] with

Table 3. Statistical parameters of final genomes assemblies of the new lineages of *Bradyrhizobium* for taxonomic purposes

Strain	Size	No. of Contigs	N50	Coverage	G+C content (mol%)
***Bradyrhizobium cenepequi* CNPSo 4026^T**	8 472 857	188	149 882	74×	62.3
***Bradyrhizobium semiaridum* WSM 1704^T**	6 712 655	120	194 653	119×	65.1
***Bradyrhizobium hereditatis* WSM 1738^T**	7 871 253	61	418 770	75×	62.0
***Bradyrhizobium australafricanum* WSM 4400^T**	9 684 669	221	125 914	75×	63.1

default parameters and Genome-to-Genome Distance Calculator version 2.1 [63], with the recommended 'formula 2' (identities/high-scoring pairs length), and the values are indicated in Table 4. The genome of CNPSo 4026^T revealed low relatedness with the genomes of the closer *Bradyrhizobium* species, with values equal or lower than 82.65% of ANI and 25.50% of dDDH. WSM 1704^T showed higher genomic similarity (88.27% of ANI and 35.40% of dDDH) to *B. ivorense* CI-1B^T isolated from *Cajanus cajan* in West Africa [59]. Strain WSM 1738^T shared 87.59% of ANI and 34.30% of dDDH with *B. archetypum* WSM 1744^T. Finally, WSM 4400^T shared 94.88% of ANI and 58.80% of dDDH with *B. elkanii* USDA 76^T, a soybean nodulating strain used as inoculant [64, 65], 89.96% of ANI and 36.70% of dDDH with *B. brasilense* UFLA03-321^T, isolated from *V. unguiculata* in Brazilian soils [66], and 95.46% of ANI and 62.20% of dDDH with *B. pachyrhizi* PAC48^T, isolated from *Pachyrhizus erosus* in Costa Rica [67]. Among the strains described in this study, the values ranged from 81.95 to 87.18% for ANI and from 24.40 to 33.0% for dDDH (Table 4). Considering that the four strains of this study showed values below the cut-off values for species delineation of 95–96% for ANI and 70% for dDDH [61, 63], the genomic analyses confirmed that CNPSo 4026^T, WSM 1704^T and WSM 1738^T from Western Australia and WSM 4400^T from South Africa represent novel *Bradyrhizobium* species.

The automatic annotation from RAST showed that strain CNPso 4026^T possesses the nodulation genes possibly organized in a symbiotic island starting with a *nodD*, putative *fixJ*, *nodABCSUIJ*, a pseudogene of sulfotransferase and *nolNO*, while the *nodZ* was not found. *nodZ* is an important gene for Nod factor synthesis since it is related to the fucosylation of the core lipochitooligosaccharide [68] and it has been pointed out as a host-specific nodulation gene [69]. The absence of the *nodZ* gene in strain CNPSo 4026^T may indicate that the strain uses different strategies to modify the Nod factor and establish the nodulation, which should be carefully investigated in further studies. The nodulation region of WSM 1738^T presented two copies of *nodD*, putative *fixJ*, followed by *nodABCSUIJ*, *nolNO* and a putative *nodZ*. StrainWSM 4400^T showed *nodD2D1ABCSUIJ*, *nolNO* and a putative *nodZ*. As commented before, we did not find *nod* genes in the genome of WSM 1704^T, which can be related to the smaller size of the genome of this strain.

The SEED platform [60] was used to estimate the G+C genome content, defined as 62.3, 65.1, 62.0 and 63.1 mol% for CNPSo 4026^T, WSM 1704^T, WSM 1738^T and WSM 4400^T, respectively.

PHENOTYPIC CHARACTERIZATION

Morphophysiological analyses were carried out and compared among strains CNPSo 4026^T, WSM 1704^T, WSM 1738^T, WSM 4400^T, *B. archetypum* WSM 1744^T and *B. elkanii* USDA 76^T. The tests were performed using modified-YMA medium at 28 °C [27], and when the strains were cultivated in different conditions this is described. Congo red was used in the modified-YMA medium to verify colony morphology after 7–10 days of growth. The physiological features were given according to adaptations from Hungria *et al.* [70] by growth in medium containing bromothymol blue as an indicator for, indicating acid, neutral or alkaline reaction; with 1% (w/v) NaCl; at 37 °C, at pH 4.0 and pH 8.0; growth on Luria–Bertani (LB) medium. Urease activity was evaluated using 2% (w/v) urea and the pH indicator phenol red. The API 50CH kit platform (bioMérieux) was used to determine carbohydrate metabolism, with bacteria grown in modified-YM-minus-mannitol with bromothymol blue. Tolerance of antibiotics was analysed by the disc-diffusion technique [71] using ampicillin (10 µg), bacitracin (10 U), cefuroxime (30 µg), chloramphenicol (30 µg), nalidixic acid (30 µg), neomycin (30 µg), penicillin (10 U), streptomycin (10 µg), tetracycline (30 µg) and erythromycin (15 µg). All tests were conducted in duplicate and the differential phenotypical features among the strains from this study and closest species are described in Table 5.

The most contrasting morphophysiological features observed were that strains WSM 1704^T and WSM 1738^T were able to grow on modified-YMA at 37 °C in 4 and 10 days, respectively, indicating tolerance to high temperature, especially WSM 1704^T. Another interesting feature is that strain CNPSo 4026^T was able to grow weakly on modified-YMA with 1% (w/v) NaCl, a trait not commonly found in *Bradyrhizobium*. The strains of this study presented the ability to grow well on modified-YMA with pH 4.0 and 8.0, except for strain WSM 1738^T, which grew weakly at pH 4. Concerning carbohydrate metabolism, strain CNPSo 4026^T was unable to use glycerol and D-mannitol, whereas WSM 1738^T was only unable to use D-mannitol, while other strains were able to weakly use both C sources. Even though glycerol and D-mannitol are commonly used as C sources in YMA culture medium, it is worth mentioning that the growth conditions are different between the API 50CH platform and agar culture medium in Petri plates; therefore, it is common to find some incongruences in patterns of C-source utilization, and this also raises doubts about the usefulness of using platforms such as the API system to describe physiological features.

Nodulation and nitrogen fixation abilities were evaluated 30 days after inoculation of the strains on *Glycine max* (commercial cultivar 'BRASMAX Potência RR) and *Macroptilium atropurpureum* (commonly known as 'siratro') that were grown under controlled glasshouse conditions in Leonard jars with sterilized sand, vermiculite (2:1, v/v), and N-free nutrient solution [72]. Strains CNPSo 4026^T, WSM 1738^T and WSM 4400^T formed effective nodules, presenting red or pink colour, on siratro, whereas only WSM 4400^T was able to nodulate soybean, but nodules were not as effective as in siratro, verified by the pale green colour of the leaves. Previous studies have confirmed that WSM 4400^T forms effective nodules in *Vigna unguiculata* (data not shown). Even though strain WSM 1704^T was isolated from nodules of *T. gardneri* [26], the strain was unable to nodulate siratro and soybean. We were also unable to amplify or find the main nodulation and nitrogen-fixation genes in the genome WSM 1704^T. However,

Table 4. Average nucleotide identity (ANI) and digital DNA–DNA hybridization (dDDH) values among new lineages of *Bradyrhizobium* and closely related *Bradyrhizobium* species

	ANI (%)	dDDH (%)	ANI (%)	dDDH (%)	ANI (%)	dDDH (%)	ANI (%)	dDDH (%)
Strains	***Bradyrhizobium cenepequi* CNPSo 4026^T**		***Bradyrhizobium semiaridum* WSM 1704^T**		***Bradyrhizobium hereditatis* WSM 1738^T**		***Bradyrhizobium australafricanum* WSM 4400^T**	
***B. cenepequi* CNPSo 4026^T (JAGKJI000000000)**	–	–	82.65	25.20	82.10	24.60	82.64	25.40
***B. semiaridum* WSM 1704^T (JAGKJJ000000000)**	82.65	25.20	–	–	81.96	24.40	87.18	33.00
***B. hereditatis* WSM 1738^T (JAGKJK000000000)**	82.10	24.60	81.95	24.40	–	–	81.95	24.50
***B. australafricanum* WSM 4400^T (JAGKJL000000000)**	82.64	25.40	87.18	33.00	81.95	24.50	–	–
B. archetypum WSM 1744^T (JAAVLW000000000)	82.18	24.60	82.00	24.40	87.59	34.30	82.10	24.60
B. brasilense UFLA03-321^T	82.58	25.40	87.21	33.10	82.06	24.60	89.96	36.70
B. elkanii USDA 76^T (ARAG01000000)	82.63	25.50	87.18	33.20	81.99	24.50	94.88	58.80
B. ivorense CI-1B^T (CAADFC000000000)	82.65	25.40	88.27	35.40	82.13	24.50	87.32	33.20
B. pachyrhizi PAC 48^T (LFIQ00000000)	82.61	25.30	87.11	32.90	82.03	24.30	95.46	62.20

Table 5. Distinctive phenotypical properties of new lineages of *Bradyrhizobium* and closely related strains

Strains: 1, *Bradyrhizobium cenepequi* CNPSo 4026^T; 2, *Bradyrhizobium semiaridum* WSM 1704^T; 3, *Bradyrhizobium hereditatis* WSM 1738^T; 4, *Bradyrhizobium australafricanum* WSM 4400^T; 5, *Bradyrhizobium archetypum* WSM 1744^T; 6, *Bradyrhizobium elkanii* USDA 6^T. +, Positive growth; w, weak growth; –, no growth.

Charactheristic	1	2	3	4	5*	6†
Carbon source utilization:						
Glycerol	–	w	w	w	w	w
Erythritol	–	–	w	w	w	–
L-Arabinose	–	+	+	+	w	+
D-Ribose	w	+	+	+	w	+
D-Xylose	w	+	w	+	w	+
D-Adonitol	–	+	w	+	+	w
Methyl β-D-xylopyranoside	–	–	w	–	+	–
D-Galactose	–	w	w	+	w	+
D-Glucose	–	w	w	w	w	w
D-Fructose	–	w	w	w	w	w
D-Mannose	w	w	w	+	w	+
L-Sorbose	–	w	w	w	+	–
Dulcitol	–	w	+	–	w	w
Inositol	–	w	–	+	w	–
D-Mannitol	–	w	–	w	w	w
D-Sorbitol	–	w	+	w	w	w
Methyl α-D-mannopyranoside	–	–	–	w	w	–
Methyl α-D-glusopyranoside	–	+	–	w	w	–
N-Acetylglicosamine	–	–	+	w	w	–
Amygdalin	–	w	–	–	w	–
Arbutin	–	+	–	–	w	–
Aesculin ferric citrate	+	+	+	+	+	w
Salicin	–	+	–	–	w	–
Cellobiose	–	+	w	–	w	–
Maltose	–	–	+	+	w	–
Lactose	–	–	+	+	w	–
Melibiose	–	–	–	+	+	–
Trehalose	w	–	w	–	w	–
Inulin	–	–	w	–	w	–
Melezitose	–	w	–	–	w	–
Raffinose	–	–	–	w	w	–
Glycogen	+	+	–	+	+	–
Xylitol	–	+	w	–	+	w
Gentiobiose	–	w	w	+	+	–

Continued

Table 5. Continued

Charactheristic	1	2	3	4	5*	6†
Turanose	–	–	–	W	W	–
D-Lyxose	+	+	W	+	W	+
D-Tagatose	–	–	–	W	W	–
D-Fucose	W	+	W	+	W	+
L-Fucose	+	+	W	+	+	+
D-Arabitol	–	+	–	+	+	W
L-Arabitol	–	+	–	W	+	W
Potassium gluconate	+	+	+	+	+	–
Potassium 2-keto-gluconate	+	+	+	+	+	–
Potassium 5-keto-gluconate	+	+	+	+	+	–
Growth at/in:						
pH 4	+	+	W	+	W	ND
37 °C	–	+	+	–	+	–
1% NaCl	W	–	–	–	–	–
Tolerance to antibiotics (μg disc^{-1}):						
Ampicillin (10)	–	+	+	+	+	ND
Neomycin (30)	+	+	+	W	+	–
Penicillin G (10 U)	+	+	+	+	+	ND
Tetracycline (30)	+	+	–	+	+	+
Streptomycin (10)	–	–	+	–	–	+
Cefuroxima (30)	–	+	–	+	+	+

*Data obtained from Helene *et al.* [42].
†Data obtained from Helene *et al.* [37].

in the genome WSM 1704^T, we did find the *nfeD* gene related to nodulation efficiency and competitiveness according to the host plant, *nifU* gene involved in the mobilization of Fe-S cluster synthesis and repair and, *fixA* which is normally part of the *fixABCX* operon, and it is required for nitrogenase activity. Therefore, further studies are needed to investigate the mechanisms involving the nodulation with the original host, or the possible loss of the symbiotic ability of this strain during the evolution process.

Based on the extensive polyphasic study presented here, we propose the description of four novel *Bradyrhizobium* species, for which we suggest the following names: *Bradyrhizobium cenepequi* sp. nov. (type strain CNPSo 4026^T), *Bradyrhizobium semiaridum* sp. nov. (type strain WSM 1704^T), *Bradyrhizobium hereditatis* sp. nov. (type strain WSM 1738^T) and *Bradyrhizobium australafricanum* sp. nov. (type strain WSM 4400^T), isolated from different regions of Western Australia and South Africa. In addition, the symbiotic gene phylogenies allow the description of three new symbiovars: cenepequi, glycinis and cajani, contributing to knowledge about the evolutionary history of symbiotic relationships.

DESCRIPTION OF *BRADYRHIZOBIUM CENEPEQUI* SP. NOV.

