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FABIANE PAULITSCH

ANÁLISE POLIFÁSICA DE ESTIRPES BRASILEIRAS DE BETA-RIZÓBIOS DO GÊNERO *Paraburkholderia*

Londrina
2021

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

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Londrina, 23 de junho de 2021.

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“Não fui eu que lhe ordenei? Seja forte e corajoso! Não se apavore, nem se desanime, pois o Senhor, o seu Deus, estará com você por onde você andar". Josué 1:9

PAULITSCH, FABIANE. **Análise polifásica de estirpes brasileiras de beta-rizóbios do gênero *Paraburkholderia***. 2021. 218 f. Tese (Doutorado em microbiologia) – Universidade Estadual de Londrina, Londrina, 2021.

RESUMO

Um grupo restrito de bactérias diazotróficas presentes no solo é capaz de realizar simbiose com plantas da familia Fabaceae, levando à formação de nódulos radiculares, especializados no processo de Fixação Biológica do Nitrogênio (FBN). Esses microrganismos são coletivamente chamados de rizóbios e estão presentes nas classes Alfa e Betaproteobacteria. Dentre as Betaproteobacterias, o gênero *Paraburkholderia* se destaca pela sua diversidade, encontrada especialmente no Brasil e na África do Sul. No sentido de melhor compreender a diversidade e evolução de rizóbios do gênero *Paraburkholderia*, quatro estudos foram conduzidos. Utilizando a taxonomia polifásica, foram descritas as novas espécies P. guartelaensis (Estudo 1), *P. atlantica* e *P. franconis* (Estudo 2). A espécie *P. guartelaensis* foi isolada de nódulos de *Mimosa gymnas* em uma região de ecótono entre a Mata Atlântica e o Cerrado. As análises filogenéticas revelaram que as estirpes em estudo apresentam uma posição isolada em relação as outras espécies conhecidas do gênero: apresentou como espécies mais próximas *P. oxyphila* na análise do 16S RNAr, compartilhando 98,7–98,9% de IN (identidade nucleotídica), e *P. nodosa* Br3437^T na análise de sequências multilocus (MLSA), apresentando menos de 95,7% de IN. Análises comparativas baseadas em sequenciamento genômico resultaram em valores inferiores aos sugeridos para a delimitação de espécie, respaldando a descrição da nova espécie. No mesmo sentido, o estudo 2 descreve as espécies *P. atlantica* e *P. franconis*, isoladas de solos da Mata Atlantica no estado do Rio de Janeiro, utilizando como plantas-iscas *Mimosa pudica* e *Phaseolus vulgaris*. As estirpes de *P. atlantica* apresentaram maior IN na filogenia do 16S RNAr (99,6%) e MLSA (96,5%) com *P. piptadeniae* enquanto as estirpes de P. franconis apresentaram como espécie mais próxima *P. tuberum*, com IN de 99% e 96% nas filogenias do 16S RNAr e MLSA, respectivamente. Nas análises genômicas, as estirpes de *P. atlantica* e *P. franconis* apresentaram os valores de 94,4% e 92,7% de identidade genômica com as espécies mais próximas. O estudo 3 identificou simbiovares dentre as espécies de rizóbios do gênero *Paraburkholderia*. Os genes simbióticos *nodA*, *nodC* e *nifH* foram submetidos à análises filogenéticas, revelando a presença de pelo menos cinco simbiovares distintos dentro do gênero *Paraburkholderia*, com alto suporte estatístico. Os simbiovares sugeridos foram denominados como mimosae, atlantica, tropicalis, piptadeniae e africana. Por fim, o estudo 4 tem como objetivo realizar uma revisão sobre *Paraburkholderia* nodulíferas com ênfase em estirpes brasileiras. As informações foram relacionadas com a descoberta da capacidade de nodulação e FBN pelas bactérias desse gênero, o histórico taxônomico, a distribuição mundial das espécies, a presença de simbiovares, características filogenéticas, ecológicas e o potencial agrobiotecnológico. Os referidos estudos demonstram que o Brasil é um rico reservatório de espécies nodulíferas de *Paraburkholderia* e nossos resultados contribuíram para compreensão das relações evolutivas dentre as espécies do gênero e com seus hospedeiros. Por fim, os quatro estudos reforçam a grande diversidade genotípica e metabólica de rizóbios do gênero *Paraburkholderia*, em especial no Brasil, que pode ser considerado um importante centro de diversidade do gênero e fonte de bactérias com potenciais inovadores.

Palavras-chave: taxônomia polifásica; beta-rizóbios; nodulação; simbiovar.

PAULITSCH, Fabiane. **Polyphasic analysis of Brazilian beta-rhizobia of the *Paraburkholderia* genus.** 2021. 218 p. Thesis (PhD in Microbiology) – State University of Londrina, Londrina, 2021.

ABSTRACT

A restricted group of diazotrophic bacteria that inhabit the soil is able to perform symbiosis with plants of the Fabaceae family, leading to the development of an organ in the root called nodule, specialized in the Biological Nitrogen Fixation (BNF) process. These microorganisms are collectively called rhizobia and can be found in the Alpha and Betaproteobacteria classes. Among the Betaproteobacteria, the *Paraburkholderia* genus stands out for its diversity, especially in Brazil and South Africa. In order to better understand the diversity and evolution of rhizobia of the genus *Paraburkholderia*, four studies were conducted. A polyphasic approach was conducted resulting in description of the new species *P. guartelaensis* (study 1), *P. atlantica* and *P. franconis* (study 2). The *P. guartelaensis* species was isolated from *Mimosa gymnas* nodules in an ecotone region between the Atlantic Forest and the Cerrado biomes. The phylogenetic analysis revealed that the strains under study presented an isolated position in relation to other known species of the genus, with *P. oxyphila* as the closest species in the 16S rRNA analysis, sharing 98.7–98.9% of NI (nucleotide identity), and *P. nodosa* Br3437^T in the Multilocus Sequence Analysis (MLSA), presenting less than 95.7% of NI. Comparative analysis based on genomic sequencing resulted in values lower than those suggested for species delimitation, supporting the description of the new species. Similarly, the second study describes *P. atlantica* and *P. franconis* species, isolated from Atlantic Forest soils in the Rio de Janeiro state, using *Mimosa pudica* and *Phaseolus vulgaris* as trap plants. The *P. atlantica* strains showed the higher NI with *P. piptadeniae* in the phylogeny of 16S rRNA (99.6%) and MLSA (96.5%), while *P. franconis* strains presented *P. tuberum* as the closest species, with NI of 99% and 96% in the 16S rRNA and MLSA phylogenies, respectively. In the genomic analysis, the *P. atlantica* and *P. franconis* strains presented 94.4% and 92.7% of genomic identity with the closest species. The third identified symbiovars among the rhizobia species in the *Paraburkholderia* genus. The phylogenetic analysis of the symbiotic genes *nodA*, *nodC* and *nifH* revealed the presence of at least five distinct symbiovars within *Paraburkholderia*, with high statistical support. The proposed symbiovars were named as mimosae, atlantica, tropicalis, piptadeniae and africana. Finally, study 4 aims to carry out a review of nodulating *Paraburkholderia* emphasizing Brazilian strains. The gathered information is based in the discovery of the nodulation capacity and FBN by bacteria of this genus, the taxonomic history, the world distribution of the species, the presence of symbiovars, phylogenetic and ecological characteristics and the agrobiotechnological potential. These studies demonstrate that Brazil is a rich reservoir of nodulating *Paraburkholderia* species and our results contributed to the understanding of the evolutionary relationships among the species of the genus and with their hosts plants. Finally, the four studies reinforce the great genotypic and metabolic diversity of rhizobia of the *Paraburkholderia* genus, especially in Brazil, which can be considered an important center of diversity of the genus and source of bacteria with innovative potential.

Keywords: polyphasic taxonomy; beta-rhizobia; nodulation; symbiovar.

LISTA DE ILUSTRAÇÕES

LISTA DE TABELAS

LISTA DE ABREVIATURAS E SIGLAS

FBN	Fixação Biológica do Nitrogênio
sv.	Simbiovar
THG	Transferência horizontal de genes
l.s.	*Lato sensu*
s.s	*Stricto sensu*
MLSA	*Multilocus Sequence Analysis*
OGRI	*Overall Genome Related Index*
ANI	*Average Nucleotide Identity*
HDDd	Hibridação DNA DNA digital
ABT	Azul de Bromotimol
UFC	Unidades formadoras de colônias
PCR	*Polymerase Chain Reaction*

SUMÁRIO

1. INTRODUÇÃO

O nitrogênio (N) é o principal componente da atmosfera em sua forma molecular (N_2) e é um constituinte essencial de várias biomoléculas. Apesar de tal abundância, as plantas requerem uma fonte de N assimilável biologicamente, em geral nas formas de nitrato e amônia, que são limitadas no solo e podem restringir o crescimento vegetal. Algumas espécies de procariotos são diazotróficos, ou seja, realizam a conversão enzimática do N_2 em uma forma passível de assimilação pelas plantas, em um processo denominado fixação biológica de nitrogênio (FBN).

Os rizóbios são organismos diazotróficos presentes no solo que possuem a capacidade singular de estabelecer simbiose usualmente com plantas da família Fabaceae (=Leguminosae). O sucesso de tal associação deve-se à formação de estruturas especializadas, normalmente localizadas nas raízes ou caules, denominadas nódulos, no interior dos quais é mantido um constante suprimento de fontes de carbono e regulação da baixa pressão de oxigênio, permitindo que os rizóbios realizem a FBN e disponibilizem o N biologicamente assimilável diretamente aos tecidos vegetais.

Ao longo de muitos anos, acreditava-se que os rizóbios eram obrigatoriamente pertencentes à classe Alfaproteobacteria (alfa-rizóbios), sendo considerados como rizóbios “clássicos”. Entretanto, há exatos vinte anos, pesquisadores demonstraram, pela primeira vez, a capacidade de nodulação por microrganismos da classe Betaproteobacteria (beta-rizóbios) (MOULIN et al., 2001). Atualmente os beta-rizóbios se encontram dentro dos gêneros *Paraburkholderia*, *Cupriavidus* e *Trinickia* (DOBRITSA; SAMADPOUR, 2016; ESTRADA-DE LOS SANTOS et al., 2018; VANDAMME; COENYE, 2004).

O Brasil apresenta uma grande diversidade de beta-rizóbios do gênero *Paraburkholderia*, os quais são principalmente encontrados em associação com espécies endêmicas e nativas de *Mimosa*, sendo considerado um reservatório de espécies simbióticas do gênero.

Devido à recente descoberta dos beta-rizóbios em relação aos alfa-rizóbios, a taxonomia de espécies simbióticas de *Paraburkholderia* ainda é pouco elucidada, especialmente em estirpes isoladas de seu hospedeiro original. Dessa forma, estudos taxonômicos utilizando a abordagem polifásica são essenciais para a identificação e posterior classificação desses microrganismos. Além disso, estudos relacionados à filogenia e à capacidade simbiótica promovem uma melhor compreensão das relações evolutivas entre rizóbios e plantas hospedeira.

2. OBJETIVOS

2.1 OBJETIVO GERAL

Analisar estirpes brasileiras de *Paraburkholderia*, provenientes das regiões Sul e Sudeste do Brasil, quanto às suas características fenotípicas, genotípicas, filogenéticas e simbióticas.

2.2 OBJETIVOS ESPECÍFICOS

1) Caracterizar fenotípicamente estirpes de *Paraburkholderia* isoladas de nódulos de *Mimosa* spp. e *Phaseolus vulgaris* através da análise de atributos morfo-quimiofisiológicos (Estudos 1 e 2);

2) Avaliar a diversidade intraespecífica dos isolados por meio da técnica de BOX-PCR (Estudos 1 e 2);

3) Inferir relações evolutivas dos isolados por meio da análise filogenética do 16S RNAr, *Multilocus Sequence Analysis* (MLSA) e a similaridade nucleotídica utilizando o *Average Nucleotide Identity* (ANI) e Hibridização DNA-DNA digital (HDDd), no intuito de determinar a posição taxonomica das estirpes de *Paraburkholderia* estudadas (Estudos 1 e 2);

4) Constatar a capacidade simbiótica dos isolados por meio de testes de nodulação em casa de vegetação e filogenia dos genes simbióticos (Estudos 1 e 2);

5) Determinar simbiovares dentre espécies simbióticas de *Paraburkholderia* conhecidas, mediante análises filogenéticas dos genes *nod*, *nif* e *fix* (Estudo 3);

6) Comparar a filogenia de genes constitutivos e simbióticos de *Paraburkholderia* para a compreensão da aquisição evolutiva dos genes simbióticos (Estudo 3);

7) Reunir e analisar informações sobre o gênero *Paraburkholderia* e suas representantes simbióticas (Estudo 4);

8) Discutir sobre a descoberta de beta-rizóbios e sua implicação na rizobiologia (Estudo 4);

9) Expor o processo de transição das espécies ambientais do gênero *Burkholderia* para o gênero *Paraburkholderia* (Estudo 4);

10) Elucidar características filogenéticas, ecológicas e o potencial agrobiotecnológico de *Paraburkholderia* simbióticas brasileiras (Estudo 4);

3. REVISÃO BIBLIOGRÁFICA

3.1 A FIXAÇÃO BIOLÓGICA DO NITROGÊNIO (FBN)

3.1.1 A importância do nitrogênio

O solo é um grande reservatório nutricional, pois armazena diversos nutrientes essenciais para a formação de biomoléculas e para o metabolismo dos seres que ali habitam. Nesse contexto, o nitrogênio (N) é requerido em grande quantidade, pois é um componente de macromoléculas como enzimas, proteínas, ácidos nucleicos, clorofila (pigmento fundamental na fotossíntese), dentre outros compostos orgânicos indispensáveis, desempenhando um papel importantíssimo no metabolismo dos seres vivos (OHYAMA, 2010). Devido a sua importância biológica, o N é um fator limitante do crescimento vegetal na maioria dos solos e, quando em escassez, as plantas exibem clorose com amarelamento nas folhas, retardo no crescimento, assim como aceleração da senescência (BOTTOMLEY; MYROLD, 2015; OHYAMA, 2010).

A maior fonte de nitrogênio da biosfera, se encontra na forma gasosa N_2 (dinitrogênio), representando a proporção de 78% da totalidade dos gases atmosféricos; entretanto, não é assimilável pela maioria dos organismos, incluindo as plantas (ROBERTSON; GROFFMAN, 2015). Para que as plantas possam assimilar o nitrogênio, é necessário que ocorra a quebra da forte tripla ligação ($N{\equiv}N$) entre os dois átomos de N para a obtenção de formas reativas passivas de assimilação, o que ocorre mediante um alto gasto energético; dessa forma, o N é capaz de se ligar com carbono, hidrogênio e oxigênio, formando as biomoléculas que compõem a vida (BOTTOMLEY; MYROLD, 2015).

As principais formas de N inorgânicas assimiláveis pelas plantas são formas oxidadas como o nitrato (NO_3^-) e formas reduzidas como a amônia (NH_3), o íon amônio NH_4^+ e aminas (BURÉN; RUBIO, 2018; LINDSTRÖM; MOUSAVI, 2020).

Existem processos naturais que reduzem o N_2, como a ação de raios, combustão, vulcanismo e a fixação biológica do nitrogênio (FBN). Entretanto, existem formas artificiais de reduzir o N_2 em amônia, como o processo para produção de fertilizantes nitrogenados conhecido como Haber–Bosch, que ocorre em níveis de pressão e temperatura altos, além de possuir um custo financeiro elevado (VOJVODIC et al., 2014). As consequências negativas do uso excessivo de fertilizantes nitrogenados são alarmantes, pois levam à poluição do solo, dos lençóis freáticos, da água e do ar, além de influenciarem no aumento do efeito estufa (WANG et al., 2019).

A FBN contribui com a maior proporção de incorporação de nitrogênio no planeta, atingindo uma média de 258 milhões de toneladas de N por ano, sendo considerada o processo biológico mais importante depois da fotossíntese (VIEIRA, 2017; WANG et al., 2019). A FBN é realizada por um grupo restrito de procariotos diazotróficos presentes nos domínios Bacteria e Archaea, que podem apresentar estilo de vida livre, associativo, fototrófico ou simbiótico (BOTTOMLEY; MYROLD, 2015).

A FBN ocorre através da ação do complexo enzimático chamado nitrogenase. A nitrogenase mais comumente estudada é a molibidênio-dependente, composta por duas melatoproteínas: a ferro proteína (Fe-proteína) também conhecida como dinitrogenase redutase e a molibdênio-ferro proteína (FeMo-proteína), também denominada de dinitrogenase (BURÉN et al., 2020; HOFFMAN et al., 2014). A nitrogenase é altamente sensível ao O_2, uma vez que a molécula pode interagir com o componente Fe das proteínas. A Fe-proteína tem como principal função ser doadora de elétrons para FeMo-proteína, que possui o sítio de ligação e redução do substrato (BURÉN et al., 2020; HOFFMAN et al., 2014). Juntas, catalisam a redução de N_2 em NH_3, por intermédio de uma reação que depende da transferência de elétrons e proteínas carreadoras, assim como da energia liberada pela hidrólise de MgATP (BURÉN et al., 2020; HOFFMAN et al., 2014). Primeiramente, o N_2 liga-se à nitrogenase, em seguida, uma carreadora de elétrons (ferredoxina ou flavodoxina) doa elétrons à Fe-proteína reduzindo-a, a qual hidrolisa 2 moléculas de ATP e reduz a FeMo-proteína, que acumulará 1 elétron por vez, até concentrar 8 deles; o substrato se liga ao sítio ativo da FeMo, onde é reduzido. Em condições ideais, a estequiometria da reação catalítica da redução de N_2 em duas moléculas de amônia seria correspondente à equação a seguir:

$$N_2 + 8^{e-} + 8H^+ + 16\ \text{Mg-ATP} \rightarrow 2NH_3 + H_2 + 16\ \text{Mg-ADP} + 16\ \text{Pi}$$

A FBN é extremamente vantajosa em diversos sentidos, porém, é um processo que requer gasto energético. São necessárias 16 moléculas de ATP para reduzir uma molécula de N_2, resultando em duas moléculas de amônia (KUMAR et al., 2020).

3.1.2 Genes envolvidos na FBN

Para que a FBN aconteça, é necessária a presença do complexo enzimático da nitrogenase (Figura 1), por intermédio do qual o N_2 será reduzido a amônia e assim passará a ser assimilável pelas plantas.

Os genes *nif* são os responsáveis pela síntese do complexo da nitrogenase. Três genes em específico codificam os componentes essenciais do complexo enzimático. O gene *nifH* codifica o homodímero Fe-proteína, enquanto os genes *nifD* e *nifK* codificam a subunidade α e β da FeMo-proteína, respectivamente (BURÉN et al., 2020; KUMAR et al., 2020; WANG et al., 2019). Em adição, é necessária a ação de outros genes *nif*, como os *nifENB* que estão envolvidos na síntese do cofator de FeMo e o gene *nifA* , cuja proteína regula positivamente os genes *nif* e *fix* (BOYD; PETERS, 2013; DE BRUIJN, 2015; WANG et al., 2019).

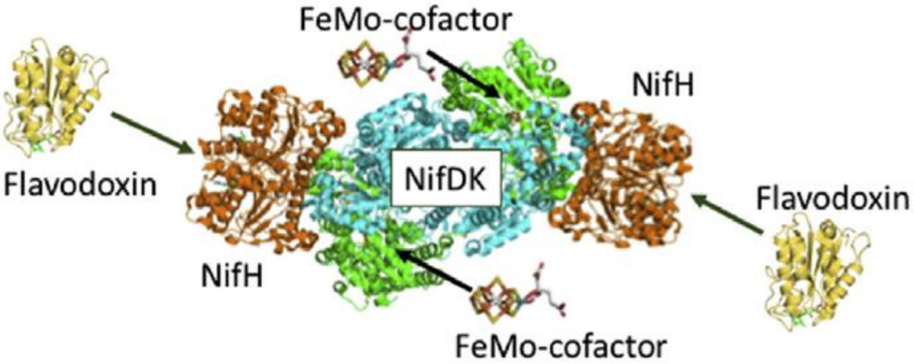

Figura 1. O complexo enzimático da nitrogenase. Fonte: Mus et al. (2019)

Os genes *fix* desempenham papeis essenciais para a FBN e estão envolvidos principalmente no transporte de elétrons para a nitrogenase, em mecanismos de regulação da transcrição dos genes *nif* e, também, na regulação do nível de oxigênio na célula (EDGREN; NORDLUND, 2004; VAN DOMMELEN; VANDERLEYDEN; MICHIELS, 2013). Existem diversos genes *fix* que cumprem diferentes funções na FBN. Dentre eles, os genes *fixABC* codificam flavoproteínas envolvidas na transferência de elétrons para a nitrogenase, essenciais para o seu funcionamento, enquanto que os genes *fixNOQP* são necessários para a síntese e atividade do complexo citocromo oxidase, o qual permite respiração eficiente e geração de ATP em um ambiente de baixo oxigênio (VAN DOMMELEN; VANDERLEYDEN; MICHIELS, 2013).

3.2 BÁCTERIAS SIMBIÓTICAS FIXADORAS DE NITROGÊNIO

Dentre os organismos diazotróficos, destaca-se um grupo de bactérias presentes no solo capazes de estabelecer uma associação simbiótica com espécies de plantas leguminosas da Família Fabaceae, coletivamente chamadas de rizóbios. Essas bactérias são, em sua maioria, de vida livre e habitam as regiões da rizosfera; mas quando infectam plantas hospedeiras induzem a formação de estruturas especializadas na FBN, denominadas nódulos, que se encontram principalmente nas raízes das plantas e, mais raramente, nos caules.

O termo “rizóbio” é derivado do gênero *Rhizobium*, criado em 1889 para reclassificar as primeiras bactérias isoladas de nódulos radiculares anteriormente classificadas como *Bacillus radicicola*. O único gênero perdurou por décadas, abrigando novas espécies. Em 1982, o gênero *Bradyrhizobium* (Brady, do grego *bradus*=lento) foi descrito contendo isolados que apresentavam características bastante diferentes, especialmente crescimento lento em meio contendo manitol como fonte de carbono (JORDAN, 1982). Com o avanço tecnológico, novas metodologias moleculares foram implementadas na taxonomia bacteriana, levando à descrição de novas espécies. Dentre as metodologias, destaca-se a utilização de sequências parciais de genes ribossomais para inferir relações filogenéticas, proposta pelo microbiologista Carl Woese, que levou à classificação dos três grandes domínios da vida, usados atualmente, em Archaea, Bacteria e Eurkarya (WOESE, 1987; WOESE; KANDLER; WHEELIS, 1990). Atualmente os rizóbios são classificados dentro do domínio Bacteria; Filo Proteobacteria, nas classes: Alfaproteobacteria e Betaproteobacteria.

Os rizóbios pertencentes à classe Alfaproteobacteria (alfa-rizóbios) abrigam espécies conhecidas como “rizóbios clássicos”, uma vez que foram os primeiros a serem descobertos e, devido a sua importância ambiental e agronômica, foram amplamente estudados. Por mais de um século acreditou-se que todos os rizóbios eram pertencentes exclusivamente à classe Alfaproteobacteria, porém, em 2001, dois autores demonstraram que bactérias de dois diferentes gêneros (*Burkholderia* e *Ralstonia*) pertencentes à classe Betaproteobacteria (beta-rizóbios) eram capazes de estabelecer simbiose com plantas leguminosas e fixar N_2 (CHEN et al., 2001; MOULIN et al., 2001).

Atualmente os alfa-rizóbios encontram-se classificados nos gêneros *Rhizobium*, *Allorhizobium*, *Azorhizobium, Bradyrhizobium, Mesorhizobium, Agrobacterium, Aminobacter, Blastobacter*, *Devosia, Ensifer* (=*Sinorhizobium*)*, Methylobacterium*, *Microvirga, Neorhizobium, Ochrobactrum, Pararhizobium, Phyllobacterium,* e *Shinella.* Os beta-rizóbios pertencem aos gêneros *Paraburkholderia*, *Cupriavidus* e *Trinickia* (HASSEN; LAMPRECHT; BOPAPE, 2020; PARTE et al., 2020; VELÁZQUEZ et al., 2017; ESTRADA DE LOS SANTOS, et al., 2018)*.*

Até o momento, todos os rizóbios descritos são bacilos Gram-negativos, não formadores de esporos, e movem-se com um ou mais flagelos polares ou peritríquios.

3.3 INTERAÇÃO SIMBIÓTICA RIZÓBIO-LEGUMINOSA

A principal contribuição para o processo da FBN se dá através da simbiose rizóbio-leguminosa e, portanto, contribui com os maiores níveis de incorporação de N nos ambientes terrestres (HUNGRIA; MENDES, 2015). O sucesso envolvendo a simbiose entre rizóbio e leguminosa é atribuído ao nódulo, uma estrutura especializada na FBN, que se encontra principalmente nas raízes das plantas e, mais raramente, nos caules.

A fixação do N_2 envolve um grande gasto energético para o rizóbio, em retorno a planta lhe oferece fontes de carbono provenientes da fotossíntese e proteção no interior do nódulo (VIEIRA, 2017).

A família das leguminosas (=Fabaceae) se caracteriza pelo seu fruto em forma de vagem, contendo sementes com alto teor nutritivo, servindo de alimento para seres humanos e animais. Além disso, inclui plantas ornamentais, fonte de madeira, pasto, forrageiras e usos medicinais (LLAMAS; ACEDO, 2016). É a terceira maior família de Angiospermas, possui distribuição global, sendo encontrada nos principais biomas; a família conta com, aproximadamente, 770 gêneros e mais de 19.500 espécies (LEWIS et al., 2013; LPWG, 2013; LPWG et al., 2017). Um levantamento com plantas da família Fabaceae realizado no Brasil, considerando espécies de importância econômica, ecológica e filogenética, indicou 2.694 espécies (em 210 gêneros diferentes) registradas, sendo 1.458 espécies (54%) endêmicas do Brasil (LEWIS, 2016).

A família Fabaceae era previamente classificada em três subfamílias: Papilionoideae, Mimosoideae e Caesalpinioideae. Recentemente, com base em análises filogenéticas envolvendo o gene *matK*, foi proposta a readequação das subfamílias; dessa forma, atualmente são classificadas em Duparquetioideae, Cercidoideae, Detarioideae, Dialioideae, Caesalpinioideae e Papilionoideae (LPWG et al., 2017). Na nova classificação, a antiga subfamília Mimosoideae passou a constituir um clado distinto dentro de Caesalpinioideae, enquanto que as novas subfamílias Duparquetioideae, Cercidoideae, Detarioideae, Dialioideae são oriundas da antiga subfamília Caesalpinioideae (LPWG et al., 2017). Discussões sobre a real posição filogenética de Mimosoideae ainda permanecem.

A nodulação é encontrada principalmente em Papilionoideae e no clado Mimosoide; as subfamílias Duparquetioideae, Cercidoideae, Detarioideae, Dialioideae não apresentam espécies nodulíferas (DOYLE, 2016; SPRENT; ARDLEY; JAMES, 2017).

3.4 O PROCESSO DE NODULAÇÃO

O nódulo é uma estrutura altamente especializada na FBN e surgiu após milhões de anos de evolução entre os rizóbios e as leguminosas. A simbiose rizóbio-leguminosa obteve tamanho sucesso que tanto as plantas quanto os rizóbios, sofrem uma série de modificações estruturais e fisiológicas para que a nodulação e a posterior FBN ocorra. Esse órgão desenvolvido pela planta apresenta diversas funções, como abrigo para seus simbiontes e a manutenção de um ambiente de microaerofilia. A microaerofilia no interior dos nódulos é primordial, uma vez que a enzima nitrogenase presente nos rizóbios é extremamente sensível ao O_2. Dentro dos nódulos, é encontrada a leghemoglobina, proteína que tem como função o sequestro do O_2, impedindo que esse se associe com à Fe da nitrogenase, evitando a sua desnaturação (LARRAINZAR et al., 2020). Também, visto que os rizóbios são bactérias aeróbias, outra importante função da leghemoglobina é controlar a difusão de O_2 para os bacteroides (LARRAINZAR et al., 2020).

O desenvolvimento do nódulo depende de um diálogo molecular bastante específico e coordenado entre macro e microssimbiontes. A infecção ocorre via pelos radiculares, entrada por fissura ou intercelular (dentre as células da epiderme da raiz) (MADSEN et al., 2010; SHARMA et al., 2020; WANG et al., 2019). O tipo de nodulação mais comum e estudado é o de infecção através dos pelos radiculares e, portanto, suas etapas estão ilustradas na Figura 2 e serão exemplificadas a seguir.

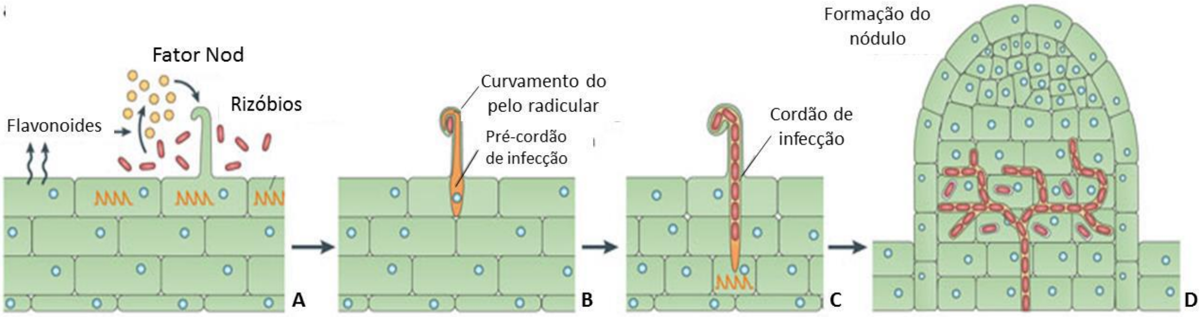

Figura 2: O processo de nodulação e organogênese do nódulo radicular. Fonte: modificado de Oldroyd et al. (2013). A – Diálogo molecular entre micro e macro simbiontes através de compostos flavonoides e fatores de nodulação. B – Encurvamento do pelo radicular e início da invaginação para formação do cordão de infecção. C – Migração dos rizóbios para o interior das células do córtex através do cordão de infecção. D – Formação do nódulo, rizóbios endocitados em células corticais.

Em condições de baixos níveis de nitrogênio no solo, a planta produz moléculas que atuam como sinalizadores moleculares, principalmente flavonoides, que são exsudados pela raiz (LIU; MURRAY, 2016). Rizóbios presentes na rizosfera, detectam a presença desses sinais, que os atrai por quimiotaxia até a raiz, além de desencadear a produção dos fatores de nodulação (fator Nod). Os fatores Nod secretados pelos rizóbios são lipo-quito-oligosacarídeos que cumprem um papel essencial à nodulação pois, interagem com receptores com domínios extracelulares do tipo LysM presentes na superfície dos pelos radiculares. Quando o fator Nod específico é reconhecido e compatível, uma cascata de sinalização é desencadeada na leguminosa, levando ao encurvamento dos pelos radiculares, à divisão das células do córtex da raiz e ao início da formação do meristema do nódulo (VIEIRA, 2017).

Os rizóbios então se multiplicam e se aderem ao redor dos pelos radiculares, que se encurvam e ocorre a dissolução da parede celular provocando uma invaginação, dando origem ao cordão de infecção, por onde os rizóbios migrarão até as células corticais (OLDROYD, 2013; VIEIRA, 2017). O cordão de infecção se estende até as células do córtex, que estão em multiplicação para a formação do nódulo primário. Com a formação do nódulo definitivo, as bactérias são liberadas dentro das células vegetais, onde são circundadas por uma membrana vegetal. Dentro dessa membrana, os rizóbios apresentam uma aparência pleomórfica chamada de bacteroide, que passa por transformações fisiológicas e morfológicas para poder fixar o N_2. Além disso, as diferentes características apresentadas pelos bacteroides estão relacionadas à planta hospedeira e à espécie de bactéria; dentre as características morfológicas mais comuns, ocorrem a perda da viabilidade reprodutiva e o aumento significativo na dimensão celular (“inchaço”) (OONO et al., 2010).

Os nódulos encontrados em leguminosas assumem principalmente duas formas e são classificados de acordo com o seu modo de desenvolvimento, podendo ser determinados ou indeterminados. Os nódulos determinados são caracterizados por seu meristema transitório, suas células se originam do córtex central e apresentam forma esférica; enquanto que os indeterminados possuem um meristema permanente, se originam de células corticais internas adjacentes à endoderme e apresentam forma alongada (OLDROYD et al., 2011; WANG et al., 2019). Ambos são funcionais na FBN e o tipo de nódulo é determinado pela planta hospedeira, geralmente leguminosas da mesma tribo apresentam o mesmo tipo de nódulo.

3.4.1 GENES ENVOLVIDOS NA NODULAÇÃO

Como discutido anteriormente, a nodulação é um processo específico que depende de um diálogo molecular para acontecer. Os rizóbios apresentam genes essenciais para o processo de nodulação, contendo mais de 30 genes diferentes, dentre eles, *nod*, *noe* e *nol* (WANG et al., 2019). Os genes *nod* possuem funções tanto estruturais quanto regulatórias e são primordiais no diálogo molecular e na consequente compatibilidade com o hospedeiro.

A proteína *nodD* é um regulador transcricional expresso constitutivamente que, em presença de flavonoides compatíveis, se liga a regiões conservadas denominadas "caixas box" e promovem a expressão dos genes de nodulação (*nod*), regulando a ativação dos genes estruturais *nodABC,* que por sua vez codificam a estrutura central do fator Nod (PERRET; STAEHELIN; BROUGHTON, 2000; WANG et al., 2019).

O centro do fator Nod é codificado pelo gene *nodC,* uma N-acetil-glucosaminiltransferase que pode apresentar variações no tamanho da cadeia. O gene *nodB* codifica uma deacetilase que remove o grupo N-acetil em uma terminação não redutora, e o gene *nodA* codifica uma aciltransferase que liga a cadeia acil no oligossacarídeo deacetilado (PERRET; STAEHELIN; BROUGHTON, 2000). Modificações e substituições nas terminações redutoras ou não redutoras dos fatores Nod (como metilação, glicosilação, sulfatação) são realizadas por proteínas espécie-especificas, codificadas por outros genes *nod* (ANDREWS; ANDREWS, 2017; VARMA; TRIPATHI; PRASAD, 2020). Tais variações são bastante específicas para diferentes espécies de planta hospedeira e, portanto, são significativas nas interações simbióticas (ANDREWS; ANDREWS, 2017; VARMA; TRIPATHI; PRASAD, 2020).

Análises filogenéticas baseadas em sequências de DNA de genes simbióticos específicos, como *nodC* e *nifH*, vêm sendo utilizadas na determinação de simbiovares em espécies de rizóbios. O termo "simbiovar" foi proposto por Rogel et al. (2011) como um equivalente ao termo "biovar" ou "patovar", utilizado para identificar estirpes da mesma espécie que apresentassem diferenças bioquímicas e enzimáticas; quando aplicado a rizóbios, o termo simbiovar se refere à identificação de subgrupos simbioticamente distintos dentro de uma mesma espécie (ROGEL; ORMEÑO-ORRILLO; MARTINEZ ROMERO, 2011). Os primeiros simbiovares foram baseados em ensaios de inoculação cruzada, nos quais as diferenças entre os grupos de rizóbios eram definidas pela habilidade de nodular ou não uma mesma espécie de legume. Porém, tanto os rizóbios, como as leguminosas podem apresentar promiscuidade, associando-se com mais de uma espécie. Além disso, os genes simbióticos podem ser facilmente transferidos por transferência horizontal de genes (THG), ultrapassando as barreiras de espécies bacterianas. De fato, espécies diferentes de rizóbios que possuem genes simbióticos similares podem nodular a mesma espécie de leguminosa. Portanto, atualmente os simbiovares são definidos a partir da filogenia de genes simbióticos como o *nodC*, *nodA* e *nifH*, sendo que filogenia do gene *nodC* é definida como critério mínimo na definição de simbiovares (PEIX et al., 2015). A importância da utilização do gene *nodC* se dá pelo fato de que o seu produto compõe a estrutura central do fator Nod, estando diretamente ligado com a especificidade de hospedeiros vegetais (PEIX et al., 2015).

3.5 O GÊNERO *PARABURKHOLDERIA*

3.5.1 Transição de *Burkholderia* à *Paraburkholderia*

O gênero *Paraburkholderia* é resultado da reclassificação de *Burkholderia lato sensu* (l.s.). *Burkholderia* l.s. caracteriza-se por ser cosmopolita e metabolicamente versátil (COMPANT et al., 2008), abrigando espécies ambientais e patógenos de humanos e animais, sendo bastante conhecida e estudada devido a sua importância clínica (BALDER et al., 2010; CHEWAPREECHA et al., 2017; VANDAMME et al., 2000; VERMIS et al., 2004).

Em 1992, através da filogenia do gene ribossomal 16S RNAr e hibridação DNA-DNA, o gênero *Burkholderia* foi proposto por Yabuuchi et al. (1992), para realocar sete estirpes que apresentavam uma posição filogenética distinta dentre o gênero *Pseudomonas* (YABUUCHI et al., 1992). O gênero ganhou importância por apresentar espécies patogênicas oportunistas em pacientes imunodeprimidos com fibrose cística (ISLES et al., 1984; MARTINA et al., 2018; ROJAS-ROJAS et al., 2019); essas espécies são conhecidas por fazerem parte do "*Burkholderia cepacia complex*" (BCC) (VANDAMME et al., 1997). Posteriormente, novas espécies foram descritas e incluídas no gênero, apresentando espécies com potencial biotecnológico e importância ambiental, como promotoras do crescimento vegetal (COMPANT et al., 2008; DEPOORTER et al., 2016; ESMAEEL et al., 2018).