Bradyrhizobium cenepequi [ce.ne.pe'qui. N.L. gen. n. *cenepequi*, arbitrarily formed from the acronym CNPq (Conselho Nacional de Pesquisa, Brazilian National Council for Scientific and Technological Development); in honour of the 60 years this public institution that finances research in Brazil, including international projects of collaboration].

Cells are Gram-stain-negative, aerobic, and non-spore-forming. Culture on modified-YMA medium with pH 6.8–7.0 and Congo red resulted in slightly pink colonies, with less than 1 mm diameter, circular shape, translucent, and with low mucus production and gummy consistency after 7 days of growth at 28 °C. The strain shows alkaline reaction on modified-YMA with bromothymol

blue and positive urease activity. CNPSo 4026^T grows well both at pH 4.0 and pH 8.0 after 7 days. The strain is unable to grow on solid LB medium and when incubated at 37 °C, and presented weak growth on modified-YMA containing 1% (w/v) NaCl. CNPSo 4026^T is able to use D-arabinose, L-xylose, aesculin ferric citrate, starch, glycogen, D-lyxose, L-fucose, potassium gluconate, potassium 2-ketogluconate and potassium 5-ketogluconate; weakly uses D-ribose, D-xylose, D-mannose, L-rhamnose, trehalose and D-fucose; but is unable to use glycerol, erythritol, L-arabinose, D-adonitol, methyl β-D-xylopyranoside, D-galactose, D-glucose, D-fructose, L-sorbose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, *N*-acetylglucosamine, arbutin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, inulin, melezitose, raffinose, xylitol, gentiobiose, turanose, D-tagatose, D-arabitol and L-arabitol. The strain is tolerant to bacitracin (10 U), chloramphenicol (30 µg), erythromycin (15 µg), nalidixic acid (30 µg), neomycin (30 µg), penicillin G (10 U) and tetracycline (30 µg); it is sensitive to ampicillin (10 µg), cefuroxime (30 µg) and streptomycin (10 µg). The strain is able to form effective nitrogen-fixing nodules on *Macroptilium atropurpureum* and *Vigna unguiculata*, but does not nodulate *Glycine max*.

The type strain, CNPSo 4026^T (=WSM 4798^T=LMG 31653^T), was isolated from root nodules of *Vigna unguiculata* used as trapping host in Western Australian soils. The DNA G+C content of strain CNPSo 4026^T is 62.3 mol%.

DESCRIPTION OF *BRADYRHIZOBIUM SEMIARIDUM* SP. NOV.

Bradyrhizobium semiaridum (se.mi.a'ri.dum. L. pref. *semi*, half; L. masc. adj. *aridus*, dry; N.L. neut. adj. semiaridum, half-dry).

Cells are Gram-stain-negative, aerobic and non-spore-forming. Culture on modified-YMA medium with pH 6.8–7.0 and Congo red resulted in white colonies, with less than 1 mm diameter, circular shape, opacity, and low mucus production and viscous consistency after 7 days of growth at 28 °C. The strain shows alkaline reaction on modified-YMA with bromothymol blue and positive urease activity. WSM 1704^T grows well at pH 4.0 and pH 8.0 after 7 days, and also when incubated at 37 °C after 4 days. The strain is unable to grow on solid LB medium and on modified-YMA containing 1% (w/v) NaCl. WSM 1704^T is able to use D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, methyl α-D-mannopyranoside, arbutin, aesculin ferric citrate, salicin, cellobiose, starch, glycogen, xylitol, D-lyxose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium 2-ketogluconate and potassium 5-ketogluconate; weakly uses glycerol, D-galactose, D-glucose, D-fructose, mannose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, amygdalin, melezitose and gentibiose; but is unable to use erythritol, methyl β-D-xylopyranoside, methyl α-D-mannopyranoside, *N*-acetylglicosamine, maltose, lactose, melibiose, sucrose, trehalose, inulin, raffinose, turanose and D-tagatose. The strain is tolerant to 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) and tetracycline (30 µg); it is sensitive to streptomycin (10 µg). The strain was isolated from nodules of *Tephrosia gardneri*, and was not able to nodulate *Macroptilium atropurpureum* or *Glycine max*.

The type strain, WSM 1704^T (=CNPSo 4028^T=LMG 31654^T), was isolated from nodules of *Tephrosia gardneri*, in Carnarvon, WA. The DNA G+C content of strain WSM 1704^T is 65.1 mol%.

DESCRIPTION OF *BRADYRHIZOBIUM HEREDITATIS* SP. NOV.

Bradyrhizobium hereditatis (he.re.di.ta'tis. L. gen. n. *hereditatis*, of heritage. To highlight the importance of preservation of World Heritage Parks, such as the Cape Range National Park, WA, a source of biodiversity hotspots).

Cells are Gram-stain-negative, aerobic and non-spore-forming. Culture on modified-YMA medium with pH 6.8–7.0 and Congo red resulted in slightly pink colonies, with less than 1 mm diameter, circular shape, translucent and low mucus production and gummy consistency after 7 days of growth at 28 °C. The strain shows alkaline reaction on modified-YMA with bromothymol blue and positive urease activity. WSM 1738^T grows well at pH 8.0 after 7 days and when incubated at 37 °C after 10 days but grows weakly at pH 4.0. The strain is unable to grow on solid LB medium nor on modified-YMA containing 1% (w/v) NaCl. Strain WSM 1738^T is able to use D-arabinose, L-arabinose, D-ribose, L-xylose, dulcitol, D-sorbitol, *N*-acetylglicosamine, aesculin ferric citrate, maltose, lactose, starch, potassium gluconate, potassium 2-ketogluconate and potassium 5-ketogluconate; it weakly uses glycerol, erythritol, D-xylose, D-adonitol, methyl β-D-xylopyranoside, D-galactose, D-glucose, D-fructose, D-mannose, L-sorbose, L-rhamnose, cellobiose, trehalose, inulin, xylitol, gentibiose, D-lyxose, D-fucose and L-fucose; it is unable to use inositol, D-mannitol, methyl α-D-mannopyranoside, methyl α-D-glusopyranoside, amygdalin, arbutin, salicin, melibiose, sucrose, melezitose, raffinose, glycogen, turanose, D-tagatose, D-arabitol and L-arabitol. The strain is tolerant to ampicillin (10 µg), bacitracin (10 U), chloramphenicol (30 µg), erythromycin (15 µg), nalidixic acid (30 µg), neomycin (30 µg), penicillin G (10 U) and streptomycin (10 µg); it is sensitive to cefuroxime (30 µg) and tetracycline (30 µg). The strain was isolated from effective nodules of *Indigofera* sp. and is able to form effective nitrogen-fixing nodules on *Macroptilium atropurpureum*, but not on *Glycine max*.

Strain WSM 1738^T (=CNPSo 4025^T=LMG 31652^T) was isolated from nodules of *Indigofera* sp., in Cape Range National Park, WA. The DNA G+C content of strain WSM 1738^T is 62.0 mol%.

DESCRIPTION OF *BRADYRHIZOBIUM AUSTRALAFRICANUM* SP. NOV.

Bradyrhizbium australafricanum (aus.tral.a.fri.ca'num. L. masc. adj. *australis*, southern; L. masc. adj. *africanus*, African; N.L. neut. adj. *australafricanum*; of or pertaining to South Africa, the source of the strain).

Cells are Gram-stain-negative, aerobic and non-spore-forming. Culture on modified-YMA medium with pH 6.8–7.0 and Congo red resulted in slightly pink colonies, with less than 1 mm diameter, circular shape, opacity and low mucus production and gummy consistency after 7 days of growth at 28 °C. The strain shows alkaline reaction on modified-YMA with bromothymol blue and positive urease activity. WSM 4400^T grows well at pH 4.0 and pH 8.0 after 7 days. The strain is unable to grow on solid LB medium, on modified-YMA containing 1% (w/v) NaCl and when incubated at 37 °C after ten days. WSM 4400^T is able to use D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, D-galactose, D-mannose, inositol, aesculin ferric citrate, maltose, lactose, melibiose, starch, glycogen, gentiobiose, L-lyxose, D-fucose, L-fucose, D-arabitol, potassium gluconate, potassium 2-ketogluconate and potassium 5-ketogluconate; it weakly uses glycerol, erythritol, D-glucose, D-fructose, L-sorbose, L-rhamnose, D-mannitol, D-sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, *N*-acetylglicosamine, raffinose, turanose, D-tagatose and L-arabitol; it is unable to use methyl β-D-xylopyranoside, dulcitol, amygdalin, arbutin, salicin, cellobiose, sucrose, trehalose, inulin, melezitose and xylitol. 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); but is moderately sensitive to neomycin (30 µg); it is sensitive to streptomycin (10 µg). The strain was isolated from nodules of *Glycine* sp. and is able to form effective nitrogen-fixing nodules in *Macroptilium atropurpureum* and less-effective nodules in *Glycine max*.

The type strain, WSM 4400^T (=CNPSo 4015^T=LMG 31648^T), isolated from nodules of *Glycine* sp. in the Amathole District, South Africa. The DNA G+C content of strain WSM 4400^T is 63.1 mol%.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

References

1. **Kumar N**, **Srivastava P**, **Vishwakarma K**, **Kumar R**, **Kuppala H**, ***et al***. The *Rhizobium*–plant symbiosis: state of the art. In: **Varma A**, **Tripathi S** and **Prasad R** (eds). *Microbe Symbiosis*. Singapore: Springer Nature; 2020. pp. 1–20.
2. **Wang ET**, **Chen WF**, **Tian CF**, **Young JPW**, **Chen WX**. Symbiosis between *Rhizobia* and legumes. In: **Wang ET**, **Chen WF**, **Tian CF**, **Young JPW** and **Chen WX** (eds). *Ecology and Evolution of Rhizobia*. Singapore: Springer Nature; 2019. pp. 3–15.
3. **Shamseldin A**. The role of different genes involved in symbiotic nitrogen fixation - review. *Glob J Biotechnol Biochem* 2013;8:84–94.
4. **Lloret L**, **Martínez-Romero E**. Evolución y filogenia de rhizobium. *Rev Latinoam Microbiol* 2005;47:43–60.
5. **Black M**, **Moolhuijzen P**, **Chapman B**, **Barrero R**, **Howieson J**, ***et al***. The genetics of symbiotic nitrogen fixation: comparative genomics of 14 rhizobia strains by resolution of protein clusters. *Genes (Basel)* 2012;3:138–166.
6. **Hungria M**, **Menna P**, **Marçon Delamuta JR**. Bradyrhizobium, the ancestor of all rhizobia: phylogeny of housekeeping and nitrogen-fixation genes. In: **Bruijn FJ** (eds). *Biological Nitrogen Fixation*. Hoboken, NJ: Wiley; 2015. pp. 191–202.
7. **Sprent JI**, **Ardley J**, **James EK**. Biogeography of nodulated legumes and their nitrogen-fixing symbionts. *New Phytol* 2017;215:40–56.
8. **VanInsberghe D**, **Maas KR**, **Cardenas E**, **Strachan CR**, **Hallam SJ**, ***et al***. Non-symbiotic *Bradyrhizobium* ecotypes dominate North American forest soils. *ISME J* 2015;9:2435–2441.
9. **Piromyou P**, **Greetatorn T**, **Teamtisong K**, **Okubo T**, **Shinoda R**, ***et al***. Preferential association of endophytic bradyrhizobia with different rice cultivars and its implications for rice endophyte evolution. *Appl Environ Microbiol* 2015;81:3049–3061.
10. **de Castilho CL**, **Volpiano CG**, **Ambrosini A**, **Zulpo L**, **Passaglia L**, ***et al***. Growth-promoting effects of *Bradyrhizobium* soybean symbionts in black oats, white oats, and ryegrass. *Braz J Microbiol* 2021;52:1451–1460.
11. **Trinick MJ**. Structure of nitrogen-fixing nodules formed by *Rhizobium* on roots of *Parasponia andersonii* Planch. *Can J Microbiol* 1979;25:565–578.
12. **Giraud E**, **Moulin L**, **Vallenet D**, **Barbe V**, **Cytryn E**, ***et al***. Legumes symbioses: absence of *Nod* genes in photosynthetic bradyrhizobia. *Science* 2007;316:1307–1312.
13. **Menna P**, **Hungria M**. Phylogeny of nodulation and nitrogen-fixation genes in *Bradyrhizobium*: supporting evidence for the theory of monophyletic origin, and spread and maintenance by both horizontal and vertical transfer. *Int J Syst Evol Microbiol* 2011;61:3052–3067.
14. **Rogel MA**, **Ormeño-Orrillo E**, **Martinez Romero E**. Symbiovars in rhizobia reflect bacterial adaptation to legumes. *Syst Appl Microbiol* 2011;34:96–104.
15. **Vinuesa P**, **León-Barrios M**, **Silva C**, **Willems A**, **Jarabo-Lorenzo A**, ***et al***. *Bradyrhizobium canariense* sp. nov., an acid-tolerant endosymbiont that nodulates endemic genistoid legumes (*Papilionoideae: Genisteae*) from the Canary Islands, along with *Bradyrhizobium japonicum* bv. *genistearum*, *Bradyrhizobium genospecies* alpha and *Bradyrhizobium genospecies* beta. *Int J Syst Evol Microbiol* 2005;55:569–575.
16. **Guerrouj K**, **Ruíz-Díez B**, **Chahboune R**, **Ramírez-Bahena M-H**, **Abdelmoumen H**, ***et al***. Definition of a novel symbiovar (sv. retamae)

within *Bradyrhizobium retamae* sp. nov., nodulating *Retama sphaerocarpa* and *Retama monosperma*. *Syst Appl Microbiol* 2013;36:218–223.

17. **Bejarano A**, **Ramírez-Bahena M-H**, **Velázquez E**, **Peix A**. *Vigna unguiculata* is nodulated in Spain by endosymbionts of Genisteae legumes and by a new symbiovar (vignae) of the genus *Bradyrhizobium*. *Syst Appl Microbiol* 2014;37:533–540.
18. **Cobo-Díaz JF**, **Martínez-Hidalgo P**, **Fernández-González AJ**, **Martínez-Molina E**, **Toro N**, ***et al***. The endemic *Genista versicolor* from Sierra Nevada National Park in Spain is nodulated by putative new *Bradyrhizobium* species and a novel symbiovar (sierranevadense). *Syst Appl Microbiol* 2014;37:177–185.
19. **Ramírez-Bahena MH**, **Flores-Félix JD**, **Chahboune R**, **Toro M**, **Velázquez E**, ***et al***. *Bradyrhizobium centrosemae* (symbiovar centrosemae) sp. nov., *Bradyrhizobium americanum* (symbiovar phaseolarum) sp. nov. and a new symbiovar (tropici) of *Bradyrhizobium viridifuturi* establish symbiosis with *Centrosema* species native to America. *Syst Appl Microbiol* 2016;39:378–383.
20. **Delamuta JRM**, **Menna P**, **Ribeiro RA**, **Hungria M**. Phylogenies of symbiotic genes of *Bradyrhizobium* symbionts of legumes of economic and environmental importance in Brazil support the definition of the new symbiovars pachyrhizi and sojae. *Syst Appl Microbiol* 2017;40:254–265.
21. **Msaddak A**, **Rejili M**, **Durán D**, **Rey L**, **Palacios JM**, ***et al***. Definition of two new symbiovars, sv. *lupini* and sv. *mediterranense*, within the genera *Bradyrhizobium* and *Phyllobacterium* efficiently nodulating *Lupinus micranthus* in Tunisia. *Syst Appl Microbiol* 2018;41:487–493.
22. **Bromfield ESP**, **Cloutier S**. *Bradyrhizobium septentrionale* sp. nov. (sv. septentrionale) and *Bradyrhizobium quebecense* sp. nov. (sv. septentrionale) associated with legumes native to Canada possess rearranged symbiosis genes and numerous insertion sequences. *Int J Syst Evol Microbiol* 2021;71.
23. **Avontuur JR**, **Palmer M**, **Beukes CW**, **Chan WY**, **Coetzee MPA**, ***et al***. Genome-informed *Bradyrhizobium* taxonomy: where to from here? *Syst Appl Microbiol* 2019;42:427–439.
24. **Ormeño-Orrillo E**, **Martínez-Romero E**. A genomotaxonomy view of the *Bradyrhizobium* genus. *Front Microbiol* 2019;10:1334.
25. **Ferraz Helene LC**, **O'Hara G**, **Hungria M**. Characterization of *Bradyrhizobium* strains indigenous to Western Australia and South Africa indicates remarkable genetic diversity and reveals putative new species. *Syst Appl Microbiol* 2020;43:126053.
26. **Yates RJ**, **Howieson JG**, **Nandasena KG**, **O'Hara GW**. Root-nodule bacteria from indigenous legumes in the north-west of Western Australia and their interaction with exotic legumes. *Soil Biol Biochem* 2004;36:1319–1329.
27. **Hungria M**, **O'Hara GW**, **Zilli JE**, **Araujo RS**, **Deaker R**, ***et al***. Isolation and growth of growth of *Rhizobia*. In: *Working with Rhizobia*. Canberra: Australian Centre for International Agriculture Reserch, 2016. pp. 39–60.
28. **Delamuta JRM**, **Ribeiro RA**, **Araújo JLS**, **Rouws LFM**, **Zilli JÉ**, ***et al***. *Bradyrhizobium stylosanthis* sp. nov., comprising nitrogen-fixing symbionts isolated from nodules of the tropical forage legume *Stylosanthes* spp. *Int J Syst Evol Microbiol* 2016;66:3078–3087.
29. **Kumar S**, **Stecher G**, **Tamura K**. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Mol Biol Evol* 2016;33:1870–1874.
30. **Edgar RC**. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* 2004;32:1792–1797.
31. **Schwarz G**. Estimating the dimension of a model. *Ann Statist* 1978;6:461–464.
32. **Felsenstein J**. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 1985;39:783–791.
33. **Hedges SB**. The number of replications needed for accurate estimation of the bootstrap P value in phylogenetic studies. *Mol Biol Evol* 1992;9:366–369.
34. **Hall TA**. BioEdit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. *Nucleic Acids Symp Ser* 1999;41:95–98.
35. **Menna P**, **Barcellos FG**, **Hungria M**. Phylogeny and taxonomy of a diverse collection of *Bradyrhizobium* strains based on multilocus sequence analysis of the 16S rRNA gene, ITS region and *glnII*, *recA*, *atpD* and *dnaK* genes. *Int J Syst Evol Microbiol* 2009;59:2934–2950.
36. **Kim M**, **Oh HS**, **Park SC**, **Chun J**. Towards a taxonomic coherence between average nucleotide identity and 16S rRNA gene sequence similarity for species demarcation of prokaryotes. *Int J Syst Evol Microbiol* 2014;64:346–351.
37. **Glaeser SP**, **Kämpfer P**. Multilocus sequence analysis (MLSA) in prokaryotic taxonomy. *Syst Appl Microbiol* 2015;38:237–245.
38. **Helene LCF**, **Klepa MS**, **O'Hara G**, **Hungria M**. *Bradyrhizobium archetypum* sp. nov., *Bradyrhizobium australiense* sp. nov. and *Bradyrhizobium murdochi* sp. nov., isolated from nodules of legumes indigenous to Western Australia. *Int J Syst Evol Microbiol* 2020;70:4623–4636.
39. **Klepa MS**, **Helene LCF**, **O'Hara G**, **HungriaM**. *Bradyrhizobium agreste* sp. nov.,*Bradyrhizobium glycinis* sp. nov. and *Bradyrhizobium diversitatis* sp. nov., isolated from a biodiversity hotspot of the genus *Glycine* in Western Australia. *Int J Syst Evol Microbiol* 2021;71:004742.
40. **Durán D**, **Rey L**, **Mayo J**, **Zúñiga-Dávila D**, **Imperial J**, ***et al***. *Bradyrhizobium paxllaeri* sp. nov. and *Bradyrhizobium icense* sp. nov., nitrogen-fixing rhizobial symbionts of Lima bean (*Phaseolus lunatus* L.) in Peru. *Int J Syst Evol Microbiol* 2014;64:2072–2078.
41. **Peix A**, **Ramírez-Bahena MH**, **Velázquez E**, **Bedmar EJ**. Bacterial associations with legumes. *CRC Crit Rev Plant Sci* 2014;34:17–42.
42. **Chang YL**, **Wang JY**, **Wang ET**, **Liu HC**, **Sui XH**, ***et al***. *Bradyrhizobium lablabi* sp. nov., isolated from effective nodules of *Lablab purpureus* and *Arachis hypogaea*. *Int J Syst Evol Microbiol* 2011;61:2496–2502.
43. **Durán D**, **Rey L**, **Navarro A**, **Busquets A**, **Imperial J**, ***et al***. *Bradyrhizobium valentinum* sp. nov., isolated from effective nodules of *Lupinus mariae-josephae*, a lupine endemic of basic-lime soils in Eastern Spain. *Syst Appl Microbiol* 2014;37:336–341.
44. **Grönemeyer JL**, **Bünger W**, **Reinhold-Hurek B**. *Bradyrhizobium namibiense* sp. nov., a symbiotic nitrogen-fixing bacterium from root nodules of *Lablab purpureus*, hyacinth bean, in Namibia. *Int J Syst Evol Microbiol* 2017;67:4884–4891.
45. **Simões-Araújo JL**, **Leite J**, **Marie Rouws LF**, **Passos SR**, **Xavier GR**, ***et al***. Draft genome sequence of *Bradyrhizobium* sp. strain BR 3262, an effective microsymbiont recommended for cowpea inoculation in Brazil. *Braz J Microbiol* 2016;47:783–784.
46. **Tampakaki AP**, **Fotiadis CT**, **Ntatsi G**, **Savvas D**. Phylogenetic multilocus sequence analysis of indigenous slow-growing rhizobia nodulating cowpea (*Vigna unguiculata* L.) in Greece. *Syst Appl Microbiol* 2017;40:179–189.
47. **Colombi E**, **Perry BJ**, **Sullivan JT**, **Bekuma AA**, **Terpolilli JJ**, ***et al***. Comparative analysis of integrative and conjugative mobile genetic elements in the genus *Mesorhizobium*. *Microb Genom* 2021;7:000657.
48. **De Meyer SE**, **Briscoe L**, **Martínez-Hidalgo P**, **Agapakis CM**, **de-Los Santos PE**, ***et al***. Symbiotic *Burkholderia* species show diverse arrangements of nif/fix and nod genes and lack typical high-affinity cytochrome cbb3 oxidase genes. *Mol Plant Microbe Interact* 2016;29:609–619.
49. **Okubo T**, **Piromyou P**, **Tittabutr P**, **Teaumroong N**, **Minamisawa K**. Origin and evolution of nitrogen fixation genes on symbiosis islands and plasmid in *Bradyrhizobium*. *Microbes Environ* 2016;31:260–267.
50. **Klepa MS**, **Urquiaga MC de O**, **Somasegaran P**, **Delamuta JRM**, **Ribeiro RA**, ***et al***. *Bradyrhizobium niftali* sp. nov., an effective nitrogen-fixing symbiont of partridge pea [*Chamaecrista fasciculata* (Michx.) Greene], a native caesalpinioid legume broadly distributed in the USA. *Int J Syst Evol Microbiol* 2019;69:3448–3459.
51. **Beukes CW**, **Stępkowski T**, **Venter SN**, **Cłapa T**, **Phalane FL**, ***et al***. Crotalarieae and Genisteae of the South African Great Escarpment are nodulated by novel *Bradyrhizobium* species with unique and diverse symbiotic loci. *Mol Phylogenet Evol* 2016;100:206–218.

52. **Remigi P, Zhu J, Young JPW, Masson-Boivin C**. Symbiosis within symbiosis: evolving nitrogen-fixing legume symbionts. *Trends Microbiol* 2016;24:63–75.
53. **Paulitsch F, Delamuta JRM, Ribeiro RA, da Silva Batista JS, Hungria M**. Phylogeny of symbiotic genes reveals symbiovars within legume-nodulating *Paraburkholderia* species. *Syst Appl Microbiol* 2020;43:126151.
54. **Wang R, Chang YL, Zheng WT, Zhang D, Zhang XX, *et al***. *Bradyrhizobium arachidis* sp. nov., isolated from effective nodules of *Arachis hypogaea* grown in China. *Syst Appl Microbiol* 2013;36:101–105.
55. **Aserse AA, Woyke T, Kyrpides NC, Whitman WB, Lindström K**. Draft genome sequences of *Bradyrhizobium shewense* sp. nov. ERR11^T and *Bradyrhizobium yuanmingense* CCBAU 10071^T. *Stand Genomic Sci* 2017;12:1–14.
56. **Martins da Costa E, Azarias Guimarães A, Soares de Carvalho T, Louzada Rodrigues T, de Almeida Ribeiro PR, *et al***. *Bradyrhizobium forestalis* sp. nov., an efficient nitrogen-fixing bacterium isolated from nodules of forest legume species in the Amazon. *Arch Microbiol* 2018;200:743–752.
57. **Urquiaga MC de O, Klepa MS, Somasegaran P, Ribeiro RA, Delamuta JRM, *et al***. *Bradyrhizobium frederickii* sp. nov., a nitrogen-fixing lineage isolated from nodules of the caesalpinioid species *Chamaecrista fasciculata* and characterized by tolerance to high temperature *in vitro* *Int J Syst Evol Microbiol* 2019;69:3863–3877.
58. **Li YH, Wang R, Sui XH, Wang ET, Zhang XX, *et al***. *Bradyrhizobium nanningense* sp. nov., *Bradyrhizobium guangzhouense* sp. nov. and *Bradyrhizobium zhanjiangense* sp. nov., isolated from effective nodules of peanut in Southeast China. *Syst Appl Microbiol* 2019;42:126002.
59. **Fossou RK, Pothier JF, Zézé A, Perret X**. *Bradyrhizobium ivorense* sp. nov. as a potential local bioinoculant for *Cajanus cajan* cultures in Côte d'Ivoire. *Int J Syst Evol Microbiol* 2020;70:1421–1430.
60. **Overbeek R, Olson R, Pusch GD, Olsen GJ, Davis JJ, *et al***. The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). *Nucleic Acids Res* 2014;42:D206-14.
61. **Chun J, Oren A, Ventosa A, Christensen H, Arahal DR, *et al***. Proposed minimal standards for the use of genome data for the taxonomy of prokaryotes. *Int J Syst Evol Microbiol* 2018;68:461–466.
62. **Rodriguez-R LM, Konstantinidis KT**. The enveomics collection: a toolbox for specialized analyses of microbial genomes and metagenomes. *PeerJ Preprints* 2016.
63. **Meier-Kolthoff JP, Auch AF, Klenk H-P, Göker M**. Genome sequence-based species delimitation with confidence intervals and improved distance functions. *BMC Bioinformatics* 2013;14:1–14.
64. **Kuykendall LD, Saxena B, Devine TE, Udell SE**. Genetic diversity in *Bradyrhizobium japonicum* Jordan 1982 and a proposal for *Bradyrhizobium elkanii* sp. nov. *Can J Microbiol* 1992;38:501–505.
65. **Reeve W, van Berkum P, Ardley J, Tian R, Gollagher M, *et al***. High-quality permanent draft genome sequence of the *Bradyrhizobium elkanii* type strain USDA 76^T, isolated from *Glycine max* (L.) Merr. *Stand Genomic Sci* 2017;12.
66. **Martins da Costa E, Azarias Guimarães A, Pereira Vicentin R, de Almeida Ribeiro PR, Ribas Leão AC, *et al***. *Bradyrhizobium brasilense* sp. nov., a symbiotic nitrogen-fixing bacterium isolated from Brazilian tropical soils. *Arch Microbiol* 2017;199:1211–1221.
67. **Ramírez-Bahena MH, Peix A, Rivas R, Camacho M, Rodríguez-Navarro DN, *et al***. *Bradyrhizobium pachyrhizi* sp. nov. and *Bradyrhizobium jicamae* sp. nov., isolated from effective nodules of *Pachyrhizus erosus*. *Int J Syst Evol Microbiol* 2009;59:1929–1934.
68. **Stacey G, Luka S, Sanjuan J, Banfalvi Z, Nieuwkoop AJ, *et al***. *nodZ*, a unique host-specific nodulation gene, is involved in the fucosylation of the lipooligosaccharide nodulation signal of *Bradyrhizobium japonicum*. *J Bacteriol* 1994;176:620–633.
69. **López-Lara IM, Blok-Tip L, Quinto C, Garcia ML, Stacey G, *et al***. *NodZ* of *Bradyrhizobium* extends the nodulation host range of *Rhizobium* by adding a fucosyl residue to nodulation signals. *Mol Microbiol* 1996;21:397–408.
70. **Hungria M, Chueire L de O, Coca RG, Megías M**. Preliminary characterization of fast growing rhizobial strains isolated from soyabean nodules in Brazil. *Soil Biol Biochem* 2001;33:1349–1361.
71. **Bauer AW, Kirby WMM, Sherris JC, Turck M**. Antibiotic susceptibility testing by a standardized single disk method. *Am J Clin Pathol* 1966;45:493–496.
72. **Yates R, Howieson J, Hungria M, Bala A, O'Hara G, *et al***. Authentication of rhizobia and assessment of the legume symbiosis in controlled plant growth systems. In: *Working with Rhizobia*. Canberra: Australian Centre for International Agricultural Research, 2016. pp. 73–108.