A partir disso, estudos filogenéticos baseados no gene 16S RNAr revelaram a existência de dois subgrupos dentro do gênero, um contendo espécies de importância ambiental (Grupo A) e outro contendo espécies de importância clínica

(Grupo B) (ESTRADA-DE LOS SANTOS; BUSTILLOS-CRISTALES; CABALLERO-MELLADO, 2001; GILLIS et al., 1995; GYANESHWAR et al., 2011; VANDAMME et al., 2000). Gyaneshwar et al. (2011) sugeriu que o grupo A deveria ser reclassificado em um novo gênero, ao qual propôs o nome *Caballeronia*, sendo apoiado pelo estudo de Zuleta et al. (2014) (GYANESHWAR et al., 2011; ZULETA et al., 2014). Devido à importância das espécies ambientais com potencial biotecnológico, era imprescindível averiguar se essas poderiam apresentar ou obter genes patogênicos, portanto, esforços para a compreensão da real posição taxonômica dos grupos A e B foram feitos.

Estudos robustos utilizando genes constitutivos através da técnica de MLSA (*Multilocus Sequence Analysis*) confirmaram os dois subgrupos dentro o gênero *Burkholderia*, de acordo com a sugestão prévia (ESTRADA-DE LOS SANTOS et al., 2013; PAYNE et al., 2005; SPILKER et al., 2009; TAYEB et al., 2008). Poucos anos depois, Sawana, Adelou e Gupta (2014) realizaram uma análise filogenômica de 45 espécies de *Burkholderia* l.s. e propuseram que as espécies ambientais fossem reclassificadas no novo gênero *Paraburkholderia*, enquanto que os patógenos permaneceriam como *Burkholderia stricto sensu* (s.s.) (SAWANA; ADEOLU; GUPTA, 2014). Por conseguinte, o gênero *Paraburkholderia* foi validado, abrigando as espécies ambientais do previamente chamado grupo A; enquanto que estirpes que apresentaram uma posição filogenética intermediaria entre *Paraburkholderia* e *Burkholderia* s.s. foram incluídas no novo gênero *Caballeronia* (OREN; GARRITY, 2015; DOBRITSA; SAMADPOUR, 2016).

O gênero *Paraburkholderia* é, atualmente, composto por mais de 85 espécies ambientais, isoladas em sua maioria de solos, incluindo solos vulcânicos e contaminados, ou em associação com plantas (BOURNAUD et al., 2017; CHEN et al., 2007; LV et al., 2016; WEBER; KING, 2017). Diversas espécies apresentam potencial biotecnológico, como de biorremediação, na solubilização de fosfato (GAO et al., 2019) e compostos aromáticos (LEE; JEON, 2018), atividade antifúngica (HUO et al., 2018) e fixação biológica do nitrogênio (CHOI; IM, 2018; DALL'AGNOL et al., 2016; DE MEYER et al., 2013a).

A reclassificação das espécies ambientais foi de extrema importância para desvinculá-las das espécies patogênicas. Embora não tenham sido detectados genes relacionados à patogenia em *Paraburkholderia,* estudos realizados para averiguar a possibilidade da presença ou obtenção via THG desses genes ainda são escassos. Angus e colaboradores (2014) demonstraram que as espécies que realizam relações simbióticas com plantas não possuem os genes necessários para desencadear patogenia em mamíferos (ANGUS et al., 2014). Enfim, recomenda-se que, para a utilização de espécies com finalidades biotecnológicas, estudos devem ser realizados para analisar o potencial patogênico das mesmas (KAUR; SELVAKUMAR; GANESHAMURTHY, 2017).

3.5.2 *PARABURKHOLDERIA* NODULÍFERAS

Há 20 anos, o grande paradigma da rizobiologia foi quebrado. Por muito tempo acreditou-se que as espécies de rizóbios estavam necessariamente dentro da classe Alfaproteobacteria. Em 2001, porém, Moulin e colaboradores demonstraram, pela primeira vez, que bactérias da classe Betaproteobacteria (*Burkholderia* l.s.) eram capazes de nodular leguminosas, contendo os genes *nodACB* e *nifH,* envolvidos na nodulação e a fixação do N_2 (MOULIN et al., 2001).

O gênero *Burkholderia,* que atraía atenção por sua grande diversidade metabólica e espécies promotoras do crescimento vegetal, mais uma vez tornou-se alvo de estudos após a descoberta do potencial biotecnológico das espécies simbióticas e, com isso, novas espécies foram descritas (CHEN et al., 2001, 2003; VANDAMME et al., 2002; YATES et al., 2004). Dentre as 85 espécies descritas de *Paraburkholderia* até o momento, 21 são rizóbios (LPSN, 2021) isolados de várias localidades geográfica. Contudo, são dois os principais centros de diversificação do gênero *Paraburkholderia*: a América do Sul e a África do Sul.

Na América do Sul, as *Paraburkholderia* nodulíferas foram predominantemente isoladas no Brasil, associadas com espécies nativas e endêmicas de *Mimosa* spp., nos biomas Cerrado e Caatinga, sendo consideradas os seus simbiontes preferidos (DOS REIS JR et al., 2010; CHEN et al., 2006, 2007, 2008; SHEU et al., 2013; SIMON et al., 2011). Estudos envolvendo o isolamento de *Paraburkholderia* de diversas espécies de *Mimosa* no Brasil central sugerem que a associação íntima entre ambas é resultado de um evento de coevolução e diversificação (BONTEMPS et al., 2010; DOS REIS JR et al., 2010; MOULIN et al., 2015). Todavia, Bournaud et al. (2013) conduziram um estudo em 9 estados brasileiros inseridos no bioma Mata Atlântica, no qual revelaram que estirpes de *Paraburkholderia* foram os principais simbiontes isolados de plantas do grupo *Piptadenia* (BOURNAUD et al., 2013). A partir dos resultados obtidos, duas novas espécies, *Paraburkholderia piptadeniae* e *P. ribeironis,* foram descritas (BOURNAUD et al., 2017). Neste mesmo sentido, Silva et al. (2018) mostraram que espécies brasileiras de *Calliandra* são noduladas por diferentes estirpes do gênero *Paraburkholderia* (SILVA et al., 2018).

O segundo centro de diversificação do gênero *Paraburkholderia* ocorre na África do Sul, mais especificamente na Região Floral do Cabo, dentro do bioma Fynbos, onde se associam particularmente a plantas endêmicas da subfamília Papilionoideae (BEUKES et al., 2013; DE MEYER et al., 2018; HOWIESON et al., 2013; LEMAIRE et al., 2016a). Diversos estudos envolvendo a diversidade filogenética das estirpes nodulíferas de Papilionoideae Sul Africanas resultaram na descrição de muitas espécies nos últimos anos, apontando a região como um reservatório de espécies simbióticas de *Paraburkholderia* (DE MEYER et al., 2013b, 2013a, 2014; STEENKAMP et al., 2015; VANDAMME et al., 2002).

Apesar de não se saber ao certo como e onde, estima-se que a associação que deu origem a simbiose rizóbio-leguminosa, é proveniente do início do Devoniano (416-350 Ma), possivelmente derivada da simbiose entre micorrizas arbusculares e plantas; da qual supõe-se que os genes envolvidos na nodulação por rizóbios são provenientes (SPRENT; ARDLEY; JAMES, 2017; WALKER et al., 2015). Hipóteses apontam que bactérias nodulíferas da classe Betaproteobacteria evoluíram de ancestrais ambientais/endofíticos; estima-se que há 50 milhões de anos, pouco depois do surgimento das leguminosas (60 Ma); esses organismos ancestrais já possuíam genes *nif* e receberam seus genes *nod* de um doador desconhecido (MOULIN et al., 2015). Essas espécies, então, encontraram leguminosas ancestrais, se tornaram seus simbiontes e coevoluíram com as mesmas (MOULIN et al., 2015). Fatores geográficos também parecem estar envolvidos na evolução dos genes simbióticos. Um trabalho realizado por De Meyer e colaboradores (2016), baseado na filogenia de genes *nif*, *nod* e *fix*, mostra que a história evolutiva desses genes de *Paraburkholderia* da América do Sul e da África do Sul são diferentes, indicando origens distintas (DE MEYER et al., 2016). Portanto, acredita-se que a aquisição dos genes simbióticos através da THG ocorreu após a diversificação das linhagens de *Paraburkholderia* (DE MEYER et al., 2016).

Um dos fatores que influencia a relação simbionte-hospedeiro é o pH do solo. Em relação ao gênero *Mimosa*, em solos considerados ácidos, *Paraburkholderia* são encontrados como os simbiontes preferenciais (DOS REIS JR et al., 2010; DE CASTRO PIRES et al., 2018); no México, onde os solos são alcalinos, o gênero *Mimosa* se associa com Alfaproteobactérias (*Rhizobium* e *Ensifer*) (BONTEMPS et al., 2016), enquanto que no Uruguai, em solos também alcalinos a associação ocorre com *Cupriavidus* (PLATERO et al., 2016). Na África do Sul, onde *Paraburkholderia* é o simbionte preferido de leguminosas Papilionoides endêmicas da região, os solos também são ácidos (LEMAIRE et al., 2016b). A “preferência” na associação de *Paraburkholderia* com leguminosas provavelmente se deve a mecanismos de tolerância à acidez do solo, resultando em uma vantagem adaptativa do gênero, permitindo a predição biogeográfica de espécies nodulíferas de *Paraburkholderia* (STOPNISEK et al., 2014).

3.6 TAXONOMIA POLÍFÁSICA NA DESCRIÇÃO DE NOVAS ESPÉCIES BACTERIANAS

Os microrganismos desempenham um papel essencial no funcionamento do meio ambiente, seja realizando a ciclagem de nutrientes, na promoção do crescimento vegetal, na biorremediação por meio da degradação de compostos poluentes, na ação antimicrobiana e/ou patogênica, entre outros. Para compreender a biodiversidade de microrganismos é necessário inferir suas relações evolutivas e ecológicas.

A taxonomia é a ciência responsável pela classificação, identificação e nomenclatura dos seres vivos, que se interligam gerando um sistema de organização hierárquico importantíssimo (Domínio, filo, classe, ordem, família, gênero e espécie), facilitando a identificação de microrganismos de importância ambiental, clínica e biotecnológica. Dentro da taxonomia bacteriana, a classificação tem como função ordenar os organismos de forma hierárquica de acordo com a similaridade dentro dos taxa; a nomenclatura segue o conjunto de regras estabelecidas para nomear um organismo e, no caso dos procariotos, segue-se o Código Internacional de Nomenclatura de Procariotos (PARKER; TINDALL; GARRITY, 2019); a identificação busca determinar a posição de um organismo dentro de classificações já existentes.

No início da taxonomia bacteriana, a utilização de características fenotípicas e morfológicas era aplicada para a classificação das espécies. Durante mais de 100 anos a taxonomia se viu em constante discussão e mudança, devido à expressão de características fenotípicas e fisiológicas similares em grupos bacterianos distintos. Além disso, a falta do compartilhamento de informações entre microbiologistas, aliada à aplicação de diferentes métodos para identificação bacteriana, resultaram na formação de grupos heterogêneos e em diversos erros na classificação e nomenclatura dos mesmos. Com isso, havia um descontentamento dos microbiologistas, devido à falta de objetividade e propósito nas metodologias recomendadas (ROSSELLO-MORA; AMANN, 2001).

Na década de 1950, em paralelo ao desenvolvimento dos computadores, surgiu a taxonomia numérica, que analisava a similaridade dos dados fenotípicos de diferentes linhagens através de coeficientes numéricos. Coincidentemente na mesma época, abordagens quimiotaxonômicas (por ex: análises da composição da parede celular e de lipídios) ganharam força e foram incorporadas na mesma abordagem, refletindo em uma melhor resolução taxonômica (SNEATH; SOKAL, 1973). Entretanto, tais abordagens não permitem uma análise das relações genéticas e filogenéticas entre os organismos.

No início dos anos 1960, com o avanço tecnológico e o desenvolvimento de técnicas de biologia molecular, a utilização de sequências nucleotídicas (DNA e RNA) na busca pela correta classificação de todos os seres vivos foi estabelecida. Inicialmente a composição do DNA era comparada entre duas espécies, através da porcentagem do conteúdo G+C (G= guanina, C= citosina) mol/%; caso apresentassem baixa similaridade, eram consideradas espécies diferentes. Todavia, os resultados obtidos pela comparação do conteúdo G+C mostrou-se superficial, sendo necessária uma técnica mais informativa. Então, a hibridização DNA-DNA foi desenvolvida e se tornou padrão na taxonomia bacteriana, devido a sua capacidade de produzir grupos taxonômicos robustos (BRENNER et al., 1969; ROSSELLO-MORA; AMANN, 2001; TINDALL et al., 2010).

A grande revolução da taxonomia bacteriana ocorreu, de fato, com o sequenciamento genético, possibilitado a amplificação e a utilização de sequências da subunidade menor do ribossomo, o 16S RNAr, considerada chave para a inferência filogenética de bactérias em espécies, gêneros e famílias (WOESE, 1987). De acordo com Woese (1987), o RNA é uma molécula considerada como um “cronômetro molecular”, devido ao seu alto grau de conservação funcional e as mudanças nas posições das sequências de bases nucleotídicas que ocorrem em taxas muito distintas entre microrganismos, permitindo a medida de relações filogenéticas. A utilização das sequências de RNAr foi tão significativa, que a análise dos genes 16S RNAr de bactérias e arqueas e o 18S RNAr de organismos eucariotos resultaram na atual classificação da árvore da vida, dividida nos domínios Archaea, Eukarya e Bacteria (WOESE; KANDLER; WHEELIS, 1990). Nesse estudo, Woese (1990) conseguiu pela primeira vez fornecer um arcabouço filogenético para bactérias e arqueas, além de possibilitar o entendimento das relações filogenéticas entre procariotos e eucariotos. Desde então, a utilização de metodologias moleculares baseadas na filogenia de DNA e RNA foram amplamente aceitas e recomendadas na taxonomia (ROSSELLÓ-MÓRA, 2012).

Atualmente, a descrição de uma nova espécie bacteriana é determinada através da integração de um conjunto de técnicas, que reúnem dados fenotípicos, genotípicos e filogenéticos, devendo refletir as relações evolutivas entre os procariotos. A integração dessas técnicas na determinação de espécies é denominada “Taxonomia polifásica”, e a sua utilização se tornou um consenso dentro da taxonomia bacteriana (VANDAMME et al., 1996).

A espécie é a unidade básica da taxonomia e ainda não há um consenso em relação à real definição de espécie bacteriana, porém, Rosselló-Mora e Amann (2015), baseando-se em características fenotípicas, filogenéticas e genômicas, definem uma espécie bacteriana como “uma categoria que circunscreve populações monofiléticas e genômicas e fenotipicamente coerentes de indivíduos que podem ser claramente discriminadas de outras entidades por meio de parâmetros padronizados”. Tal definição é considerada pragmática e útil até os dias atuais.

As técnicas utilizadas devem ser altamente padronizadas e valores de referência são empregados para uma definição de espécie mais precisa. Portanto, até a década passada determinava-se que, para que duas estirpes fossem consideradas da mesma espécie elas deveriam apresentar valor igual ou superior a 70% de similaridade na hibridização DNA-DNA, 97% de identidade na sequência do gene ribossomal 16S RNAr, e um máximo de 2% na amplitude do conteúdo G+C, sendo que as características fenotípicas devem estar de acordo com as genotípicas (GILLIS et al., 2015; THOMPSON et al., 2015).

Entretanto, diversas técnicas anteriormente caracterizadas como padrão-ouro da taxonomia, como por exemplo, a hibridização DNA-DNA, caíram em desuso devido a divergências na reprodutibilidade, alto custo e a falta de um banco de dados para a comparação dos resultados obtidos (ROSSELLÓ-MÓRA, 2012; THOMPSON et al., 2015). Com os avanços tecnológicos, novas metodologias taxonômicas foram propostas, principalmente análises filogenéticas de sequências nucleotídicas com base em genes constitutivos e do genoma total bacteriano. As informações provenientes do sequenciamento genético geram dados confiáveis que são compartilhados em bancos de dados (por exemplo: NCBI, IMG, EMBL-EBI) permitindo o compartilhamento dos mesmos à nível mundial.

Em resumo, as novas classificações bacterianas são resultado de um trabalho laborioso com métodos padronizados, contando com uma série de análises de caracteres genéticos, morfológicos, químicos e metabólicos.

3.6.1 METODOLOGIAS COMUMENTE UTILIZADAS E RECOMENDADAS

Como citado anteriormente, a taxonomia bacteriana se baseia na caracterização fenotípica, genotípica e filogenética do isolado.

As informações fenotípicas têm sido utilizadas desde o início da taxonomia bacteriana e atualmente são importantes para uma rápida identificação de gênero ou família bacteriana, especialmente em análises clínicas. Visando a descrição de espécies bacterianas, as análises fenotípicas têm como função complementar as informações genotípicas e filogenéticas. Dessa forma, uma colônia pura é caracterizada através de suas características morfológicas, fisiológicas e bioquímicas (RAINA et al., 2018). Em relação à morfologia, são analisadas: a forma celular, a formação de endósporo, presença de flagelo, corpos de inclusão, coloração de Gram e características da colônia (diâmetro, forma, borda, coloração, produção de muco, entre outros); nas análises fisiológicas e bioquímicas, se analisa o crescimento bacteriano quanto a diferentes temperaturas e pH, concentração de sais, presença de agentes antimicrobianos (geralmente antibióticos conhecidos), fontes de carbono e ação enzimática (VANDAMME et al., 1996). Dentro das análises fenotípicas, a quimiotaxonomia é altamente recomentada, pois utiliza constituintes celulares para classificação bacteriana, como composição da parede celular, ácidos graxos, ácidos micólicos, exopolissacarídeos, lipídeos polares, quinonas isoprenóides e análise de proteínas totais celulares (RAINA et al., 2018). Além disso, a metodologia de espectrometria de massa pelo MALDI-TOF-MS (caracterização de proteínas/peptídeos) cria uma verdadeira impressão digital bacteriana. Os dados de espectrometria de massa são submetidos a bancos de dados, onde é possível realizar a comparação com outras estirpes, tendo uma resolução de classificação em gênero e é bastante útil para análises intraespecíficas (TOMACHEWSKI et al., 2018)

Uma das grandes dificuldades encontradas nos métodos fenotípicos é quanto à veracidade dos dados obtidos, visto que. apesar de haver uma padronização nas técnicas, muitas vezes resultados distintos são obtidos por diferentes pesquisadores. As metodologias fenotípicas são bastante laboriosas e sugere-se que sejam realizadas com repetições (geralmente triplicatas), exigindo uso de tempo e material. No entanto, a maior desvantagem dos métodos fenotípicos é relacionada à expressão gênica, pois o mesmo organismo pode apresentar caracteres fenotípicos distintos quando em diferentes condições ambientais; assim como organismos de espécies diferentes podem apresentar caracteres fenotípicos muito similares nas mesmas condições ambientais, gerando confusões quanto à relevância dessas informações para a diferenciação dos isolados. Ainda, as análises fenotípicas não permitem a realização de inferências evolutivas dentre os organismos estudados, nesse caso, métodos mais informativos são requeridos.

No que diz respeito aos métodos genotípicos, estes se baseiam nas informações derivadas de ácidos nucleicos (DNA e RNA), gerando dados confiáveis e elucidativos. Dependendo do nível de resolução procurada, diferentes técnicas são utilizadas. Polimorfismos encontrados nas sequências de DNA do genoma bacteriano podem ser usados para a identificação da diversidade genotípica dos isolados microbianos. Metodologias chamadas de “DNA *fingerprinting*” são bastante utilizadas para uma primeira triagem das amostras em estudo. Um bom exemplo são as baseadas em elementos repetitivos do genoma, como o BOX-PCR, muito aplicado em estudos de microrganismos ambientais ou da área médica, no qual ocorre a amplificação das sequências dos elementos boxA, que se localizam em diferentes posições do genoma em cada organismo, gerando uma “impressão digital” distinta para cada isolado, permitindo a análise da diversidade intraespecífica dos organismos estudados e a identificação de clones bacterianos (KOEUTH; VERSALOVIC; LUPSKI, 1995; TINDALL et al., 2010).

A determinação da porcentagem molar de guanina e citosina (G+C) é um método genotípico fundamental na descrição padrão de uma espécie bacteriana. A variação do conteúdo não deve ser maior que 3% dentro de uma espécie bem definida e 10% em um gênero bem definido (MOORE et al., 2010; VANDAMME et al., 1996)

O sequenciamento do gene ribomossomal 16S RNA é usualmente realizado como o primeiro passo na identificação de um novo organismo. O gene 16S RNAr é um marcador molecular eficaz para fins taxonômicos devido a sua onipresença em todos os procariotos, além de ser funcionalmente estável e estar pouco sujeito à THG (RAMASAMY et al., 2014). Diversos valores de referência foram sugeridos na utilização de sequências 16S RNAr para a delimitação de espécies ao longo dos anos (STACKEBRANDT; JONAS, 2006). Atualmente, para que duas estirpes sejam consideradas da mesma espécie, o valor de similaridade atribuído à sequência completa do gene 16S RNAr deve ser igual ou superior a 98,7% (KIM et al., 2014). Contudo, certos gêneros bacterianos apresentam uma conservação muito alta destas sequências, não permitindo a diferenciação de espécies. Em virtude da alta conservação apresentada, as informações fornecidas pela sequência do gene 16S RNAr permitem uma resolução muito baixa para o nível de espécie, sendo altamente recomendada apenas para a identificação ao nível de gênero bacteriano (ZHI et al., 2012).

Genes bacterianos conhecidos como constitutivos (*housekeeping*) são aqueles que desempenham funções fundamentais no metabolismo celular. Estes genes apresentam uma taxa de mutação maior em comparação com genes ribossomais, mas apresentam conservação suficientemente alta para conter informações evolutivas valiosas, revelando relações filogenéticas mais específicas. No entanto, a utilização de apenas um único gene na análise filogenética, pode não indicar a verdadeira posição taxonômica bacteriana, devido a que diferentes genes podem apresentar diferentes histórias evolutivas, além de que são suscetíveis à THG (HAYASHI SANT'ANNA et al., 2019). Portanto, a técnica de MLSA (*MultiLocus Sequence Analysis*) se baseia no sequenciamento e concatenação de sequencias de múltiplos genes (*loci*) constitutivos, sendo proposta a utilização de cinco ou mais genes, com o intuito de tamponar os efeitos da utilização de apenas um gene (GEVERS et al., 2005; GLAESER; KÄMPFER, 2015). O MLSA é bastante recomendando para a diferenciação de espécies intimamente relacionadas, visto que muitas vezes os genes escolhidos dentro de táxons diferentes podem apresentar taxas de evolução muito distintas. Os genes constitutivos escolhidos, dentro do mesmo táxon, devem ser onipresentes e apresentar uma única cópia no genoma, sendo importante evitar genes nos quais a recombinação pode conferir uma vantagem seletiva (virulência, codificação de antígenos), ou genes intimamente relacionados (GEVERS et al., 2005; GLAESER; KÄMPFER, 2015). Devido ao poder de resolução filogenético, o MLSA é eficaz na identificação e reclassificação de espécies bacterianas (BEUKES et al., 2019; DALL'AGNOL et al., 2017; HELENE; O'HARA; HUNGRIA, 2020; LOPES-SANTOS et al., 2017; RIBEIRO et al., 2009). Os genes mais comumente empregados na identificação e filogenia de rizóbios são: *recA*, *atpD*, *glnII*, *rpoB*, *gyrB* e *dnaK* (DE LAJUDIE et al., 2019).

Konstantinidis, Ramette e Tiedje (2006) sugerem um valor de referência para a delimitação de espécies de 96% de similaridade na concatenação de genes constitutivos, entretanto, esses valores são estimados, devido a que diversos autores sugerem novos valores de referência para a técnica, indicando a utilização de valores espécie-específicos (KONSTANTINIDIS; RAMETTE; TIEDJE, 2006). Por exemplo, estudos mostram que, em *Vibrio* sp., o valor de similaridade deve ser maior ou igual à 95% no MLSA (THOMPSON et al., 2001), enquanto que, para espécies de *Pseudomonas* e *Bradyrhizobium*, o valor de referência sugerido é ≥ 97% (DURÁN et al., 2014; GOMILA et al., 2015).

Dentro da taxonomia de rizóbios, é necessário comprovar a capacidade de nodulação e fixação de nitrogênio do organismo estudado. São diversos os genes simbióticos envolvidos no processo de nodulação e FBN, dentre eles, os mais estudados são *nodABC*, responsáveis pela formação do fator Nod; e o gene *nifH*, responsável pela formação da enzima nitrogenase (DE LAJUDIE et al., 2019). Os genes *nodC*, *nodA* e *nifH* são os mais indicados para o estudo de espectro de hospedeiros e definição de simbiovares (PEIX et al., 2015; ROGEL; ORMEÑO-ORRILLO; MARTINEZ ROMERO, 2011; VELÁZQUEZ et al., 2017). Ainda, o estudo comparativo de genes constitutivos e simbióticos revela os possíveis eventos de aquisição desses genes, assim como sua origem, permitindo uma melhor compreensão da história evolutiva desses microrganismos e da simbiose rizóbio-leguminosa (DE MEYER et al., 2016).

Apesar de estar praticamente em desuso, por ser uma técnica muito laboriosa, com difícil reprodução exata e comparação de resultados, a hibridização DNA-DNA foi, por mais de 50 anos, considerada “padrão ouro” na taxonomia bacteriana (ROSSELLÓ-MÓRA, 2012). O princípio da técnica se baseia nos níveis de pareamento e dissociação de fragmentos de DNA provenientes de duas estirpes diferentes. Para que as estirpes sejam consideradas da mesma espécie elas devem compartilhar 70% ou mais de similaridade na hibridação DNA – DNA (HDD) e 5º C ou menos de ΔTm (temperatura de *melting*, ou seja, desnaturação).

Com o advento de novas tecnologias de sequenciamento genético (*Next-Generation Sequencing)*, o sequenciamento completo do genoma (WGS, *Whole Genome Sequencing*) bacteriano tem mudado gradualmente a taxonomia bacteriana (CHUN; RAINEY, 2014; MEIER-KOLTHOFF; KLENK; GÖKER, 2014; RAINA et al., 2018). As sequências do genoma total fornecem muitas informações sobre a história evolutiva dos organismos, sendo possível utilizá-las para a discriminação de espécies (CHUN; RAINEY, 2014; MEIER-KOLTHOFF; KLENK; GÖKER, 2014; RAINA et al., 2018). Dessa forma, diversos autores sugerem a substituição da HDD na taxonomia bacteriana por técnicas análogas, utilizando o genoma total bacteriano em metodologias *in sílico*, que indicam valores de similaridade compartilhada entre genomas, chamadas de *Overall Genome Related Index* (OGRI) (CHUN et al., 2018; CHUN; RAINEY, 2014; ROSSELLÓ-MÓRA; AMANN, 2015)

Dentre os OGRIs, duas metodologias vêm sendo amplamente aplicadas na mimetização *in sílico* da HDD experimental e na determinação de novas espécies, a *Average Nucleotide Identity* (ANI) e a HDD digital (HDDd) (CHUN et al., 2018; CHUN; RAINEY, 2014; HAYASHI SANT'ANNA et al., 2019; MAHATO et al., 2017; MEIER-KOLTHOFF et al., 2013; RAINA et al., 2018; ROSSELLÓ-MÓRA; AMANN, 2015). O valor de HDDd é calculado pela métrica *Genome-to-Genome Distance Calculator* (GGDC) a qual avalia a distância entre os genomas de dois organismos, convertendo esses dados em valores de similaridade e, assim, determinando a relação entre as espécies (AUCH et al., 2010; MEIER-KOLTHOFF et al., 2013). O valor de referência para a determinação de espécies é o mesmo que o do HDD experimental, devendo ser ≥ 70% entre os organismos para que sejam considerados da mesma espécie (MEIER-KOLTHOFF et al., 2013).

Finalmente, o ANI é uma medida de similaridade genômica, conferida através da comparação nucleotídica entre as regiões codificantes do genoma de duas estirpes (KONSTANTINIDIS; TIEDJE, 2005). Konstantinidis & Tiedje (2005) mostraram, pela primeira vez, que o ANI é uma medida robusta de distância evolutiva, pois os valores de ANI se correlacionam bem com os valores de HDD. Inicialmente, a sequência do genoma da primeira estirpe chamada "*query*" é dividida em fragmentos de sequências de 1020 pb de comprimento. Logo, cada fragmento é pesquisado contra a sequência do genoma total da segunda estirpe "*subject*" usando o programa BLASTn do NCBI (National Center for Biotechnology Information). Neste processo, o programa BLASTn calcula os valores de identidade nucleotídica entre os fragmentos da estirpe "*query*" e o genoma da estirpe "*subject*" (GORIS et al., 2007; RAINA et al., 2018). O valor indicado para a delimitação de espécies de 70% na HDD e HDDd correspondem a ≥ 95-96% no ANI (GORIS et al., 2007; RICHTER; ROSSELLO-MORA, 2009). Devido a sua utilidade na identificação e classificação de microrganismos, o banco de dados NCBI utiliza a metodologia para verificar as atribuições de espécies relatadas nas diversas submissões de sequências genômicas (CIUFO et al., 2018).

Dentre as estirpes consideradas como uma nova espécie, uma deve ser selecionada para ser a representante da espécie, chamada de estirpe tipo (*type strain*). Essa estirpe será comparada com outras estirpes tipo representantes de diferentes espécies bacterianas, de modo a facilitar os estudos taxonômicos.

Após a realização de todas as etapas para a identificação de uma nova espécie bacteriana, uma série de regras determinadas pelo Comitê Internacional de Sistemática de Procariontes (ICSP) (https://www.the-icsp.org/), órgão que supervisiona a nomenclatura de procariotos, devem ser seguidas. Dentro do ICSP, o subcomitê em Taxonomia de Rizóbios e Agrobacteria (DE LAJUDIE et al., 2019) acredita que para uma descrição adequada de um novo gênero ou espécie de rizóbio, os padrões mínimos necessários são:

1) A sequência do genoma da estirpe tipo deve estar disponível em banco de dados, seguindo os padrões propostos por Chun et al (2018) para a taxonomia de procariotos.

2) Apresentação de dados baseados em sequências genômicas que diferenciem a espécie proposta (estirpe tipo) de outras espécies já descritas.
3) A variação molecular intraespecífica deve ser apresentada utilizando estirpes claramente diferentes.
4) A apresentação de dados fenotípicos úteis para o cultivo do rizóbio e compreensão de sua ecologia, devem ser fornecidos para um conjunto de estirpes que representam a variação na espécie.
5) Fornecer dados sobre a capacidade simbiótica entre rizóbio e planta através de testes de nodulação (infectividade, ativação dos nódulos, gama de hospedeiros) e filogenia de genes simbióticos (*nodC* e *nifH*).

Da mesma forma, as regras atuais para a taxonomia bacteriana geral, estabelecidas no Código Internacional de Nomenclatura de Procariotos (PARKER; TINDALL; GARRITY, 2019) devem ser seguidas e incluem os seguintes requisitos obrigatórios:

6) O nome da espécie deve estar de acordo com Código Internacional de Nomenclatura de Procariotos (PARKER; TINDALL; GARRITY, 2019)
7) A estirpe tipo deve ser depositada em pelo menos duas coleções de culturas reconhecidas em dois países diferentes, onde subculturas devem estar disponíveis.
8) As descrições devem ser publicadas no *International Journal of Systematic and Evolutionary Microbiology* (IJSEM), ou outras revistas microbiológicas e, posteriormente, serem incluías na lista de validação da IJSEM.

Dessa maneira, a taxonomia polifásica bacteriana baseada na utilização de técnicas tradicionais e modernas se mostra eficaz na determinação de novas espécies. Seguramente, novas metodologias de análises *in sílico* do genoma total bacteriano serão propostas no futuro, trazendo novas possibilidades e padrões para a taxonomia bacteriana, elucidando cada vez mais as relações evolutivas desses seres que desempenham papéis essenciais na manutenção da vida.

4. METODOLOGIA

4.1 ESTIRPES UTILIZADAS

As estirpes bacterianas utilizadas nos Estudos 1, 2 e 3 são provenientes de dois estudos prévios realizados por Paulitsch et al. (2018) e Dall'Agnol et al. (2017). Ambos os estudos caracterizam a diversidade de rizóbios.

Em um estudo conduzido no Parque Estadual do Guartelá (Tibagi-PR), 29 estirpes foram coletadas de nódulos de *Mimosa gymnas*. A partir de análises filogenéticas do 16S RNAr e MLSA, um grupo contendo seis estirpes demonstrou se tratar de uma potencial nova espécie dentre o gênero *Paraburkholderia* (PAULITSCH et al., 2019). Desta forma, o grupo foi selecionado para a realização de uma análise polifásica (Tabela 1).

Similarmente, um estudo de diversidade conduzido com solos da Mata Atlântica no estado do Rio de Janeiro, através da utilização de plantas iscas (*Phaseolus vulgaris* e *Mimosa pudica*), diversos grupos apresentaram posições isoladas em análises filogenéticas do 16S RNAr+*recA* e no MLSA, representando potenciais novas espécies. Dentre eles, cinco estirpes do grupo psp1 e três do grupo psp6 foram escolhidas para a análise polifásica (Tabela 1).

As estirpes de ambos os estudos estão depositadas na Coleção de Microrganismos Multifuncionais da Embrapa Soja: Bactérias Diazotróficas e Promotoras do Crescimento de Plantas (Coleção WFCC #1213, WDCC # 1054), em Londrina, Brasil, na qual receberam a denominação CNPSo. Além disso, as estirpes provenientes do estudo de Paulitsch et al. (2019) estão depositadas na coleção de culturas "National Collection of Rhizobial Strains of Uruguay" (WFCC Collection # 1082), em Montevideo, Uruguai. E as estirpes do estudo proveniente de Dall'Agnol et al. (2017) estão depositadas no *“Laboratoire de Symbioses Tropicales et Méditerranéenes* (ABIP/LSTM)” em Montpellier, França; na “*US Collection*” da Universidade de Sevilha, Espanha; e na “*Belgian Coordinated Collections of Microorganisms*/ LMG *Bacteria Collection*” em Gante, Bélgica.

As bactérias foram cultivadas a 28ºC em meio de cultura YMA-(*yeast-mannitol-agar*) modificado (HUNGRIA et al., 2016) contendo o indicador vermelho Congo, colônias puras foram selecionadas para estocagem. Todas as estirpes foram crescidas em meio líquido YM-modificado e, posteriormente, liofilizadas ou criopreservadas em criotubos contendo 30% glicerol (v/v) a - 80ºC e - 150ºC.

Tabela 1. Nomenclatura, planta hospedeira e origem geográfica das estirpes utilizadas nos estudos 1, 2 e 3.

Estirpe	Sinônimo	Planta hospedeira/ planta isca	Origem geográfica
		Estirpes Estudo 1	
CNPSo 3008^T	U13000^T, G29.01^T	*Mimosa gymnas*	Tibagi, PR 24°33'52.75"S/50°15'10.27"O
CNPSo 2995	G10.01		Tibagi, PR 24°33'51.73"S/50°15'10.79"O
CNPSo 3003	G20.01		Tibagi, PR 24°33'52.75"S/50°15'10.27"O
CNPSo 3016	G25.02		Tibagi, PR 24°33'52.75"S/50°15'10.27"O
CNPSo 3023	G29.04		Tibagi, PR 24°33'52.75"S/50°15'10.27"O
CNPSo 3248	G32.01		Tibagi, PR 24°33'51.73"S/50°15'10.79"O

Estirpes Estudo 2 (Psp1)			
CNPSo 3157^T	ABIP 241^T/ LMG 31644^T	*Piptadeniae gonoacantha*/ *Mimosa pudica*	Búzios, RJ 22°26’426"S/41°51'419O
CNPSo 3191	STM 10535		Rio das Ostras, RJ 22°45'345"S/41°57'890"W
CNPSo 3199	STM 10611		Búzios, RJ 22°26'426"S/41°51'419"O
CNPSo 3200	STM 10605		Búzios, RJ 22°26'426"S/41°51'419"O
CNPSo 3201	STM 10628		Búzios, RJ 22°26'426"S/41°51'419"O
Estirpes Estudo 2 (Psp6)			
CNPSo 3155^T	ABIP 239^T/ LMG 31643^T	*Piptadeniae gonoacantha*/ *Mimosa pudica*	Búzios, RJ 22° 26'426S / 41° 51'419O
CNPSo 3150	ABIP 236		Búzios, RJ 22°26'426"S/41°51'419"O
CNPSo 3196	STM10835	*Piptadeniae gonoacantha*/ *Phaseolus vulgaris*	Búzios RJ 22°26'426"S/41°51'419"O

4.2 ANÁLISES FENOTÍPICAS

Nos Estudos 1 e 2 as características morfológicas das colônias foram analisadas de acordo com a manifestação do crescimento, tamanho, forma, elevação, borda, superfície, produção de muco, consistência, detalhes ópticos, cromogênese e alteração de pH do meio, de acordo com Vincent (1970) e Somasegaran e Hoben (1994), com algumas modificações.

As estirpes foram cultivadas a 28 °C por 4 dias, em diferentes condições, e para confiabilidade dos resultados, todos os testes foram realizados em duplicata.

Inicialmente as estirpes foram semeadas em placa de Petri contendo meio YMA-modificado com vermelho Congo (HUNGRIA et al., 2016) e, após 4 dias, foram analisadas. Em relação à manifestação do crescimento, foi observada a taxa da formação de colônias isoladas em placa de Petri, em crescimento rápido (até 3 dias) e intermedário (4 a 5 dias). Mediu-se o diâmetro de três colônias em milímetros com a utilização de paquímetro, e a média dos valores obtidos foi registrada.