International Journal of Systematic and Evolutionary Microbiology

SUPPLEMENTARY MATERIAL

***Bradyrhizobium cenepequi* sp. nov., *Bradyrhizobium semiaridum* sp. nov., *Bradyrhizobium hereditatis* sp. nov. and *Bradyrhizobium australafricanum* sp. nov., symbionts of different leguminous plants of Western Australia and South Africa and definition of three novel symbiovars**

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Table S1. Accession numbers of 16S rRNA, *atpD*, *dnaK*, *glnII*, *gyrB*, *recA* and *rpoB* used in this study.

Strains	16S rRNA	*atpD*	*dnaK*	*glnII*	*gyrB*	*recA*	*rpoB*
***B. cenepequi* CNPSo 4026^T**	**JAGKJI000000000**	**JAGKJI000000000**	**MK674811**	**MK860838**	**MK860859**	**MK863439**	**JAGKJI000000000**
***B. semiardium* WSM 1704^T**	**JAGKJJ000000000**	**JAGKJJ000000000**	**MK674812**	**MK860839**	**MK860860**	**MK863440**	**JAGKJJ000000000**
***B. hereditatis*WSM 1738^T**	**JAGKJK000000000**	**JAGKJK000000000**	**MK674816**	**MK860843**	**MK860864**	**MK863444**	**JAGKJK000000000**
***B. australafricanum* WSM 4400^T**	**MK676054**	**JAGKJL000000000**	**MK674809**	**MK860836**	**MK860857**	**MK863437**	**JAGKJL000000000**
B. agreste CNPSo 4010^T	MK676048	MT683853	MK674804	MK860831	MK860852	MK863432	JACCHP000000000
B. algeriense RST89^T	PYCM01000001.1	KF956544.1	FJ264922.1	FJ264924.1	PYCM01000001.1	FJ264927.1	-
B. americanum CMVU44^T	KU991833.1	KC247125.1	-	KX012942.1	-	KC247141.1	-
B. amphicarpaeae 39S1MB^T	KP768779	KP768547.1	CP029426	KP768605.1	KP768721.1	KF615002.1	KP768663.1
B. arachidis CCBAU 051107^T	NZ_FPBQ00000000.1	HM107217.1	JX437668.1	HM107251.1	JX437675.1	HM107233.1	JX437682.1
B. archetypum WSM 1744^T	MK676065	MT070745	MK674820	MK860847	MK860868	MK863448	JAAVLW000000000
B. australiense WSM 1791^T	MK676067	MT070746	MK674822	MK860849	MK860870	MK863450	JAAVLX000000000
B. betae LMG 21987^T	AY372184	FM253129	AY923046.1	AB353733.1	FM253217	AB353734.1	FM253260
B. brasilense UFLA03-321^T	NZ_MPVQ00000000.1	KF452730.1	KF452791.1	NZ_MPVQ00000000	KF452827.1	KT793142.1	KF452879.1
B. cajani AMBPC1010^T	KY349447.1	NZ_WQNE01000000	NZ_WQNE00000000	KY349442.1	NZ_WQNE00000000	KY349440.1	NZ_WQNE01000005.1
B. campsiandrae UFLA 01-1174^T	JAANIH000000000	JAANIH000000000	KT793131.1	JAANIH000000000	KT793134.1	KT793146.1	JAANIH000000000
B canariense LMG 22265^T	AJ558025.1	AY386739.1	AY923047.1	AY386765.1	FM253220	FM253177	FM253263
B. centrolobii BR 10245^T	KF927049.1	NZ_LUUB01000000	KX527928.1	KX527991.1	KX528004.1	KX527954.1	KF983827.3
B. centrosemae A9^T	KC247115.1	KC247129.1	-	KX012940.1	-	KC247145.1	-
B. coscmicum 58S1^T	KP768789.1	CP041656	CP041656	KP768615.1	KP768731.1	KF615104.1	KP768673.1
B. cytisi CTAW11^T	EU561065.2	GU001613.1	JQ945184.1	GU001594.1	JN186292.1	GU001575.1	JN186288.1
B. daqingense CCBAU 15774 ^{T}	NZ_VLKL01000000	HQ231289.1	KF962684.1	HQ231301.1	JX437669.1	HQ231270.1	JX437676.1
B. denitrificans LMG 8443^T	X66025.1	FM253153.1	KF962685.1	HM047121.1	FM253239.1	FM253196.1	FM253282.1
B. diazoefficiens USDA 110^T	NC_004463.1	NC_004463.1	NC_004463.1	NC_004463.1	NC_004463.1	NC_004463.1	NC_004463.1
B. diversitatis CNPSo 4019^T	MK676046	MT683855	MK674802	MK860829	MK860850	MK863430	JACEGD000000000
B. elkanii USDA 76^T	U35000	AY386758.1	AY328392.1	AY599117.1	AM418800	AY591568.1	AM295348
B. embrapense CNPSo 2833^T	AY904773.1	HQ634875	KP234519.2	GQ160500	HQ634891	HQ634899	HQ634910.1
B. erythrophlei CCBAU 53325^T	KF114645.1	-	MG811656.1	KF114693.1	KF114717.1	KF114669.1	MG811654.1

B. ferriligni CCBAU 51502^T	KJ818096.1	-	MG811657.1	KJ818099.1	KJ818102.1	KJ818112.1	MG811655.1
B. forestalis INPA54B^T	NZ_PGVG00000000.1	KF452722.1	KF452796.1	NZ_PGVG00000000	KF452831.1	KF452867.1	PGVG00000000.1
B. frederickii CNPSo 3426^T	MK672937	SPQS00000000	SPQS00000000	MK682688	MK682721	MK682710	MK682699.1
B. ganzhouense RITF806^T	JQ796661.2	JX277182.1	KP420023.1	JX277110.1	KP420022.1	JX277144.1	-
B. glycinis CNPSo 4016^T	MK676047	MT683854	MK674803	MK860830	MK860851	MK863431	JACCHQ000000000
B. guangdongense CCBAU 51649^T	CP030051.1	KC508916.1	KC508964.1	KC509023.1	KC509072.1	KC509269.1	KC509318.1
B. guangxiense CCBAU 53363^T	NZ_CP022219.1	KC508926.1	KC508974.1	KC509033.1	KC509082.1	KC509279.1	KC509328.1
B. guangzhouense CCBAU 51670^T	CP030053.1	KC508902.1	KC508950.1	KC509008.1	KC509057.1	KC509254.1	CP030053.1
B. hipponense aSej3^T	MK458611.1	VSTH00000000	VSTH00000000	VSTH00000000	VSTH00000000	VSTH00000000	VSTH00000000
B. huanghuaihaiense CCBAU 23303^T	HQ231463.1	HQ231682.1	JX437665.1	HQ231639.1	JX437672.1	HQ231595.1	HQ587647.1
B. icense LMTR 13^T	KF896156	KF896192	KF896182	KF896175	KF896201	JX943615	NZ_CP016428.1
B. ingae BR 10250^T	KF927043.1	KY753593.1	KF927055.1	KF927067.1	KF927079.1	KF927061.1	KF927073.1
B. iriomotense EK05^T	AB300992	AB300994.1	JF308944.1	AB300995	AB300997	AB300996	HQ587646.1
B. ivorense CI-1B^T	KX396570.1	CAADFC000000000	MK376326	MH756157	MH756161	MK376330	KX388393.1
B. japonicum USDA 6^T	X66024	AM168320	AM168362	AF169582	AM418801	AM182158	AM295349
B. jicamae PAC68^T	AY624134	FJ428211	JF308945.1	FJ428204	HQ873309.1	HM047133.1	HQ587647
B. kavangense 14-3^T	KP899562.1	KY753592.1	KR259949.1	KM378446.1	KX661397.1	KM378399.1	KM378311.1
B. lablabi CCBAU 23086^T	NZ_LLYB00000000.1	GU433473.1	KF962687.1	GU433498.1	JX437670.1	GU433522.1	JX437677
B. liaoningense LMG 18230^T	AF208513	AY386752.1	AY923041.1	AY386775.1	FM253223	AY591564.1	FM253266
B. lupini USDA 3051^T	KM114861.1	KU738808.1	-	KM114862.1	-	KM114866.1	-
B. macuxiense BR 10303^T	NZ_LNCU00000000.1	NZ_LNCU01000024.1	KX527932.1	KX527995.1	KX528008.1	KX527958.1	KX527969.1
B. manausense BR 3351^T	HQ641226.2	NZ_LJYG01000000	KF786001.1	KF785986.1	KF786000.1	KF785992.1	KF785998.1
B. mercantei SEMIA 6399^T	FJ025102.1	NZ_MKFI01000000	KX690617.1	MK860844	KX690623.1	KX690615.1	NZ_MKFI01000000.1
B. murdochi WSM 1741^T	MK676062	MT070743	MK674817	MK860844	MK860865	MK863445	AXAU00000000
B. namibiense 5-10^T	KX661401.2	KX661387.1	KP402058.1	KM378440.1	KX661393.1	KM378377.1	KM378306.1
B. nanningense CCBAU 53390^T	NZ_LBJC01000082.1	KC508921.1	KC508969.1	KC509028.1	KC509077.1	KC509274.1	KC509323.1
B. neotropicale BR 10247^T	NZ_LSEF00000000.1	NZ_LSEF01000000	KJ661693.1	KJ661700.1	KJ661707.1	KJ661714.1	KF983829.1
B. niftali CNPSo 3448^T	SPQT00000000.1	SPQT00000000	SPQT00000000	MK675791	MK675794	MK675797	MK675800.1
B. nitroreducens TSA1^T	AB542368.1	NZ_LFJC00000000	NZ_LFJC01000003.1	NZ_LFJC00000000.1	NZ_LFJC00000000.1	NZ_LFJC00000000.1	NZ_LFJC00000000.1