Para a análise da alteração de pH do meio, as estirpes foram inoculadas em meio YMA-modificado contendo o indicador de pH azul de bromotimol. A acidificação do meio resulta na alteração da coloração original a amarela, a alcalização na coloração azul e a coloração verde (coloração original do meio) indica a não alteração de pH do meio.

As características de forma, elevação, tipo de borda e superfície das colônias foram analisadas com o auxílio de lupa com luz incendente superior, sempre comparando três colônias isoladas. Observaram-se as colônias quanto as suas formas circulares, ovais (elípitcas) ou irregulares; tipo de borda lisas, onduladas ou filamentosa; superfície lisa ou rugosa; e a elevação da colônia, observada lateralmente, como plana (crescimento rente à placa) ou convexa (pulvinadas/drop-like), quando crescem consideravelmente acima do meio de cultura.

Em relação à produção de muco, analisou-se o tamanho da colônia, dentre as opções de pouca produção (tamanho ≤ 1,9 mm), produção moderada (tamanho entre 2 a 3,9 mm) e produção abundante (tamanho ≥ 4 mm).

A consistência da massa de crescimento das colônias foi determinada por meio da utilização de alça de platina flambada em bico de Bulsen e após resfriada foi encostada delicadamente sobre uma colônia isolada, sendo classificadas como aquosas (se desmancham ao entrar em contato com a alça de platina), secas (pouca produção de muco), gomosas (podem ser arrastadas pela placa sem se desmanchar deixando um rastro) e viscosas (formam um fio elástico ao tocá-las).

Os detalhes ópticos foram analisados com a utilização de um fundo branco e lupa, fez-se um risco com caneta de retroprojetor embaixo da placa de Petri, exatamente onde a colônia com maior crescimento se localiza. As placas foram viradas para a orientação original e observou-se se a colônia era opaca (não permite a visualização do risco feito) ou translúcida (permite a visualização do risco).

A cromogênese da colônia foi avaliada em placa de Petri contendo YMA-modificado acrescido de vermelho Congo, podendo a massa de crescimento apresentar a coloração branca, rosada, avermelhada (centro) ou vermelha. Da mesma forma, em YMA-modificada contendo azul de bromotimol as colônias podem apresentar coloração amarelo claro, amarelo escuro, rosa ou azul.

Com o propósito de observar os paramêtros fisiológicos das estirpes, avaliou-se o crescimento em YMA-modificado em diferentes condições, utilização de fontes de carbono, tolerância a antibióticos e atividade da enzima urease, sendo os resultados avaliados como positivos (+), negativos (-) ou intermedários (±), bem (HOWIESON; DILWORTH, 2016; VANDAMME et al., 1996).

A determinação da capacidade de crescimento dos isolados em condições de alta salinidade ocorreu em meio YMA-modificado contendo 1% de NaCl. O crescimemto em diferentes pHs foi estimado em meio YMA-modificado com pH 8,0 e 4,0; o crescimento em alta temperatura foi avaliado a 37°C. Avaliou-se também o crescimento em meio Luria-Bertani sólido a 28°C por 4 dias (HUNGRIA et al., 2016).

O teste de tolerância à antibióticos foi realizado pelo método de disco-difusão proposto por Bauer e Kirby (1966). Inicialmente, cultivou-se o pré-inoculo em YM-modificado líquido durante 2 dias em agitação de 100 rpm. Logo, 100 mL do pré inóculo foram espalhados em meio YMA-modificado com o auxílio de alça Drigalski. Em cada placa de Petri colocaram-se dois discos de antibióticos com distanciamento suficiente para a avalição da formação de halos de inibição. Oito antibióticos foram testados: tetraciclina (30 µg), bacitracina (0.04 U), clorofenicol (30 µg), eritromicina (10 µg), neomicina (30 µg), ácido nalidíxico (30 µg), estreptomicina (15 µg), e cefuroxina (30 µg). O tamanho dos halos em milimetros é interpretado com o auxílio de uma tabela (BAUER; KIRBY, 1966), que indica se o microrganismo é resistente (+), moderadamente resistente (±) ou sensível (-) ao antibiótico submetido.

A atividade da enzima urease foi observada em meio YMA-modificado acrescido de 2% de ureia com indicador vermelho de fenol. A presença da coloração rosada no meio significa degradação da ureia (+), ou seja, presença de urease; pouca alteração na coloração indica baixa presença e, por tanto, baixa degradação (±) e a inalteração da coloração do meio (amarela) demonstra ausência da atividade enzimática (-).

Para avaliar a utilização de fontes de carbono, o crecimento das estirpes foi avaliado em 49 tipos diferentes de carbono, por meio do kit API 50CH (BioMérieux), em duplicata. Primeiramente, foi realizado o pré-inoculo dos isolados em meio YM-modificado líquido, por 2 dias, a 100 rpm. Logo, o pré-inóculo foi centrigufado a 6.000 rpm durante 10 min, e o sobrenadante descartado. O *pellet* formado foi ressuspendido em 5 mL de solução salina 0,85% e centrifugado novamente nas condições anteriores. O sobrenadante resultante foi novamente descartado e o *pellet* ressuspendido em 2 mL de água destilada estéril. Em outro frasco contendo 5 mL de água destilada estéril, com o auxilio de um conta-gotas, inseriu-se aos poucos a solução contendo as células bacterianas, até atingir a escala de turbidez 3 de Mcfarland, correspondente a 900 UFC x10^6 /mL (SUTTON, 2011). O dobro do número de gotas inseridas para se chegar à escala 3 de Mcfarland foi adicionado a um novo frasco contendo YM-modificado sem a fonte de carbono manitol e acrescido de azul de bromotimol. O kit API 50CH contém galerias separadas para cada fonte de carbono, nas quais 230 µL do inóculo (YM modificado+azul de bromotimol) foram adicionados e, em seguida, as galerias foram seladas com 2 a 3 gotas de vaselina estéril. O mesmo teste foi realizado para estirpes-tipo próximas, visando comparações. Após 4 dias de crescimento, os resultados foram resgistrados, sendo que a primeira galeria representa o controle, ou seja, nela não há nenhuma fonte de carbono disponível. Devido à presença de azul de bromotimol, a alteração intensa da cor do meio em amarela (ácida) ou azul (básica) indica a utilização da fonte de carbono da galeria analisada (+), a alteração de cor fraca, representa pouca utilização da fonte de carbono (±) e, quando a coloração permaneçe igual a do controle, não ocorreu a metabolização da fonte de carbono (-).

Por último, a caracterização bioquímica se deu através do perfil dos ácidos graxos celulares em parceiria com a Embrapa Meio Ambiente, onde as análises foram realizadas. Tanto as estirpes em estudo quanto as estirpes-tipo próximas foram submetidas à análise. Todas as estirpes foram crescidas em meio YMA-modificado e seus perfis de ácidos graxos foram obtidos por meio de cromotografia gasosa, utilizando o MIDI (Microbial Identification System) (SASSER, 2006) conforme descrito por Delamuta et al. (2013).

4.3 ANÁLISES GENOTÍPICAS E FILOGENÉTICAS

4.3.1 Extração de DNA total

A extração do DNA genômico das estirpes utilizadas nos estudos 1, 2 e 3 foi realizada por meio do kit *DNeasy Blood and Tissue Kit* (Qiagen), seguindo as instruções do fabricante. A confirmação da integridade dos DNAs extraídos foi verificada por meio de eletroforese em gel de agarose 1%, corado em solução de brometo de etídio na concentração 0,00005 % e observado sob luz ultravioleta. Após a verificação, as amostras com bandas significativas foram armazenadas em freezer a -20ºC para conservação e utilizadas nas reações de PCR (*Polymerase* C*hain Reaction*) e sequenciamento do genoma total bacteriano.

4.3.2 DNA *fingerprinting* - BOX-PCR

O perfil genotípico das estirpes dos Estudos 1 e 2 foi obtido pela amplificação de regiões repetitivas do genoma bacteriano (boxA), utilizando o iniciador BOX-A1R (VERSALOVIC et al., 1994; KASCHUK et al., 2006). A reação de PCR foi conduzida com 13,8 µL de água ultra pura estéril; 5,0 µL de dNTPs, (1,5 mmol/L de cada nucleotídeo); 2,5 µL de tampão 10X (500 mM KCl; 200 mM Tris–HCl, pH 8,4); 1,5 µL de MgCl (50 mmol/L); 1,0 µL de iniciador (50 pmol/µL); 1,0 µL de DNA (50 ng/µL) e 0,2 µL de Taq DNA polimerase (5 U/µL), resultando em um volume final de 25 µL. Após a amplificação, as amostras foram submetidas à eletroforese em gel de agarose 1,5% para a separação das bandas por aproximadamente 5 horas. Logo, o gel foi corado em solução contendo brometo de etídio, visualizado sob luz ultravioleta e foto documentado em alta qualidade.

A foto do gel de BOX-PCR foi utilizada no programa Bionumerics (Applied Mathematics, Kortrijk, Bélgica, v.7.6) para a identificação das bandas de cada estirpe. Posteriormente, construiu-se um dendograma de similaridade, aplicando o algorítimo UPGMA (*Unweighted Pair-Group Method with Arithmetic mean*) (SNEATH; SOKAL, 1973) e o coeficiente de Jaccard com 1% de tolerância para o Estudo 1 e 3% de tolerância no Estudo 2 (JACCARD, 1912).

4.3.3 Amplificação de genes constitutivos e simbióticos

Todas as estirpes utilizadas nos Estudos 1 e 2, tiveram seu gene ribossomal 16S RNAamplificado. A reação foi conduzida com 35,3 μL de água ultrapura estéril; 3,0 μL de dNTPs, (1,5 mmol/L de cada nucleotídeo); 5,0 μL de tampão 10X (500 mM KCl; 200 mM Tris–HCl, pH 8,4); 1,5 μL de $MgCl_2$ (50 mmol/L); 1,5 μL do iniciador *forward* (10pmol/ μL) e 1,5 μL do iniciador *reverse* (10pmol/ μL), 2,0 μL de DNA (50 ng/μL) e 0,2 μL de Taq DNA polimerase (5 U/μL) resultando no volume final de 50 μL.

Os genes constitutivos *recA, gyrB, trpB, gltB* e *atpD* foram amplificados para todas as estirpes do Estudo 1. Entretando, as sequências dos genes *recA*, *gyrB*, *trpB* e *gltB* utilizadas no Estudo 2 já haviam sido amplificadas por Dall'Agnol et al. (2017) e foram retiradas do banco de dados NCBI. A reação de PCR para a amplificação de genes do Estudo 1 foi conduzida com 35,3 μL de água ultrapura estéril; 3,0 μL de dNTPs, (1,5 mmol/L de cada nucleotídeo); 5,0 μL de tampão 10X (500 mM KCl; 200 mM Tris–HCl, pH 8,4); 1,5 μL de $MgCl_2$ (50 mmol/L); 1,5 μL do iniciador *forward* (10pmol/ μL) e 1,5 μL do iniciador *reverse* (10pmol/ μL); 2,0 μL de DNA (50 ng/μL) e 0,2 μL de Taq DNA polimerase (5 U/μL), resultando no volume final de 50 μL.

O genes *nodC* das estirpes dos Estudos 1 e 2 foram amplificado e sequenciados em estudos anteriores (DALL'AGNOL et al., 2017; PAULITSCH et al., 2019).

Os genes simbióticos *nodA* e *nifH* foram amplificados para as estirpes utilizadas no Estudo 3. A reação de PCR foi conduzida com 34,3 µL de água ultrapura estéril; 3,0 µL de dNTPs, (1,5 mmol/L de cada nucleotídeo); 5,0 µL de tampão 10X (500 mM KCl; 200 mM Tris–HCl, pH 8,4); 1,5 µL de $MgCl_2$ (50 mmol/L); 2 µL do iniciador *forward* (10pmol/ µL) e 2 µL do iniciador *reverse* (10pmol/ µL); 2,0 µL de DNA (50 ng/µL) e 0,2 µL de Taq DNA polimerase (5 U/µL), resultando no volume final de 50 µL.

As condições de amplificação por PCR e os iniciadores utilizados de todos os genes estão descritos na Tabela 2.

Após a amplificação as amotras foram submetidas à eletroforese em gel de agarose 1% para verificação da amplificação e a integridade do produto de PCR. As amostras cuja verificação foi positiva foram purificadas com o *kit PureLink Quick PCR Purification* (Invitrogen), seguindo as recomendações do fabricante. Para verificação da pureza, as amostras foram novamente submetidas à eletroforese nas mesmas condições descritas anteriormente.

Tabela 2. Condições de amplificação e iniciadores utilizados nos Estudos 1, 2 e 3.

Gene	**Iniciador**	**Sequência (5' - 3')**	**Condições de PCR**	**Referências**
16S rRNA	fD1	AGAGTTTGATCCTGGCTCAG	2 min 95°C, 30 x (15 s 94°C, 45 s 93°C, 45 s 55°C, 2 min 72°C) and 5 min 72°C	(WEISBURG et al., 1991)
	rD1	AAGGAGGTGATCCAGCC		
	362f*	CTCCTACGGGAGGCAGCAGTG GGG	Sequenciamento 1 min 96 ºC, 35 x	(MENNA et al., 2006)

	786f*	CGAAAGCGTGGGGAGCAAACAGG	(15 s 96ºC, 15 s 50ºC, 4 min 60ºC)	
recA	*recA*-BurkF	AGGACGATTCATGGAAGAWAGC	2 min 95 ºC, 35 x (30 s 94ºC, 30 s 58ºC, 1 min 72ºC) e 5 min 72ºC	(SPILKER et al., 2009)
	recA-BurkR	GACGCACYGAYGMRTAGAACTT		
gyrB	gyrB-BurkF	ACCGGTCTGCAYCACCTCGT		
	gyrB-BurkR	YTCGTTGWARCTGTCGTTCCACTGC		
trpB	trpB-BurkF	CGCGYTTCGGVATGGARTG		
	trpB-BurkR	ACSGTRTGCATGTCCTTGTCG		
gltB	gltB-BurkF	CTGCATCATGATGCGCAAGTG		
	gltB-BurkR	CITGCCGCGGAARTCGTTGG		
atpD	atpD-BurkF	ATGAGTACTRCTGCTTTGGTAGAAGG		
	atpD-BurkR	CGTGAAACGGTAGATGTTGTCG		
nodC	nodCBurkF	TRATYGAYATGGAATACTGGC	3 min 95°C, 35 X (30 s 95°C, 30 s 55°C, 45 s 55°C, 45 s 72°C) e 7 min 72°C	(BOURNAUD et al., 2013)
	nodCBurkR	CAGCGGAYATMGTCATTGA		
nodA	Burk nodA-for	CCRACMGGGBCKTTYAAYGC	5 min 95°C, 40 X (30 s 95°C, 30 s 50°C, 30 s 72°C) e 7 min 72°C	(BONTEMPS et al., 2016)
	Burk nodA-rev	TCACAGCTCHGGVCCGTTSG		
nifH	nifH-F	AAAGGYGGWATCGGYAARTCCAC	2 min 95°C, 40 X (30 s 94°C, 30 s 60°C, 1,5 min 72°C) e 7 min 68°C	(LOIRET et al., 2004)l
	nifH-R	TTGTTSGCSGCRTACATSGCCAT		

BOX-PCR	A1R	CTACGGCAAGGCGACGCTGACG	7 min 94°C, 35 x (1 min 94°C, 1 min 52°C, 8 min 72°C) 16 min 65°C	(VERSALOVIC et al., 1994)

4.3.4 Sequenciamento dos genes constitutivos e simbióticos

As amostras purificadas dos genes obtidos anteriormente foram novamente amplificadas para o sequenciamento. A reação de PCR consistiu na adição de 3 µL de DNA (15µgmL-1), 1,3 µL de BigDye, 1 µL de iniciador (3,2 pmol), 2,5 µL de tampão 10x (500 mM KCl; 200mM Tris–HCl, pH 8,4) e 12,2 µL de H_2O ultrapura estéril, totalizando um volume final de 20 µL por reação, reação conduzida em placas de 96 poços. A amplificação por PCR foi realizada em termociclador, com o seguinte ciclo para todos os genes: 96 °C por 1 min, seguido de 35 ciclos de 96 °C por 15 s, 50 °C por 15 s e 60 °C por 4 min (DELAMUTA et al., 2017).

Após a amplificação, os produtos foram purificados de acordo com Menna et al. (2006), com algumas modificações. Em todos os poços foram adicionados 2 µL de acetato de amônio estéril (7,5 M) e 65 µL de etanol absoluto (temperatura ambiente). Logo, a placa foi selada, homogeneizada e passou por centrifugação a 4000 rpm durante 45 min. Descartou-se o sobrenadante resultante, invertendo-se a placa em papel absorvente. Foram adicionados 150 µL de etanol 70% em todos os poços e a placa foi centrifugada a 400 rpm por 20 min. Novamente a placa foi invertida para descarte do sobrenadante e submetida a nova centrifugação em rotação mais baixa (300 rpm) durante 20 segundos para a retirada de quaisquer resíduos de sobrenadante. A placa foi colocada em estufa a 37 °C para secagem durante 30 min.

Após a secagem total da placa, os *pellets* foram ressuspendidos com 10 µL de formamida. A placa foi então aquecida em termociclador à 95º C durante 5 min e depois resfriada por 2 min em gelo. Por fim, a placa com as amostras foi submetida ao sequenciador ABI 3500XL (Applied Biosystems).

Os dados de sequenciamento foram processados no programa Bionumerics (v.7.6), onde as sequências foram corrigidas manualmente. A correta fase de leitura (*Open Read Frame*) das sequências de cada gene constitutivo foi verificada pelo *Expasy Translate Tool* (https://web.expasy.org/translate) (ARTIMO et al., 2012). Depois, as sequências de todos os genes (constitutívos e simbióticos) foram depositadas no banco de dados NCBI, acessível mundialmente.

4.3.5 Filogenia dos genes constitutivos e simbióticos

Para inferir as relações evolutivas dentre os genes obtidos nos Estudos 1, 2 e 3, as sequências nucleotídicas de cada gene individual foram comparadas com as sequências depositadas no NCBI através da ferramenta BLASTn. O software MEGA 7 (KUMAR; STECHER; TAMURA, 2016) foi utilizado para analisar as sequências nucleotídicas obtidas nos estudos citados acima e de espécies proximas (retiradas de bancos de dados online). As sequências foram alinhadas por meio do algorítimo MUSCLE (EDGAR, 2004) e, em seguida, cortadas, obtendo o mesmo comprimento para todas as sequências em análise. Para a construção da árvore filogenética, o modelo matemático Máxima verossimilhança (*Maximum Likelihood* -ML) foi aplicado (FELSENSTEIN, 1981). O melhor modelo evolutivo para construção da filogenia de cada gene foi escolhido por meio do programa MEGA, utilizando o suporte estatístico avaliado por *bootstrap* (FELSENSTEIN, 1985) com 1000 repetições (HEDGES, 1992)

Para a abordagem do MLSA dos Estudos 1, 2 e a análise de genes simbióticos do Estudo 3, os genes foram concatenados com o auxilio do programa *Seaview* (GOUY; GUINDON; GASCUEL, 2010).

O programa Bioedit (v. 7.0.4.1) (HALL, 1999) foi empregado para a obtenção da identidade nucleotídica dentre estirpes dos Estudos 1, 2 e 3 e estirpes próximas utilizadas nas filogenias, através das sequências nucleotídicas dos genes individuais e concatenados.

4.3.6 Sequenciamento do genoma bacteriano

Nos Estudos 1 e 2, uma estirpe foi escolhida como representante (estirpe tipo) dos grupos estudados. O DNA total bacteriano das estirpes CNPSo 3008 (Estudo1), CNPSo 3157 e CNPSo 3155 (Estudo 2) foi utilizado para o sequenciamento e construção da biblioteca genômica no sequenciador Illumina MiSeq. As amostras foram preparadas com o *kit* Nextera XT (*Nextera DNA Library Preparation Kits*), de acordo com as recomendações do fabricante. Logo, o *genome draft* foi montado com o *pipeline* A5-miseq *de novo* (Illumina). O *genome draft* foi enviado para o NCBI, para anotação automática. O genoma também foi submetido aos programas Rast (AZIZ et al., 2008) e Quast (GUREVICH et al., 2013), nos quais foram caracterizados quanto ao tamanho do genoma, N_{50}, cobertura, número de *contigs* e conteúdo G+C.

4.3.7 Determinação das relações genômicas por ANI e HDDd

Nos Estudos 1 e 2 os genomas das estirpes representantes de cada nova espécie (CNPSo 3008, CNPSo 3157, CNPSo 3155) foram comparados com sequências genômicas de estirpes-tipo próximas, retiradas de bancos de dados (JGI/IMG/R, NCBI), para analisar as relações entre eles. Para tanto, duas metodologias *in silico* foram utilizadas, o ANI (*Average Nucleotide Identity*) e a hibridação DNA-DNA digital. Os valores de ANI foram calculados a partir da submissão das sequências genômicas em formato FASTA ao programa *ANI calculator*. Também, com a utilização das sequências em formato FASTA, os valores de HDDd foram obtidos pelo *Genome-to-Genome Distance Calculator* v 2.1, adotando a Fórmula: 2 (*identities* / *High Score Pairs length*) recomendada pelo próprio programa.

Ambos os *softwares* estão disponíveis gratuitamente e *online* nos *sites* http://enve-omics.ce.gatech.edu/ani/ (ANI) e http://ggdc.dsmz.de/ggdc.php# (HDDd).

4.4 CAPACIDADE E EFICIÊNCIA DE NODULAÇÃO

A capacidade simbiótica das estirpes e o espectro de hospedeiros foram testados em feijoeiro (*Phaseolus vulgaris*), *Mimosa pudica* e siratro (*Macroptilium atropurpureum*) nos Estudos 1 e 2 e em soja (*Glycine max*) apenas no Estudo 1.

Tanto as sementes de siratro, como as de *Mimosa pudica* necessecitaram de processos para a quebra de dormência para o processo de germinação. As sementes de siratro foram levadas à capela de exaustão onde foram submersas em ácido sulfúrico e agitadas em movimentos circulares durante 10 min, o ácido foi descartado e as sementes passaram por cinco lavagens consecutivas com água destilada estéril. Já as sementes de *Mimosa pudica* sofreram escarificação mecânica, sendo levemente lixadas.

Todas as sementes foram esterilizadas superficialmente em etanol 70% por 1 min e, em seguida, embebidas em hipoclorito de sódio comercial (2–3%) por 5 min e lavadas 5 vezes em água destilada estéril.

As sementes de feijoeiro e soja foram pré germinadas em papel germitest umidecido com água destilada estéril, enquanto que as sementes de *Mimosa pudica* e siratro foram pré germinadas em placa de Petri contendo meio ágar-água 1 % (ágar 10 g/L) e cobertas com papel aluminio para a proteção contra luminosidade. Todas as sementes foram incubadas em germinador a 28 °C durante 2 dias.

O teste foi realizado com a utilização de sacos de polipropileno contendo papel germitest e solução livre de nitrogênio (BROUGHTON; DILWORTH, 1971), conduzido em triplicata. Dentro de fluxo lâminar, as mudas foram transplantadas para os sacos de polipropileno com o auxílio de pinça esterelizada. As estirpes foram cultivadas em meio YM-modificado líquido até a fase exponencial e utilizadas como inóculo um dia após a transferência das mudas. A estirpe *Paraburkholderia nodosa* Br3437^T foi utilizada como controle positivo para *Mimosa pudica* e siratro e a estirpe *Rhizobium tropici* CIAT 899 para feijoeiro e *Bradyrhizobium japonicum* USDA 110^T para soja. Um dia após o inóculo, as plantas foram levadas à casa de vegetação, onde o feijoeiro, siratro e soja permaneceram por 30 dias e *Mimosa pudica* por 40 dias.

Passados os 30 a 40 dias, as plantas foram retiradas dos sacos e analisadas. Observaram-se a presença ou ausência de nódulos e a presença de nódulos eficientes na fixação do N_2 das estirpes. Para a avaliação da eficiência, dois ou três nódulos de cada planta foram selecionados e cortados para a observação da presença ou ausência de leghemoglobina, com coloração típica rósea ou avermelhada.

Figura 3. Teste de nodulação em saco de polipropileno utilizando feijoeiro, siratro e *Mimosa pudica*.
Fonte: Autor

4.5 REVISÃO BIBLIOGRÁFICA DE ESPÉCIES BRASILEIRAS DE *PARABURKHOLDERIA* NODULÍFERAS

O Estudo 4 se refere a uma revisão sobre espécies descritas de *Paraburkholderia* nodulíferas isoladas no Brasil. Para isso, uma extensa pesquisa foi realizada, selecionando artigos científicos que trouxessem informações sobre o tema. O foco da pesquisa se baseou na busca de informações sobre o marco da descoberta de bactérias nodulíferas na classe Betaproteobacteria, o histórico taxônomico envolvido na reclassificação de *Burkholderia* l.s. em *Paraburkholderia*, as hipóteses evolutivas e distribuição mundial de *Paraburkholderia* nodulíferas, os simbiovares dentro do gênero, as *Paraburkholderia* isoladas do Brasil e o potencial agrobiotecnológico das mesmas.

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6. ESTUDO 1

PAULITSCH, F. et al. *Paraburkholderia guartelaensis* sp. nov., a nitrogen-fixing species isolated from nodules of *Mimosa gymnas* in an ecotone considered as a hotspot of biodiversity in Brazil. **Archives of Microbiology**, p. 1–12, 2019.

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ORIGINAL PAPER

Paraburkholderia guartelaensis sp. nov., a nitrogen-fixing species isolated from nodules of *Mimosa gymnas* in an ecotone considered as a hotspot of biodiversity in Brazil

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Abstract

A polyphasic approach was used to infer the phylogenetic position of six nitrogen-fixing symbiotic bacteria isolated from *Mimosa gymnas* nodules grown in an ecotone between the Brazilian biomes of Atlantic Forest and Cerrado, considered as a hotspot of biodiversity. The 16S rRNA gene phylogeny indicated the highest similarity with *Paraburkholderia oxyphila* (98.7–98.9%), but similar values were found with other *Paraburkholderia* species. The multilocus sequence analysis (MLSA) of five (*recA, gyrB, trpB, gltB,* and *atpD*) housekeeping genes indicated that the CNPSo strains represent a novel lineage, sharing less than 95.7% of nucleotide identity (NI) with other *Paraburkholderia* species, being more closely related to *P. nodosa*. Genome parameters were analyzed for strain CNPSo 3008^T, and DNA–DNA hybridization revealed a maximum of 55.9% of DNA–DNA relatedness with *P. nodosa*, while average nucleotide identity with the two closest species was of 93.84% with *P. nodosa* and of 87.93% with *P. mimosarum*, both parameters confirming that the strain represents a new species. In the analysis of the nodulation *nodC* gene, all CNPSo strains showed the highest similarity with *P. nodosa*, and nodulation tests indicated host specificity with *Mimosa*. Other phylogenetic, physiological, and chemotaxonomic properties were evaluated. All data obtained support the description of the novel species *Paraburkholderia guartelaensis* sp. nov., with CNPSo 3008^T (=U13000^T=G29.01^T) indicated as the type strain.

Keywords Biological nitrogen fixation · Nodulation · MLSA · ANI · dDDH

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Abbreviations

NI	Nucleotide identity
ANI	Average nucleotide identity
DDH	DNA–DNA hybridization
dDDH	Digital DNA–DNA hybridization
MLSA	Multilocus sequence analysis

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Introduction

Rhizobia is the common name used for diazotrophic symbiotic bacteria and most of them (16 genera) belong to the subdivision Alphaproteobacteria, while only *Paraburkholderia*, *Cupriavidus,* and *Trinickia* belong to the Betaproteobacteria (Velázquez et al. 2017; Estrada-de los Santos et al. 2018). *Paraburkholderia* were previously classified as *Burkholderia* and belong to the *Burkholderiaceae* family, in the order *Burkholderiales*. The genus *Burkholderia* was first proposed by Yabuuchi et al. (1992), with the purpose of transferring seven species of the genus *Pseudomonas* to the new genus, with *Burkholderia cepacia* representing the type species. The genus *Burkholderia* is outstanding by some properties, including a high level of diversity, occupying several niches (soil, water, plants, and hospitals), possessing great metabolic diversity and with many strains showing biotechnological potential (Compant et al. 2008; Eberl and Vandamme 2016).

In the last decade, the phylogeny of the *Burkholderia* sensu lato has been discussed, leading to a major reclassification. Based on the phylogenetic analysis of the 16S rRNA and housekeeping genes, it has been shown that the genus comprises at least two major clades, one composed of clinically important and phytopathogenic members, and the other one with environmental species (Gyaneshwar et al. 2011; Estrada-De Los Santos et al. 2013). First, a detailed phylogenomic study of 45 *Burkholderia* species resulted in the proposal of two new genus, *Paraburkholderia* and *Caballeronia*, to allocate the clade of environmental species, with the reclassification of 11 *Burkholderia* species (including *B. andropogonis*) into the new genus *Paraburkholderia* (Sawana et al. 2014; Dobritsa and Samadpour 2016). Soon after, Lopes-Santos et al. (2017) performed a study based on the analysis of 16S rRNA gene sequences and multilocus sequence analysis (MLSA) to clarify the taxonomic position of *B. andropogonis,* that was incongruent with the *Paraburkholderia* genus, and the results led to the description of the new genus *Robbsia* (Lopes-Santos et al. 2017). Following, studies performing a robust phylogenomic analysis using amino acid and nucleotide sequences revealed two other distinct groups of *Burkholderia*, leading to the proposal of the genera *Mycetohabitans* and *Trinickia* (Beukes et al. 2017; Estrada-de los Santos et al. 2018).

Currently, *Paraburkholderia* is composed of 74 recognized species from the most varied environments, including agricultural soils, volcanic soils, water, and plants (Chen et al. 2007; Farh et al. 2015; Weber and King 2017; LPSN 2019). Some *Paraburkholderia* species are able to establish nitrogen-fixing symbiosis with plants of the Fabaceae family (Barrett and Parker 2005; 2006; Chen et al. 2005, 2006; Elliott et al. 2007a, b; Gyaneshwar et al. 2011; Sheu et al. 2013, 2015; Dall'Agnol et al. 2016; Bournaud et al. 2017; Paulitsch et al. 2019), contributing to plant growth and to the N input in terrestrial ecosystems.

Nowadays, there are 16 *Paraburkholderia* species with nitrogen-fixing strains (Velázquez et al. 2017). Some of the known *Paraburkholderia* have been isolated from root nodules of plants of the Mimosoideae family, especially of the genus *Mimosa* (Chen et al. 2006, 2007, 2008; Sheu et al. 2012, 2013), and most of these isolates were obtained in Brazil.

Brazil is considered as a hotspot of biodiversity, including its microbiota (Myers et al. 2000). In the Fabaceae family, *Mimosa* represents the second largest genus of the subfamily Mimosoideae, with more than 540 species, distributed essentially in neotropical regions (Barneby 1991). It is worth mentioning that recently, a reclassification has been suggested, and the mimosas would fit into the subfamily Caesalpinioideae, in the clade Mimosoideae–Caesalpinieae–Cassieae (MCC) (LPWG 2017). The main radiation center of the *Mimosa* genus is located in Brazil, many of which are endemic, particularly in high altitude areas (Simon et al. 2011). Most studies of mimosoid symbionts conducted in Brazil reveal that *Paraburkholderia* are the preferred symbionts (Chen et al. 2005; Bontemps et al. 2010; dos Reis Junior et al. 2010). The country also represents one of the main centers of diversity of the *Paraburkholderia* genus due to the ability to nodulate many species of *Mimosa*, *Calliandra,* and *Piptadeniae* plants (Chen et al. 2007; Bournaud et al. 2013; Sheu et al. 2013; Moulin et al. 2015; Dall'Agnol et al. 2016; Silva et al. 2018; Paulitsch et al. 2019). In contrast, *Mimosa* from other American countries such as Mexico are mainly nodulated by alpha-rhizobia, while in Uruguay, the main symbionts are *Cupriavidus*. Apparently, soil conditions are the main responsible for this arrangement (Bontemps et al. 2016; Platero et al. 2016). In addition, a second center for legume-nodulating *Paraburkholderia* has been described in the Fynbos biome in South Africa (Elliott et al. 2007a; Garau et al. 2009). However, the South American symbionts differ from the South African mainly for their ability to nodulate Papilionoideae legumes and not been able to nodulate *Mimosa*; other properties also indicate a unique origin for the nodulating *Paraburkholderia* species of South Africa (Beukes et al. 2013; Howieson et al. 2013; De Meyer et al. 2013a, b, 2014; Liu et al. 2014; Lemaire et al. 2015, 2016a; b; Steenkamp et al. 2015).

In this study, we describe a polyphasic analysis performed with *Paraburkholderia* strains isolated from root nodules of the Brazilian native and endemic *Mimosa gymnas* species. A new species was identified, for which the name *Paraburkholderia guartelaensis* sp. nov., is proposed.

Materials and methods

Strains, culture conditions, and DNA extraction

Twenty-nine strains isolated from root nodules of *M. gymnas* at the Guartelá State Park, an ecotone of the "Campos Gerais", located in Tibagi, Paraná State, southern Brazil have been previously characterized, and six of them (CNPSo 2995, CNPSo 3003, CNPSo 3008, CNPSo 3016, CNPSo 3023, and CNPSo 3248) formed a separated group in the MLSA analysis (Paulitsch et al. 2019). These six strains (Table S1) are now studied in depth, including a more detailed MLSA analysis; the geographic coordinates of isolation of each stain are also indicated in Table S1. All strains are deposited at the "Diazotrophic and Plant Growth Promoting Bacteria Culture Collection of Embrapa Soja" (WFCC Collection # 1213, WDCM Collection # 1054), in Londrina, State of Paraná, Brazil, and at the "National Collection of Rhizobial Strains of Uruguay" (WFCC Collection # 1082), in Montevideo, Uruguay.

Strains were grown at 28 °C from 2 to 7 days on modified-yeast extract–mannitol–agar (YMA) medium (Hungria et al. 2016). Stock cultures were maintained on modified-YMA at 4 °C, and for long-term storage strains were both cryopreserved in modified-YM (without agar) with 30% (v/v) glycerol at − 80 °C and − 150 °C, and lyophilized. Total genomic DNA of the strains was extracted using the DNeasy Blood and Tissue Kit (Qiagen), following the manufacturer's instructions.

Phylogeny

For the taxonomic characterization, the genomic DNA was used for PCR amplification and sequencing of the 16S rRNA and housekeeping genes (*recA*, *gyrB*, *trpB*, *gltB,* and *atpD*). To verify evolutionary relationships regarding symbiotic genes of CNPSo strains, a fragment of *nodC* was amplified and sequenced. Primers and amplification conditions used are listed in Table S2. The PCR products were purified using the PureLink™ Quick PCR Purification Kit (Invitrogen™), following the manufacturer's instructions and sequenced using an ABI 3500xL (Applied Biosystems®). In addition, we retrieved sequences from the GenBank database. Accession numbers for the gene sequences obtained in this study and for the genes retrieved from the GenBank database are shown in the phylograms and/or in Table S3.

To infer the phylogenetic position of the CNPSo strains, all sequences were aligned with MUSCLE (Edgar 2004) and the phylogenetic trees for the 16S rRNA, housekeeping and *nodC* genes were constructed using the MEGA6 (Tamura et al. 2013), with the Maximum-Likelihood (ML) algorithm. To determine the phylogeny of the 16S rRNA gene, the Tamura Nei model (Tamura and Nei 1993) with gamma-distributed invariant sites (G + I) (Tamura 1992) was used. For the MLSA (*recA*, *gyrB*, *trpB*, *gltB,* and *atpD*), the distance model General Time Reversible (Waddell and Steel 1997) with G + I was used, while for the *nodC* gene, the Tamura 3-parameter (Tamura et al. 2013) model with + I was used. The statistical support for the trees was evaluated by bootstrap analysis with 1000 re-samplings (Felenstein 1985). Nucleotide identity (NI) was calculated with Bioedit (v. 7.2.5) (Hall 1999).

Genome features

Genotypic fingerprintings of BOX-PCR were obtained for the CNPSo strains and the closest type strains of *Paraburkholderia*, including *P. nodosa* BR3437^T, *P. mimosarum* PAS44^T, and *P. diazotrophica* JPY461^T, using the primers and conditions specified in Table S2. A dendrogram was built with the profiles using the software Bionumerics (Applied Mathematics, Kortrijk, Belgium, v. 7.6), applying the UPGMA algorithm (Unweighted Pair-Group Method with Arithmetic mean) (Sneath and Sokal 1973) and the Jaccard coefficient (Jaccard 1912), with 1% of tolerance.

For the genome analyses, total DNA of strain CNPSo 3008^T was used to build a genome library using the Nextera XT kit, according to the manufacturer's instructions. The genomic library processing was realized on the MiSeq platform (Ilumina) at Embrapa Soja. The draft genome was assembled with the A5-MiSeq pipeline (de novo) v.20140604. Genome size was estimated with RAST v.2.0 (Aziz et al. 2008) and confirmed with QUAST v.2.0 (Gurevich et al. 2013). The genome sequences of the closely related species *P. nodosa* BR3437^T (JAFA01000001.1), *P. mimosarum* PAS44^T (AXAN01000001.1), *P. oxyphila* (NZ_BAYD01000014), and *P. diazotrophica* JPY461^T (NZ_FNYE00000000.1) were retrieved from the GenBank. ANI comparisons were evaluated with ANI calculator (available at https://enve-omics.ce.gatech.edu/ani/). For the estimation of digital DNA–DNA hybridization (dDDH), a digital bioinformatics method was used that allows comparisons in silico of the DDH (Meier-Kolthoff et al. 2013, 2014). All pairwise values with the closest species were estimated by GGDC v2.1 using the recommended 'Formula 2′ (https://ggdc.dsmz.de/distcalc2.php).