B. oligotrophicum LMG 10732^T	JQ619230.1	JQ619232.1	KF962688.1	JQ619233.1	KF962697.1	JQ619231.1	KF962713.1
B. ottawaense OO99^T	JN186270.1	HQ455212.1	JF308816.1	HQ587750.1	HQ873179.1	HQ587287.1	HQ587518.1
B. pachyrhizi PAC48^T	AY624135	FJ428208	JF308946.1	FJ428201.1	HQ873310.1	HM047130.1	HQ587648.1
B. paxllaeri LMTR 21^T	AY923031	KF896186	AY923038	KF896169	KF896195	JX943617	KP308154.1
B. quebequense 66S1MB^T	KP768782.1	KP768550.1	JABWSX000000000	KP768608.1	KP768724.1	KF615025.1	KP768666.1
B. retamae Ro19^T	NZ_LLYA00000000.1	FJ428208	KF896184.1	KC247108	KF896204.1	KF962711.1	KF962714.1
B. rifense CTAW71^T	EU561074.2	GU001617.1	JQ945187.1	GU001604.1	KC569466.1	GU001585.1	KC569468.1
B. ripae WR4^T	MF593081.1	-	MF593102.1	MF593086.1	MF593094.1	MF593090.1	MF593098.1
B. sacchari BR 10280^T	KF113091.3	KX065107.1	KX065103.1	KX065099.1	LWIG00000000	KX065095.1	-
B. septentrionale 1S1^T	KP768787.1	KP768555.1	JABXFA000000000	KP768613.1	KP768729.1	KF615049.1	KP768671.1
B. shewense ERR11^T	NZ_FMAI01000001.1	NZ_FMAI01000000.1	NZ_FMAI00000000	JQ809893.1	NZ_FMAI01000013.1	NZ_FMAI01000022.1	JQ810006.1
B. stylosanthis BR 446^T	NZ_LVEM01000003.1	NZ_LVEM00000000	KU724145	KU724148	KU724151	KU724163.1	KU724166
B. subterraneum 58 2-1^T	KP308152.1	KX661391.1	KP308157.1	KM378484.1	KX661396.1	KM378397	KM378349.1
B. symbiodeficiens 85S1MB^T	KP768783.1	KP768551.1	CP029427	KP768609.1	KP768725.1	KF615036.1	KP768667.1
B. tropiciagri CNPSo 1112^T	AY904753.1	FJ390968	FJ391008.1	FJ391048	HQ634890	FJ391168	HQ634909.1
B. uaiense UFLA 03-164^T	NZ_VKHP01000993.1	KF452739.1	KF452780.1	NZ_VKHP00000000	KT793133.1	KT793144.1	NZ_VKHP00000000
B. valentinum LmjM3^T	JX514883.2	JX518561.2	NZ_LLXX01000028.1	JX518575	NZ_LLXX01000044.1	JX518589.2	-
B. vignae 7-2^T	NZ_RDQF00000000.1	KX683215.1	KR259951.1	KM378443.1	KX683216.1	KM378374.1	KM378308.1
B. viridifuturi SEMIA 690^T	FJ025107.1	NZ_LGTB01000039.1	KR149128	KR149131.1	KR149134.1	KR149140	KU724169.1
B. yuanmingense LMG 21827^T	AF193818	AY386760.1	AY923039.1	AY386780.1	FM253226	AM168343	FM253269
B. zhanjiangense CCBAU 51778^T	NZ_CP022221.1	KC508911.1	KC508959.1	KC509017.1	KC509066.1	KC509263.1	KC509312.1
B. elkanii SEMIA 5019	AF237422.2	-	-	FJ391030.1	-	FJ391150.1	-
B. elkanii SEMIA 587	AF234890.2	-	-	FJ391025.1	-	FJ391145.1	-
B. diazoefficiens SEMIA 5080	AF234889.2	-	-	FJ391037.1	-	FJ391157.1	-
B. japonicum SEMIA 5079	AF234888.2	-	-	FJ391036.1	-	FJ391156.1	-
B. pachyrhizi BR 3262	-	-	-	KF828816.1	-	KF828817.1	-
Bradyrhizobium sp. GV137	KF483532.1	-	-	-	-	-	-
Bradyehizobium sp. VUCR24	LT630292.1	-	-	LT630312.1	-	LT630304.1	-
Bradyrhizobium sp. VULI21	LT630290.1	-	-	LT630311.1	-	LT630303.1	-
Bradyrhizobium sp. VULI11	LT630288.1	-	-	LT630310.1	-	LT630302.1	-
Bradyrhizobium sp. VUPME10	HG940529.1	-	-	-	-	-	-

Table S2. Nucleotide Identity (NI) among new lineages of *Bradyrhizobium* and closely related species, based on the sequences of symbiotic genes (*nodC* and *nifH*).

Nucleotide Identity		
Strains	***nodC* (335 bp)**	***nifH* (205 bp)**
	***B. cenepequi* CNPSo 4026^T**	
B. valentinum LmjM3^T	91.6	94.6
B. lablabi CCBAU 23086^T	91.3	93.6
B.retamae Ro19 ^{T}	91.3	93.6
	***B. hereditatis*WSM 1738^T**	
B. lablabi CCBAU 23086^T	98.5	98
B. murdochi WSM 1741^T	98.5	99.5
B. paxllaeri LMTR 21^T	97.6	98
B. namibiense 5-10 ^{T}	96.4	97.5
B. icense LMTR 13^T	95.5	97
B. retamae Ro19 ^{T}	95.5	96.5
B. archetypum WSM 1744^T	95.2	97
B. australiense WSM 1791^T	95.2	95.1
B. valentinum LmjM3^T	95.2	97
B. algeriense RST91^T	94.6	97
	***B. australafricanum* WSM 4400^T**	
B. pachyrhizi BR 3262	99.1	99.5
Bradyrhizobium sp. VULI21	98.8	99.5
Bradyrhizobium sp. VUCR24	96.1	99.5
Bradyrhizobium sp. STM 3062	95.8	94.1
Bradyrhizobium sp. VULI11	95.2	-
Bradyrhizobium sp. VUPME10	95.2	99.5

Table S3. Novel symbiovars in *Bradyrhizobium* genus.

Symbiovar	Strains	Original host	Host range	Geographical origin	NI among each other in *nodC* phylogeny	References
cenepequi	*Bradyrhizobium cenepequi* CNPSo 4026^T	*Vigna unguiculata*	*Macroptilium atropurpureum*	Australia	≤ 91.6 %	Helene et al. [25]; this study
glycinis	*B. glycinis* CNPSo 4016^T	*Glycine tabacina*	*M. atropurpureum*	Australia	≤ 92.5 %	Helene et al. [25]; Klepa et al. [39]
cajani	*B. agreste* CNPSo 4010^T	*Glycine clandestina*	*M. atropurpureum*	Australia	91.3 - 96.4 %	Helene et al. [25]; Klepa et al. [39]
	B. arachidis CCBAU 051107^T	*Arachidis hypogaea*	*Lablab purpureus*	China	92.8 - 98.8 %	Wang et al. [54]
	B. cajani AMBPC1010^T	*Cajanus cajan*	ND	Dominican Republic	92.2 - 98.8 %	Araújo et al. [75]
	B. forestalis INPA54B^T	*Inga* sp.	*M. atropurpureum; Phaseolus lunatus; Stizolobium aterrimum*	Brazil	92.8 - 98.8 %	Costa et al. [56]
	B. frederickii CNPSo 3426^T	*Chamaecrista fasciculata*	*M. atropurpureum*	United States	93.2 - 99.7 %	Urquiaga et al. [57]
	B. kavangense 14-3^T	*V. unguiculata*	*Vigna subterranea; L. purpureus; A. hypogaea*	Namibia	91.6 - 94.9 %	Grönemeyer et al. [72, 73]
	B. niftali CNPSo 3448^T	*C. fasciculata*	*M. atropurpureum*	United States	94.3 - 99.7 %	Klepa et al. [50]
	B. shewense ERR11^T	*Erythrina brucei*	*Indigofera arrecta, A. hypogaea*	Ethiopia	93.4 - 97 %	Aserse et al. [55]
	B. subterraneum 58 2-1^T	*A. hypogaea*	*V. unguiculata; V. subterranea; L. purpureus*	Namibia	93.7 - 96.4 %	Grönemeyer et al. [72; 74]
	B. vignae 7-2^T	*V. unguiculata*	*V. subterranea; L. purpureus; A. hypogaea*	Namibia	91.3 - 94 %	Grönemeyer et al. [72; 75]
	B. zhanjiangense CCBAU 51778^T	*A. hypogaea*	*L. purpureus; A. hypogaea; Vigna radiata*	China	93.4 - 96.4 %	Li et al. [58]

Legends of Supplementary Figures

Figure S1. Maximum likelihood phylogeny based from *atpD* alignment (398 bp), using the GTR: General Time Reversible +G+I model by MEGA v.7. Accession numbers are indicated in parentheses and in Table S1. The novel species are shown in bold. Bootstrap values >70 % are indicated at the nodes. *Xanthobacter autotrophicus* Py2 was used as outgroup. Bar indicates five substitutions per 100 nucleotide positions.

Figure S2. Maximum likelihood phylogeny based from *dnaK* alignment (221 bp), using the TN93: Tamura-Nei +G model by MEGA v.7. Accession numbers are indicated in parentheses and in Table S1. The novel species are shown in bold. Bootstrap values >70 % are indicated at the nodes. *Xanthobacter autotrophicus* Py2 was used as outgroup. Bar indicates five substitutions per 100 nucleotide positions.

Figure S3. Maximum likelihood phylogeny based from *glnII* alignment (504 bp), using the GTR: General Time Reversible +G model by MEGA v.7. Accession numbers are indicated in parentheses and in Table S1. The novel species are shown in bold. Bootstrap values >70 % are indicated at the nodes. *Xanthobacter autotrophicus* Py2 was used as outgroup. Bar indicates two substitutions per 100 nucleotide positions.

Figure S4. Maximum likelihood phylogeny based from *gyrB* alignment (553 bp), using the GTR: General Time Reversible +G model by MEGA v.7. Accession numbers are indicated in parentheses and in Table S1. The novel species are shown in bold. Bootstrap values >70 % are indicated at the nodes. *Xanthobacter autotrophicus* Py2 was used as outgroup. Bar indicates five substitutions per 100 nucleotide positions.

Figure S5. Maximum likelihood phylogeny based from *recA* alignment (360 bp), using the TN93: Tamura-Nei +G+I model by MEGA v.7. Accession numbers are indicated in parentheses and in Table S1. The novel species are shown in bold. Bootstrap values >70 % are indicated at the nodes. *Xanthobacter autotrophicus* Py2 was used as outgroup. Bar indicates five substitutions per 100 nucleotide positions.

Figure S6. Maximum likelihood phylogeny based from *rpoB* alignment (439 bp), using the TN93: Tamura-Nei +G model by MEGA v.7. Accession numbers are indicated in parentheses and in Table S1. The novel species are shown in bold. Bootstrap values >70 % are indicated at the nodes. *Xanthobacter autotrophicus* Py2 was used as outgroup. Bar indicates two substitutions per 100 nucleotide positions.

Figure S7. Maximum likelihood phylogeny based from alignment of the *atpD+dnaK+glnII+gyrB+recA+rpoB* complete sequences concatenated (11,676 bp), using the GTR: General Time Reversible +G+I model by MEGA v. 7. Accession numbers are indicated in parentheses. The novel species are shown in bold. Bootstrap values >70 % are indicated at the nodes. *Xanthobacter autotrophicus* Py2 was used as outgroup. *Xanthobacter autotrophicus* Py2 was used as outgroup. Bar indicates five substitutions per 100 nucleotide positions.

Figure S8. Maximum likelihood phylogeny based from *nifH* alignment (205 bp), using the T92: Tamura 3-Parameter +G+I model by MEGA v.7. Accession numbers are indicated in parentheses. The novel species are shown in bold. Bootstrap values >70 % are indicated at the nodes. Bar indicates two substitutions per 100 nucleotide positions.

Figure S9. Maximum likelihood phylogeny based from 16S rRNA alignment (1,314 bp), using the T92: Tamura 3-Parameter +G+I model by MEGA v. 7. Accession numbers are indicated in parentheses and in Table S1. The novel species are shown in bold. Bootstrap values >70 % are indicated at the nodes. *Xanthobacter autotrophicus* Py2 was used as outgroup. Bar indicates one substitution per 100 nucleotide positions.

Figure S10. Maximum likelihood phylogeny based from alignment of the *glnII+recA* partial sequences concatenated (864 bp), using the GTR: General Time Reversible +G+I model by MEGA v. 7. Accession numbers are indicated in Table S1. The novel species are shown in bold. Bootstrap values >70 % are indicated at the nodes. *Xanthobacter autotrophicus* Py2 was used as outgroup. Bar indicates two substitutions per 100 nucleotide positions.

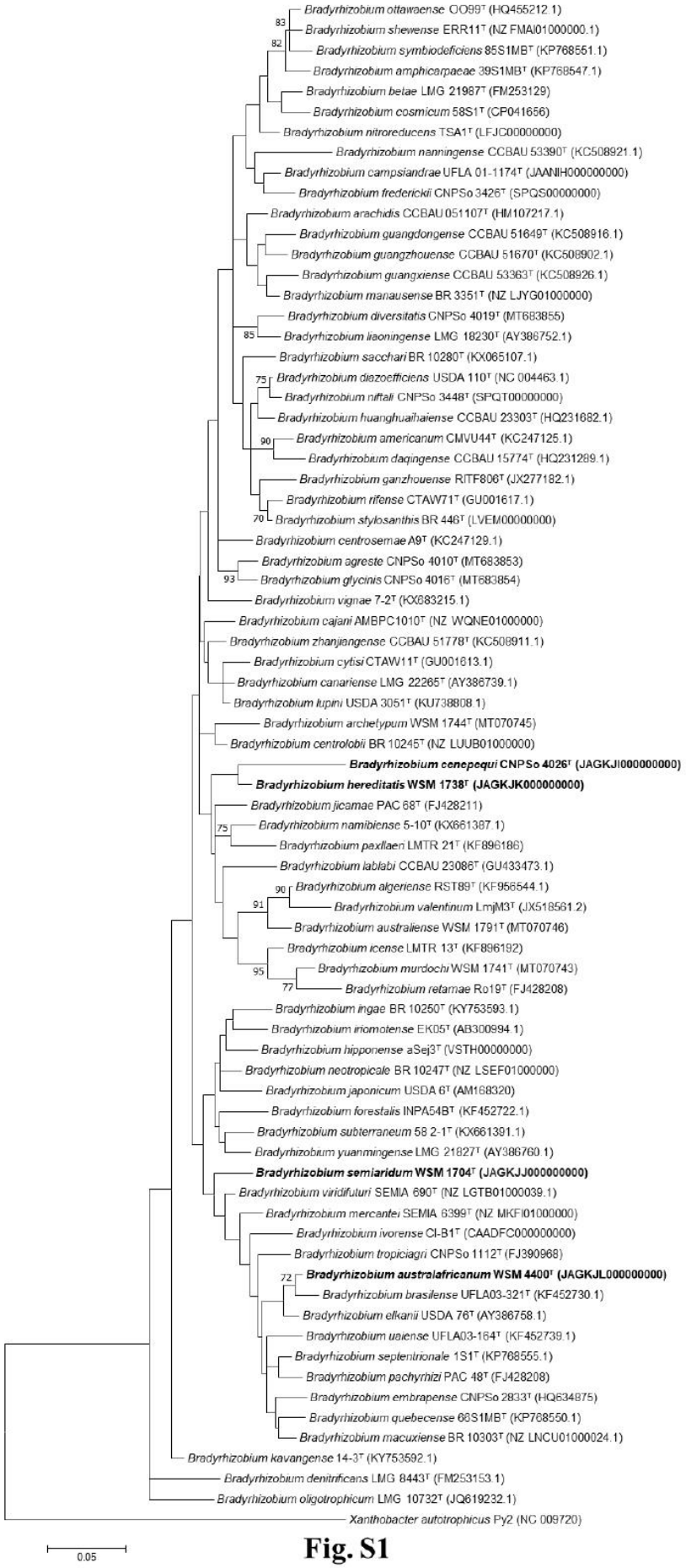
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Fig. S1

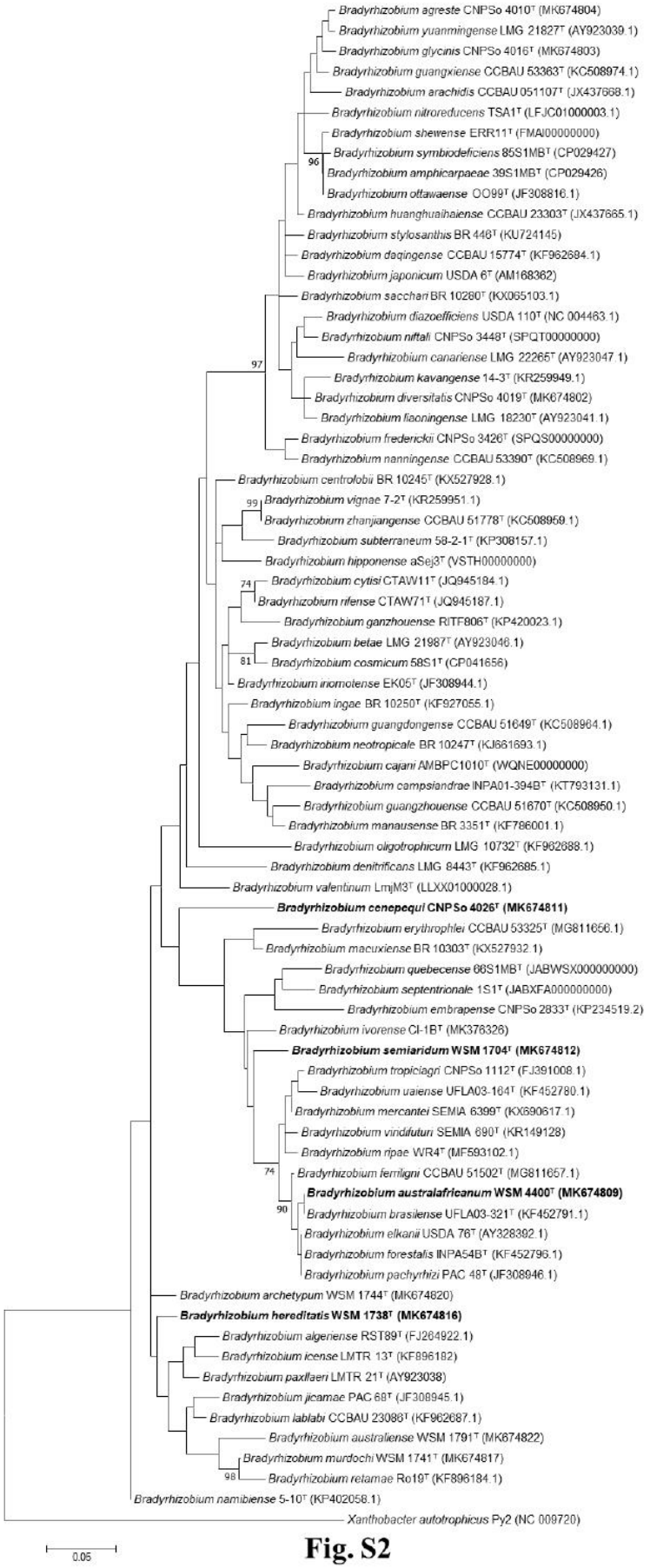
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Fig. S2

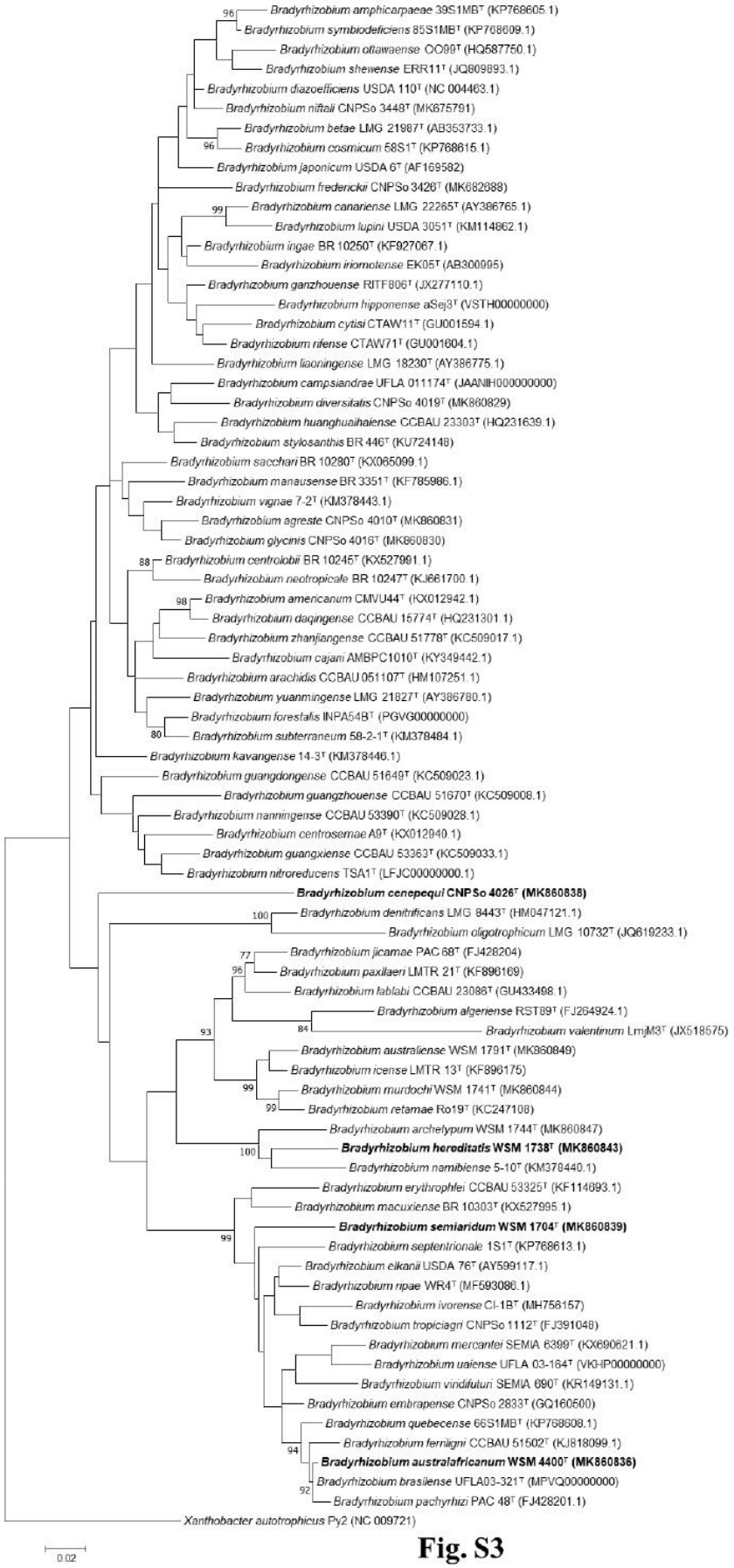
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Fig. S3

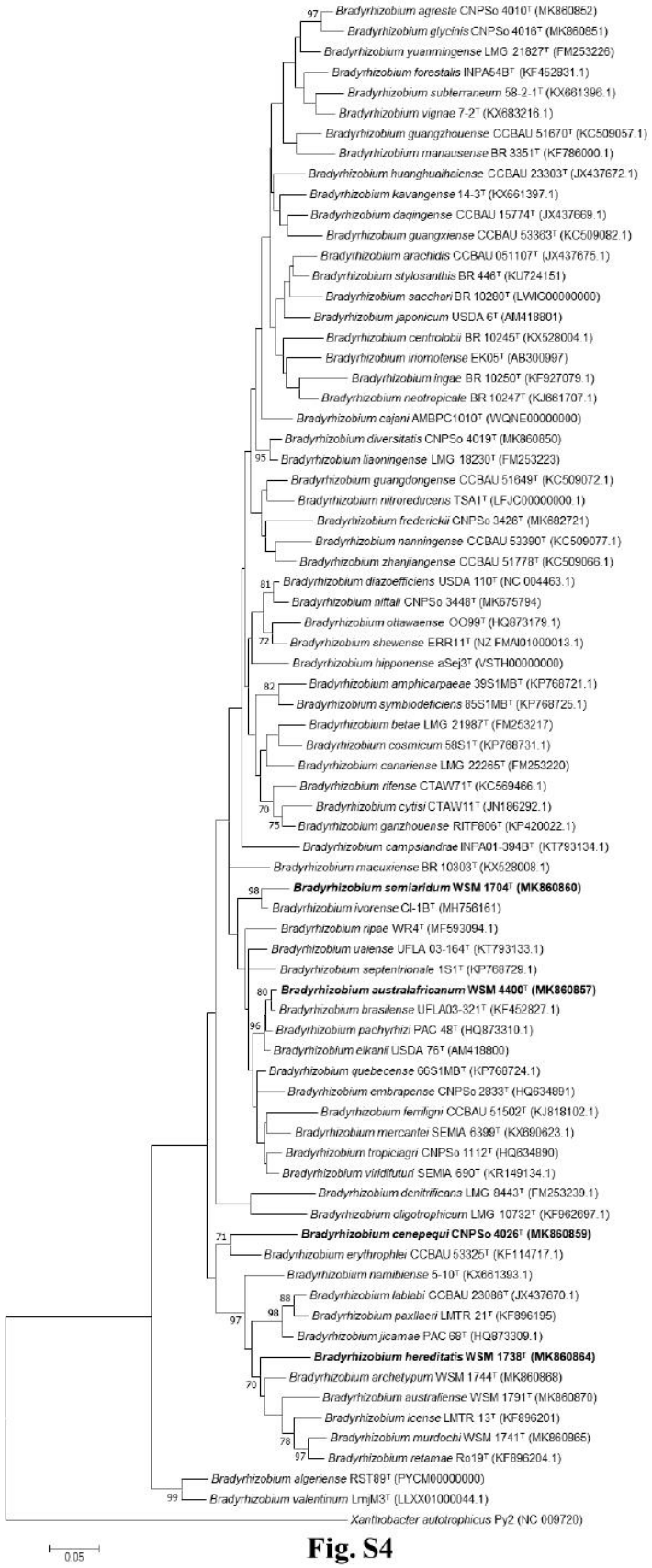
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Fig. S4

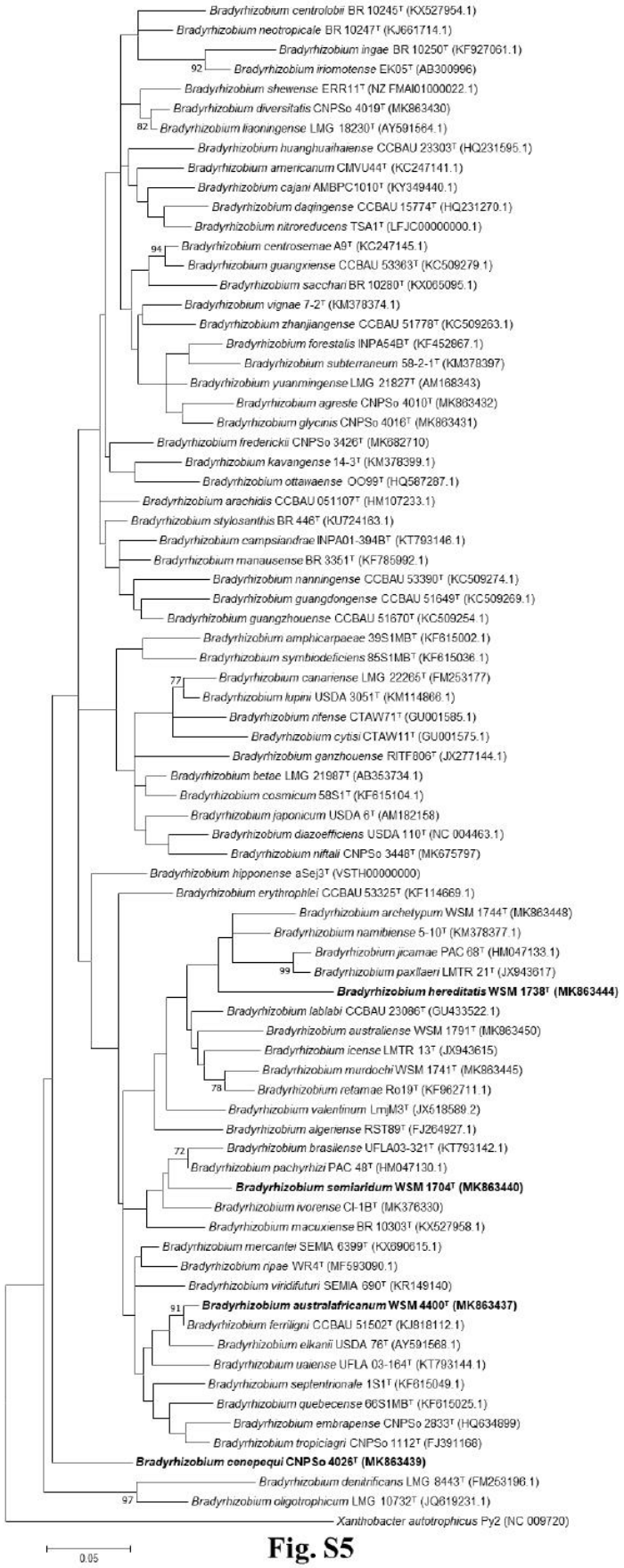
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Fig. S5