To determine the DNA G + C content of the CNPSo 3008^T strain, the proportion of G + C bases was calculated with QUAST (Gurevich et al. 2013).

Chemotaxonomy

Strain CNPSo 3008^T and the related species *P. nodosa* BR3437^T, *P. mimosarum* PAS44^T and *P. diazotrophica* JPY461^T were grown in modified-YMA until the end of the exponential growth phase (4 days) when the cellular fatty acids profiles were determined using the MIDI Sherlock Microbial Identification System (version 6.1) and the FAME library TSBA6. The analyses were performed in a GC Agilent model 6850 with an Ultra 2 column (25 m length, ID 0.2 mm, film f 0.33 µm), detector FID, carrier gas hydrogen (30 mL/min), and make-up gas nitrogen (30 mL/min), automatic injector series 7683 (liner 19,251–60,540), injection of 2 µL, and run of 25 min. Injector temperature of 250 °C and of the detector of 300 °C. Program of the oven temperature: initial of 170 °C, raising 5 °C/min until 260 °C (hold for 18 min), and then raised at 40 °C/min until 310 °C (hold for 1.5 min).

Physiological characteristics

Phenotypic characterization was performed with strains CNPSo 3008^T, *P. nodosa* BR3437^T, *P. mimosarum* PAS44^T, and *P. diazotrophica* JPY461^T. Unless indicated otherwise, the strains were grown at 28 °C for 4 days under different conditions and the tests were performed in duplicate. Modified-YMA medium (Hungria et al. 2016) with bromothymol blue was used to evaluate acid/alkaline reaction. Growth at pH 4.0 and 8.0, with 1% NaCl, and at 37 °C was evaluated in modified-YMA, and the capacity to grown in solid Luria–Bertani medium (LB) (Hungria et al. 2016), both at 28 °C for 4 days. For the evaluation of urease, strains were grown in modified-YMA with 2% urea and phenol red as indicator. Carbon source utilization was evaluated using modified-YM without mannitol as the basal medium with the API 50CH kit (BioMérieux). Tolerance to antibiotics was evaluated in modified YMA through the disc-diffusion method with the following antibiotics (per disc): tetracycline (30 µg), bacitracin (0.04 U), chloramphenicol (30 µg), erythromycin (10 µg), neomycin (30 µg), nalidixic acid (30 µg), streptomycin (15 µg), and cefuroxime (30 µg).

Host-range nodulation ability

Nodulation ability was evaluated in *Phaseolus vulgaris* (common bean), *Macroptilium atropurpureum* (siratro), *Glycine max* (soybean), and *Mimosa pudica*. The siratro seeds were scarified with concentrated sulphuric acid for 10 min and *M. pudica* seeds were lightly sanded. Seeds were surface sterilized in 70% ethanol for 1 min and then soaked in commercial sodium hypochlorite (2–2.5%) for 5 min, followed by washing five times in sterile distilled water. Seeds were pre-germinated for 2 days in 1% water agar and incubated at 28 °C. Tests were performed in triplicate for each strain and conducted in sterile polypropylene bags containing germitest paper and N-free nutrient solution (Broughton and Dilworth 1971). The strains were grown in modified-YM medium for 4 days and used as inocula 1 day after seedling transfer. The seedlings were grown in a greenhouse (28 °C) for 30 days for common bean, siratro, and soybean and 40 days for *M. pudica*. Nodulation was then evaluated as well as nodule effectiveness by the examination of the internal color of the nodules.

Results and discussion

Phylogenetic analysis of the 16S rRNA and housekeeping genes

The phylogram built with the sequences of the 16S rRNA gene (1299 bp) clustered all CNPSo strains in a well distinct clade; to get a better definition, Fig. 1 shows the relationships with the closest *Paraburkholderia* species, while Fig. S1 (1310 bp) displays all described species. The CNPSo strains shared 99.6–100% of nucleotide identity (NI) with each other in the 16S rRNA gene and of 94.2–98.9% with other described species of the genus *Paraburkholderia* (Table S4), showing the highest identity with *P. oxyphila*. High NI values of 16S rRNA in *Paraburkholderia* have been reported due to the high conservation of this gene in the genus (Lv et al. 2016; Bournaud et al. 2017; Choi and Im 2018; Huo et al. 2018). However, despite conserved, it was possible to observe that the CNPSo strains formed a distinct group in relation to the other *Paraburkholderia* species (Fig. 1; Fig. S1).

Considering the lower power of discrimination of the 16S rRNA gene in the *Paraburkholderia* genus, we proceeded with the MLSA analysis, which has been broadly adopted in phylogenetic and taxonomic studies within this genus, including the recent reclassification of the environmental *Paraburkholderia* species (Sawana et al. 2014; Dobritsa and Samadpour 2016). Five housekeeping genes were sequenced and used in our study (*recA, gyrB, trpB, gltB,* and *atpD*), and single trees were generated for each of them (Figs. S2–S6, respectively), and in all of them, the CNPSo strains were grouped in a different cluster than the other species. For the MLSA, a phylogenetic tree was built with the partial concatenated sequences of the five housekeeping genes (2280 bp). The CNPSo strains were clustered in a separated group from all other *Paraburkholderia* species, with 100% bootstrap support, with *P. nodosa* as the closest species (Fig. 2). These data reinforce that the CNPSo strains compose a new species. Another tree was built with the complete sequences of the five housekeeping genes (10,717 bp) retrieved from available *Paraburkholderia*-type strain genomes and from

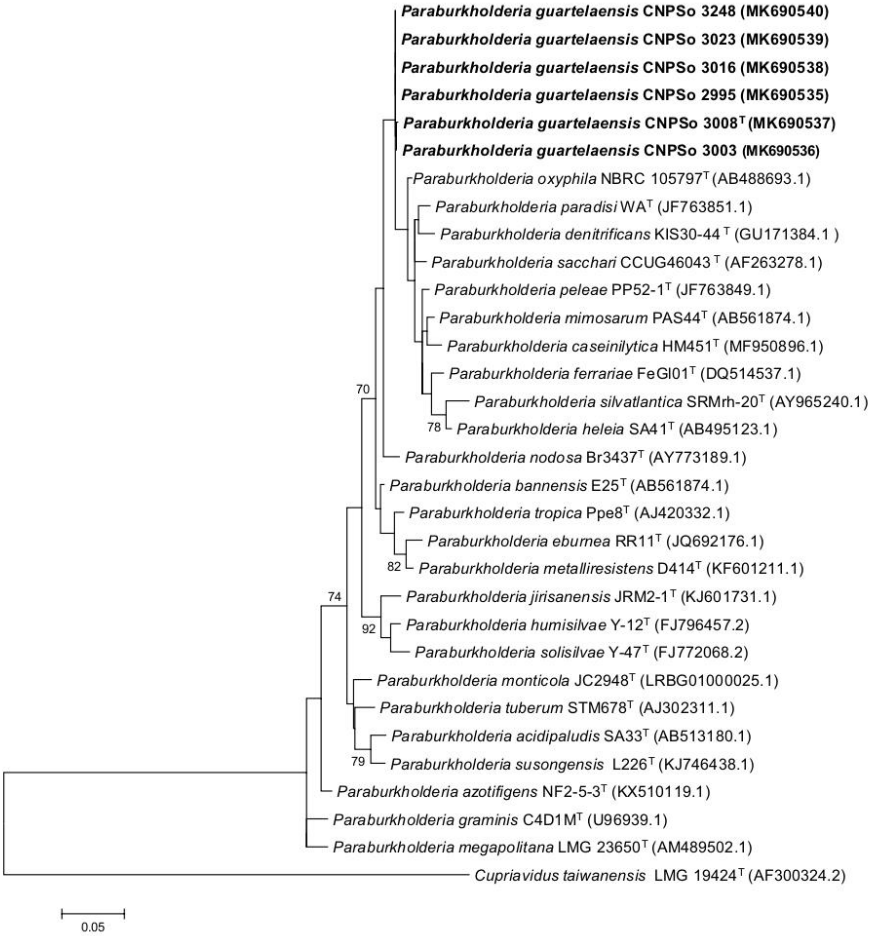

Fig. 1 Maximum-likelihood phylogeny of the 16S rRNA gene (1299 bp) between CNPSo strains and most closely related *Paraburkholderia* species. Bootstrap values > 70% are indicated at the nodes. Accession numbers are indicated in parentheses. Strains of the novel species are shown in boldface. *Cupriavidus taiwanensis* LMG 19424^T was used as outgroup. Bar indicates five substitution per 100 nucleotide positions

the CNPSo 3008^T genome (Fig. S7). The MLSA confirmed the taxonomic position of CNPSo 3008^T that did not group with any *Paraburkholderia*-type strain and is closely related to *P. nodosa*. Interestingly, the type strains of *P. sediminicola* and *P. terricola* grouped together in the MLSA, suggesting that they belong to the same species (Fig. S7).

Nucleotide identities (NI) of single and concatenated housekeeping genes are shown in Table S4. In the MLSA analysis of partial gene sequences, the CNPSo strains shared 100% of similarity with each other, and with closely related species, the values ranged from 85.2 to 95.7%. *P. nodosa* was the closest species, sharing 95.7% of NI with CNPSo 3008^T and, in relation to *P. oxyphila*, which showed the highest NI in the 16S rRNA, the NI for the concatenated genes was of 94.3% (Table S4). In the MLSA analysis with the complete sequences of the housekeeping genes, the strain CNPSo 3008^T shared 97% of NI with *P. nodosa*.

NI values obtained are lower than the suggested threshold of 96% for concatenated orthologous genes, indicative of new bacterial species (Konstantinidis et al. 2006) and

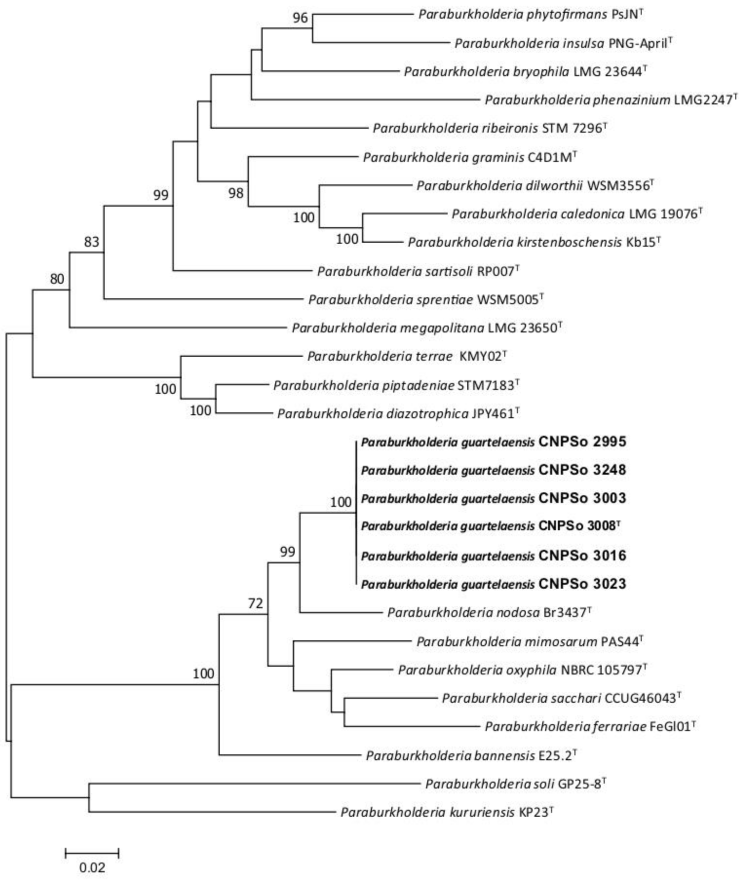

Fig. 2 Maximum-likelihood phylogeny based on the concatenated gene sequences (*recA* + *gyrB* + *trpB* + *gltB* + *atpD*) (2280 bp) showing the relationships of novel species of *Paraburkholderia* (in bold) and other members of the genus. Bootstrap values > 70% are indicated at the nodes. Accession numbers are indicated in Table S3. Bar indicates two substitutions per 100 nucleotide positions

supporting the proposal that the CNPSo strains represent a new species within the genus; however, these values were suggested considering partial sequences of concatenated genes, a usual procedure in the MLSA analysis. Nevertheless, currently, most *Paraburkholderia* genomes are available and the use of complete gene sequences allows an increase in the number of nucleotides analyzed, and, possibly, the NI threshold value for *Paraburkholderia* species delimitation should be reassessed.

Genomic features

A draft genome sequence of the CNPSo 3008^T strain was obtained and deposited at the NCBI (SMOD00000000, Biosample SAMN11089664). The shotgun sequences of CNPSo 3008^T allowed a genome coverage of 95-fold, assembled in 173 contigs, and N_{50} was calculated as 172,576 bp. The genome size of CNPSo 3008^T was estimated at 9,532,701 bp.

The average nucleotide identity (ANI) has been increasingly used due to its potential to estimate genome relatedness in prokaryotic taxonomy. According to Richter and Rosselló-Móra (2009), ANI values of 95–96% are comparable to 70% DNA–DNA hybridization (DDH), establishing the analysis of draft genomes as an alternative to DDH. The ANI values between CNPSo 3008^T and the closest species in the MLSA analysis, *P. nodosa* BR3437^T, *P. oxyphyla* NBRC 105797^T, *P. mimosarum* PAS44^T, *P. sacchari* CCUG46043^T, and *P. bannensis* E25.2^T were of 93.84%, 85.31%, 87.93%, 84.98%, and 84.33%, respectively; with *P. diazotrophica* JPY461^T ANI was of 81.87% (Table 1). Therefore, the ANI results endorse the proposal of a novel species for CNPSo strains, since the ANI values are below

Table 1 ANI and digital DNA–DNA hybridization (dDDH) values (percentages) between *Paraburkholderia guartelaensis* CNPSo 3008 ^{T} and related *Paraburkholderia* species

Strain used as reference	*P. guartelaensis* CNPSo 3008^T	
	ANI (%)	dDDH (%)
Paraburkholderia nodosa BR3437^T	93.84	55.90
Paraburkholderia oxyphila NBRC 105797^T	85.31	27.80
Paraburkholderia mimosarum PAS44^T	87.93	34.30
Paraburkholderia sacchari CCUG46043^T	84.98	27.30
Paraburkholderia bannensis E25.2^T	84.33	26.20
Paraburkholderia diazotrophica JPY461^T	81.87	23.30

the threshold (<95–96%) suggested for species delineation (Richter and Rosselló-Móra 2009).

We also performed an in silico dDDH between CNPSo 3008^T and the closest strains in the MLSA analyses, and the highest values were confirmed with *P. nodosa* BR3437^T, *P. mimosarum* PAS44^T, and *P. oxyphyla* NBRC 105797^T with DNA–DNA relatedness of 55.9%, 34.3%, and 27.8%, respectively (Table 1), again below the threshold for species delimitation (Rosselló-Móra et al. 2011).

The G + C content of CNPSo 3008^T was estimated at 63.72 mol%, fitting within the expected range for the *Paraburkholderia* genus (Dobritsa and Samadpour 2016).

The rep-PCR analysis has been successfully used to detect diversity among rhizobial strains (Menna et al. 2009), and high intraspecies genetic diversity among the CNPSo strains was observed in the BOX-PCR analysis, with the strains being clustered at a final level of similarity of only 35%. The other *Paraburkholderia* species used for comparison showed very distinct genomic profiles from the CNPSo strains (Fig. S8).

Phylogeny of *nodC* gene and host-range nodulation ability

The phylogeny of nodulation genes is commonly used in studies related to rhizobia aiming at the investigation of the evolutionary story of the nodulation ability. The *nodC* gene was chosen due to its key role in the Nod factors synthesis, affecting host specificity. Considering the partial sequences of the *nodC* gene (400 bp), all CNPSo strains were grouped in the same cluster as *P. nodosa* BR3437^T, followed by *P. mimosarum* PAS44^T, with high bootstrap support (Fig. 3). In addition, we retrieved the whole *nodC* sequences of the type strains with available genomes and of strain CNPSo 3008^T and the phylogenetic tree obtained with 1184 bp confirmed the relationships obtained with the partial sequences (Fig. S9).

The CNPSo strains shared among each other 100% of NI in the symbiotic *nodC* gene, revealing a monophyletic

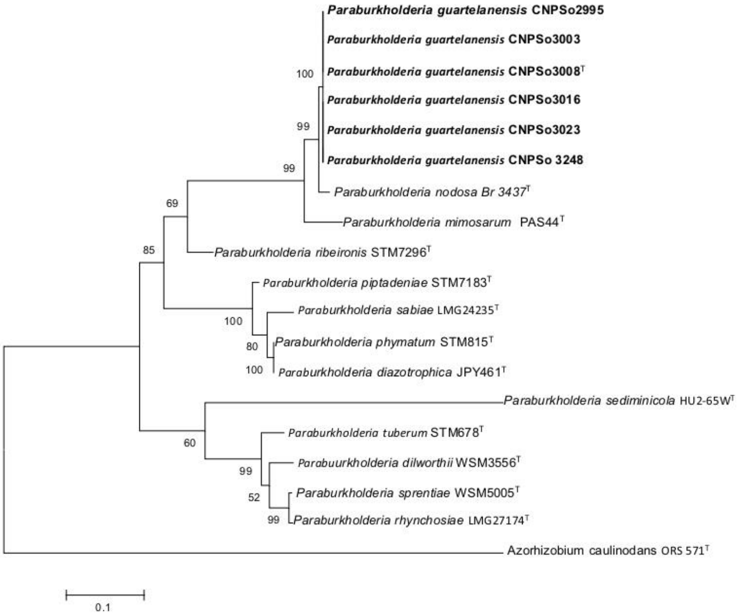

Fig. 3 Maximum-likelihood phylogeny based on *nodC* gene (400 bp) among novel *Paraburkholderia* species (in bold) and other members of the genus. Bootstrap values > 70% are indicated at the nodes. Accession numbers are indicated in Table S3. *Azorhizobium caulinodans* ORS 571^T was used as outgroup. Bar indicates one substitution per 100 nucleotide positions

character; the NI with *P. nodosa* BR3437^T was of 98.2% and with *P. mimosarum* PAS44^T of 93.4% (Table S4). Interestingly, *P. nodosa* BR3437^T was isolated in Brazil from *Mimosa bimucronata* and *Mimosa scabrella* nodules (Chen et al. 2007), and it is possible that they share a close evolutionary history of nodulation ability. In addition, it is important to remind that *P. oxyphila* NBRC 105797^T differs in this property from CNPSo strains, because it does not carry nitrogen-fixation genes (Otsuka et al. 2011).

In relation to the host-range nodulation ability, all CNPSo strains were able to nodulate common bean and siratro, but were unable to nodulate soybean; however, in both common bean and siratro, the nodules were ineffective in fixing nitrogen. All CNPSo strains were able to nodulate *Mimosa pudica*, and were effective in fixing nitrogen, showing nodules with the typical internal pink color due to the presence of an active leghemoglobin, except for strains CNPSo 3003 and CNPSo 3016, that were not as effective as the other CNPSo strains. Some examples of typical nodules obtained in the evaluation are shown in Fig. S10. Therefore, the evaluation of nodulation in some host plants confirmed the reported host-specificity of *Paraburkholderia* with *Mimosa* spp. in Brazil, suggesting millions of years of co-evolution between these plants and their symbionts (Bontemps et al. 2010; de Castro Pires et al. 2018).

Chemotaxonomic characteristics

Only slight differences were observed in the cellular fatty acid profile of CNPSo 3008^T strain when compared to the closely related species *P. nodosa* BR3437^T, *P. mimosarum* PAS44^T, and *P. diazotrophica* JPY461^T (Table S5). The CNPSo 3008^T strain presented in greater proportions the following fatty acids: $C_{16:O}$ (21.86%), C19:0 cyclo ω8c (19.79%), $C_{17:0}$ cyclo (16.19%) and Summer Feature 8 (15.15%). In smaller proportions were found Summer Feature 2 (6.88%), $C_{16:0}$ 3OH (5.91%), $C_{14:0}$ (4.66%), $C_{16:0}$ 2OH (3.51%), Summer Feature 3 (2.44%), $C_{16:1}$ 2OH (1.85%) and $C_{18:0}$ (1.76%), that was also observed in *P. diazotrophica* JPY461^T (1.09%).

Physiology

The main phenotypic differences between *P. guartelaensis* CNPSo 3008^T, *P. nodosa* BR3437^T, *P. mimosarum* PAS44^T, and *P. diazotrophica* JPY461^T were that CNPSo 3008^T grows in modified-YMA with 1% NaCl and LB medium, is tolerant to the antibiotic cefuroxime, and differs in the utilization of 37 carbon sources in relation to the closest type species. More details are shown in Table 2. Contrasting with the high intraspecies genetic diversity observed in the BOX-PCR analysis (Fig. S7), there were no significant phenotypic differences among *P. guartelaensis* strains,

Table 2 Phenotypic comparisons of CNPSo 3008^T strain and related type strains of the genus *Paraburkholderia*

Characteristics	1	2	3	4
Growth at/in/with				
1% NaCl	+	w	−	−
37 °C	−	−	+	+
Urea 2%	+	−	+	+
LB	+	−	−	−
Tolerance to antibiotic				
Cefuroxime (30 µg per disc)	+	−	−	−
Carbohydrates				
Glycerol	w	w	+	−
Erythritol	w	−	−	−
L-Arabinose	+	w	+	+
D-Ribose	+	+	w	w
L-Xylose	+	+	w	w
D-Adonitol	+	w	w	w
D-Galactose	+	+	+	w
D-Glucose	+	+	+	w
D-Fructose	+	w	+	w
D-Mannose	+	+	+	w
L-Sorbose	−	−	w	−
L-Rhamnose	+	+	w	+
Dulcitol	−	−	w	−
D-Mannitol	+	w	w	w
D-Sorbitol	+	w	w	w
N-acetylglucosamine	−	−	+	+
Arbutin	w	−	w	−
Esculin ferric citrate	−	−	+	w
Salicin	w	−	w	−
D-Cellobiose	w	w	w	−
D-Maltose	−	−	+	−
D-Lactose	w	w	w	−
D-Melibiose	−	−	+	−
D-Saccharose	w	+	+	+
D-Trehalose	+	w	+	+
D-Melezitose	−	−	w	−
D-Raffinose	−	w	+	w
Glycogen	w	w	w	+
Gentiobiose	w	+	+	−
D-Turanose	−	−	w	−
D-Lyxose	w	w	+	w
D-Tagatose	−	−	w	−
L-Fucose	+	w	w	−
D-Arabitol	w	w	+	w
L-Arabitol	w	w	w	+
Potassium 2-ketogluconate	−	−	+	+
Potassium 5-ketogluconate	+	+	+	w

Strains: 1 *P. guartelaensis* CNPSo 3008^T, 2 *P. nodosa* BR3437^T, 3 *P. mimosarum* PAS44^T, 4 *P. diazotrophica* JPY461^T. All data were obtained with two biological replicates. Growth (+), no growth (−), weakly positive (w) indicating high stability of phenotypic properties among the strains (Table S6).

In conclusion, the results of phylogenetic analyses, genome sequencing, DNA fingerprinting, and physiological features reveal that CNPSo strains differ from closely related species of *Paraburkholderia*, representing a novel species of the genus. The name *Paraburkholderia guartelaensis* sp. nov., is proposed for the new taxon, with CNPSo 3008^T (= $U13000^T$ = $G29.01^T$) nominated as the type strain.

Description of *Paraburkholderia guartelaensis* sp. nov.

Paraburkholderia guartelaensis [*guar.te.la.en'sis.* N.L. fem. adj. guartelaensis of Guartelá State Park, in Tibagi, Paraná State, Brazil, where the bacteria have been isolated from].

Cells are Gram-stain-negative, aerobic, rod-shaped. Colonies in modified-YMA (yeast extract–mannitol–agar) medium with Congo red are circular, opaque, with low production of mucus, rose-colored and measure 1.7–2.8 in diameter within 4 days of incubation at 28 °C. All strains produce acid reaction in modified-YMA with bromothymol blue. Optimum growth occurs at pH 6.8 and 28 °C. Strains are able to grow in LB (Luria–Bertani) medium, in modified-YMA with pH 4.0 and 8.0 and 1% NaCl, but are unable to grow at 37 °C. They are positive for urease activity. In the API test, they are able to grow with the following carbohydrates: D-arabinose, L-arabinose, D-ribose, L-xylose, D-adonitol, D-galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, D-mannitol, D-sorbitol, D-trehalose, starch, D-fucose, L-fucose, potassium gluconate, and potassium 5-ketogluconate. They grow weakly with glycerol, erythritol, D-xylose, inositol, arbutin, salicin, D-cellobiose, D-lactose, D-saccharose, glycogen, xylitol, gentiobiose, D-lyxose, D-arabitol, and L-arabitol and they are unable to grow with methyl-β D-xylopyranoside, L-sorbose, dulcitol, methyl-α D-mannopyranoside, methyl-α D-glucopyranoside, *N*-acetylglucosamine, amygdalin, esculin ferric citrate, D-maltose, D-melibiose, inulin, D-melezitose, D-raffinose, D-turanose, D-tagatose, and potassium 2-ketogluconate. Strains are tolerant (per disc) to the antibiotics bacitracin (0.04 U) and chloramphenicol (30 µg) and sensitive to tetracycline (30 µg), nalidixic acid (30 µg), cefuroxime (30 µg), erythromycin (10 µg), streptomycin (15 µg), and neomycin (30 µg). The major cellular fatty acids are $C_{16:O,}$ $C_{19:0}$ cyclo ω8c, $C_{17:0}$ cyclo, and Summer Feature 8 ($C_{18:1}$ ω7c). The G + C content in the DNA of CNPSo 3008^T is 63.72 mol%. The strains produce effective nitrogen-fixing nodules in *M. gymnas* and *Mimosa pudica*, ineffective nodules in *Macroptilum atropurpureum* and *Phaseolus vulgaris* and do not nodulate *Glycine max*.

The type strain is CNPSo 3008^T (= $U13000^T$ = $G29.01^T$), isolated from nodules of *M. gymnas* in the Guartelá State Park, Tibagi, Paraná State, Brazil (Latitude 24°33′52.75″S, Longitude 50°15′10.27″O).

The following new sequences have been deposited in GenBank database:

16S rDNA of CNPSo 2995 (MK690535), CNPSo 3003 (MK690536), CNPSo 3008^T (MK690537), CNPSo 3016 (MK690538), CNPSo 3023 (MK690539), and CNPSo 3248 (MK690540).

Genome sequences of *Paraburkholderia guartelaensis* CNPSo 3008^T (SMOD00000000, Biosample SAMN11089664).

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

Ethics approval and consent to participate The authors declare no ethical conflicts; authors declare that they have consented to participate in the manuscript and publish it.

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Archives of Microbiology

Paraburkholderia guartelaensis **sp. nov., a nitrogen-fixing species isolated from nodules of** ***Mimosa gymnas*** **in an ecotone considered as a hotspot of biodiversity in Brazil**

SUPPLEMENTARY MATERIAL

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Table S1 Strains used in this study

Species/strains name	Other strain nomenclature	Original host species	Geographical origin	Reference
P. guartelanensis CNPSo 2995	G10.01	*M. gymnas*	Brazil	Area 3*, Paulitsch et al. (2019)
P. guartelanensis CNPSo 3003	G20.01	*M. gymnas*	Brazil	Area 2*, Paulitsch et al. (2019)
P. guartelanensis CNPSo 3008^T	U13000, G29.01	*M. gymnas*	Brazil	Area 2,
P. guartelanensis CNPSo 3016	G25.02	*M. gymnas*	Brazil	Area 2*, Paulitsch et al. (2019)
P. guartelanensis CNPSo 3023	G29.04	*M. gymnas*	Brazil	Area 2*, Paulitsch et al. (2019)
P. guartelanensis CNPSo 3248	G32.01	*M. gymnas*	Brazil	Area 3*, Paulitsch et al. (2019)
P. nodosa Br 3437^T	BCRC 17575^T, LMG 23741^T	*M. bimucronata* and *M. scabrella*	Brazil	Chen et al. (2007)
P. mimosarum PAS44^T	CCUG 54296^T, LMG 23256^T	*M. pigra*	Taiwan	Chen et al. (2006)
P. diazotrophica JPY461^T	KCTC 23308^T, LMG 26031^T	*M. candollei*	Brazil	Sheu et al. (2013)

*Area 2 (Latitude 24°33'52.75"S, Longitude 50°15'10.27"O), Area 3 (Latitude 24°33'51.73"S, Longitude 50°15'10.79"O).

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Table S2 Primers and PCR conditions used in this study

Gene	***Primer***	**Sequence (5’ - 3’)**	**PCR conditions**	**Reference**
16S rRNA	fD1	AGAGTTTGATCCTGGCTCAG	2 min 95°C, 30 x (15s 94°C, 45s 93°C, 45s 55°C, 2min 72°C) and 5 min 72°C	Weisburg et al. (1991)
	rD1	AAGGAGGTGATCCAGCC		
	362f*	CTCCTACGGGAGGCAGCAGTGGGG	*1 min 96 ºC, 35 x (15s 96ºC, 15s 50ºC, 4min 60ºC)	Menna et al. (2006)
	786f*	CGAAAGCGTGGGGAGCAAACAGG		
recA	*recA*-BurkF	AGGACGATTCATGGAAGAWAGC	2 min 95 ºC, 35 x (30s 94ºC, 30s 58ºC, 1min 72ºC), 5 min 72ºC	Spilker et al. (2009)
	recA-BurkR	GACGCACYGAYGMRTAGAACTT		
gyrB	gyrB-BurkF	ACCGGTCTGCAYCACCTCGT	2 min 95 ºC, 35 x (30s 94ºC, 30s 58ºC, 1min 72ºC), 5 min 72ºC	Spilker et al. (2009)
	gyrB-BurkR	YTCGTTGWARCTGTCGTTCCACTGC		
trpB	trpB-BurkF	CGCGYTTCGGVATGGARTG	2 min 95 ºC, 35 x (30s 94ºC, 30s 58ºC, 1min 72ºC), 5 min 72ºC	Spilker et al. (2009)
	trpB-BurkR	ACSGTRTGCATGTCCTTGTCG		
gltB	gltB-BurkF	CTGCATCATGATGCGCAAGTG	2 min 95 ºC, 35 x (30s 94ºC, 30s 58ºC, 1min 72ºC), 5 min 72ºC	Spilker et al. (2009)
	gltB-BurkR	CITGCCGCGGAARTCGTTGG		
atpD	atpD-BurkF	ATGAGTACTRCTGCTTTGGTAGAAGG	2 min 95 ºC, 35 x (30s 94ºC, 30s 58ºC, 1min 72ºC), 5 min 72ºC	Spilker et al. (2009)
	atpD-BurkR	CGTGAAACGGTAGATGTTGTCG		
nodC	nodCBurkF	TRATYGAYATGGAATACTGGC	3 min 95°C, 35 X (30s 95°C, 30s 55°C, 45s 55°C, 45s 72°C) and 7 min 72°C	Bournaud et al. (2013)
	nodCBurkR	CAGCGGAYATMGTCATTGA		
BOX-PCR	AIR1	ATGTAAGCTCCTGGGGATTCAC	7 min 94°C, 35 x (1min 94°C, 1min 52°C, 8 min 72°C) 16min 65°C	Versalovic et al. (1994)

*used for sequencing reaction

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Table S3 Accession numbers of the sequences used for the phylogenetic analysis

Strain	16S rRNA	*recA*	*gyrB*	*trpB*	*gltB*	*atpD*	*nodC*
P. guartelaensis CNPSo 2995	MK690535	KY498207	KY498191	MH687395	MH687407	MH687401	KY498167
P. guartelaensis CNPSo 3003	MK690536	KY498212	KY498198	MH687396	MH687408	MH687402	KY498171
P. guartelaensis CNPSo 3008	MK690537	NZ_SMOD01000007.1	NZ_SMOD01000009.1	NZ_SMOD01000011.1	NZ_SMOD01000026.1	NZ_SMOD01000009	NZ SMOD01000053.1
P. guartelaensis CNPSo 3016	MK690538	KY498217	KY498200	MH687398	MH687410	MH687404	KY498179
P. guartelaensis CNPSo 3023	MK690539	KY498219	KY498203	MH687399	MH687411	MH687405	KY498183
P. guartelaensis CNPSo 3248	MK690540	KY498222	KY498206	MH687400	MH687412	MH687406	KY498183
P. acidipaludis SA33	AB513180.1	NZ_BAXZ01000005.1	NZ_BAXZ01000002.1	NZ_BAXZ01000036.1	NZ_BAXZ01000027.1	NZ_BAXZ01000001.1	ND
P. aromaticivorans BN5	MF817715.1	ND	ND	ND	ND	CP022989.1	ND
P. aspalathi VG1C	KC817488.1	FPBH01000010.1	NZ_FPBH01000006.1	NZ FPBH01000008.1	LT708285.1	LT708240.1	ND
P. azotifigens NF2-5-3	KX510119.1	ND	ND	ND	ND	ND	ND
P. bannensis E25.2	AB561874.1	BAYA01000012.1	NZ_BAYA01000004.1	BAYA01000007.1	BAYA01000022.1	BAYA01000004.1	ND
P. bryophila LMG23644	AM489501.1	NZ_QLTK01000002.1	NZ_QLTK01000008.1	NZ_QLTK01000010.1	QLTK01000013.1	NZ_QLTK01000008.1	ND
P. caffeinilytica CF1	KT607985.1	CP031467.1	CP031467.1	NZ_CP031466.1	NZ_CP031467.1	CP031467.1	ND
P. caledonica LMG19076	AF215704.1	NZ_BAYE01000012.1	NZ_BAYE01000004.1	NZ_BAYE01000018.1	NZ_BAYE01000017.1	NZ_BAYE01000004.1	ND
P. caribensis MWAP64	Y17009.1	CP026101.1	CP026101.1	CP026102.1	NZ_CP013102.1	CP013102.1	ND
P. caryophylli ATCC 25418	AB021423.1	ND	ND	ND	ND	HQ398420.1	ND
P. caseinilytica HM451	MF950896.1	ND	ND	ND	ND	ND	ND

P. denitrificans KIS30-44	GU171384.1	ND	ND	ND	ND	ND	ND
P. diazotrophica JPY461	NR_117848.1	FNYE01000008.1	NZ_FNYE01000053.1	NZ_FNYE01000028.1	NZ_FNYE01000032.1	LT708234.1	NZ_FNYE01000044.1
P. dilworthii WSM3556	HQ698908.1	NZ_AWZT01000011.1	AWZT01000003.1	NZ_AWZT01000049.1	NZ_AWZT01000013.1	NZ_AWZT01000003.1	NZ_AWZT01000045.1
P. dipogonis DL7	JX009148.2	SNVI01000001.1	SNVI01000001.1	SNVI01000002.1	NZ_SNVI01000001.1	SNVI01000001.1	NZ_SNVI01000008.1
P. eburnea RR11	JQ692176.1	ND	ND	ND	ND	NZ_PQGA01000010.1	ND
P. endofungorum HKI 456	AM420302.1	ND	ND	ND	ND	NZ_PRDW01000011.1	ND
P. ferrariae FeGl01	DQ514537.1	NZ_BAYB01000011.1	NZ_BAYB01000021.1	NZ_BAYB01000001.1	NZ_BAYB01000028.1	NZ_BAYB01000023.1	ND
P. fungorum LMG 16225	AF215705.1	ND	ND	ND	ND	ND	ND
P. ginsengisoli KMY03	AB201286.1	HQ398579.1	KU723581.1	ND	HQ398485.1	HQ398437.1	ND
P. ginsengiterrae DCY85	KF915802.1	NZ_LXKA01000022.1	KX233702.1	NZ_LXJZ01000209.1	NZ_LXKA01000065.1	NZ_LXJZ01000101.1	ND
P. graminis C4D1M	U96939.1	NZ_ABLD01000009.1	NZ_ABLD01000012.1	NZ_ABLD01000002.1	ABLD01000026.1	NZ_ABLD01000014.1	ND
P. heleia SA41	AB495123.1	NZ_BBJH01000021.1	ND	ND	ND	NZ_BBJH01000020.1	ND
P. hospita LMG 20598	AY040365.1	CP026105.1	CP026105.1	CP026106.1	LT708278.1	LT708233.1	ND
P. humisilvae Y-12	FJ796457.2	ND	ND	ND	ND	ND	ND
P. insulsa PNG-April	KF733462.1	NZ_PVZM01000016.1	NZ_PVZM01000029.1	NZ_PVZM01000004.1	PVZM01000022.1	NZ_PVZM01000051.1	ND
P. jirisanensis JRM2-1	KJ601731.1	ND	ND	ND	ND	ND	ND
P. kirstenboschensis Kb15	NR_146352.1	NZ JRZC01000422.1	NZ JRZC01000001.1	NZ JRZC01000751.1	LT708286.1	NZ_JRZC01000493.1	NZ_JRZC01000835.1
P. kururiensis KP23	NR_024721.1	NZ_RJZE01000007.1	NZ_RJZE01000024.1	NZ RJZE01000028.1	RJZE01000029.1	NZ_RJZE01000024.1	ND

P. megapolitana LMG 23650	AM489502.1	NZ_FOQU01000003.1	NZ_FOQU01000008.1	NZ FOQU01000004.1	NZ_FOQU01000013.1	NZ_FOQU01000008.1	ND
P. metalliresistens D414	KF601211.1	ND	ND	ND	ND	ND	ND
P. metrosideri DNBP6-1	JF763856.1	ND	ND	ND	ND	ND	ND
P. mimosarum PAS44	AB561874.1	NZ_BBJJ01000016.1	AXAN01000006.1	NZ_BBJJ01000001.1	AXAN01000030.1	AXAN01000006.1	NZ_BBJJ01000051.1
P. monticola JC2948	LRBG01000025.1	LRBG01000003.1	LRBG01000004.1	LRBG01000009.1	NZ_LRBG01000010.1	LRBG01000004.1	ND
P. nodosa Br3437	AY773189.1	NZ_JAFA01000018.1	JAFA01000005.1	NZ_JAFA01000004.1	NZ_JAFA01000023.1	HQ398444.1	NZ_JAFA01000055.1
P. oxyphila NBRC 105797	AB488693.1	NZ_BAYD01000014.1	NZ_BAYD01000080.1	NZ_BAYD01000038.1	NZ BAYD01000021.1	NZ_BAYD01000046.1	ND
P. pallidirosea DHOK13	KP938221.1	KR092136.1	KR092135.1	ND	ND	ND	ND
P. panaciterrae DCY85-1	KF999960.1	KX233696.1	KX233697.1	ND	ND	ND	ND
P. paradisi WA	JF763851.1	KY305130.1	ND	ND	ND	ND	ND
P. peleae PP52-1	JF763849.1	2756170199**					ND
P. phenazinium LMG2247	U96936.1	NZ_FNCJ01000005.1	NZ_FNCJ01000001.1	NZ_FNCJ01000023.1	LT708280.1	LT708235.1	ND
P. phenoliruptrix LMG22037	AY435213.1	HQ398589.1	HQ849207.1	ND	HQ398495.1	HQ398447.1	ND
P. phymatum STM815	AJ302312.1	CP001043.1	CP001043.1	NC_010623.1	NC_010622.1	NC_010622.1	NC_010627.1
P. phytofirmans PsJN	NR_042931.1	CP001052	NC_010681.1	NC_010676.1	CP001052.1	CP001052.1	ND
P. piptadeniae STM7183	LN875219.1	NZ_CYGY02000074.1	CYGY02000002.1	NZ_CYGY02000034.1	CYGY02000043.1	NZ_CYGY02000070.1	CYGY02000180.1
P. rhizosphaerae WR43	AB365791.2	ND	ND	ND	ND	ND	ND

P. rhizoxinica HKI 454	AJ938142.1	ND	ND	ND	ND	ND	ND
P. rhynchosiae LMG27174	EU219865.1	PNXY01000005.1	PNXY01000017.1	PNXY01000024.1	PNXY01000009.1	PNXY01000017.1	PNXY01000002.1
P. ribeironis STM 7296	NR_156098.1	CYGX02000047.1	CYGX02000034.1	NZ CYGX02000021.1	CYGX02000009.1	NZ_CYGX02000034.1	NZ_CYGX02000044.1
P. sabiae LMG24235	AY773186.1	EU294397.1	LT632431.1	ND	HQ398497.1	HQ398449.1	ND
P. sacchari CCUG46043	AF263278.1	JTDB01000022.1	JTDB01000003.1	NZ_JTDB01000018.1	NZ_JTDB01000019.1	JTDB01000003.1	ND
P. sartisoli RP007	AF061872.1	LT708244.1	NZ FNRQ01000005.1	NZ FNRQ01000008.1	NZ_FNRQ01000010.1	LT708236.1	ND
P. sediminicola HU2-65W	EU035613.1	NZ_FNIU01000013.1	NZ_FNIU01000005.1	NZ_FNIU01000012.1	FNIU01000045.1	NZ_FNIU01000005.1	NZ_FNIU01000026.1
P. silvatlantica SRMrh-20	AY965240.1	CU633749.1	HQ849213.1	ND	ND	ND	ND
P. soli GP25-8	DQ465451.1	PNYB01000004.1	PNYB01000045.1	NZ_PNYB01000035.1	PNYB01000014.1	NZ_PNYB01000023.1	ND
P. solisilvae Y-47	FJ772068.2	ND	ND	ND	ND	ND	ND
P. sordicola SNU 020123	AF512827.1	ND	ND	ND	ND	ND	ND
P. sprentiae WSM5005	HF549035.1	AXBN01000087.1	AXBN01000095.1	AXBN01000039.1	AXBN01000088.1	CP017561.1	NZ_CP017565.1
P. susongensis L226	KJ746438.1	FXAT01000009.1	FXAT01000003.1	FXAT01000017.1	FXAT01000015.1	NZ_FXAT01000003.1	ND
P. symbiotica JPY345	HM357233.1	NZ_PTIR01000007.1	NZ_PTIR01000010.1	NZ_PTIR01000017.1	PTIR01000013.1	NZ_PTIR01000010.1	NZ_PTIR01000032.1
P. terrae KMY02	AB201285.1	CP026111.1	CP026111.1	CP026112.1	NZ_BBJK01000103.1	CP026111.1	ND
P. terricola CCUG44527	AY040362.1	FRAB01000012.1	FRAB01000005.1	FRAB01000009.1	LT708282.1	LT708237.1	ND
P. tropica	AJ420332.1	FNZM01000020.1	FNZM01000005.1	FNZM01000004.1	LT708283.1	LT708238.1	ND

Ppe8							
P. tuberum STM678	AJ302311.1	2512047030**					ND
P. xenovorans LB400	U86373.1	CP008760.1	CP008760.1	CP008762.1	CP000270.1	CP008760.1	ND
P. zhejiangensis OP-1	HM802212.1	ND	ND	ND	ND	ND	ND
Cupriavidus taiwanensis LMG19424	AF300324.2	CU633749.1	CU633749.1	CU633749.1	NZ CYGY02000070.1	NC_010528.1	ND
Azorhizobium caulinodans ORS 571	ND	ND	ND	ND	ND	ND	AP009384.1

* ND, Not Determined.

** Genome deposited at the JGI/IMG/R

Table S4 Nucleotide identity (NI, %) of the 16S rRNA and housekeeping genes between *Paraburkholderia guartelanensis* CNPSo $3008^{T,}$ CNPSo 3003, CNPSo 3016, CNPSo 3023 and CNPSo 3248 strains and close *Paraburkholderia* species. Length of aligned regions: 16S rRNA (1,310 bp), partial sequences of *recA* (276 bp), *gyrB* (574 bp), *trpB* (568 bp), *gltB* (299 bp), *atpD* (611 bp) and MLSA (2,280 bp), complete sequences *recA* (1,025 pb), *gyrB* (2,475 pb), *trpB* (1,194 bp), *gltB* (4,646 bp), *atpD* (1,377 bp), MLSA (10,717 bp). ND: not determined

	16S rRNA	*recA*	*gyrB*	*trpB*	*gltB*	*atpD*	MLSA Parcial sequences	MLSA Complete sequences	*nodC*
within *P. guartelaensis*	99.6-100	100	96.5-100	100	100	100	100	-	100
	between *P. guartelaensis* and:							**Between CNPSo 3008 and:**	
P. acipaludis SA33	96.9-97	92.3	89.4- 89.9	ND	ND	ND	ND	93.6	ND
P. aromaticivorans BN5	95.9-96.2	ND	ND	ND	ND	89.8	ND	ND	ND
P. aspalathi LMG 27731		87.3	86.5	85	ND	ND	ND	87.7	ND
P. azotifigens NF2-5-3	96.3-96.5	ND	ND	ND	ND	ND	ND	ND	ND
P. bannensis E25.2	98.2-98.3	91.3	92.2-92.4	92.2	95.9	93	92.8	94	ND
P. bryophila LMG23644	96-96.2	89.1	83.1-83.5	83.9	85.2	89.3	85.9	87.5	ND
P. caffeinilytica CF1	95.9-96.2	ND	85.8-86.3	ND	ND	89.5	ND	87.8	ND
P. caledonica LMG19076	96.3-96.6	86.5	85.6-86.2	81.8	84.2	89.7	85.6	87.5	ND
P. caribensis MWAP64	95.9-96.1	ND	ND	ND	ND	ND	ND	89.2	ND
P. caryophylli ATCC 25418	95-95.1	ND	ND	ND	ND	89.5	ND	ND	ND
P. caseinilytica HM451	97.8-97.9	ND	ND	ND	ND	ND	ND	ND	ND
P. denitrificans KIS30-44	97.6-97.8	ND	ND	ND	ND	ND	ND	ND	ND

P. diazotrophica JPY461	95.7-95.9	88.7	85.3-86.4	88.2	80.9	91.1	87.5	88.9	77.1
P. dilworthii WSM3556	95.9-96.1	88	86.2-86.2	84.3	83.2	89.7	86.4	87.7	73
P. dipogonis DL7	96.2-96.4	ND	ND	ND	ND	ND	ND	87.5	70.1
P. eburnea RR11	96.9-96.9	ND	ND	ND	ND	95.7	ND	ND	ND
P. endofungorum HKI 456	94.2-94.3	ND	ND	ND	ND	84.5	ND	ND	ND
P. ferrariae LMG23612	97.2-97.4	93.4	92.2-92.6	92.4	90.3	94.3	92.4	92.8	ND
P. fungorum LMG 16225	95.9-96.1	ND	ND	ND	ND	ND	ND	ND	ND
P. ginsengisoli KMY03	95.5-95.7	88.4	85.8-86.5	ND	84.9	90.2	ND	ND	ND
P. ginsengiterrae DCY85	95.4-95.6	ND	ND	ND	ND	89.5	ND	88	ND
P. graminis C4D1M	96.3-96.6	90.9	85.1-85.3	85.3	84.6	89.7	86.9	88.2	ND
P. heleia SA41	97.4-97.6	97.8	ND	ND	ND	94.6	ND	ND	ND
P. hospita LMG 20598	95.9-96	ND	ND	ND	ND	ND	ND	89.3	ND
P. humisilvae Y-12	96.6-96.8	ND	ND	ND	ND	ND	ND	ND	ND
P. insulsa PNG-April	96.1-96.3	86.5	86-86.3	83.6	84.6	88.4	86	ND	ND
P. jirisanensis JRM2-1	96.5-96.7	ND	ND	ND	ND	ND	ND	ND	ND
P. kirstenboschensis Kb15	95.6-95.9	88.7	85.8-86.2	82.7	84.9	89.8	86.3	88	69.9
P. kururiensis KP23	96.2-96.3	88.7	85.8-86.5	86.4	79.9	91.3	86.9	88.6	ND
P. megapolitana LMG 23650	96.3-96.5	90.9	83.8-84.4	87.5	83.6	91.3	87.4	87.7	ND
P. metalliresistens D414	97.6-97.7	ND	ND	ND	ND	ND	ND	ND	ND
P. metrosideri DNBP6-1	95.5-95.7	ND	ND	ND	ND	ND	ND	ND	ND
P. mimosarum PAS44	98-98.2	95.2	93.5-94.2	92.4	91.6	95.5	93.7	94.8	93.4
P. monticola JC2948	97.2-97.4	91.3	ND	ND	ND	91.8	ND	88.6	ND
P. nodosa Br3437	98.1-98.2	98.1	95.6-99.1	96.3	92.6	95.7	95.7	97	98.2
P. oxyphila NBRC 105797	98.7-98.9	95.2	93.7-95.1	95	92.9	94.6	94.3	94.8	ND
P. pallidirosea DHOK13	95.9-96.2	89.4	84	ND	ND	ND	ND	ND	ND
P. panaciterrae	95.8-95.9	89.1	86.7	ND	ND	ND	ND	ND	ND

DCY85-1									
P. paradisi WA	97.9	93.8	ND	ND	ND	ND	ND	ND	ND
P. peleae PP52-1	98.3-98.5	ND	ND	ND	ND	ND	ND	95.8	ND
P. phenazinium LMG2247	95.6-95.9	89.1	80.6 - 81	84.3	83.9	89.1	85.2	87.3	ND
P. phenoliruptrix LMG22037	96.1-96.3	90.5	86.3 - 86.5	ND	83.6	91.6	ND	88.9	ND
P. phymatum STM815	96.2-96.5	88.7	83.5 - 83.6	83.8	80.2	ND	ND	88.9	ND
P. phytofirmans PsJN	96.3-96.5	88.9	85.3	82.9	84.2	89	85.6	87.6	ND
P. piptadeniae STM7183	96.3-96.5	89.4	85.4 - 86.3	86.4	81.2	91.1	87.2	88.8	78.4
P. rhizosphaerae WR43	96.7-96.9	ND	ND	ND	ND	ND	ND	ND	ND
P. rhizoxinica HKI 454	94.3-94.4	ND	ND	ND	ND	ND	ND	ND	ND
P. rhynchosiae LMG27174	95.9-96.2	87.6	85.8 - 86.4	85.2	ND	ND	ND	87.9	73
P. ribeironis STM 7296	95.5-95.8	90.5	84.7	82.5	85.9	89.7	86.3	88	84.2
P. sabiae LMG24235	96.2-96.4	87.3	85.3 - 85.8	ND	79.9	91.8	ND	ND	76.3
P. sacchari CCUG46043	98-98.1	94.5	92.6 - 94.2	93.8	89.9	95	93.4	93.9	ND
P. sartisoli RP007	94.6-94.8	89.4	86.2 -87	86.7	85.2	89.7	87.7	88.3	ND
P. sediminicola HU2-65W	95.8-96	89.4	ND	84.5	86.6	90	ND	88.1	65.9
P. silvatlantica SRMrh-20	97.2-97.3	97.8	94.8-97.1	ND	ND	ND	ND	ND	ND
P. soli GP25-8	94.9-95	89.1	85.8-86.5	84.1	80.6	90.6	86.3	86.8	ND
P. solisilvae Y-47	96.5-96.6	ND	ND	ND	ND	ND	ND	ND	ND
P. sordicola SNU 020123	94.7-94.8	ND	ND	ND	ND	ND	ND	ND	ND
P. sprentiae WSM5005	95.8-96	91.3	86 - 86.5	85.2	85.6	90.4	87.5	87.9	73
P. susongensis L226	96.2-96.5	90.9	86.5-86.7	ND	ND	ND	ND	89.1	ND
P. symbiotica JPY345	95.6-05.8	ND	ND	84.6	ND	86.7	ND	86.1	72.2
P. terrae KMY02	95.9-96.1	85.5	83.5-84.9	86.7	81.6	91.3	86.6	89.1	ND
P. terricola CCUG44527	96-96.2	89.4	87.6-87.8	ND	85.9	89.7	ND	88.1	ND
P. tropica Ppe8	97.7-97.9	ND	93.5 - 93.9	ND	94.3	95.2	ND	94.3	ND
P. tuberum STM678	96.7-96.9	87.3	85.3 - 86	85.2	84.9	ND	ND	88.4	73.3
P. xenovorans LB400	95.5-95.7	ND	ND	ND	92.9	89.8	ND	87.6	ND

P. zhejiangensis OP-1	94.9-95.2	ND	ND	ND	ND	ND	ND	ND	ND
Cupriavidus taiwanensis LMG19424	90.9-91	83.3	76.4 - 76.6	86.2	76.2	84.7	ND	ND	ND
Azorhizobium caulinodans ORS 571	ND	ND	ND	ND	ND	ND	ND	ND	59.6

***ND, Not Determined**

Table S5 Fatty acid profile (%) of *Paraburkholderia guartelanensis* CNPSo 3008^T and related *Paraburkholderia* species

Fatty acid	***P. guartelanensis*** **CNPSo 3008^T**	***P. nodosa*** **Br 3437^T**	***P. mimosarum*** **PAS44^T**	***P. diazotrophica*** **JPY461^T**
$C_{14:0}$	4.66	4.20	6.12	4.55
$C_{16:O}$	21.86	22.68	25.89	25.50
$C_{17:0}$ cyclo	16.19	15.89	22.01	19.51
$C_{16:1}$ 2OH	1.85	1.84	1.85	1.43
$C_{16:0}$ 2OH	3.51	3.51	5.23	4.55
$C_{16:0}$ 3OH	5.91	5.70	7.40	6.25
$C_{18:0}$	1.76			1.09
$C_{17:0}$ iso 3OH				1.29
$C_{19:0}$ cyclo ω8c	19.79	12.65	15.16	18.94
Summer Feature 2*	6.88	6.87	9.26	6.76
Summer Feature 3#	2.44	3.99	1.96	1.87
Summer Feature 8$^\Delta$	15.15	22.66	5.11	8.26

* Summed Feature 2: $C_{12:0}$ aldehyde/unknown 10.928/$C_{14:0}$ 3OH/$C_{16:1}$ iso I
# Summed Feature 3: $C_{16:1}$ ω7c/$C_{16:1}$ ω6c
$^\Delta$ Summed Feature 8: $C_{18:1}$ ω7c

Table S6 Phenotypic characteristics of *Paraburkholderia guartelanensis* strains

Characteristics	**CNPSo strains**					
	2995	3003	3008^T	3016	3023	3248
Growth at/in/with:						
1 % Nacl	+	+	+	+	+	+
37 °C	-	-	-	-	-	w
pH 4	+	+	+	+	+	+
pH 8	+	+	+	+	+	+
Urea 2 %	+	+	+	+	+	+
LB	+	+	+	+	+	+
Tolerance to (µg per disc):						
Tetracyclin (30)	-	-	-	-	-	-
Bacitracin (0.04 U)	+	+	+	+	+	+
Chloramphenicol (30)	-	-	-	-	-	-
Erythromycin (15)	-	-	-	-	-	-
Neomycin (30)	-	-	-	-	-	-
Nalidixic Acid (30)	-	-	-	-	-	-
Streptomycin (15)	-	-	-	-	-	-
Cefuroxine (30)	+	+	+	+	+	+

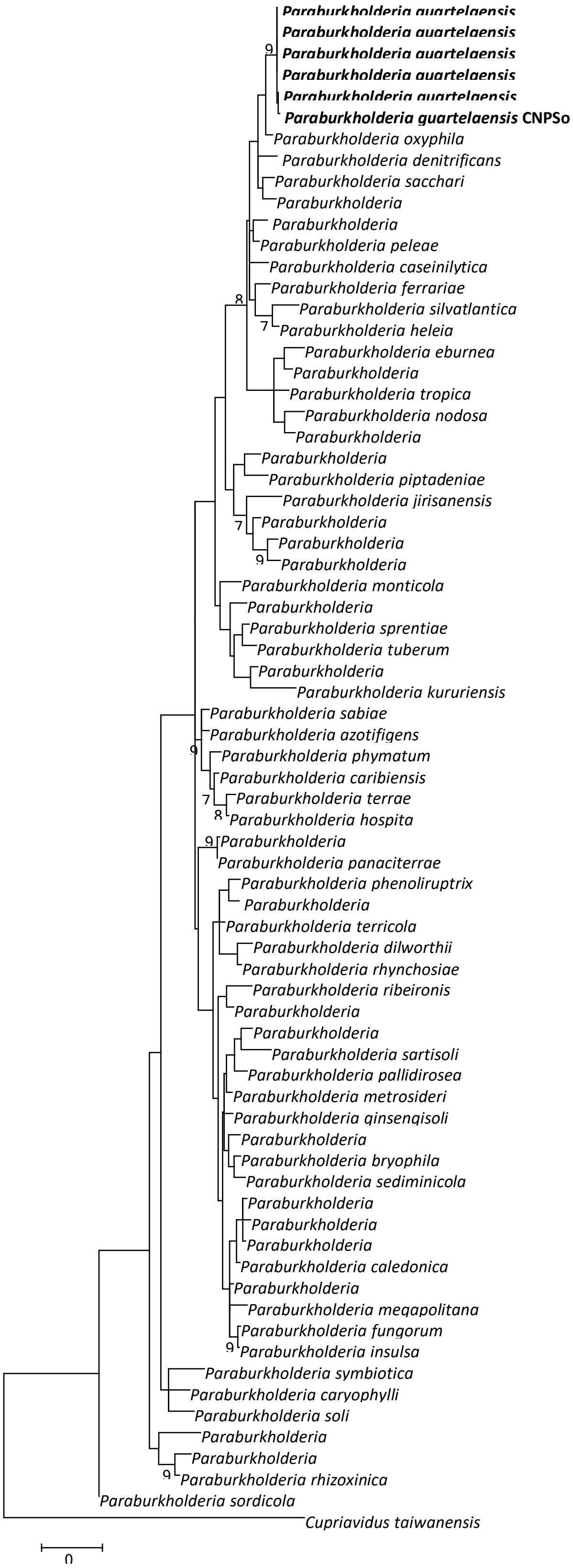


Fig. S1. Maximum likelihood phylogeny of the 16S rRNA gene (1,359 bp) between CNPSo strains and *Paraburkholderia* species. Bootstrap values >70 % are indicated at the nodes. Accession numbers are indicated in parentheses. Strains of the novel species are shown in bold. *Cupriavidus taiwanensis* LMG 19424^{T} was used as outgroup. Bar indicates five substitution per 100 nucleotide positions

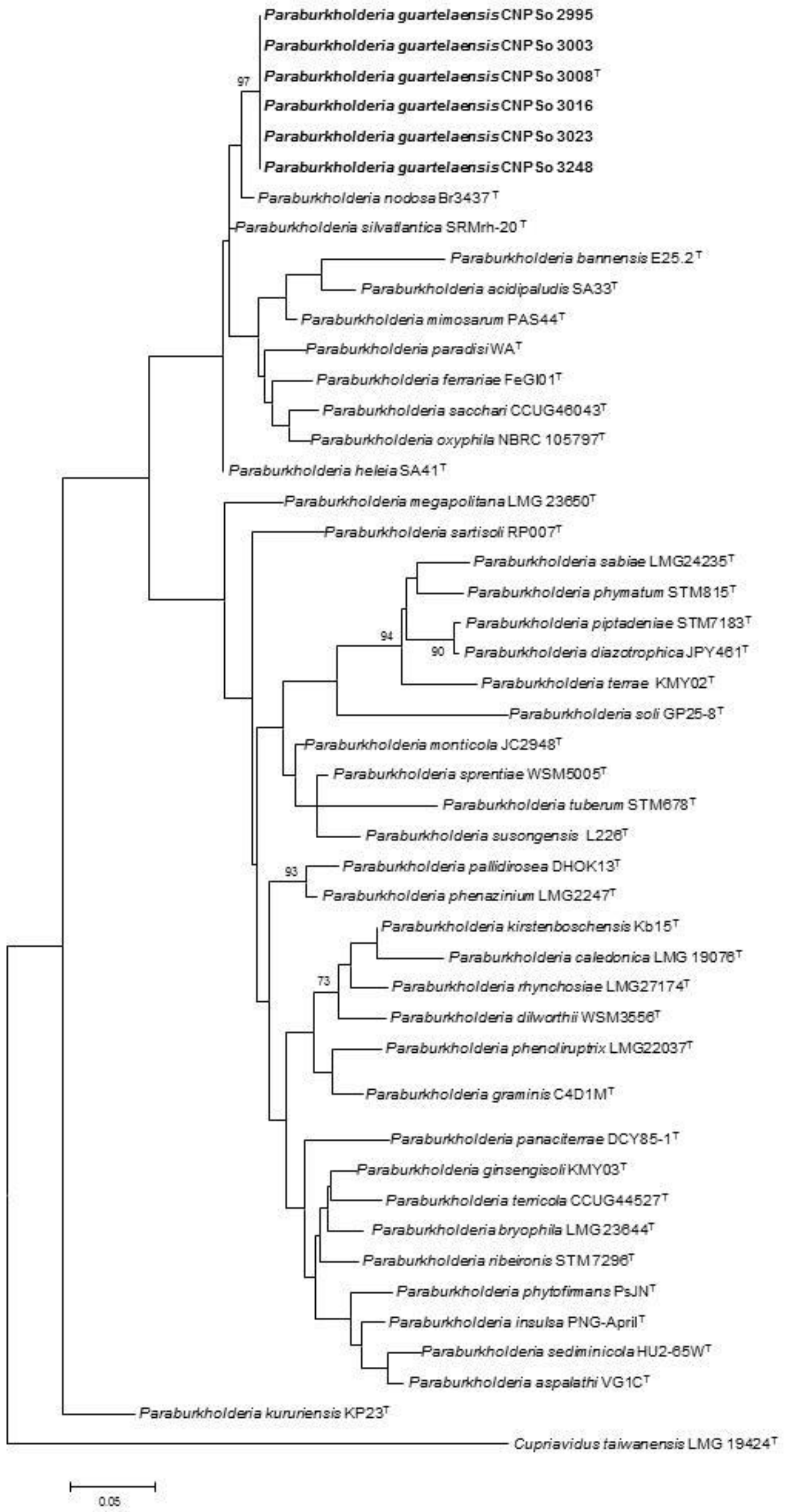


Fig. S2 Phylogenetic tree based on the partial sequences of the *recA* (276 bp) gene. Strains of the novel species are shown in bold. Bootstrap values >70 % are indicated at the nodes. Bar indicates five substitutions per 100 nucleotide positions. Accession numbers are indicated in Table S3.

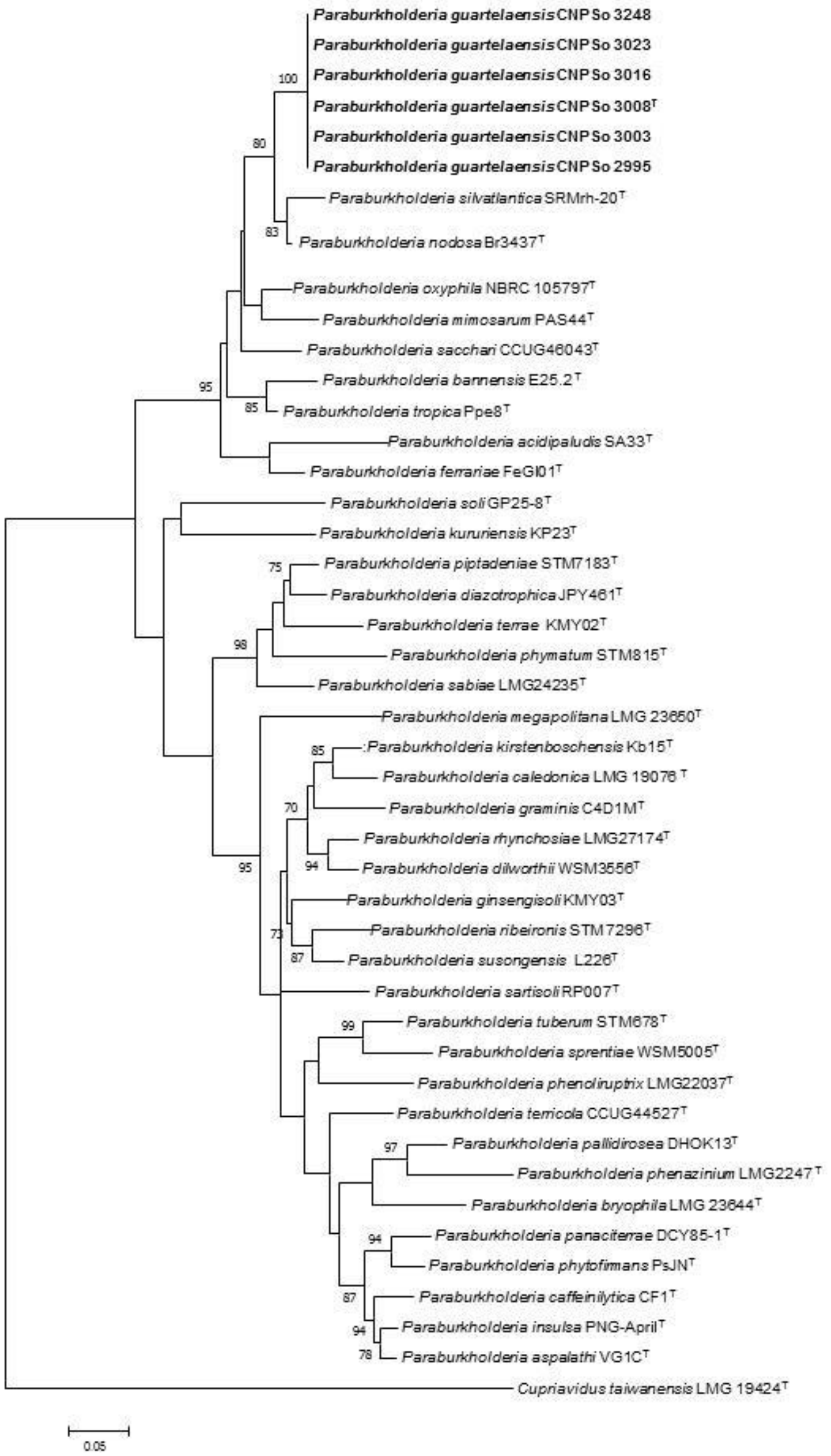


Fig S3. Phylogenetic tree based on the partial sequences of the *gyrB* (574 bp) gene. Strains of the novel species are shown in boldface. Bootstrap values >70 % are indicated at the nodes. Bar indicates five substitutions per 100 nucleotide positions. Accession numbers are indicated in Table S3.

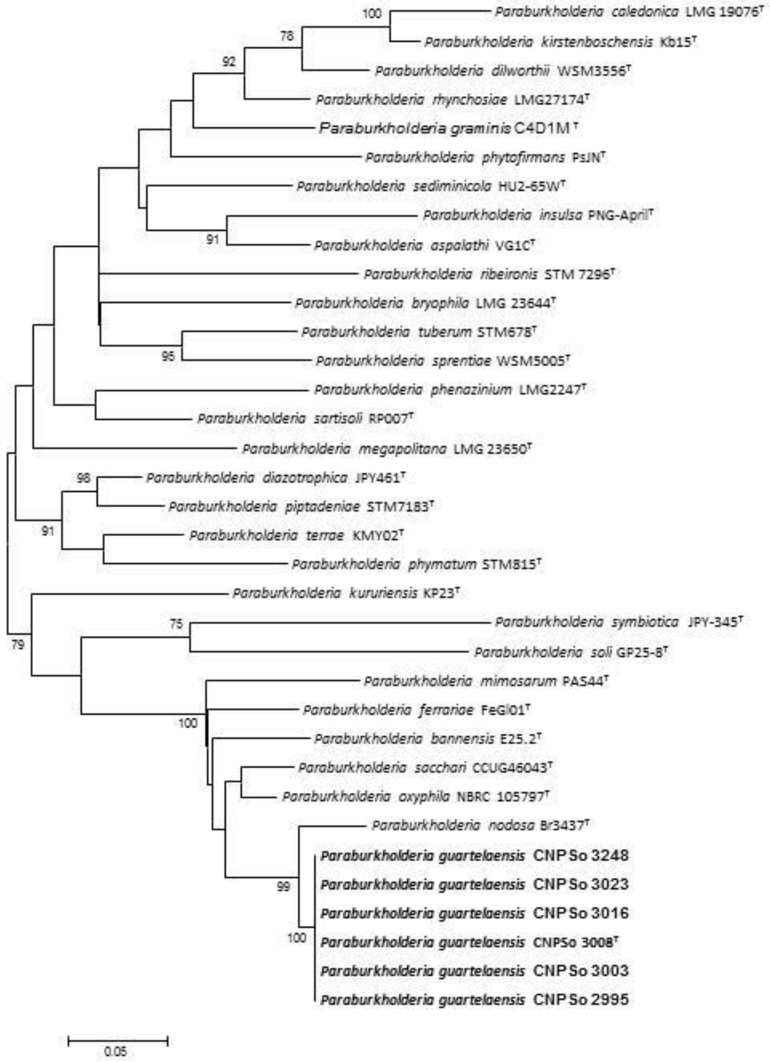


Fig S4. Phylogenetic tree based on the partial sequences of the *trpB* (568 bp) gene. Strains of the novel species are shown in boldface. Bootstrap values >70 % are indicated at the nodes. Bar indicates five substitutions per 100 nucleotide positions. Accession numbers are indicated in Table S3.

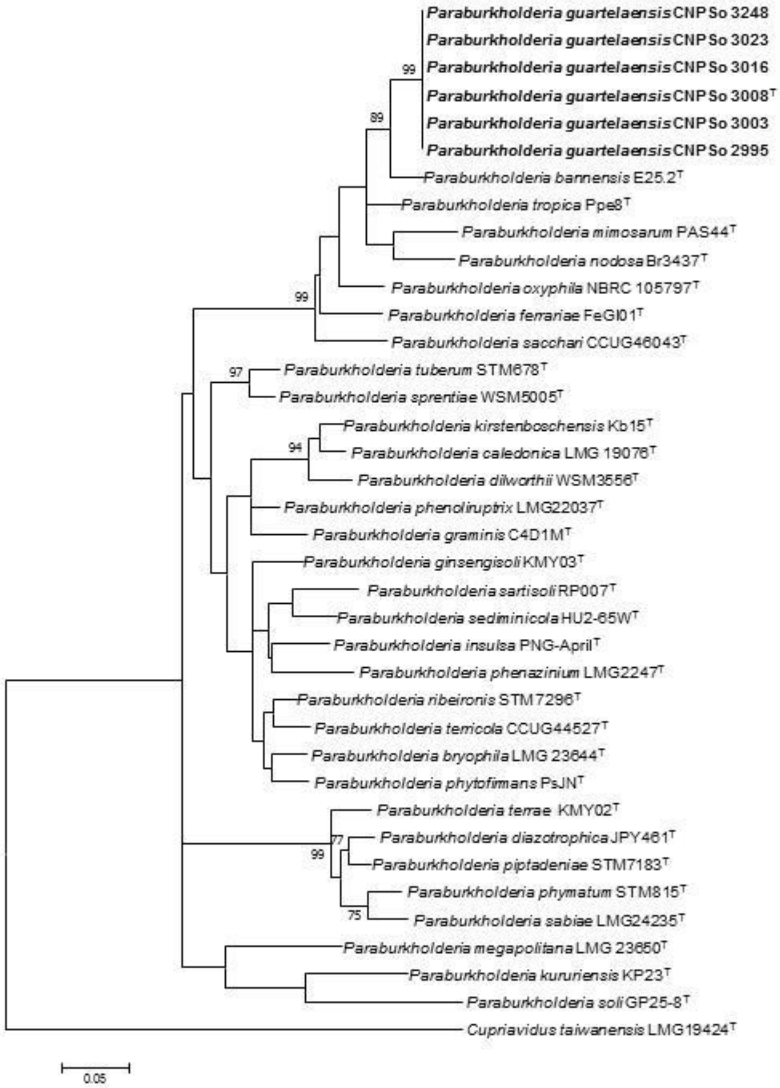


Fig S5. Phylogenetic tree based on the partial sequences of the *gltB* (299 bp) gene. Strains of the novel species are shown in boldface. Bootstrap values >70 % are indicated at the nodes. Bar indicates five substitutions per 100 nucleotide positions. Accession numbers are indicated in Table S3.

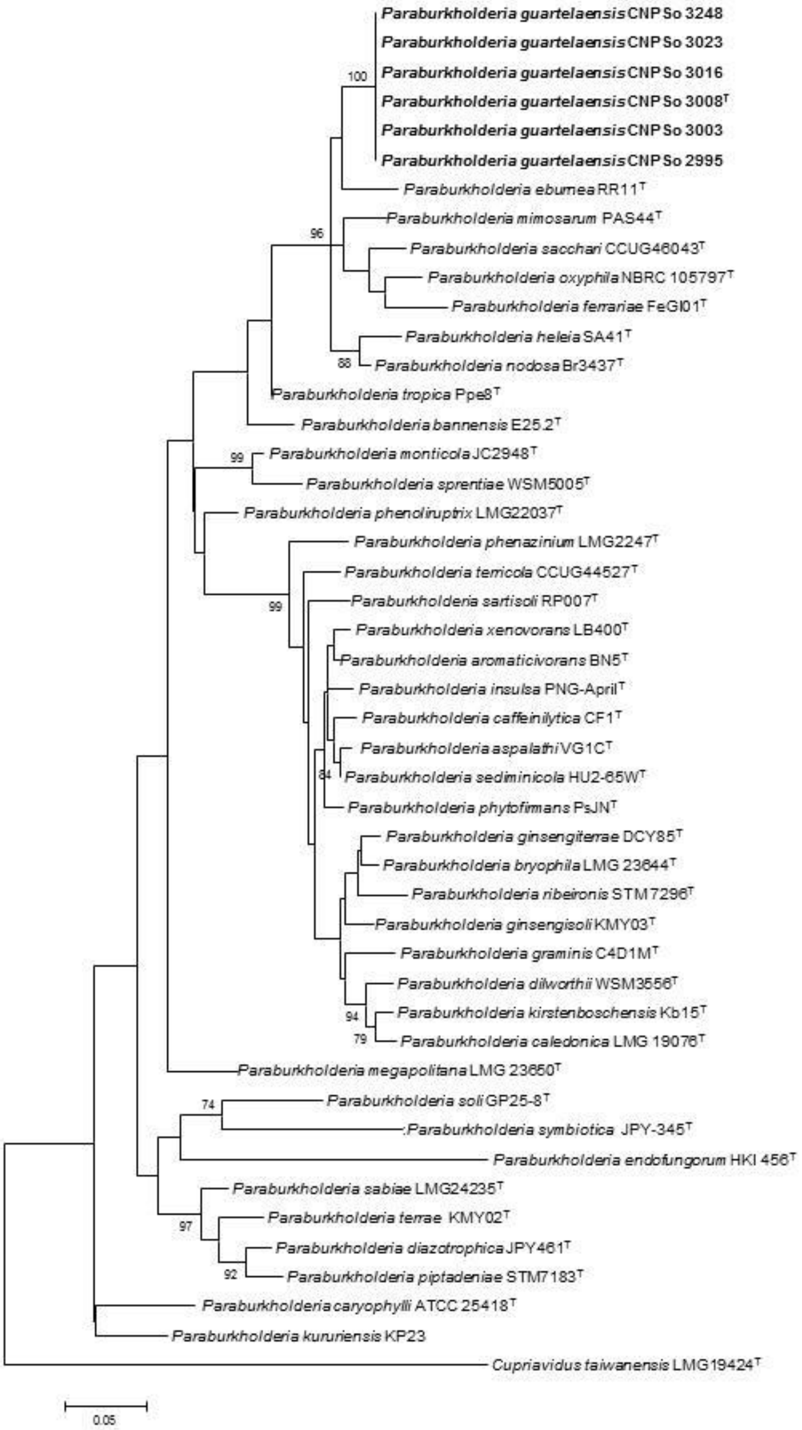


Fig. S6 Phylogenetic tree based on the partial sequences of the *atpD* (611 bp) gene. Strains of the novel species are shown in boldface. Bootstrap values >70 % are indicated at the nodes. Bar indicates five substitution per nucleotide position. Accession numbers are indicated in Table S3.