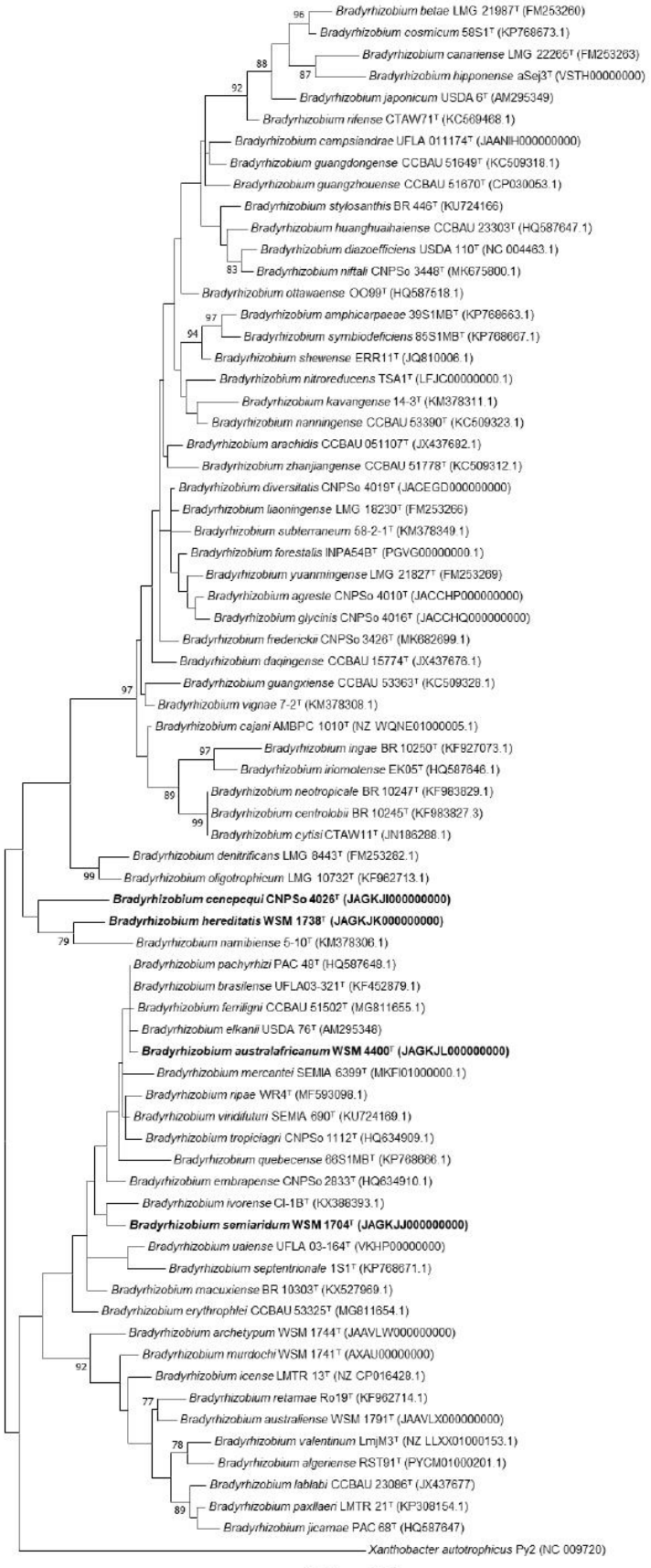
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Fig. S6

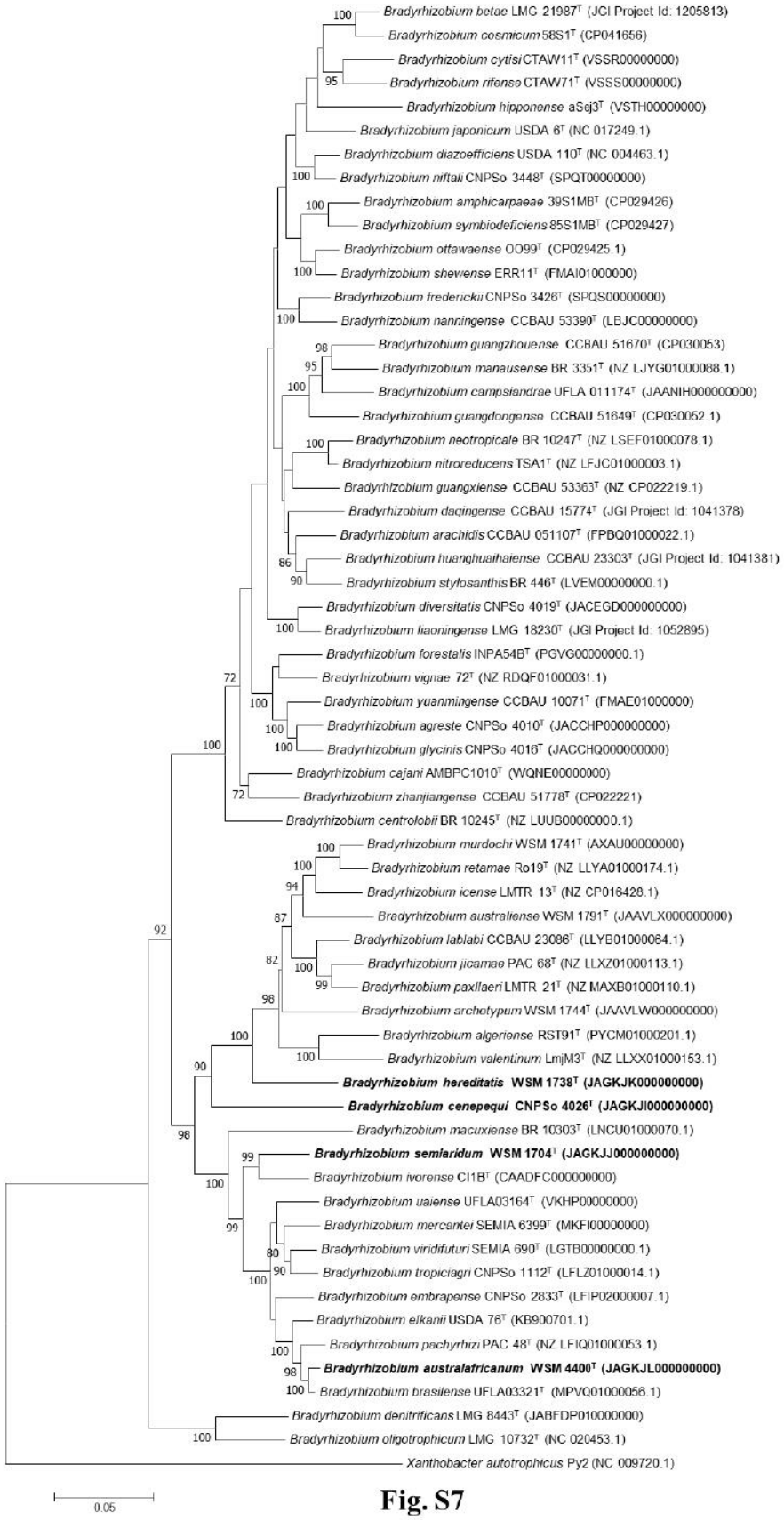
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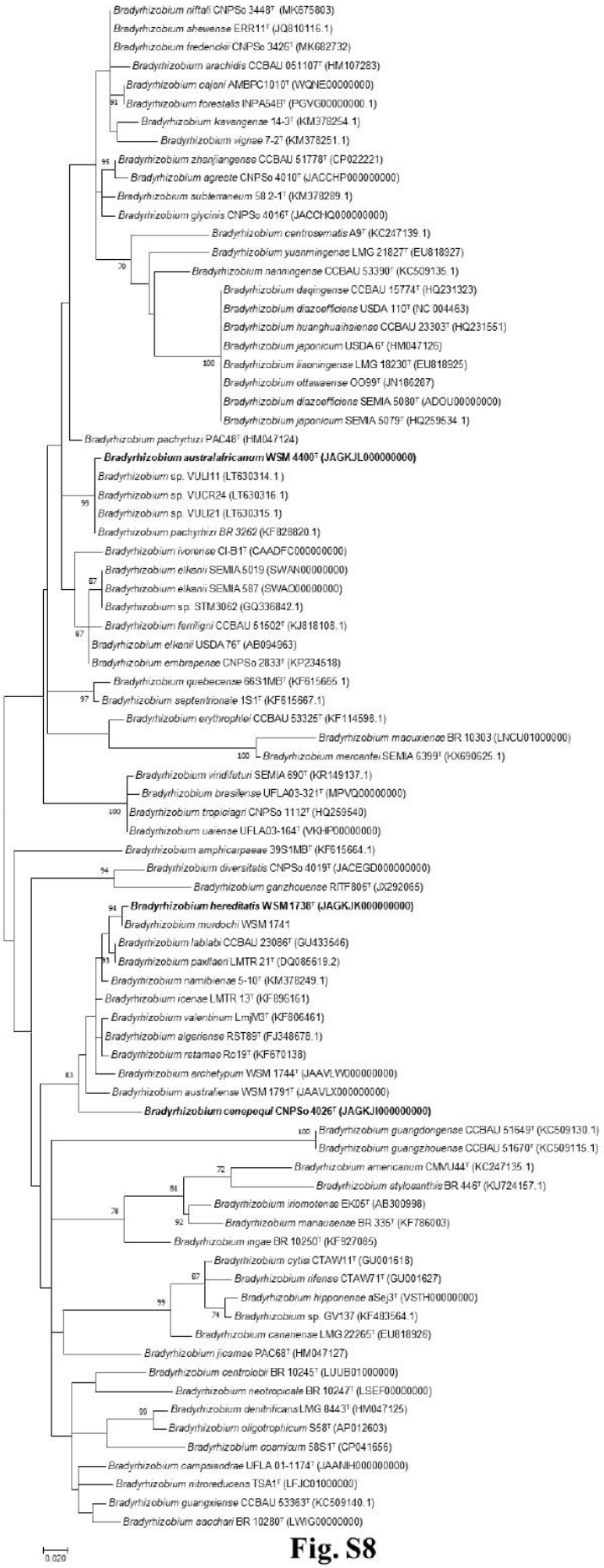
Fig. S7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Bradyrhizobium campsiandrae UFLA 01-1174T (JAANIH000000000)
Bradyrhizobium nitroreducens TSA1T (LFJC01000000)
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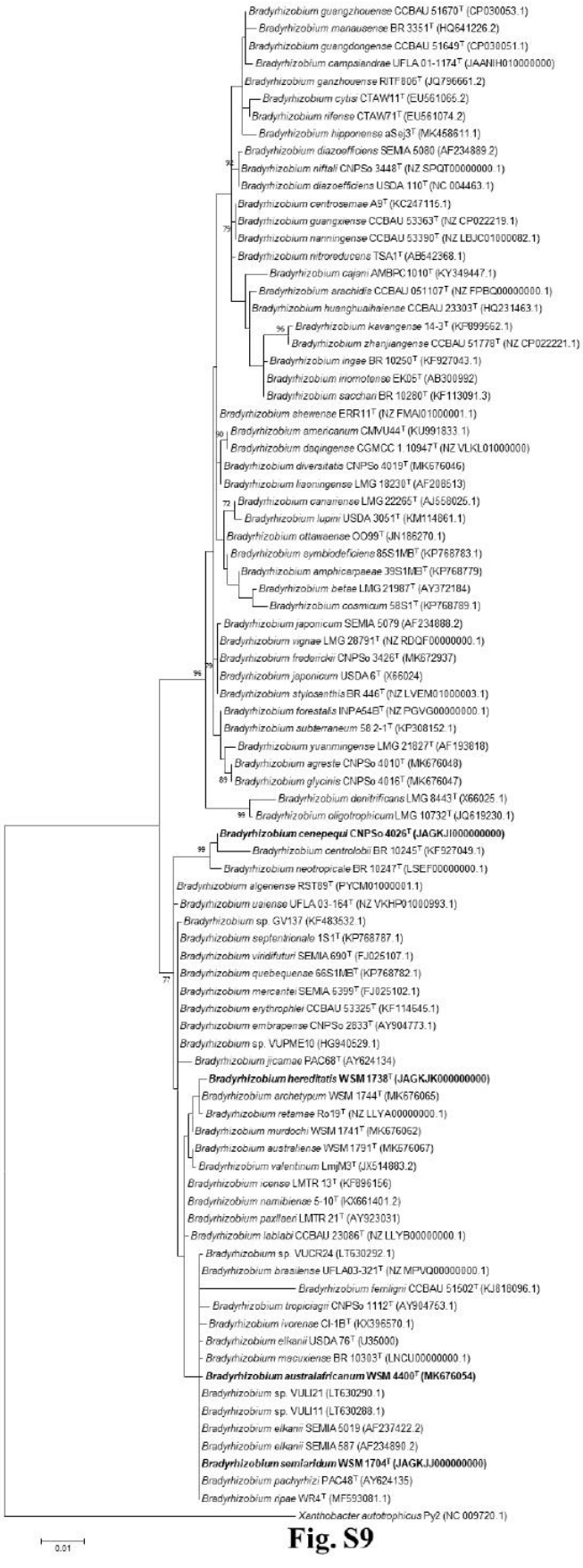
0.020

Fig. S8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Bradyrhizobium zhanjiangense CCBAU 51778T (NZ CP022221.1)
Bradyrhizobium ingae BR 10250T (KF927043.1)
Bradyrhizobium iriomotense EK05T (AB300992)
Bradyrhizobium saochan BR 10280T (KF113091.3)
Bradyrhizobium shewense ERR11T (NZ FMAI01000001.1)
Bradyrhizobium americanum CMVU44T (KU991833.1)
Bradyrhizobium daqingense CGMCC 1.10947T (NZ VLKL01000000)
Bradyrhizobium diversitatis CNPSo 4019T (MK676046)
Bradyrhizobium liaoningense LMG 18230T (AF208513)
Bradyrhizobium canariense LMG 22265T (AJ558025.1)
Bradyrhizobium lupini USDA 3051T (KM114861.1)
Bradyrhizobium ottawaense OO99T (JN186270.1)
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Bradyrhizobium glycinis CNPSo 4016T (MK676047)
Bradyrhizobium denitrificans LMG 8443T (X66025.1)
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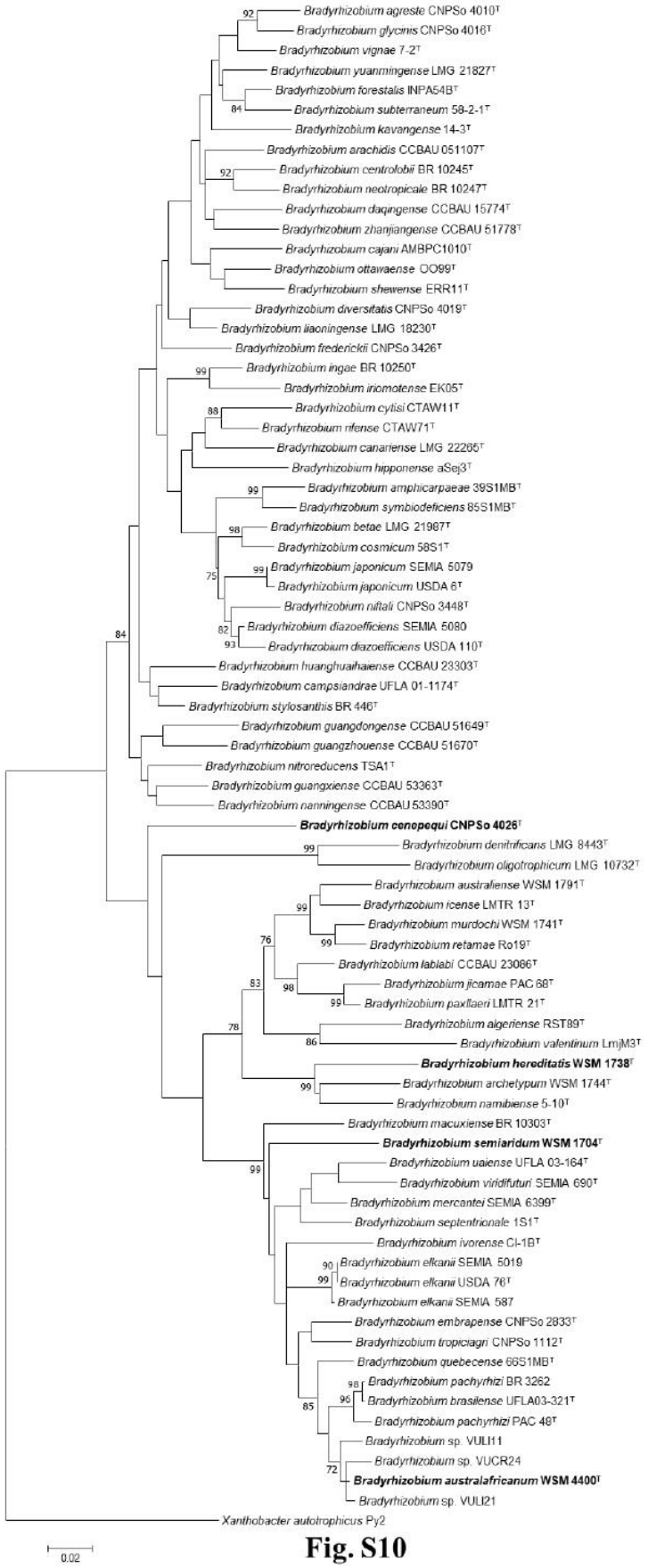
Fig. S9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Bradyrhizobium glycinis CNPSo 4016T
Bradyrhizobium vignae 7-2T
Bradyrhizobium yuanmingense LMG 21827T
Bradyrhizobium forestalis INPA54BT
Bradyrhizobium subterraneum 58-2-1T
Bradyrhizobium kavangense 14-3T
Bradyrhizobium arachidis CCBAU 051107T
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Bradyrhizobium japonicum SEMIA 5079
Bradyrhizobium japonicum USDA 6T
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Bradyrhizobium retamae Ro19T
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Bradyrhizobium brasilense UFLA03-321T
Bradyrhizobium pachyrhizi PAC 48T
Bradyrhizobium sp. VULI11
Bradyrhizobium sp. VUCR24
Bradyrhizobium australafricanum WSM 4400T
Bradyrhizobium sp. VULI21
Xanthobacter autotrophicus Py2
0.02

Fig. S10

REFERENCES

25. Helene LCF, O'Hara G, Hungria M. Characterization of *Bradyrhizobium* strains indigenous to Western Australia and South Africa indicates remarkable genetic diversity and reveals putative new species. Syst Appl Microbiol. 2020;43:126053.

39. Klepa MS, Helene LCF, O'Hara G, Hungria M. *Bradyrhizobium agreste* sp. nov., *Bradyrhizobium glycinis* sp. nov. and *Bradyrhizobium diversitatis* sp. nov., isolated from a biodiversity hotspot of the genus *Glycine* in Western Australia. Int J Syst Evol Microbiol. 2021;71:004742.

50. Klepa MS, Urquiaga MCDO, Somasegaran P, Delamuta JRM, Ribeiro RA, Hungria M. *Bradyrhizobium niftali* sp. nov., an effective nitrogen-fixing symbiont of partridge pea [*Chamaecrista fasciculata* (Michx.) Greene], a native caesalpinioid legume broadly distributed in the USA. Int J Syst Evol Microbiol. 2019;69:3448–3459.

54. Wang R, Chang YL, Zheng WT, Zhang D, Zhang XX, Sui XH, et al. *Bradyrhizobium arachidis* sp. nov., isolated from effective nodules of *Arachis hypogaea* grown in China. Syst Appl Microbiol. 2013;36(2):101–105.

56. Da Costa EM, Guimarães AA, Carvalho TS de, Rodrigues TL, Ribeiro PR de ALL, Willems A, et al. *Bradyrhizobium forestalis* sp. nov., an efficient nitrogen-fixing bacterium isolated from nodules of forest legume species in the Amazon. Arch Microbiol 2018;200(5):743–752.

57. Urquiaga MCO, Klepa MS, Somasegaran P, Ribeiro RA, Delamuta JRM, Hungria M. *Bradyrhizobium frederickii* sp. nov., a nitrogen-fixing lineage isolated from nodules of the caesalpinioid species *Chamaecrista fasciculata* and characterized by tolerance to high temperature in vitro. Int J Syst Evol Microbiol. 2019;69(12):3863–3877.

58. Li YH, Wang R, Sui XH, Wang ET, Zhang XX, Tian CF, et al. *Bradyrhizobium nanningense* sp. nov., *Bradyrhizobium guangzhouense* sp. nov. and *Bradyrhizobium zhanjiangense* sp. nov., isolated from effective nodules of peanut in Southeast China. Syst Appl Microbiol. 2019;42:126002.

72. Grönemeyer JL, Kulkarni A, Berkelmann D, Hurek T, Reinhold-Hurek B. Rhizobia indigenous to the Okavango region in Sub-Saharan Africa: diversity, adaptations, and host specificity. AEM. 2014;80: 7244–7257.

73. Grönemeyer JL, Hurek T, Reinhold-Hurek B. *Bradyrhizobium kavangense* sp. nov., a symbiotic nitrogen-fixing bacterium from root nodules of traditional Namibian pulses. Int J Syst Evol Microbiol. 2015; 65:4886–4894.

74. Grönemeyer JL, Chimwamurombe P, Reinhold-Hurek B. *Bradyrhizobium subterraneum* sp. nov., a symbiotic nitrogen-fixing bacterium from root nodules of groundnuts. Int J Syst Evol Microbiol. 2015;65:3241–3247.

75. Grönemeyer JL, Hurek T, Bünger W, Reinhold-Hurek B. *Bradyrhizobium vignae* sp. nov., a nitrogen-fixing symbiont isolated from effective nodules of *Vigna* and *Arachis*. Int J Syst Evol Microbiol. 2016;66:62–69.

76. Grönemeyer JL, Chimwamurombe P, Reinhold-Hurek B. *Bradyrhizobium subterraneum* sp. nov., a symbiotic nitrogen-fixing bacterium from root nodules of groundnuts. Int J Syst Evol Microbiol 2015;65:3241–3247.

77. Grönemeyer JL, Hurek T, Bünger W, Reinhold-Hurek B. *Bradyrhizobium vignae* sp. nov., a nitrogen-fixing symbiont isolated from effective nodules of Vigna and Arachis. Int J Syst Evol Microbiol 2016;66:62–69.

CONSIDERAÇÕES FINAIS

Embora classificar, nomear e identificar organismos ainda sejam os objetivos da taxonomia bacteriana desde os primeiros estudos realizados na década de 1870, as metodologias utilizadas pelos taxonomistas para atingir tais objetivos têm sido alteradas significativamente à medida que técnicas com maior poder de resolução vão surgindo. Os principais marcos da taxonomia bacteriana se deram com os avanços científicos em estudos sobre o DNA, seguidos pelo desenvolvimento de técnicas moleculares e a caracterização do gene ribossomal 16S. Mais recentemente, o aprimoramento das técnicas de sequenciamento genômico permitiu outro grande avanço na taxonomia bacteriana. O maior volume de dados vem permitindo a identificação de genes anteriormente desconhecidos, uma melhor compreensão das relações evolutivas existente entre bactérias e maior colaboração entre a comunidade científica, uma vez que sequências genômicas podem ser disponibilizadas em bancos de dados públicos. Com a taxonomia de rizóbios não foi diferente, e a evolução das técnicas aplicadas à taxonomia bacteriana permitiu a classificação e reclassificação de várias espécies de rizóbios, as quais totalizaram 226 espécies em outubro de 2021. No entanto, o número de espécies de rizóbios descritas ainda é pequeno quando comparado com a diversidade de leguminosas existentes e a vasta distribuição geográfica da família na natureza, as quais podem estar associadas com espécies desconhecidas rizóbios. Portanto, estudos que visem uma caracterização adequada das populações naturais de rizóbios são essenciais, uma vez que essas comunidades representam uma valiosa fonte de variabilidade genética e metabólica, a qual pode ser explorada na seleção de estirpes com características simbióticas desejáveis para a otimização da produtividade agrícola ou na participação de regeneração de áreas degradadas. Além disso, podem conter informações ainda desconhecidas acerca da evolução desses organismos.

Nos capítulos II, III e IV acessamos a diversidade do gênero *Bradyrhizobium* e contribuímos com a descrição de dez novas espécies utilizando uma abordagem polifásica. *B. archetypum, B. australiense* e *B. murdochi* foram isoladas das espécies de leguminosas indígenas da Austrália Ocidental, *Muelleranthus trifoliatus, Indigofera* sp., e *Rhynchosia minima*. *Bradyrhizobium*

agreste, B. glycinis e *B. diversitatis* foram isoladas de três espécies diferentes de leguminosas do gênero *Glycine, G. clandestina, G. tabacina* e *G. max*, na Austrália Ocidental. Baseados em dados filogenéticos das estirpes, corroboramos a hipótese de que a separação do supercontinente Gondwana favoreceu eventos de dispersão de leguminosas entre o continente asiático, centro de origem de *Glycine*, e o continente Australiano, atualmente maior *hotspost* de diversidade de espécies indígenas do gênero, possibilitando a entrada dos microssimbiontes conjuntamente. Por fim, descrevemos as novas espécies *B. cenepequi, B. semiaridum, B. hereditatis* e *B. australafricanum*, isoladas de *Vigna unguiculata*, *Tephrosia gardeneri*, *Indigofera* sp. e *Glycine* sp., na Austrália Ocidental e África do Sul, dentre as quais algumas apresentaram sequências de genes simbióticos nunca relatados anteriormente, possibilitando o delineamento de três novos simbiovares, sv. cenepequi, sv. glycinis e sv. cajani, o que permitiu corroborar a hipótese de que a evolução dos genes simbióticos *nodC* e *nifH* vem ocorrendo simultaneamente nas estirpes estudadas.