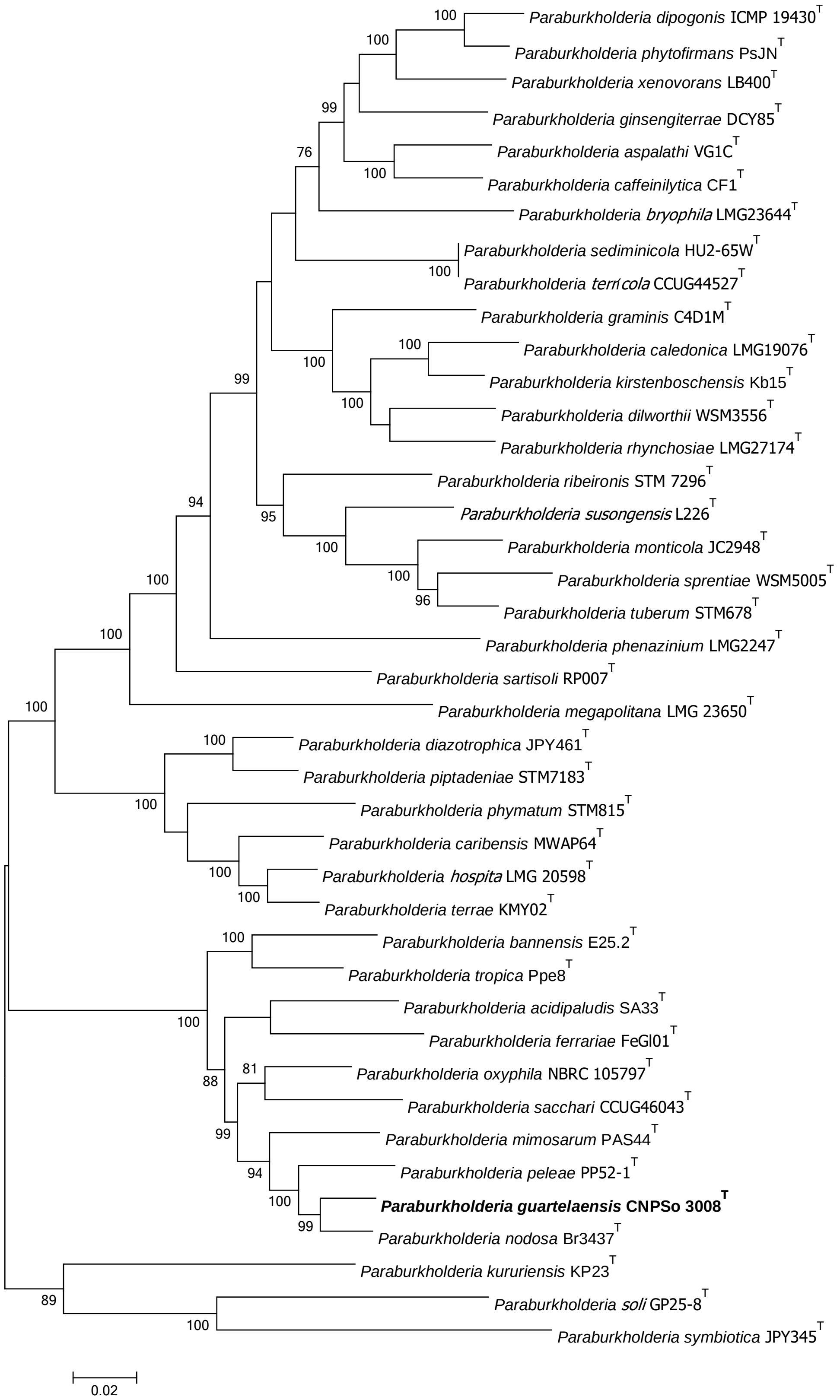
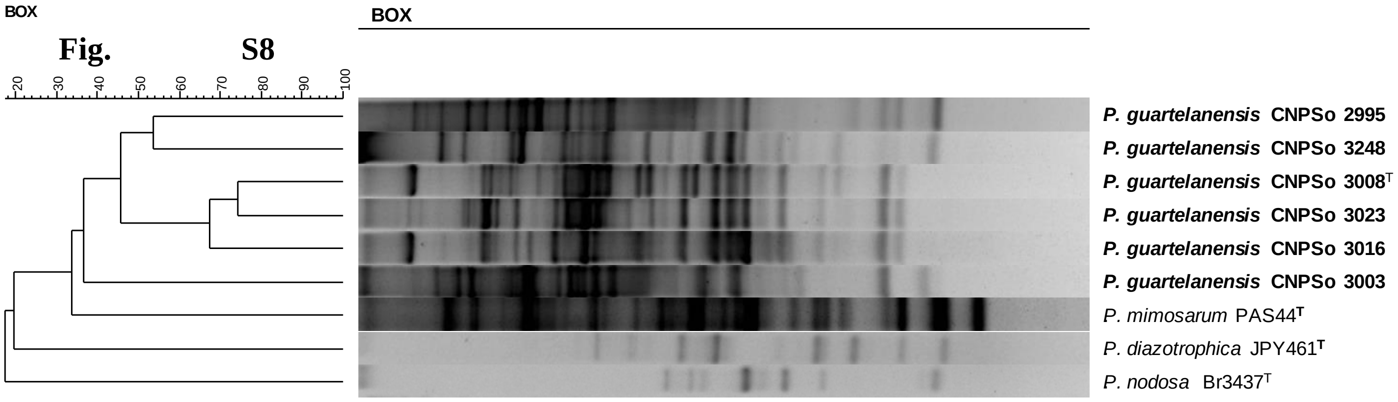


Fig. S7 Maximum likelihood phylogeny based on concatenated *recA*+*gyrB*+*trpB*+*gltB*+*atpD* (10,717 pb) gene sequences showing the relationships of novel species of *Paraburkholderia* (in boldface) and other members of the genus. Bootstrap values >70 % are indicated at the nodes. Accession numbers are indicated in Table S3. Bar indicates two substitutions per 100 nucleotide positions.

Fig. S8



Dendrogram of similarity based on the BOX-PCR profiles of the novel *Paraburkholderia guartelanensis* and related 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 1 % tolerance.

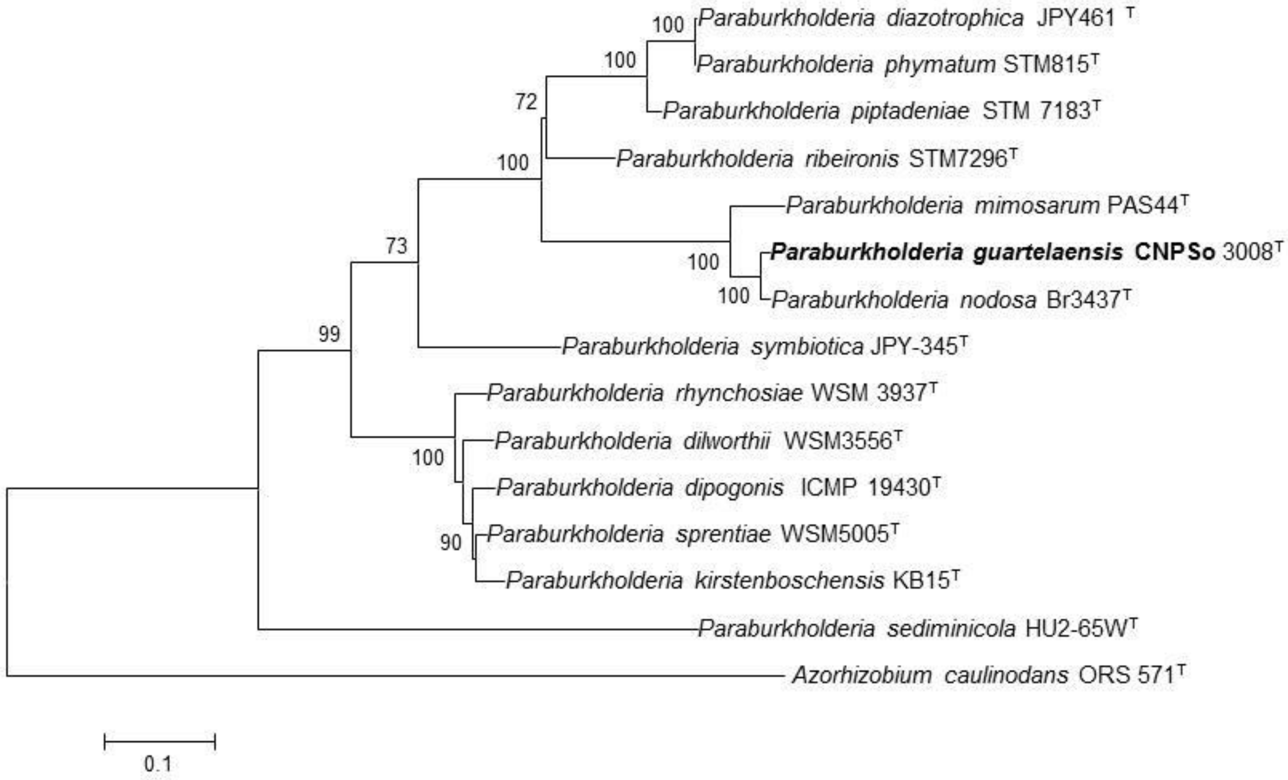


Fig. S9. Maximum-likelihood phylogeny based on the whole *nodC* gene sequences (1,184 bp) among the novel *Paraburkholderia* species (in boldface) and other members of the genus. Bootstrap values >70 % are indicated at the nodes. Accession numbers are indicated in Table S5. *Azorhizobium caulinodans* ORS 571^{T} was used as outgroup. Bar indicates one substitution per 100 nucleotide positions.

Fig. S10 Ineffective nodules formed by P*araburkholderia guartelaensis* strain CNPSo 3008^T in (a) common beans (*Phaseolus vulgaris*) and (b) siratro (*Macroptilium atropurpureum*), and effective nitrogen-fixing nodules formed in (c) *Mimosa pudica;* (d) effective nodules with internal pink color formed by by CNPSo 2995 in *Mimosa pudica*.

7. ESTUDO 2

PAULITSCH, F. et al. *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**, p. 1435-1446, 2020.

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ORIGINAL PAPER

Paraburkholderia atlantica sp. nov. and *Paraburkholderia franconis* sp. nov., two new nitrogen-fixing nodulating species isolated from Atlantic forest soils in Brazil

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Abstract

A polyphasic study was conducted with 11 strains trapped by *Mimosa pudica* and *Phaseolus vulgaris* grown in soils of the Brazilian Atlantic Forest. In the phylogenetic analysis of the 16S rRNA gene, one clade of strains (Psp1) showed higher similarity with *Paraburkholderia piptadeniae* STM7183^T (99.6%), whereas the second (Psp6) was closely related to *Paraburkholderia tuberum* STM678^T (99%). An MLSA (multilocus sequence analysis) with four (*recA*, *gyrB*, *trpB* and *gltB*) housekeeping genes placed both Psp1 and Psp6 strains in new clades, and BOX-PCR profiles indicated high intraspecific genetic diversity within each clade. Values of digital DNA–DNA hybridization (dDDH) and average nucleotide identity (ANI) of the whole genome sequences were of 56.9 and 94.4% between the Psp1 strain CNPSo 3157^T and *P. piptadeniae*; and of 49.7% and 92.7% between the Psp6 strain CNPSo 3155^T and *P. tuberum*, below the threshold for species delimitation. In the *nodC* analysis, Psp1 strains clustered together with *P. piptadeniae,* while Psp6 did not group with any symbiotic *Paraburkholderia*. Other phenotypic, genotypic and symbiotic properties were evaluated. The polyphasic analysis supports that the strains represent two novel species, for which the names *Paraburkholderia franconis* sp. nov. with type strain CNPSo 3157^T (= ABIP 241, = LMG 31644) and *Paraburkholderia atlantica* sp. nov. with type strain CNPSo 3155^T (= ABIP 236, = LMG 31643) are proposed.

Keywords Taxonomy · Phylogeny · Biological nitrogen fixation · MLSA · ANI · dDDH

Abbreviations

NI	Nucleotide identity
ANI	Average nucleotide identity
BNF	Biological nitrogen fixation
DDH	DNA–DNA hybridization
dDDH	Digital DNA–DNA hybridization

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MLSA	Multilocus sequence analysis
PCR	Polymerase chain reaction

Introduction

Free-living soil bacteria denominated diazotrophic are capable of converting molecular nitrogen (N_2) into soluble compounds, easily assimilated by plants in a process known as biological nitrogen fixation (BNF), highly contributing to the global N balance. Rhizobia are diazotrophic bacteria distinguished by the capacity of nodulating and fixing nitrogen when in symbiosis with plants of the Fabaceae family (Oldroyd et al. 2011; Ormeño-Orrillo et al. 2013).

As a result of studies based on phylogenetic analyses of the 16S rRNA and housekeeping genes and phylogenomics, species of *Burkholderia* with environmental importance were reallocated into the new genera *Paraburkholderia* and *Cupriavidus*, whereas species of clinical importance remained as *Burkholderia stricto* sensu (Gyaneshwar et al. 2011; Estrada-de los Santos et al. 2013; Sawana et al. 2014; Dobritsa and Samadpour 2016). Recently, studies with whole genome sequence data, amino acid sequences and maximum-likelihood analysis support that *Burkholderia lato* sensu comprises at least three new genera, *Robbisia* (Lopes-Santos et al. 2017), *Trinickia* and *Mycetohabitans* (Estrada-de los Santos et al. 2018).

Advancements in whole genome sequencing technology have allowed the description of several new species of *Paraburkholderia* such that currently the genus has over 70 validated species, and among them, about 20 are rhizobia (Velázquez et al. 2017; LPSN 2019). Nodulating *Paraburkholderia* are the *Mimosa* preferred symbionts in Brazil, a country considered as a center of diversity for legume-nodulating *Paraburkholderia*, especially in the Cerrado and Caatinga biomes (Chen et al. 2005; dos Reis Junior et al. 2010; Dall'Agnol et al. 2017; Paulitsch et al. 2019a). Nodulation by *Paraburkholderia* has also been reported in plants of the *Piptadenia* group (Bournaud et al. 2013) and in *Calliandra* species (Silva et al. 2018). Soil characteristics have been considered as the main factor influencing *Paraburkholderia* nodulation predominance, especially in acid pH, where many species of the genus were revealed to have competitive advantages (Stopnisek et al. 2014; de Castro Pires et al. 2018; Paulitsch et al. 2019b). Besides Brazil, other legume-nodulating *Paraburkholderia* diversity center is the Fynbos biome in South Africa, where soils are nutrient poor and acidic, leading to the association of *Paraburkholderia* with Papilionoideae endemic legumes, but in this case they do not seem to nodulate *Mimosa* (Elliott et al. 2007; Beukes et al. 2013; De Meyer et al. 2016; Lemaire et al. 2016).

In this study, we describe a polyphasic analysis performed with *Paraburkholderia* strains isolated from root nodules of *Mimosa pudica* and *Phaseolus vulgaris* used as trapping hosts when inoculated with soils of the Brazilian Atlantic Forest, considered a hotspot of biodiversity (Myers et al. 2000). The isolation and preliminary characterization of the eight strains used in this study were performed by our group (Dall'Agnol et al. 2017); now we have completed the genetic characterization and proceeded with the description of two new species, *Paraburkholderia atlantica* sp. nov. and *Paraburkholderia franconis* sp. nov.

Materials and methods

Strains, culture conditions and DNA extraction

The eight strains used in this study were previously described as belonging to two clades, Psp1 and Psp6 (Dall'Agnol et al. (2017). The Psp1 clade comprises strains CNPSo 3157^T, CNPSo 3191, CNPSo 3199, CNPSo 3200 and CNPSo 3201 and the Psp6 clade strains CNPSo 3150, CNPSo 3155^T and CNPSo 3196. Information about the CNPSo strains are available in Table S1. Dall'Agnol et al. (2017) used soils from the Brazilian Atlantic Forest as inocula and *Mimosa pudica* and *Phaseolus vulgaris* as trapping hosts. The strains were isolated from *M. pudica* root nodules, except for CNPSo 3196 that was isolated from *P. vulgaris* nodules. All strains are deposited at the "Diazotrophic and Plant Growth Promoting Bacteria Culture Collection of Embrapa Soja" (CNPSo) (WFCC Collection No. 1213, WDCM Collection No. 1054), in Londrina, Brazil, at the ABIP Collection (IRD/LSTM Montpellier, France), at the University of Seville (US Collection, Seville, Spain) and at the LMG collection (Belgium).

Strains were grown at 28 °C from 2 to 7 days on modified-yeast extract–mannitol–agar (YMA) medium (Hungria et al. 2016). Stock cultures were maintained on YMA at 4 ºC, and for long-term preservation strains were cryopreserved in liquid-modified YM with 30% (v/v) glycerol at – 80 °C and – 150 °C, and lyophilized.

Phylogeny

Total genomic DNA of CNPSo strains was extracted using the DNeasy blood & Tissue Kit (Qiagen), following the manufacturer's instructions. Total DNA was used to conduct PCR amplification and sequencing of the 16S rRNA and four housekeeping genes (*recA*, *gyrB*, *trpB* and *gltB*). In addition, *nodC* gene was amplified to evaluate the phylogenetic position of the symbiotic genes. Primers, amplification and sequencing conditions were conducted as described by Dall'Agnol et al. (2017), except for the 16S rRNA gene that was purified using the PureLink™ Quick PCR Purification Kit (Invitrogen™), following the manufacturer's instructions and sequenced using an ABI 3500xL (Applied Biosystems®). Primers and PCR conditions used in this study are listed in Table S2.

For the phylogenetic analysis of 16S rRNA, housekeeping and *nodC* genes, all sequences were aligned with MUSCLE (Edgar 2004) and phylogenies were constructed using MEGA 7 (Kumar et al. 2016) with the maximum-likelihood (ML) algorithm. For the16S rRNA phylogeny, the Tamura Nei model (Tamura and Nei 1993) with gamma-distributed invariant sites (G+I) (Tamura 1992) was used. The MLSA (*recA*, *gyrB*, *gltB* and *trpB*) was constructed with the distance model general time reversible (Waddell and Steel 1997) and gamma-distributed invariant sites (G+I).

An MLSA with nine full housekeeping genes (*recA*, *gyrB*, *gltB, trpB, rpoB, lepA, glnA, thrC* and *dnaK*) was also performed to confirm the taxonomic position of the CNPSo strains. For that, the sequences were retrieved from the whole genomes, sequenced in this study, or retrieved from databases, as will be described in the next section. For the MSLA analysis, the general time reversible model was employed (Waddell and Steel 1997), using G+I. For the *nodC* gene, the Tamura 3-parameter (Tamura et al. 2013) model with+I was used. The statistical support for the trees was evaluated by bootstrap analysis with 1,000 re-samplings (Felsenstein 1985). Nucleotide identity (NI) was calculated with Bioedit (v. 7.2.5) (Hall 1999). Accession numbers of gene sequences from this study or retrieved from the GenBank database are shown in the phylograms and/or in Table S3.

Genome features

The genomic DNA was used to analyze the BOX-PCR fingerprinting profile of the CNPSo strains and the closest type strains *Paraburkholderia phymatum* STM815^T, *Paraburkholderia tuberum* STM678^T, *Paraburkholderia diazotrophica* JPY461^T, and *Paraburkholderia sprentiae* WSM5005^T; as the type strain of *Paraburkholderia piptadeniae* STM7183^T was unavailable, we used the *P. piptadeniae* strain CNPSo 3139 as a representative for this species. Primers and amplification conditions are specified in Table S2. A dendrogram was built with the genetic profiles using the software Bionumerics (Applied Mathematics, Kortrijk, Belgium, v. 7.6), applying the UPGMA algorithm (unweighted pair-group method with arithmetic mean) (Sneath and Sokal 1973) and the Jaccard coefficient (Jaccard 1912) with 3% of tolerance.

For the genome analysis, total DNA of strains CNPSo 3155^T and CNPSo 3157^T was used to build libraries using the Nextera XT kit, according to the manufacturer's instructions. The library processing was realized on the MiSeq platform (Ilumina) at Embrapa Soja. The draft genomes were assembled with the A5-MiSeq pipeline (de novo) v.20140604. Genome sizes were estimated with RAST v.2.0 (Aziz et al. 2008) and confirmed with QUAST v.2.0 (Gurevich et al. 2013) and the sequences were deposited at the NCBI database. The type strains genomes of *P. phymatum* STM815^T (GCA_000020045.1), *P. piptadeniae* STM7183^T (NZ_CYGY00000000.2), *P. sprentiae* WSM5005^T (GCA_001865575.1) and *P. diazotrophica* JPY461^T (NZ_FNYE00000000.1) were retrieved from the GenBank database, whereas *P. tuberum* STM678^T (2,512,047,030) genome was retrieved from the JGI/IMG/R. ANI comparisons were evaluated with ANI calculator (available at <https://enve-omics.ce.gatech.edu/ani/>). An *in silico* comparison for the estimation of the DDH was conducted via digital DNA–DNA hybridization (dDDH) (Meier-Kolthoff et al. 2013, 2014). All pairwise values with the closest species were estimated by GGDC v2.1 using the recommended 'Formula 2′ (https://ggdc.dsmz.de/distcalc2.php).

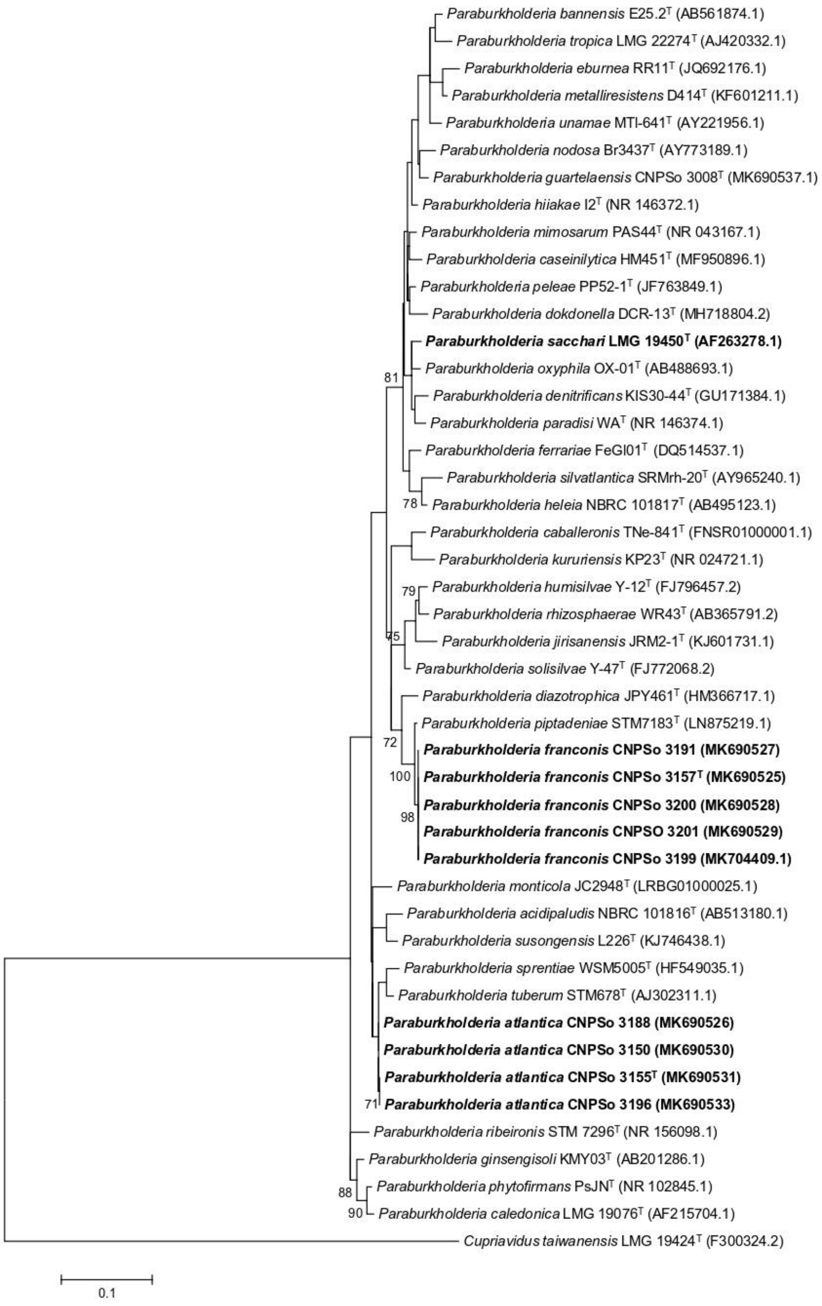
The DNA G+C contents of the CNPSo 3155 ^{T} and CNPSo 3157^T strains were calculated with QUAST (Gurevich et al. 2013).

Physiological characteristics

Phenotypic characterization was performed with CNPSo 3155^T, CNPSo 3157^T, *P. phymatum* STM815^T, *P. tuberum* STM678^T, *P. diazotrophica* JPY461^T, *P. sprentiae* WSM5005^T and *P. piptadeniae* CNPSo 3139. Strains were grown at 28 °C for 4 days under different conditions and all the tests were performed in duplicate. To evaluate acid/alkaline reaction, the strains were grown in modified-YMA medium (Hungria et al. 2016) with bromothymol blue as the pH indicator. Growth in modified YMA with pH 4.0 and 8.0 and 1% NaCl, and the capacity to grow in solid Luria–Bertani medium (LB) were also evaluated. Growth at high temperature was tested at 37 ºC in modified YMA. For evaluation of urease activity strains were grown in modified YMA with 2% urea and phenol red as indicator. Carbon source utilization was evaluated using the API 50CH kit (BioMérieux) with modified YM without mannitol as the basal medium. Disc diffusion method in modified YMA was used to evaluate the antibiotics tolerance with the following antibiotics (per disc): tetracycline (30 µg), bacitracin (0.04 U), chloramphenicol (30 µg), erythromycin (10 µg), neomycin (30 µg), nalidixic acid (30 µg), streptomycin (15 µg) and cefuroxime (30 µg).

Nodulation tests

Nodulation tests were conducted with *P. vulgaris* (common bean), *Macroptilium atropurpureum* (siratro) and *M. pudica*. To improve the germination, *M. atropurpureum* seeds were scarified with concentrated sulfuric acid for 10 min and then washed five times with sterile distilled water, and *M. pudica*


Paraburkholderia bannensis E25.2^{T} (AB561874.1)
Paraburkholderia tropica LMG 22274^{T} (AJ420332.1)
Paraburkholderia eburnea RR11^{T} (JQ692176.1)
Paraburkholderia metalliresistens D414^{T} (KF601211.1)
Paraburkholderia unamae MTI-641^{T} (AY221956.1)
Paraburkholderia nodosa Br3437^{T} (AY773189.1)
Paraburkholderia guartelaensis CNPSo 3008^{T} (MK690537.1)
Paraburkholderia hiiakae I2^{T} (NR 146372.1)
Paraburkholderia mimosarum PAS44^{T} (NR 043167.1)
Paraburkholderia caseinilytica HM451^{T} (MF950896.1)
Paraburkholderia peleae PP52-1^{T} (JF763849.1)
Paraburkholderia dokdonella DCR-13^{T} (MH718804.2)
Paraburkholderia sacchari LMG 19450^{T} (AF263278.1)
81
Paraburkholderia oxyphila OX-01^{T} (AB488693.1)
Paraburkholderia denitrificans KIS30-44^{T} (GU171384.1)
Paraburkholderia paradisi WA^{T} (NR 146374.1)
Paraburkholderia ferrariae FeGI01^{T} (DQ514537.1)
Paraburkholderia silvatlantica SRMrh-20^{T} (AY965240.1)
78
Paraburkholderia heleia NBRC 101817^{T} (AB495123.1)
Paraburkholderia caballeronis TNe-841^{T} (FNSR01000001.1)
Paraburkholderia kururiensis KP23^{T} (NR 024721.1)
79
Paraburkholderia humisilvae Y-12^{T} (FJ796457.2)
Paraburkholderia rhizosphaerae WR43^{T} (AB365791.2)
75
Paraburkholderia jirisanensis JRM2-1^{T} (KJ601731.1)
Paraburkholderia solisilvae Y-47^{T} (FJ772068.2)
Paraburkholderia diazotrophica JPY461^{T} (HM366717.1)
Paraburkholderia piptadeniae STM7183^{T} (LN875219.1)
72
Paraburkholderia franconis CNPSo 3191 (MK690527)
100
Paraburkholderia franconis CNPSo 3157^{T} (MK690525)
Paraburkholderia franconis CNPSo 3200 (MK690528)
98
Paraburkholderia franconis CNPSO 3201 (MK690529)
Paraburkholderia franconis CNPSo 3199 (MK704409.1)
Paraburkholderia monticola JC2948^{T} (LRBG01000025.1)
Paraburkholderia acidipaludis NBRC 101816^{T} (AB513180.1)
Paraburkholderia susongensis L226^{T} (KJ746438.1)
Paraburkholderia sprentiae WSM5005^{T} (HF549035.1)
Paraburkholderia tuberum STM678^{T} (AJ302311.1)
Paraburkholderia atlantica CNPSo 3188 (MK690526)
Paraburkholderia atlantica CNPSo 3150 (MK690530)
Paraburkholderia atlantica CNPSo 3155^{T} (MK690531)
71
Paraburkholderia atlantica CNPSo 3196 (MK690533)
Paraburkholderia ribeironis STM 7296^{T} (NR 156098.1)
Paraburkholderia ginsengisoli KMY03^{T} (AB201286.1)
88
Paraburkholderia phytofirmans PsJN^{T} (NR 102845.1)
90
Paraburkholderia caledonica LMG 19076^{T} (AF215704.1)
Cupriavidus taiwanensis LMG 19424^{T} (F300324.2)
0.1

◂**Fig. 1** Maximum-likelihood (ML) phylogeny based on 16S rRNA sequences (1317 bp) between Psp1 and Psp6 strains and most closely related *Paraburkholderia* species. Bootstrap values > 70% are indicated at the nodes. Accession numbers are indicated in parentheses and in Table S3. Strains of the novel species are shown in boldface. *Cupriavidus taiwanensis* LMG 19424^T was used as outgroup. Scale bar indicates one substitutions per 100 nucleotide positions

seeds were lightly sanded. Seeds were surface sterilized in 70% ethanol for 1 min, soaked in commercial sodium hypochlorite (2–2.5%) for 5 min, and washed five times in sterile distilled water. Seeds were pre-germinated for 2 days (siratro and common bean) and for 3 days (*M. pudica)* in 1% water–agar at 28 °C. Tests were conducted in triplicate in sterile polypropylene bags containing Germitest paper and N-free plant nutrient solution (Broughton and Dilworth 1971). The strains were grown in modified YM medium and inoculated at the log phase 1 day after seed transfer. Plants of common bean and siratro were grown in a glasshouse at 28 °C, for 30 days and, in the case of *M. pudica,* for 40 days. After this period, nodulation was evaluated, as well as the effectiveness of the nodules, through verification of pink color inside it.

Results and discussion

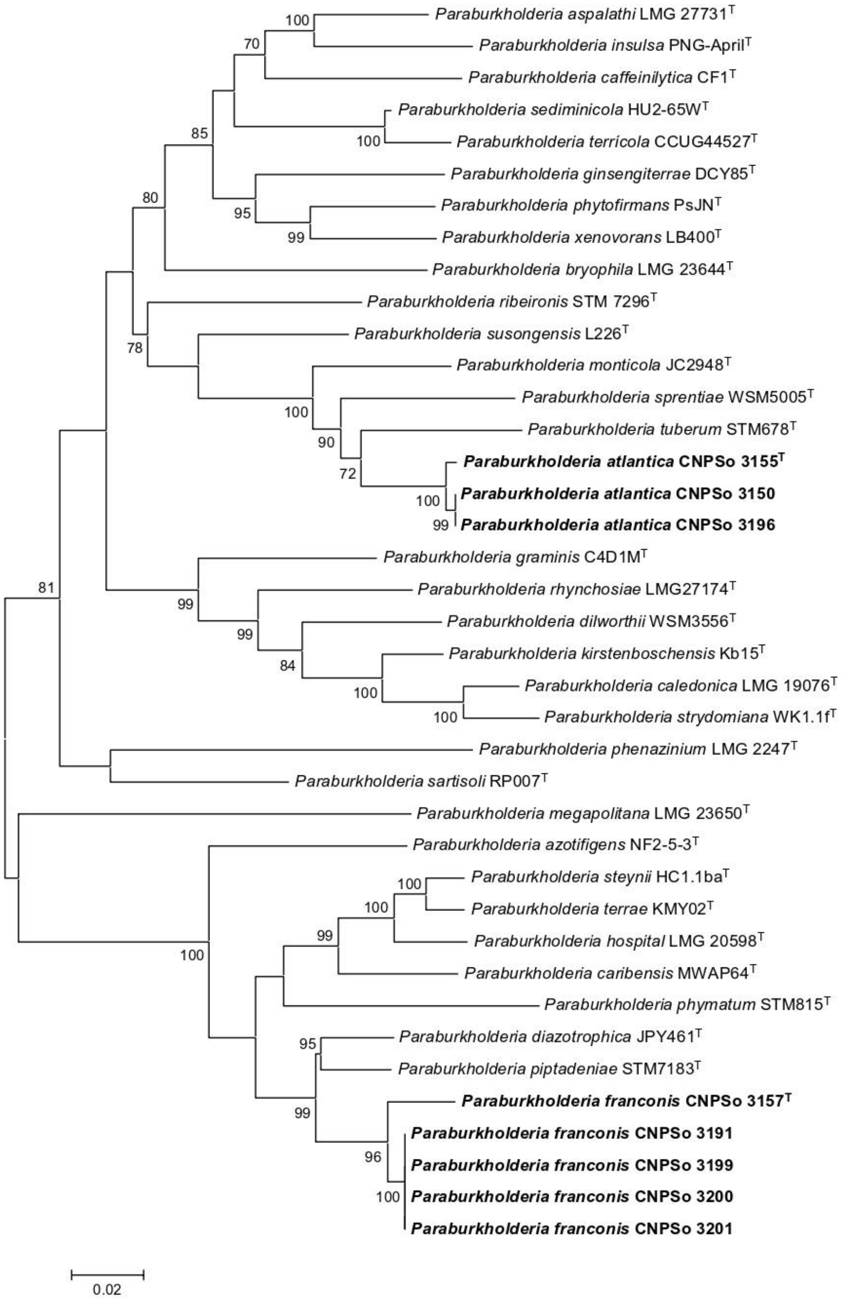
Phylogenetic analysis of the 16S rRNA and housekeeping genes

The phylogenetic trees based on 16S rRNA sequences (1317 bp) showed that the strains from the Psp1 and Psp6 clades differ from each other. The Psp1 strains clustered in a well distinct clade, with *P. piptadeniae* STM7183^T as the closest species, and the Psp6 strains also clustered separately, and their closest neighbors were *P. tuberum* STM678^T and *P. sprentiae* WSM5005^T (Fig. 1). The phylogeny was confirmed when more species were included in the analysis (Fig. S1). The Psp1 strains shared 100% of nucleotide identity (NI) with each other in the 16S rRNA gene and Psp6 strains showed 99.9–100% (Table S4). Psp1 strains shared 96.3–99.6% of NI with other nearby *Paraburkholderia* species, with the highest values with *P. piptadeniae* (99.6%) and *P. diazotrophica* (97.7%). The similarity of Psp6 clade with other related *Paraburkholderia* species ranged from 97.2 to 99%, showing higher similarity to *P. tuberum* (99%) and *P. sprentiae* (98.6%) (Table S4). Although the 16S rRNA sequence often fails in separating closely relative taxa, including the genus *Paraburkholderia* (Lv et al. 2016; Bournaud et al. 2017; Choi and Im 2018; Huo et al. 2018), strains of the Psp1 and Psp6 clades were not clustered with other *Paraburkholderia* species (Fig. 1, Fig. S1).

We proceeded with the MLSA analysis, employed not only for being a more informative tool, but also for tamponing the effects of horizontal gene transfer and gene recombination (Bournaud et al. 2017; Dall'Agnol et al. 2017; Paulitsch et al. 2019b), becoming the prefered method for classification and reclassifications of the environmental species of *Paraburkholderia* (Sawana et al. 2014; Dobritsa and Samadpour 2016). An MLSA phylogeny with the partial concatenated sequences (2,152 bp) of four housekeeping (*recA, gyrB, trpB* and *gltB*) genes was conducted with Psp1 and Psp6 strains and the closest *Paraburkholderia* species. The Psp1 strains clustered in a separated clade from all other *Paraburkholderia* species with 96% bootstrap support, and with *P. piptadeniae* and *P. diazotrophica* being the closest neighbors (Fig. 2). Psp6 strains also clustered separately from other species with high bootstrap support (100%), with *P. tuberum* and *P. sprentiae* as the closest species (Fig. 2). Individual trees for each gene were built and confirmed the phylogenetic position of Psp1 and Psp6 strains (data not shown). To obtain an even more precise taxonomic position of Psp1 and Psp6 strains, a phylogenetic tree was built with the complete sequences of nine housekeeping genes (*recA, gyrB, gltB, trpB, rpoB, lepA, glnA, thrC* and *dnaK)* (19,600 bp), retrieved from CNPSo 3157 ^{T} and CNPSo 3155^T genomes sequenced in this study and from *Paraburkholderia* genomes available in the Genbank and JGI databases (Fig. S2). The results observed in the MLSA with nine housekeeping genes (Fig. S2) were congruent with both the 16S rRNA (Fig. 1, Fig. S1) and the MLSA with four housekeeping genes (Fig. 2) phylogenies, giving support to the proposition that CNPSo 3157 ^{T} and CNPSo 3155^T represent new species of *Paraburkholderia*.

The NI of single, four and nine concatenated housekeeping genes are shown in Table S4. The Psp1 clade showed higher NI with *P. piptadeniae* in the concatenated partial sequences of four housekeeping genes and with the complete sequences of nine housekeeping genes, sharing 96.5% and 96.9% of identity, respectively. The NI values ranged from 91.6 to 96.3% (four genes) and from 92 to 96.9% (nine genes) with the other closest type strains. The Psp6 clade shared 94.6% and 96% of NI with *P. tuberum* in the MLSA with four and nine genes, respectively, and 91–94.1% (MLSA with four genes) and 92.7–95.7% (MLSA with nine genes) with the other closest type strains of *Paraburkholderia* (Table S4). It is worth mentioning that *P. piptadeniae* STM 7183^T was isolated by Bournaud et al. (2017) using soils from Rio de Janeiro. Using the same soils, Dall'Agnol et al. (2017) isolated strains CNPSo 3157^T and CNPSo 3155^T, indicating the richness of the Atlantic Forest biome in *Paraburkholderia.*

The NI values obtained between Psp1 strains and *P. piptadeniae* are higher than the suggested threshold of 96% for species delimitation (Konstantinidis et al. 2006); nevertheless, there is a genealogical coherence in all phylogenies analyzed (including the 16S rRNA phylogeny), revealing


100
Paraburkholderia aspalathi LMG 27731^T
70
Paraburkholderia insulsa PNG-April^T
Paraburkholderia caffeinilytica CF1^T
Paraburkholderia sediminicola HU2-65W^T
85
100
Paraburkholderia terricola CCUG44527^T
Paraburkholderia ginsengiterrae DCY85^T
80
Paraburkholderia phytofirmans PsJN^T
95
99
Paraburkholderia xenovorans LB400^T
Paraburkholderia bryophila LMG 23644^T
Paraburkholderia ribeironis STM 7296^T
Paraburkholderia susongensis L226^T
78
Paraburkholderia monticola JC2948^T
Paraburkholderia sprentiae WSM5005^T
100
Paraburkholderia tuberum STM678^T
90
Paraburkholderia atlantica CNPSo 3155^T
72
100
Paraburkholderia atlantica CNPSo 3150
99
Paraburkholderia atlantica CNPSo 3196
Paraburkholderia graminis C4D1M^T
81
Paraburkholderia rhynchosiae LMG27174^T
99
Paraburkholderia dilworthii WSM3556^T
99
Paraburkholderia kirstenboschensis Kb15^T
84
Paraburkholderia caledonica LMG 19076^T
100
100
Paraburkholderia strydomiana WK1.1f^T
Paraburkholderia phenazinium LMG 2247^T
Paraburkholderia sartisoli RP007^T
Paraburkholderia megapolitana LMG 23650^T
Paraburkholderia azotifigens NF2-5-3^T
100
Paraburkholderia steynii HC1.1ba^T
100
Paraburkholderia terrae KMY02^T
99
Paraburkholderia hospital LMG 20598^T
100
Paraburkholderia caribensis MWAP64^T
Paraburkholderia phymatum STM815^T
95
Paraburkholderia diazotrophica JPY461^T
Paraburkholderia piptadeniae STM7183^T
Paraburkholderia franconis CNPSo 3157^T
99
Paraburkholderia franconis CNPSo 3191
96
Paraburkholderia franconis CNPSo 3199
100
Paraburkholderia franconis CNPSo 3200
Paraburkholderia franconis CNPSo 3201
0.02

◂**Fig. 2** Maximum-likelihood (ML) phylogeny based on the concatenated gene sequences (*recA* + *gyrB* + *gltB* + *trpB*) (2152 bp) showing the relationships of novel species of *Paraburkholderia* (in bold) and other members of the genus. Bootstrap values > 70% are indicated at the nodes. Accession numbers are indicated in Table S3. Scale bar indicates two substitutions per 100 nucleotide positions

that Psp1 represents a well-defined species. Also, with the information from previous studies (Steenkamp et al. 2015; Venter et al. 2017; Paulitsch et al. 2019b), in addition to our results we suggest a reevaluation of the NI threshold value for *Paraburkholderia* species delimitation to at least 97% for MLSA analyses.

Genomic features

Genome sequences were obtained and deposited at the NCBI for strains CNPSo 3157^T (WHNP00000000, Biosample SAMN13050534) and CNPSo 3155 ^{T} (WHNQ00000000, Biosample SAMN13050733). The shotgun sequences of CNPSo 3157^T allowed a genome coverage of 110-fold, assembled in 260 contigs. The N_{50} was calculated as 135,516 bp, and the genome size was estimated at 10,047,340 bp. CNPSo 3155^T genome sequencing resulted in a genome coverage of 95-fold, assembled in 158 contigs. N_{50} was calculated as 203,420 bp and genome size was estimated at 8,855,873 bp. Both genome features were confirmed with RAST v.2.0 (Aziz et al. 2008) and QUAST v.2.0 (Gurevich et al. 2013).

Genomic methodologies, including average nucleotide identity (ANI) and DNA–DNA hybridization (dDDH), have been increasingly used in replacement of conventional DDH, with proposed value boundaries of 95–96% for ANI and 70% for dDDH (Meier-Kolthoff et al. 2013; Chun et al. 2018). For ANI comparisons, we selected the closest species based on the MLSA, resulting in ANI values between strain CNPSo 3157^T, *P. piptadeniae* STM 7183^T, *P. diazotrophica* JPY461^T, and *P. phymatum* STM815^T of 94.4%, 93.6% and 86%, respectively (Table 1). The ANI values between strain CNPSo 3155^T, *P. tuberum* STM678^T and *P. sprentiae* WSM5005^T were 92.7% and 90.6%, respectively (Table 1). The ANI values are below the threshold (< 95%) suggested for species delineation, confirming that the Psp1 and Psp6 clades represent two novel *Paraburkholderia* species.

An *in silico* dDDH between CNPSo 3157^T, CNPSo 3155^T and closely related *Paraburkholderia* species was estimated. For the CNPSo 3157^T strain, the DNA–DNA relatedness values were 56.9%, 54.4% and 29.8% with *P. piptadeniae* STM 7183^T, *P. diazotrophica* JPY461^T, and *P. phymatum* STM815^T, respectively (Table 1). For the CNPSo 3155^T strain, the highest values of dDDH were 49.7% with *P. tuberum* STM678^T and 41.7% with *P. sprentiae* WSM5005^T (Table 1). The dDDH values were also below the threshold for species delimitation (Rosselló-Móra et al. 2011), corroborating that CNPSo 3157^T and CNPSo 3155 ^{T} do not belong to any described *Paraburkholderia* species.

The G + C contents of CNPSo 3157^T and CNPSo 3155^T were estimated at 62.3 mol% and 63.0 mol%, respectively, as expected for the species of the *Paraburkholderia* genus (Dobritsa and Samadpour 2016).

The BOX-PCR analysis revealed intra- and interspecific diversity among the Psp1 and Psp6 strains, differing significantly from the other *Paraburkholderia* species used for comparison (Fig. S3).

Phylogeny of *nodC* gene and nodulation host-range ability

To investigate the evolutionary story of the nodulation ability, we performed a phylogenetic analysis of the *nodC* nodulation gene, chosen due to its role in the Nod factors synthesis, straight related to host specificity. All Psp1 strains were grouped in the same cluster together with *P. piptadeniae* STM 7183 ^{T}, with high bootstrap support (Fig. 3). The Psp6 strains clustered together with 100% bootstrap value and did not group with any other *Paraburkholderia* species (Fig. 3). Clades Psp1 and Psp6 shared similarity of 77.3% in this gene, and strains within both clades shared 100% NI with each other (data not shown). Interestingly, although CNPSo 3155^T and CNPSo 3157 T were isolated from the same geographic region, the *nodC* phylogeny revealed that they underwent separate evolutionary events in their nodulation genes (Fig. 3).

Therefore, although Psp1 and Psp6 strains have been isolated from soils of the same site of the Brazilian Atlantic Forest, several trapped by *Piptadenia* (Bournaud et al. 2017; Dall'Agnol et al. 2017), we may conclude that they show different evolutionary histories for the symbiotic genes, once the CNPSo 3157^T group, but not the CNPSo 3155^T group was related to *P. piptadeniae*. In agreement with all phylogenies conducted, strains of the Psp1 clade, including CNPSo 3157^T are closely related to *P. piptadeniae* STM 7183^T, and this relationship is confirmed in the *nodC* phylogeny, sharing 100% of NI (Fig. 3). Previous studies showed that *P. piptadenie* occupies a different position in the *nodC* phylogeny when compared to other symbiotic *Paraburkholderia* (Bournaud et al. 2013, 2017), indicating that CNPSo 3157^T and *P. piptadeniae* belong to the same symbiovar. On the contrary, CNPSo 3155^T showed the highest NI value for the *nodC* gene (82.8%) with *P. ribeironis* STM 7296^T, which was also isolated in the study by Bournaud et al. (2017).

Strains of the Psp1 and Psp6 clades showed differences in nodulation capacity. Psp1 strains were able to nodulate common bean and *M. pudica*, but unable to nodulate siratro. Nodules in common bean were effective in fixing nitrogen, with internal light pink color inside the nodule (e.g., Fig.

Table 1 ANI and digital DNA–DNA hybridization (dDDH) values (percentages) between *Paraburkholderia atlantica* sp. nov. CNPSo 3155^T (Psp6), *Paraburkholderia franconis* sp. nov. CNPSo 3157^T (Psp1) and related species of *Paraburkholderia* type strains

Strain used as reference	*P. atlantica* CNPSo 3155^T		*P. franconis* CNPSo 3157^T	
	ANI (%)	dDDH (%)	ANI (%)	dDDH (%)
P. piptadeniae	82.97%	32.40%	94.48%	56.90%
P. phymatum	82.04%	24.00%	86.07%	29.80%
P. diazotrophica	82.78%	24.80%	93.6%	54.40%
P. tuberum	92.75%	49.70%	82.62%	24.80%
P. sprentiae	90.63%	41.70%	82.41%	24.30%
P. atlantica CNPSo 3155^T	–	–	82.77%	25%

S4), although not as effective as a highly efficient *R. tropici* strain (data not shown). Except for CNPSo 3002, the other Psp1 strains formed effective pink-colored nodules in *M. pudica*. Interestingly, Psp6 strains were able to nodulate common bean, *M. pudica* and siratro. As with Psp1 strains, nodules were effective in common bean showing light internal pink color in the nodules, but ineffective in siratro. Psp6 also formed effective nodules in *M. pudica*. It is important to note that although strain CNPSo 3196 was initially isolated from common bean, it has the ability to nodulate and form effective nitrogen-fixing nodules in *M. pudica*. Examples of nodules obtained in the evaluation of strains CNPSo 3155^T and CNPSo 3157^T are shown in Fig. S4.

Many studies have shown that noduliferous *Paraburkholderia* and *Mimosa* share an evolutionary relationship,

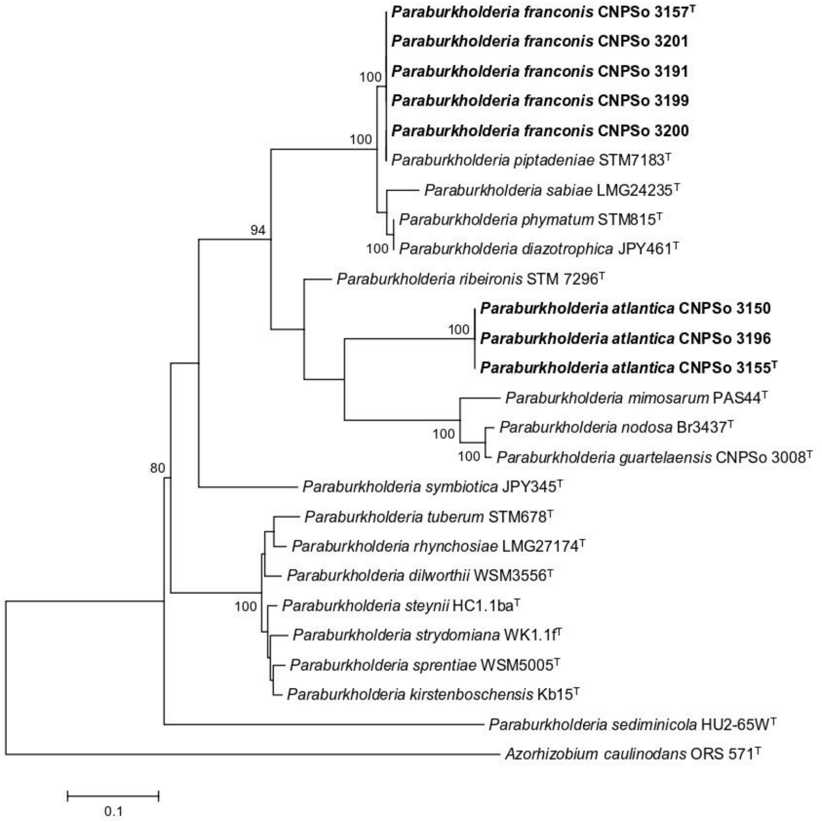


Fig. 3 Maximum-likelihood phylogeny based on sequences of *nodC* gene (347 bp) among novel *Paraburkholderia* species (in bold) and other nodulating members of the genus. Bootstrap values > 70% are indicated at the nodes. Accession numbers are indicated in Table S3. *Azorhizobium caulinodans* ORS 571^T was used as outgroup. Scale bar indicates ten substitutions per 100 nucleotide positions

suggesting coevolution. In the Cerrado and Caatinga biomes (Brazil) over 200 species of *Mimosa* can be found (Simon et al. 2011), and within them, many species are reported to be nodulated by a broad range of *Paraburkholderia* (Bontemps et al. 2010; dos Reis Junior et al. 2010). In an extensive study conducted by Bournaud et al. (2013), it was reported that *Paraburkholderia* are the most common and preferred symbiont of the *Piptadeniae* group, a genus closely related to *Mimosa* within the tribe Mimoseae. In another study conducted by Bournaud et al. (2017), the authors have shown that *Paraburkholderia* strains isolated from *Piptadenia gonoacantha* root nodules are able to nodulate and establish effective nodules with *M. pudica*. Altogether, and in addition to our study, the results reveal that *Paraburkholderia* host range and nodulation capacity are more widespread than expected.

Physiology

Strains of the Psp1 and Psp6 clades reveal similar properties in most of the analyzed tests. All strains were able to grow at 1% NaCl and under acid (pH 4.0) and basic (pH 8.0) conditions. Psp6 strains are urease positive, while Psp1 strains grew weakly or did not grow. Tolerance of antibiotics was similar for all strains, but carbon source utilization was more variable (Table S5). Comparisons of CNPSo 3157^T and CNPSo 3155^T strains and closely related *Paraburkholderia* species are shown in Table 2. Properties characterizing the new type strains CNPSo 3157^T and CNPSo 3155^T are included in the species description.

In conclusion, by using a polyphasic approach that combined phylogenetic analysis, genome sequencing, DNA fingerprinting and physiological features, it was concluded that strains positioned in Psp1 and Psp6 clades are representative of two novel species of the *Paraburkholderia* genus, for which the names *Paraburkholderia atlantica* sp. nov. and *Paraburkholderia franconis* sp. nov. are proposed, with CNPSo 3155^T and CNPSo 3157^T chosen as type strains, respectively.

Description of *Paraburkholderia atlantica* sp. nov.

Paraburkholderia atlantica (at.lan'ti.ca. L. neut. adj. atlantica pertaining to the Atlantic Forest biome of Brazil "Mata Atlântica").

Cells are Gram stain negative, aerobic, and rod shaped. Colonies in modified YMA (yeast–mannitol–agar) medium with Congo red are circular, opaque, with low production of mucus, light pink color and measure from 1.2 to 2.3 mm in diameter within 4 days of incubation at 28 °C. Strains produce acid reaction in modified YMA with bromothymol blue. Optimum growth occurs at pH 6.8 and 28 °C. Strains are able to grow at 28 °C in LB (Luria–Bertani) medium, in modified-YMA with pH 4.0 and 8.0, 1% NaCl and at 37 °C. They are positive for urease activity. Regarding carbon source, the type strain is capable of assimilating glycerol, D-glucose, L-rhamnose, *N*-acetylglucosamine, D-cellobiose, D-trehalose, amidon, glycogen, gentiobiose, D-fucose, L-fucose, potassium gluconate, potassium 2-ketogluconate and potassium 5-ketogluconate. It weakly assimilates 17 and does not assimilate 19 carbon sources (Table 2). Strains showed tolerance to the antibiotics bacitracin, chloramphenicol and cefuroxime and are sensitive to tetracycline, nalidixic acid, erythromycin, streptomycin and neomycin. The G + C content in the DNA of CNPSo 3155^T is 63.0 mol%.

The type strain is CNPSo 3155^T (= ABIP 239^T, = LMG 31643), isolated from nodules of *Mimosa pudica* grown in soil of the Brazilian Atlantic Forest, Rio de Janeiro State, Brazil.

Description of *Paraburkholderia franconis* sp. nov.

Paraburkholderia franconis (fran.co'nis. N.L. masc. gen. n. franconis, named after Dr. Antonio Avilio Franco, Brazilian researcher, who dedicated most of his work to the field of biological nitrogen fixation with legume trees).

Cells are Gram stain negative, aerobic, and rod shaped. Colonies in modified YMA (yeast–mannitol–agar) medium with Congo red are circular, opaque, with low production of mucus, light pink color and measure from 1.5 to 2.5 mm in diameter within 4 days of incubation at 28 °C. Strains produce acid reaction in modified YMA with bromothymol blue. Optimum growth occurs at pH 6.8 and 28 °C. Strains are able to grow in LB (Luria–Bertani) medium, in modified-YMA with pH 4.0 and 8.0, 1% NaCl and at 37 °C. They are negative for urease activity. The type strain is capable of assimilating: D-arabinose, D-glucose, D-fructose, D-mannose, rhamnose *N*-acetylglucosamine, D-saccharose, amidon, D-fucose, potassium gluconate and potassium 5-ketogluconate. It weakly assimilates 19 and it is unable to assimilate 20 carbon sources, respectively (Table 2). Strains are tolerant to the antibiotics (per disc) bacitracin (0.04 U), chloramphenicol (30 µg) and cefuroxime (30 µg) and sensitive to tetracycline (30 µg), nalidixic acid (30 µg), erythromycin (15 µg), streptomycin (15 µg) and neomycin (30 µg). The G + C content of CNPSo 3157^T is 62.3 mol%.

The type strain is CNPSo 3157^T (= ABIP 241^T, = LMG 31644), isolated from nodules of *Mimosa pudica* grown in soil of the Brazilian Atlantic Forest, Rio de Janeiro State, Brazil.

The following new sequences have been deposited in GenBank database:

16S rRNA of CNPSo 3157^T (MK690525.1), CNPSo 3191 (MK690527.1), CNPSo 3199 (MK690526), CNPSo 3200 (MK690528), CNPSo 3201 (MK690529), CNPSo 3150

Table 2 Phenotypic comparisons of CNPSo 3155^T and CNPSo 3157^T strain and related type strains of the genus *Paraburkholderia*

Characteristics	1	2	3	4	5	6	7
Growth at/in/with							
1% NaCl	+	+	−	−	−	−	−
37 °C	+	+	−	−	+	+	w
pH 4	+	+	w	+	+	+	+
pH 8	+	+	w	+	+	+	+
Urea 2%	+	−	+	w	+	+	−
LB	+	w	−	−	−	−	−
Tolerance to antibiotic (per disc)							
Chloramphenicol (30 µg)	+	+	−	+	−	−	w
Neomycin (30 µg)	−	−	w	−	−	w	w
Cefuroxime (30 µg)	+	+	−	−	−	+	+
Carbohydrates							
Glycerol	+	w	+	w	+	w	−
Erythritol	−	−	−	w	−	−	−
D-Arabinose	w	+	+	+	+	+	+
L-Arabinose	w	w	+	w	+	+	+
D-Ribose	w	w	w	+	w	+	w
D-Xylose	w	w	+	+	w	w	w
L-Xylose	w	w	−	+	−	w	w
D-Adonitol	w	w	+	+	w	+	w
Methyl-β D-xylopyranoside	−	−	w	−	−	−	−
D-Galactose	w	w	+	+	w	w	w
D-Glucose	+	+	+	+	w	w	w
D-Fructose	w	+	+	w	w	+	w
D-Mannose	w	+	+	+	+	w	w
L-Sorbose	−	w	−	−	−	−	−
D-Mannitol	w	w	w	+	w	w	w
Methyl-α D-mannopyranoside	−	−	w	−	−	−	−
N-acetylglucosamine	+	+	−	−	−	+	+
Amygdalin	−	−	w	−	−	−	−
Arbutin	−	−	+	−	−	−	−
Esculin ferric citrate	−	−	+	−	+	w	w
Salicin	−	−	w	−	w	w	−
D-Cellobiose	+	−	+	w	w	−	−
D-Lactose	w	−	w	w	w	−	−
D-Melibiose	−	−	−	w	w	−	−
D-Saccharose	−	+	+	+	w	w	+
D-Trehalose	+	w	w	w	w	w	+
D-Raffinose	−	−	−	w	−	−	w
Glycogen	+	w	w	w	+	+	+
Xylitol	w	−	w	w	−	w	w
Gentiobiose	+	w	+	+	+	−	−
D-Turanose	−	−	−	−	−	−	−
D-Lyxose	w	w	w	+	+	+	w
D-Tagatose	−	w	−	−	−	−	−
D-Fucose	+	+	+	+	+	+	+
L-Fucose	+	w	+	+	+	w	−
D-Arabitol	w	w	w	+	+	w	w
L-Arabitol	w	w	w	w	w	w	+
Potassium gluconate	+	+	+	+	+	+	+
Potassium 2-ketogluconate	+	−	−	−	+	+	+
Potassium 5-ketogluconate	+	+	+	+	w	w	w

Strains: 1 *P. atlantica* CNPSo 3155^T, 2 *P. franconis* CNPSo 3157^T, 3 *P. piptadeniae* CNPSo 3139, 4 *P. tuberum* STM678, 5 *P. diazotrophica* JPY461^T, 6 *P. phymatum* STM815^T, 7 *P. sprentiae* WSM5005^T. All data were obtained with two biological replicates. Growth (+), no growth (−), weakly positive (w)

(MK690530), CNPSo 3155^{T} (MK690531) and CNPSo 3196 (MK690533).

Genome sequences of: *Paraburkholderia franconis* CNPSo 3157^{T} (WHNP00000000, Biosample SAMN13050534) and *Paraburkholderia atlantica* CNPSo 3155^{T} (WHNQ00000000, Biosample SAMN13050733).

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Compliance with ethical standards

Competing interest All authors declare that they have no competing interests.

Ethics approval and consent to participate The authors declare no ethical conflicts. The authors declare that they have consented to participate in preparing the manuscript and publish it.

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Supplementary Material

Paraburkholderia atlantica sp. nov. and *Paraburkholderia franconis* sp. nov., two new nitrogen-fixing nodulating species isolated from Atlantic forest soils in Brazil

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Table S1 Strains used in this study.

Species/strains name	Other strain nomenclature	Original species grown in bulk soil / Trap -host plant	Geographical origin	Reference
Paraburkholderia franconis (Psp1)				
CNPSo 3157^{T}	ABIP 241^{T} LMG 31644^{T}	*Piptadeniae gonoacantha*/ *Mimosa pudica*	Búzios, Brazil 22° 26. 426S / 41° 51. 419W	Dall'Agnol et al. (2017)
CNPSo 3191	STM 10535	*Piptadeniae paniculata*/ *Mimosa pudica*	Rio das Ostras, Brazil 22° 45. 345S / 41° 57. 890W	Dall'Agnol et al. (2017)
CNPSo 3199	STM 10611	*Piptadeniae gonoacantha*/ *Mimosa pudica*	Búzios, Brazil 22° 26. 426S / 41° 51. 419W	Dall'Agnol et al. (2017)
CNPSo 3200	STM 10605	*Piptadeniae gonoacantha*/ *Mimosa pudica*	Búzios, Brazil 22° 26. 426S / 41° 51. 419W	Dall'Agnol et al. (2017)
CNPSo 3201	STM 10628	*Piptadeniae gonoacantha*/ *Mimosa pudica*	Búzios, Brazil 22° 26. 426S / 41° 51. 419W	Dall'Agnol et al. (2017)
Paraburkholderia atlantica (Psp6)				
CNPSo 3155^{T}	ABIP 239^{T} LMG 31643^{T}	*Piptadeniae gonoacantha*/ *Mimosa pudica*	Búzios, Brazil 22° 26. 426S / 41° 51. 419W	Dall'Agnol et al. (2017)
CNPSo 3150	ABIP 236	*Piptadeniae gonoacantha*/ *Mimosa pudica*	Búzios, Brazil 22° 26. 426S / 41° 51. 419W	Dall'Agnol et al. (2017)
CNPSo 3196	STM10835	*Piptadeniae gonoacantha*/ *Phaseolus vulgaris*	Búzios Brazil 22° 26. 426S / 41° 51. 419W	Dall'Agnol et al. (2017)
Paraburkholderia piptadeniae CNPSo 3139	ABIP 192	*Piptadeniae gonoacantha*/ *Mimosa pudica*	Búzios, Brazil 22° 26. 426S / 41° 51. 419W	Dall'Agnol et al. (2017)
Paraburkholderia piptadeniae STM STM7183^{T}	DSM 101189^{T}; LMG 29163^{T}.	*Piptadenia gonoacantha*	Cabo Frio, Brazil	Bournaud et al. (2017)
Paraburkholderia diazotrophica JPY461^{T}	KCTC 23308^{T}, LMG 26031^{T}	*Mimosa candollei*	Chapada dos Veiadeiros, Goiás State, Brazil	Sheu et al. (2013)
Paraburkholderia phymatum STM815^{T}	CCUG 47179^{T}, DSM 17167^{T}	*Machaerium lunatum*	French Guiana	Vandamme et al (2002)
Paraburkholderia tuberum STM678^{T}	CCUG 47178^{T}, DSM 18489^{T}	*Aspalathus carnosa*	South Africa	Vandamme et al (2002)

Paraburkholderia sprentiae WSM5005^T	HAMBI 3357^T, LMG 27175^T	*Lebeckia ambigua*	Western Cape of South Africa	De Meyer et al. (2013)

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Table S2 Primers and PCR conditions used in this study.

Gene	***Primer***	**Sequence (5’ - 3’)**	**PCR conditions**	**Reference**
16S rRNA	fD1	AGAGTTTGATCCTGGCTCAG	2 min 95°C, 30 x (15s 94°C, 45s 93°C, 45s 55°C, 2min 72°C) and 5 min 72°C	Weisburg et al. (1991)
	rD1	AAGGAGGTGATCCAGCC		
	362f*	CTCCTACGGGAGGCAGCAGTGGGG	*1 min 96 ºC, 35 x (15s 96ºC, 15s 50ºC, 4min 60ºC)	Menna et al. (2006)
	786f*	CGAAAGCGTGGGGAGCAAACAGG		
recA	*recA*-BurkF	AGGACGATTCATGGAAGAWAGC	2 min 95 ºC, 35 x (30s 94ºC, 30s 58ºC, 1min 72ºC), 5 min 72ºC	Spilker et al. (2009)
	recA-BurkR	GACGCACYGAYGMRTAGAACTT		
gyrB	gyrB-BurkF	ACCGGTCTGCAYCACCTCGT	2 min 95 ºC, 35 x (30s 94ºC, 30s 58ºC, 1min 72ºC), 5 min 72ºC	Spilker et al. (2009)
	gyrB-BurkR	YTCGTTGWARCTGTCGTTCCACTGC		
trpB	trpB-BurkF	CGCGYTTCGGVATGGARTG	2 min 95 ºC, 35 x (30s 94ºC, 30s 58ºC, 1min 72ºC), 5 min 72ºC	Spilker et al. (2009)
	trpB-BurkR	ACSGTRTGCATGTCCTTGTCG		
gltB	gltB-BurkF	CTGCATCATGATGCGCAAGTG	2 min 95 ºC, 35 x (30s 94ºC, 30s 58ºC, 1min 72ºC), 5 min 72ºC	Spilker et al. (2009)
	gltB-BurkR	CITGCCGCGGAARTCGTTGG		
nodC	nodCBurkF	TRATYGAYATGGAATACTGGC	3 min 95°C, 35 X (30s 95°C, 30s 55°C, 45s 55°C, 45s 72°C) and 7 min 72°C	Bournaud et al. (2013)
	nodCBurkR	CAGCGGAYATMGTCATTGA		
BOX	AIR1	ATGTAAGCTCCTGGGGATTCAC	7 min 94°C, 35 x (1min 94°C, 1min 52°C, 8 min 72°C) 16min 65°C	Versalovic et al. (1994)

*used for sequencing reaction

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Table S3 Accession number of the sequences used for the phylogenetic analysis.

Strains	16S rDNA	*recA*	*gyrB*	*gltB*	*trpB*	*rpoB*	*lepA*	*glnA*	*thrC*	*dnaK*	*nodC*
P. franconis CNPSo 3157^{T}	MK690525	KX814766	KX814597	KX814715	KX814839	WHNP00000000					KX814645
P. franconis CNPSo 3191	MK690527	KX814745	KX814587	KX814705	KX814829	ND	ND	ND	ND	ND	KX814626
P. franconis CNPSo 3199	MK704409	KX814763	KX814594	KX814712	KX814836	ND	ND	ND	ND	ND	KX814642
P. franconis CNPSo 3200	MK690528	KX814762	KX814593	KX814711	KX814835	ND	ND	ND	ND	ND	KX814641
P. franconis CNPSo 3201	MK690529	KX814768	KX814598	KX814717	KX814841	ND	ND	ND	ND	ND	KX814647
P. atlantica CNPSo 3155^{T}	MK690531	KX814764	KX814595	KX814713	KX814837	WHNQ00000000					KX814643.1
P. atlantica CNPSo 3150	MK690530	KX814760	KX814592	KX814710	KX814834	ND	ND	ND	ND	ND	KX814639
P. atlantica CNPSo 3196	MK690533	KX814798	KX814607	KX814726	KX814850	ND	ND	ND	ND	ND	KX814677
P. acidipaludis NBRC 101816^{T}	AB513180.1	NZ_BAXZ01000005.1	NZ_BAXZ01000002.1	NZ_BAXZ01000027.1	NZ_BAXZ01000036.1	ND	ND	ND	ND	ND	ND
P. aromaticivorans BN5^{T}	MF817715.1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
P. aspalathi LMG 27731^{T}	KC817488.1	FPBH01000010.1	KU723579.1	FPBH01000027.1	NZ_FPBH01000008.1	ND	ND	ND	ND	ND	ND
P. azotifigens NF2-5-3^{T}	KX510119.1	VOQS01000001.1	VOQS01000001.1	VOQS01000001.1	VOQS01000003.1	ND	ND	ND	ND	ND	ND
P. bannensis E25.2^{T}	AB561874.1	BAYA01000012.1	BAYA01000004.1	BAYA01000022.1	NZ_BAYA01000007.1	NZ_BAYA00000000.1	NZ_BAYA00000000.1	NZ_BAYA01000001.1	NZ_BAYA00000000.1	NZ_BAYA01000001.1	ND
P. bryophila LMG 23644^{T}	AM489501.1	NZ_QLTK01000002.1	NZ_QLTK01000008.1	QLTK01000013.1	NZ_QLTK01000010.1	NZ_QLTK01000013.1	NZ_QLTK01000002.1	NZ_QLTK01000001.1	NZ_QLTK01000003.1	QLTK01000022.1	ND

P. caballeronis Tne-841^{T}	FNSR01000001.1	FNSR01000001.1	FNSR01000001.1	SOCL01000023.1	FNSR01000002.1	ND	ND	ND	ND	ND	ND
P. caffeinilytica CF1^{T}	KT607985.1	CP031467.1	CP031467.1	NZ_CP031467.1	NZ_CP031466.1	ND	ND	ND	ND	ND	ND
P. caledonica LMG19076^{T}	AF215704.1	NZ_BAYE01000012.1	KU723584.1	NZ_BAYE01000017.1	NZ_BAYE01000018.1	NZ_BAYE00000000.1	JX429369.1	NZ_BAYE00000000.1	NZ_BAYE01000009.1	NZ_BAYE01000007.1	ND
P. caribensis MWAP64^{T}	Y17009.1	CP026101.1	CP026101.1	NZ_CP013102.1	CP026102.1	NZ_CP012746.1	NZ_CP013102.1	NZ_CP026101.1	NZ_CP012746.1	CP013102.1	ND
P. caseinilytica HM451^{T}	MF950896.1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
P. denitrificans KIS30-44^{T}	GU171384.1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
P. diazotrophica JPY461^{T}	NR_117848.1	NZ_FNYE01000008.1	KM655754.1	NZ_FNYE01000032.1	NZ_FNYE01000028.1	LT708250.1	NZ_FNYE01000012.1	NZ_FNYE01000009.1	NZ_FNYE01000001.1	FNYE01000026.1	NZ_FNYE01000044.1
P. dilworthii WSM3556^{T}	HQ698908.1	NZ_AWZT01000011.1	AWZT01000003.1	NZ_AWZT01000013.1	NZ_AWZT01000049.1	NZ_AWZT01000000	NZ_KI421431.1	NZ_AWZT01000003.1	AWZT01000002.1	AWZT01000006.1	NZ_AWZT01000045.1
P. dokdonella DCR-13	MH718804.2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
P. eburnea RR11^{T}	JQ692176.1	NZ_PQGA01000014.1	NZ_PQGA01000038.1	PQGA01000019.1	NZ_PQGA01000002.1	ND	ND	ND	ND	ND	ND
P. ferrariae FeGl01^{T}	DQ514537.1	NZ_BAYB01000011.1	NZ_BAYB01000021.1	NZ_BAYB01000028.1	NZ_BAYB01000001.1	NZ_BAYB01000063.1	NZ_BAYB01000016.1	NZ_BAYB01000003.1	NZ_BAYB01000006.1	NZ_BAYB01000014.1	ND
P. fungorum LMG 16225^{T}	AF215705.1	AJ549505.1	KU723574.1	ND	ND	ND	ND	ND	ND	ND	ND
P. guartelaensis CNPSo 3008^{T}	MK690537.1	SMOD01000007.1	SMOD01000009.1	NZ_SMOD01000026.1	NZ_SMOD01000011.1	SMOD00000000	SMOD00000000	SMOD00000000	SMOD01000013.1	NZ_SMOD01000006.1	NZ_SMOD01000053.1
P. ginsengisoli LMG 24044^{T}	AB201286.1	NZ_BBJF01000002.1	KU723581.1	ND	NZ_BBJF01000022.1	ND	ND	ND	ND	ND	ND
P. ginsengiterrae DCY85^{T}	KF915802.1	NZ_LXKA01000022.1	KX233702.1	NZ_LXKA01000065.1	NZ_LXJZ01000209.1	ND	ND	ND	ND	ND	ND

P. graminis C4D1M^T	U96939.1	NZ_ABLD01000009.1	NZ_ABLD01000012.1	ABLD01000026.1	NZ_ABLD01000002.1	NZ_ABLD01000060.1	ABLD01000023.1	CP024934.1	NZ_ABLD01000005.1	ABLD01000003.1	ND
P. heleia NBRC 101817^T	AB495123.1	NZ_BBJH01000021.1	NZ_BBJH01000020.1	ND	NZ_BBJH01000066.1	ND	ND	ND	ND	ND	ND
P. hiiakae I2^T	NR_146372.1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
P. hospita LMG 20598 ^{T}	CP026105.1	CP026105.1	CP026105.1	LT708278.1	CP026106.1	NZ_FNXA01000114.1	LT708257.1	NZ_CP026105.1	CP026105.1	NZ_FNXA01000034.1	ND
P. humisilvae Y-12 ^{T}	FJ796457.2	LC036348.1	LC036345.1	ND	ND	ND	ND	ND	ND	ND	ND
P. insulsa LMG 28183^T	KF733462.1	NZ_PVZM01000016.1	NZ_PVZM01000029.1	PVZM01000022.1	NZ_PVZM01000004.1	NZ_PVZM01000022.1	PVZM01000001.1	NZ_PVZM01000001.1	NZ_PVZM01000002.1	PVZM01000024.1	ND
P. jirisanensis JRM2-1 ^{T}	NR_148795.1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
P. kirstenboschensis KB15^T	NR_146352.1	NZ_JRZC01000422.1	NZ_JRZC01000001.1	LT708286.1	NZ_JRZC01000751.1	NZ_JRZC01000461.1	NZ_JRZC01000371.1	NZ_JRZC01000330.1	NZ_JRZC01000203.1	NZ_JRZC01000056.1	NZ_JRZC01000835.1
P. kururiensis KP23^T	NR_024721.1	NZ_RJZE01000007.1	NZ_RJZE01000024.1	RJZE01000029.1	NZ_RJZE01000028.1	NZ_RJZE01000029.1	NZ_RJZE01000006.1	NZ_RJZE01000002.1	NZ_RJZE01000010.1	NZ_RJZE01000014.1	ND
P. lacunae S27 ^{T}	MG745917.1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
P. megapolitana LMG 23650^T	AM489502.1	NZ_FOQU01000003.1	NZ_FOQU01000008.1	NZ_FOQU01000013.1	NZ_FOQU01000004.1	NZ_FOQU01000013.1	CP041745.1	NZ_FOQU01000005.1	NZ_FOQU01000007.1	FOQU01000002.1	ND
P. metalliresistens D414 ^{T}	KF601211.1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
P. metrosideri DNBP6-1 ^{T}	JF763856.1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
P. mimosarum PAS44^T	NR_043167.1	NZ_BBJJ01000016.1	AXAN01000006.1	AXAN01000030.1	NZ_BBJJ01000001.1	NZ_BBJJ01000097.1	AXAN01000020.1	NZ_BBJJ01000012.1	NZ_BBJJ01000008.1	AXAN01000002.1	NZ_BBJJ01000051.1
P. monticola JC2948^T	LRBG01000025.1	LRBG01000003.1	LRBG01000004.1	NZ_LRBG01000010.1	NZ_LRBG01000009.1	LRBG01000020.1	NZ_LRBG010000	NZ_LRBG010000	LRBG01000012.1	LRBG01000002.1	ND

							08.1	39.1			
P. nodosa Br3437^{T}	AY773189.1	JAFA01000018.1	JAFA01000005.1	JAFA01000023.1	NZ_JAFA01000004.1	NZ_JAFA01000073.1	HQ398539.1	NZ_JAFA01000008.1	NZ_JAFA01000011.1	JAFA01000001.1	NZ_JAFA01000055.1
P. oxyphila OX-01 ^{T}	AB488693.1	NZ_BAYD01000014.1	NZ_BAYD01000080.1	BAYD01000021.1	NZ_BAYD01000038.1	ND	ND	ND	ND	ND	ND
P. pallidirosea DHOK13^{T}	KP938221.1	KR092136.1	KR092135.1	ND	ND	ND	ND	ND	ND	ND	ND
P. panaciterrae DCY85-1 ^{T}	KF999960.1	KX233696.1	KX233697.1	ND	ND	ND	ND	ND	ND	ND	ND
P. paradisi WA ^{T}	NR_146374.1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
P. peleae PP52-1 ^{T}	JF763849.1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
P. phenazinium LMG 2247^{T}	U96936.1	NZ_FNCJ01000005.1	NZ_FNCJ01000001.1	LT708280.1	NZ_FNCJ01000023.1	LT708251.1	LT708259.1	NZ_FNCJ01000000	NZ_FNCJ01000003.1	FNCJ01000010.1	ND
P. phenoliruptrix LMG22037 ^{T}	AY435213.1	HQ398589.1	HQ849207.1	ND	ND	ND	ND	ND	ND	ND	ND
P. phymatum STM815 ^{T}	AJ302312.1	CP001043.1	CP001043.1	NC_010622.1	NC_010623.1	NC_010622.1	NC_010622.1	CP001043.1	NC_010622.1	NC_010622.1	NC_010627.1
P. phytofirmans PsJN ^{T}	NR_102845.1	CP001052.1	NC_010681.1	CP001052.1	CP001053.1	NC_010681.1	CP001052.1	CP001052.1	NC_010681.1	CP001052.1	ND
P. piptadeniae STM7183 ^{T}	LN875219.1	CYGY02000074.1	CYGY02000002.1	CYGY02000043.1	NZ_CYGY02000034.1	NZ_CYGY02000086.1	CYGY02000017.1	NZ_CYGY02000000	CYGY02000035.1	NZ_CYGY02000008.1	CYGY02000180.1
P. rhizosphaerae WR43 ^{T}	AB365791.2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
P. rhizoxinica HKI 454 ^{T}	AJ938142.1	FR687359.1	FR687359.1	FR687359.1	FR687359.1	ND	ND	ND	ND	ND	ND
P. rhynchosiae WSM3937 ^{T}	EU219865.1	PNXY01000005.1	PNXY01000017.1	PNXY01000009.1	PNXY01000024.1	ND	ND	ND	ND	ND	PNXY01000002.1
P. ribeironis STM7296 ^{T}	NR_156098.1	CYGX02000047.1	CYGX02000034.1	CYGX02000009.1	NZ_CYGX02000021.1	NZ_CYGX02000050.1	CYGX02000077.1	NZ_CYGX020000	CYGX02000060.1	NZ_CYGX020000	NZ_CYGX02000

								46.1		38.1	044.1
P. sabiae LMG 24235^T	AY773186.1	EU294397.1	LT632431.1	ND	ND	ND	ND	ND	ND	ND	ND
P. sacchari LMG 19450^T	AF263278.1	JTDB01000022.1	JTDB01000003.1	NZ_JTDB01000019.1	NZ_JTDB01000018.1	JTDB01000042.1	NZ_JTDB01000002.1	JTDB01000017.1	JTDB01000006.1	JTDB01000007.1	ND
P. sartisoli LMG 24000^T	AF061872.1	FNRQ01000001.1	NZ_FNRQ01000005.1	NZ_FNRQ01000010.1	NZ_FNRQ01000008.1	LT708252.1	LT708260.1	NZ_FNRQ01000001	NZ_FNRQ01000003.1	FNRQ01000002.1	ND
P. sediminicola LMG 24238^T	EU035613.1	NZ_FNIU01000013.1	NZ_FNIU01000005.1	FNIU01000045.1	NZ_FNIU01000012.1	ND	ND	ND	ND	ND	NZ_FNIU01000026.1
P. silvatlantica LMG 23149^T	AY965240.1	HQ849157.1	HQ849213.1	ND	ND	ND	ND	ND	ND	ND	ND
P. soli 24076^T	DQ465451.1	PNYB01000004.1	PNYB01000045.1	PNYB01000014.1	NZ_PNYB01000035.1	PNYB01000014.1	NZ_PNYB01000009.1	NZ_PNYB01000032.1	NZ_PNYB01000002.1	PNYB01000006.1	ND
P. solisilvaeY-47 ^{T}	FJ772068.2	LC036349.1	ND	ND	ND	ND	ND	ND	ND	ND	ND
P. sprentiae WSM5005^T	HF549035.1	AXBN01000087.1	AXBN01000095.1	AXBN01000088.1	AXBN01000039.1	NZ_CP017561	NZ_CP017561	NZ_CP017561.1	AXBN01000077.1	AXBN01000063.1	NZ_CP017565.1
P. steynii HC1.1ba^T	HF674712.1	NZ_MWML01000033.1	NZ_MWML01000048.1	LT708295.1	NZ_MWML01000343.1	NZ_MWML01000207.1	LT708274.1	NZ_MWML01000163.1	NZ_MWML01000220.1	NZ_MWML01000253.1	NZ_MWML01000063.1
P. strydomiana WK1.1f^T	HF674688	NZ_MWMK01000034.1	NZ_MWMK01000002.1	LT708294.1	NZ_MWMK01000026.1	NZ_MWMK01000101.1	NZ_MWMK01000037.1	NZ_MWMK01000025.1	NZ_MWMK01000063.1	NZ_MWMK01000010.1	NZ_MWMK01000078.1
P. susongensis L226^T	KJ746438.1	NZ_FXAT01000009.1	FXAT01000003.1	FXAT01000015.1	FXAT01000017.1	NZ_FXAT01000015.1	FXAT01000001.1	NZ_FXAT01000001.1	NZ_FXAT01000002.1	NZ_FXAT01000004.1	ND
P. symbiotica JPY345^T	HM357233.1	NZ_PTIR01000007.1	NZ_PTIR01000010.1	PTIR01000013.1	NZ_KB890166.1	NZ_PTIR01000013.1	NZ_PTIR01000001.1	NZ_PTIR01000018.1	NZ_PTIR01000036.1	NZ_PTIR01000014.1	NZ_PTIR01000032.1
P. telluris DHOC27^T	MF979827.1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
P. terrae KMY02^T	AB201285.1	CP026111.1	CP026111.1	NZ_BBJK01000103.1	NZ_BBJK01000137.1	CP026111.1	NZ_CP026111.1	CP026111.1	NZ_BBJK01000054.1	CP026111.1	ND

P. terrícola LMG 20594 ^{T}	AY040362.1	NZ_FRAB01000012.1	FRAB01000005.1	LT708282.1	FRAB01000009.1	ND	ND	ND	ND	ND	ND
P. tropica LMG 22274 ^{T}	AJ420332.1	FNZM01000020.1	FNZM01000005.1	LT708283.1	FNZM01000004.1	ND	ND	ND	ND	ND	ND
P. tuberum STM678 ^{T}	AJ302311.1	2512047030**									
P. unamae MTI-641 ^{T}	AY221956.1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
P. xenovorans LB-400 ^{T}	U86373.1	CP008760.1	CP008760.1	CP000270.1	CP008762.1	NZ_CP008760.1	CP008760.1	NZ_CP008760.1	CP008760.1	CP008760.1	ND
Cupriavidus taiwanensis LMG19424 ^{T}	F300324.2	CU633749.1	CU633749.1	CU633749.1	CU633749.1	ND	ND	ND	ND	ND	ND
Azorhizobium caulinodans ORS 571 ^{T}	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	AP009384.1

* ND, Not Determined.

** Genome deposited at the JGI/IMG/R

Table S4 Nucleotide identity (NI, %) of the 16S rRNA and housekeeping genes between *Paraburkholderia franconis* (Psp1 group, including CNPSo 3157^{T}), *Paraburkholderia atlantica* (Psp6 group, including CNPSo 3155^{T}) strains and closest *Paraburkholderia* species. Length of aligned regions: 16S rRNA (1,317 bp), *recA* (414 bp), *gyrB* (559 bp), *trpB* (568 bp), *gltB* (624 bp), MLSA with 4 genes (*recA*, *gyrB*, *gltB* and *trpB*) (2,152 bp) and MLSA with 9 genes (*recA*, *gyrB*, *gltB*, *trpB*, *rpoB*, *lepA*, *glnA*, *thrC* and *dnaK*) (19,600 bp). Not determined (ND).

	Nucleotide Identity (%)						
	16S	*recA*	*gyrB*	*trpB*	*gltB*	MLSA 4 genes	MLSA 9 genes
	Psp1 group						
Among strains	100	100	100	100	100	100	ND
Between strains and reference type strains:							
Paraburkholderia piptadeniae STM7183^{T}	99.6	97.1	96.4	94.5	98	96.5	96.9
Paraburkholderia diazotrophica JPY461^{T}	97.7	96.6	96.4	94.5	97.7	96.3	96.9
Paraburkholderia phymatum STM815T	96.8	93.9	91.3	89.6	94.2	91.6	93.4
Paraburkholderia caribensis MWAP64^{T}	96.3	92.7	94.9	91.1	94.1	93.2	93.6
Paraburkholderia hospita LMG 20598^{T}	97.1	92.2	94.6	91.9	93.9	93.2	94
Paraburkholderia terrae KMY02^{T}	96.9	92.9	92.8	91.7	94.2	92.7	93.4
Paraburkholderia steynii HC1.1ba^{T}	97.2	93.4	93.5	92.2	93.7	93.2	92
	Psp6 group						
Among strains	99.9-100	100	99.4-100	99.1-100	99.5-100	99.4-100	ND
Between strains and reference type strains:							
Paraburkholderia tuberum STM678^{T}	98.9-99	91	94.9-95.5	92-92.6	96.7-96.8	94.6	96
Paraburkholderia sprentiae WSM5005^{T}	98.5-98.6	97.5	92.1-92.2	90.6-90.8	96.2	93.9-94	95.1
Paraburkholderia monticola JC2948^{T}	97.8-97.9	96.8	93.1-93.3	91.3-91.7	95.7-95.9	94.1	95.7
Paraburkholderia susongensis L226^{T}	97.6	97.1	89.6-90.1	92-92.4	91.9-92.4	92.4-92.6	94.2
Paraburkholderia ribeironis STM 7296^{T}	97.2-97.3	94.9	89.4-89.9	86.6-86.7	93.9-94.2	91-91.2	92.7
	Psp6 group						

Psp1group	97.6	89.3	87-87.6	87.3	89.7-89.8	88.3-90.5	ND

	Paraburkholderia franconis CNPSo 3157^T						
Paraburkholderia atlantica. CNPSo 3155^T	97.3	89.3	87	87.3	89.7	90.3	90.1

Table S5 Phenotypic characteristics of *Paraburkholderia franconis* (Psp1) and *Paraburkholderia antlantica* (Psp6) strains.

Characteristics	Psp1 CNPSo strains				Psp6 CNPSo strains			
	3157^T	3191	3199	3200	3201	3150	3155^T	3196
Growth at/in/with:								
pH reaction in YMA + Bromothymol Blue	Acid	Acid	Acid	Acid	Acid	Acid	Acid	Acid
1% NaCl	+	+	+	+	+	+	+	+
37 °C	+	+	-	-	w	+	+	+
pH 4	+	+	+	+	+	+	+	+
pH 8	+	+	+	+	+	+	+	+
Urea 2%	-	w	-	w	w	+	+	+
LB	+	+	+	+	+	w	w	+
Tolerance (per disc):								
Tetracyclin (30 µg)	-	-	-	-	-	-	-	-
Bacitracin (0.04 U)	+	+	+	+	+	+	+	+
Chloramphenicol (30 µg)	+	+	+	+	+	+	+	+
Erythromycin (15 µg)	-	-	-	-	-	-	-	-
Neomycin (30 µg)	-	-	-	-	-	-	-	-
Nalidixic Acid (30 µg)	-	-	-	-	-	-	-	-
Streptomycin (15 µg)	-	-	-	-	-	-	-	-
Cefuroxine (30 µg)	+	+	+	+	+	+	+	+

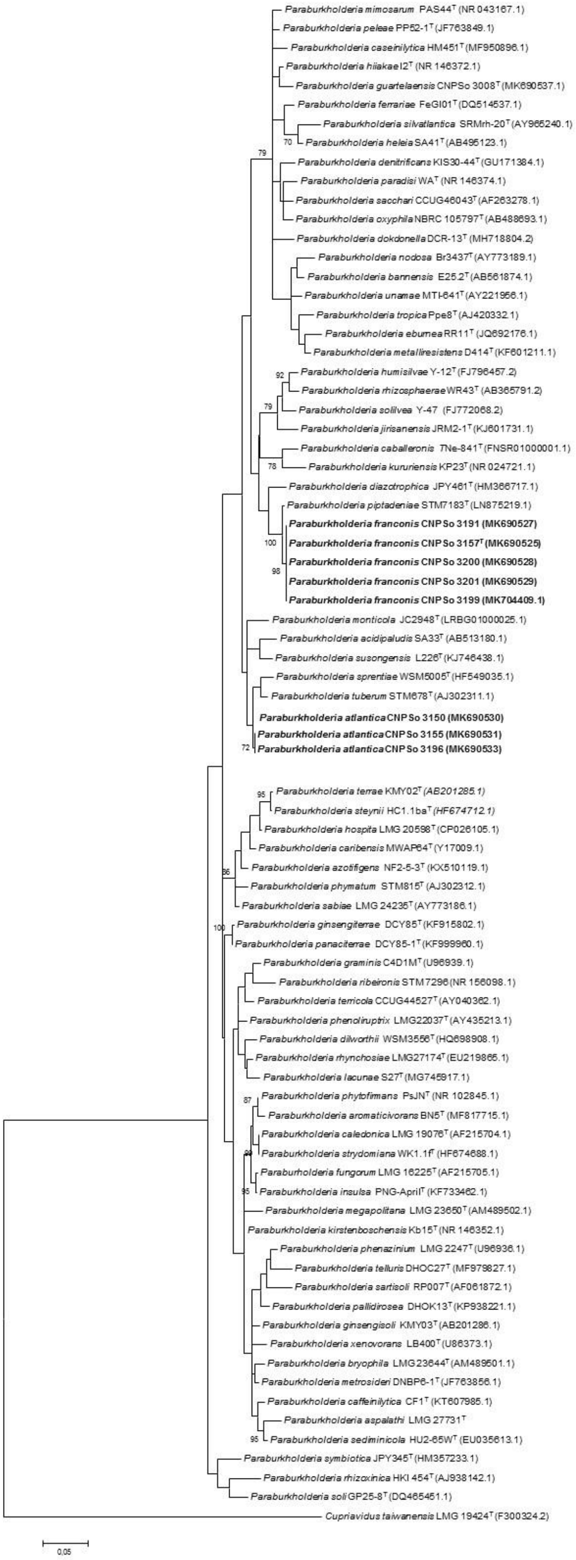

Fig. S1. Maximum-likelihood (ML) phylogeny based on 16S rRNA sequences (1,317 bp) between Psp1 and Psp6 strains and most of *Paraburkholderia* species. Bootstrap values > 70% are indicated at the nodes. Accession numbers are indicated in parentheses and in Table S3. Strains of the novel species are shown in boldface. *Cupriavidus taiwanensis* LMG 19424^{T} was used as outgroup. Scale bar indicates five substitutions per 100 nucleotide positions

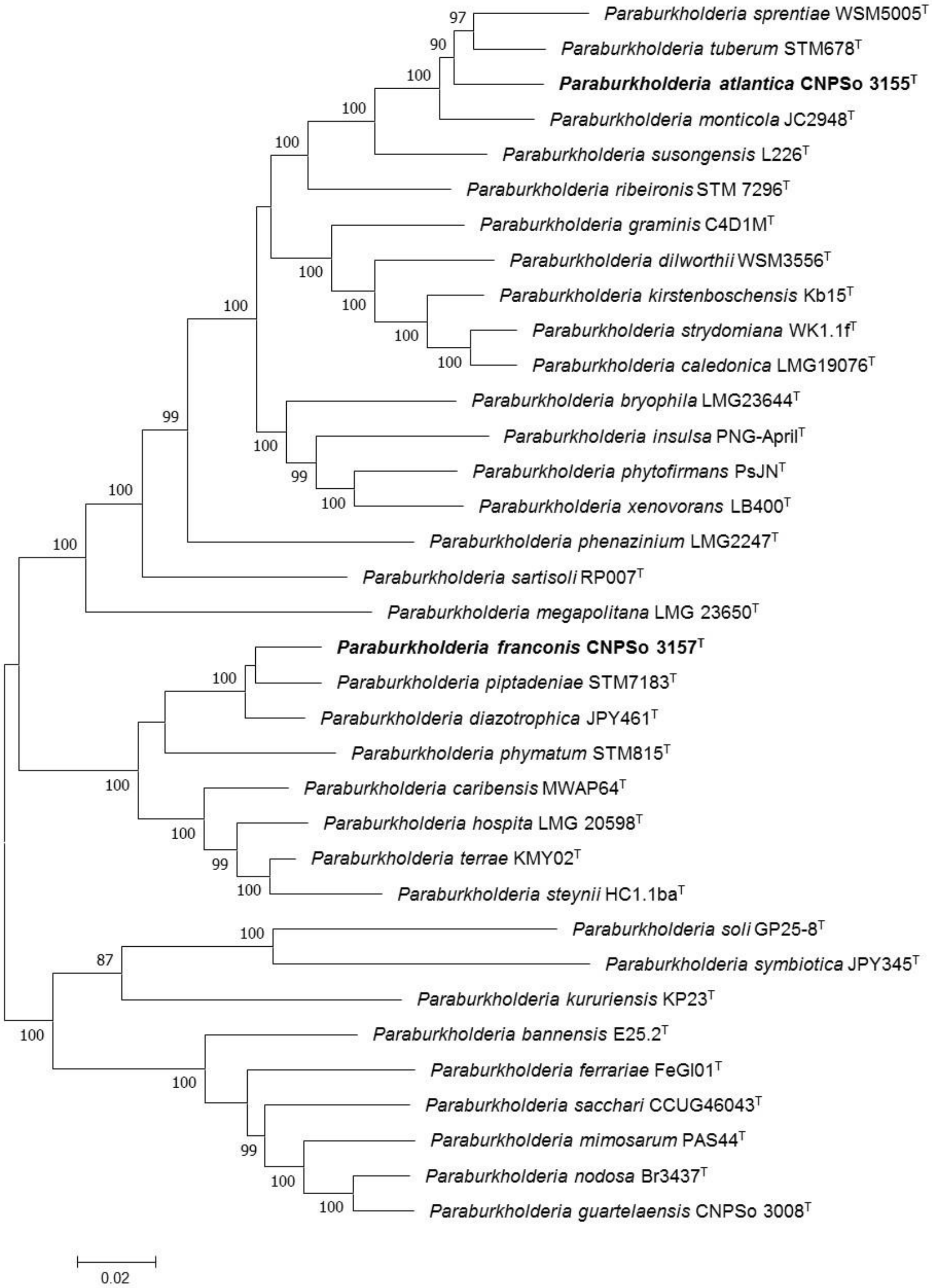

Fig S2 Maximum Likelihood (ML) phylogeny based on the concatenated aligned sequences (19,600 bp) of the *recA* (1,026 bp), *gyrB* (2,438 bp), *gltB* (4,394 bp), *trpB* (1,194 bp), *rpoB* (4,105 bp), *lepA* (1,791 bp), *glnA* (1,377 bp), *thrC* (1,446 bp) and *dnaK* (1,829 bp) genes showing the relationships between the novel *Paraburkholderia* species (in bold) and closely related members of the *Paraburkholderia* genus. Bootstrap values >70% are indicated at the nodes. Bar indicates two substitutions per 100 nucleotide positions. Accession numbers are indicated in Table S3.

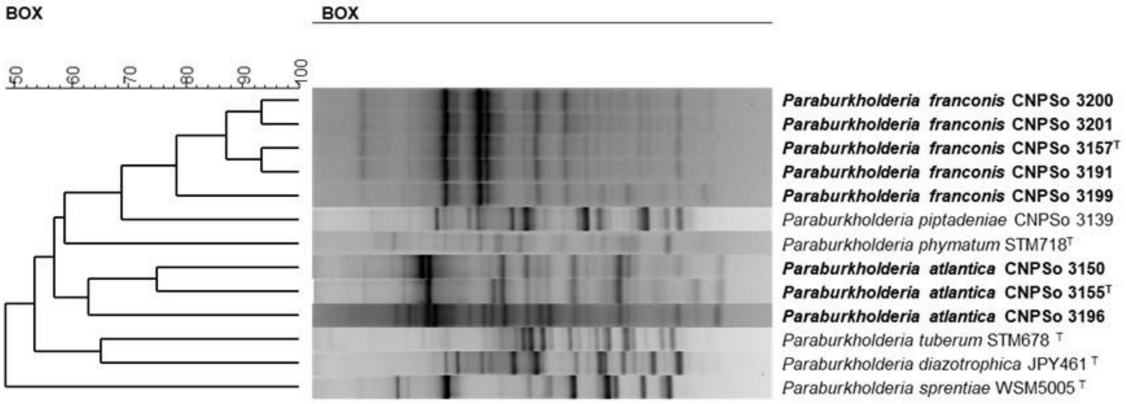

Fig. S3 Dendrogram of similarity based on BOX-PCR profiles of novel *Paraburkholderia* and related species, performed with the program Bionumerics (Applied Mathematics, Kortrijk, Belgium, v.7.6) using the UPGMA algorithm (Unweighted Pair-Group Method with Arithmetic mean) and the Jaccard coefficient, with 3% tolerance.

Fig. S4 Ineffective nodules formed by *Paraburkholderia atlantica* CNPSo 3155^T in (A) common bean (*Phaseolus vulgaris*) and (B) siratro (*Macroptilium atropurpureum*), and effective nitrogen-fixing nodules formed in (C) *Mimosa pudica;* Ineffective nodules formed by *Paraburkholderia franconis* CNPSo 3157^T in (D) common bean, absence of nodules in (E) siratro and effective nitrogen-fixing nodules formed in (F) *M. pudica.*

8. ESTUDO 3

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Phylogeny of symbiotic genes reveals symbiovars within legume-nodulating *Paraburkholderia* species

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Symbiotic genes

ABSTRACT

Bacteria belonging to the genus *Paraburkholderia* are capable of establishing symbiotic relationships with plants belonging to the Fabaceae (=Leguminosae) family and fixing the atmospheric nitrogen in specialized structures in the roots called nodules, in a process known as biological nitrogen fixation (BNF). In the nodulation and BNF processes several bacterial symbiotic genes are involved, but the relations between symbiotic, core genes and host specificity are still poorly studied and understood in *Paraburkholderia*. In this study, eight strains of nodulating nitrogen-fixing *Paraburkholderia* isolated in Brazil, together with described species and other reference strains were used to infer the relatedness between core (16S rDNA, *recA*) and symbiotic (*nod*, *nif*, *fix*) genes. The diversity of genes involved in the nodulation (*nodAC*) and nitrogen fixation (*nifH*) abilities was investigated. Only two groups, one containing three *Paraburkholderia* species symbionts of *Mimosa*, and another one with *P. ribeironis* strains presented similar phylogenetic patterns in the analysis of core and symbiotic genes. In three other groups events of horizontal gene transfer of symbiotic genes were detected. *Paraburkholderia* strains with available genomes were used in the complementary analysis of *nifHDK* and *fixABC* and confirmed well-defined phylogenetic positions of symbiotic genes. In all analyses of *nod*, *nif* and *fix* genes the strains were distributed into five clades with high bootstrap support, allowing the proposal of five symbiovars in nodulating nitrogen-fixing *Paraburkholderia*, designated as mimosae, africana, tropicalis, atlantica and piptadeniae. Phylogenetic inferences within each symbiovar are discussed.

Introduction

Diazotrophic symbiotic bacteria, generally called rhizobia, perform an important role in the nitrogen input into terrestrial systems, improving soil fertility and environmental sustainability. Rhizobia are capable of fixing atmospheric nitrogen (N_2) into available forms to plants, in a process known as biological nitrogen fixation (BNF), carried out by the enzymatic complex of nitrogenase [53]. The major contribution of the BNF process occurs in symbiotic associations with plants belonging to the Fabaceae (=Leguminosae) family, in specialized structures in the roots and occasionally in stems, called nodules [58].

Legume-rhizobia interactions initiate with a specific and coordinated molecular dialogue between the symbionts, in which the host plant exudates signals, mainly flavonoids, that will promote chemotaxis and the transcription of the regulator NodD protein in the compatible rhizobia, starting the expression of the nodulation genes that will lead to the synthesis and secretion of lipochitooligosaccharides (LCOs), called Nod factors (NF) [64,79]. When the host root hairs perceive the NFs, a series of morphological changes start that will lead to nodule organogenesis [57]

Abbreviations: NI, nucleotide identity; NF, Nod factors.

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A common core structure of the NFs results from the expression of the nodulation *nodABC* genes [1,79]. NFs are composed of a chitin-like *N*-acetyl glucosamine oligosaccharide backbone with a fatty acyl chain at the non-reducing end in most rhizobia, but different species vary in their backbone length and in the size and saturation of the fatty acyl chain. The *nodA* gene product recognizes and transfers specific fatty acids to the chitin oligomers, whereas *nodC* is involved in the synthesis and length of the backbone of glucosamine oligosaccharide [35,80].

After nodule organogenesis, rhizobia differentiate into nitrogen-fixing bacteroids, and the synthesis of the nitrogenase complex takes place. The *nif* genes are responsible for the synthesis, processing and assembly of the nitrogenase complex. The nitrogenase complex is composed by the iron-protein (encoded by *nifH*) and the molybdenum-iron protein (encoded by *nifD* and *nifK* genes), and the operon *nifHDK* is regulated by the transcriptional activator *nifA* [43,80]. The *nifA* gene also controls the expression of other genes, such as the *fixABCX* [41]. The *nif* genes do not determine host specificity; nevertheless, their phylogenies have been largely studied, as they represent important markers of symbiotic plasmids/island, contributing to the distinction between symbiovar groups [69]. Similarly, some *fix* genes are critical for nitrogen fixation. The proteins FixA, FixB, FixC and FixX have been confirmed to be involved in the electron transfer pathway dedicated to the generation of reductants for the nitrogenase activity [27].

The term symbiovar (symbiotic variant) was suggested by Rogel [69], as a parallel term to pathovar in pathogenic bacteria, to distinguish rhizobial subgroups that are symbiotically distinct within one species. Therefore, the symbiovars are defined based on the symbiotic capabilities to nodulate the host plants, distinguished by differences in host range. However, due to rhizobia and legume promiscuity, the identification of a symbiovar has been based on the analysis of symbiotic genes [63,67].

Paraburkholderia (formerly *Burkholderia*) comprise many symbiotic species isolated mostly from South Africa and South America. Brazil is considered a center of diversity for legume-nodulating *Paraburkholderia*, especially in the Cerrado, Caatinga and Atlantic Forest biomes [4,14,15,25]. *Paraburkholderia* are preferred symbionts in Brazil for *Mimosa* [4,10,25,62] although their nodulation ability has also been reported in plants of the *Piptadenia* group [7] and *Calliandra* species [72]. Another legume-nodulating *Paraburkholderia* diversity center is the Fynbos biome in South Africa, where the genus is associated with endemic Papilionoid plants [19,20,21,28,33,39,49]. Interestingly, mimosoid-nodulating and papilionoid-nodulating *Paraburkholderia* differ considerably in the host range, since South African *Paraburkholderia* seem not to be able to nodulate *Mimosa* [28,50]. Studies conducted with the comparative phylogenetic analysis of South American and South African *Paraburkholderia* indicate different evolution patterns of symbiotic genes [7,18,55].

The phylogenetic analysis of symbiotic genes is necessary for a better understanding of host range, symbiovar definition and evolutionary processes. The objective of this study was to verify the correlation between core and symbiotic genes and also with the host specificity and geographic location of *Paraburkholderia* species. Based on the results obtained, we report and describe five symbiovars of nodulating *Paraburkholderia*.

Material and methods

Strains selection, DNA extraction, gene amplification and sequencing

Eight *Paraburkholderia* strains isolated from *Mimosa* spp. and one strain isolated from *Phaseolus vulgaris* were chosen for this study. The strains were selected from previous studies carried out by our group [60,61] and are listed in Table S1. All strains are deposited at the "Diazotrophic and Plant Growth Promoting Bacteria Culture Collection of Embrapa Soja" (WFCC Collection # 1213, WDCM Collection #1054), in Londrina, State of Paraná, Brazil and in other culture collections. Stock cultures were maintained on modified-YMA medium [40] at 4 °C, and for long-term preservation strains were cryopreserved in modified-YM medium with 30% (v/v) glycerol at −80 °C and −150 °C, and lyophilized. The genomic DNA from these selected isolates was extracted using the DNeasy Blood and Tissue Kit (Qiagen), following the manufacturer's instructions.

The housekeeping genes 16S rRNA and *recA* were amplified as previously described [60]. For a better understanding of the evolutionary history of symbiotic genes, partial sequences of the nodulation genes *nodA* and *nodC* and the nitrogenase *nifH* gene were obtained. First, *nodA* and *nodC* genes were amplified according to the conditions described by Bontemps et al. [5] and Bournaud et al. [7], respectively, whereas the *nifH* gene amplification was performed with the primers and conditions described by Loiret et al. [52]. The PCR products were purified using the PureLink$^{\mathrm{TM}}$ Quick PCR Purification Kit (Invitrogen$^{\mathrm{TM}}$), following the manufacturer's instructions and sequenced using an ABI 3500xL (Applied Biosystems®). Sequences obtained in the study were submitted to the NCBI GenBank database and received the accession numbers from MT465888 to MT465925 (Table S2). In addition, we retrieved genomes and nucleotides sequences (*nifD*, *nifK*, *fixA*, *fixB*, *fixC*) of other nodulating species of *Paraburkholderia* from the GenBank and Joint Genome Portal (JGI/IMG/R) databases and the accession numbers are shown in the phylograms and/or in Table S2.

Phylogenetic analyses

The nucleotide sequences obtained in this study were assembled and corrected with the Bionumerics software (version 7.6). Multiple sequences alignments were performed with MUSCLE [26] and phylogenetic analyses were performed using MEGA 7 [44] with the maximum-likelihood (ML) algorithm.

For the 16S rRNA and the concatenated sequences of *fix* gene (*fixA*, *fixB*, *fixC*) phylogenies, the Tamura 3-parameter (T92) [76] model with gamma-distributed selection with invariant sites (G + I) was used. The *recA* and *nifH* phylogenies were performed with the T92 + G model. Phylogenies of single *nodC* and the concatenated sequences of *nodA* and *nodC* (*nodAC*) were built using T92 + I, while Kimura two-parameter + G [42] was used for the individual *nodA*. A phylogenetic tree of the concatenated *nifHDK* genes was conducted using Tamurei Nei + G model [76,77].

The statistical support for the trees was evaluated by bootstrap analysis with 1000 re-samplings [32]. Nucleotide identities (NI) were calculated using the Bioedit software (version 7.2.5) [37].

Results

Phylogenetic analysis of the 16S rRNA and recA genes

Aiming to compare core and symbiotic genes, first 16S rRNA (929 bp) (Fig. 1) and *recA* (414) (Fig. 2) phylograms were built. In the 16S rRNA phylogram, a large cluster included *Paraburkholderia* spp. strains JPY58, JPY604, STM604, JPY251, UYCP14C and CCGE1002 with *P. atlantica* strains. The phylogenies of the strains previously studied in the description of *P. franconis* (CNPSo 3191, 3200 and 3157$^{\mathrm{T}}$) and *P. atlantica* (CNPSo 3196, 3150 and 3155$^{\mathrm{T}}$) was confirmed [61]. *P. piptadeniae* STM7183$^{\mathrm{T}}$ clustered together with *P. franconis* strains as the most closely related species. The remaining strains grouped together with *P. nodosa* CNPSo 1341, isolated from the Brazilian savanna (Cerrado) [15,16], and with *P. guarte-*

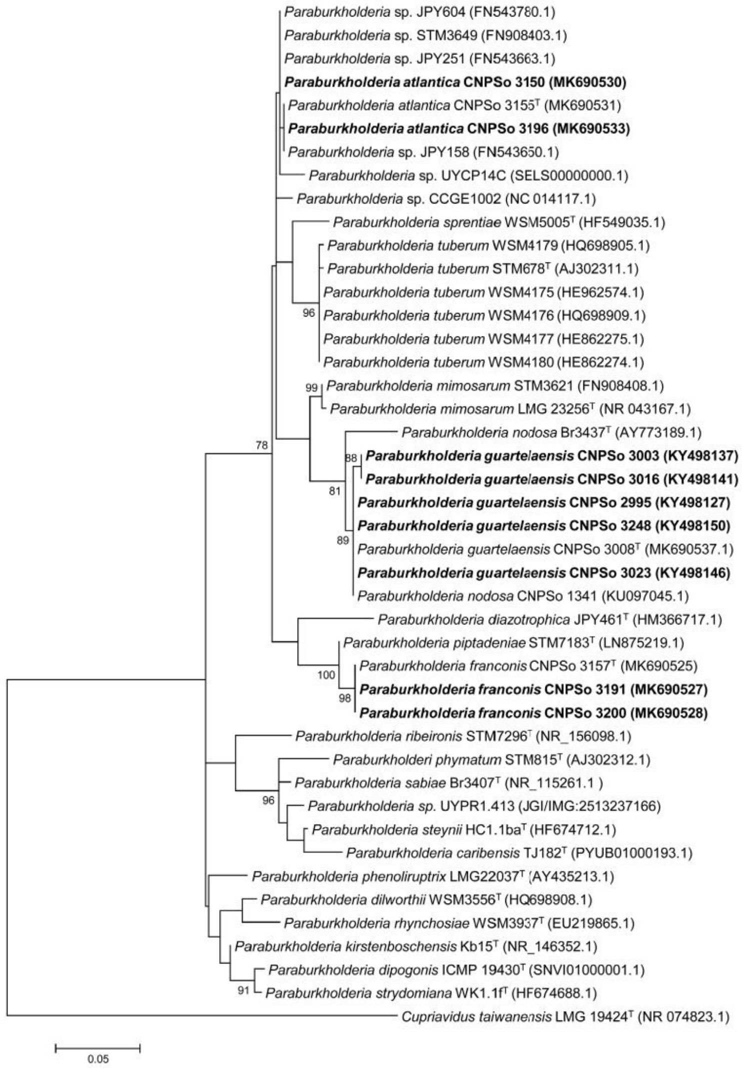

Fig. 1. Maximum likehood phylogenetic tree based on the 16S rRNA gene (929 bp) of nodulating *Paraburkholderia*. Accession numbers are indicated in parentheses and in Table S2. Strains under study are shown in boldface. Bootstrap values >70% are indicated at the nodes. *Cupriavidus taiwanensis* LMG 19424 was used as outgroup. Bar indicates five substitutions per 100 nucleotide positions.

laensis CNPSo 3008^T, in agreement with the study that indicated that the strains CNPSo 2995, CNPSo 3003, CNPSo 3016, CNPSo 3023 and CNPSo 3248 belong to this species [60]. Therefore, *P. nodosa* Br3437^T is the closest neighbor to *P. guartelaensis*, followed by *P. mimosarum* PAS44^T and *P. mimosarum* STM3621 (Fig. 1).

The phylogeny of the *recA* gene (Fig. 2) was in agreement with the 16S rRNA analysis (Fig. 1), indicating a consistent evolutionary pattern. It is worth mentioning that in both the 16S rRNA and *recA* phylograms, the strains classified as *P. tuberum*, JPY604, JPY158, STM3649, together with *Paraburkholderia* sp. CCGE1002 were more related to the newly described species *P. atlantica* than with *P. tuberum* STM678^T, indicating that they should be reclassified.

Phylogeny of Paraburkholderia *symbiotic genes*

Phylogenetic analyses including all strains from this study and available sequences of other *Paraburkholderia* type and reference strains were performed with concatenated *nodAC* gene fragments (876 bp) (Fig. 3), and with partial sequences of the *nifH* gene (326 bp) (Fig. 4). In the *nodAC* phylogram, four different clusters were observed, all with 100% of bootstrap support, while *P. ribeiro-*

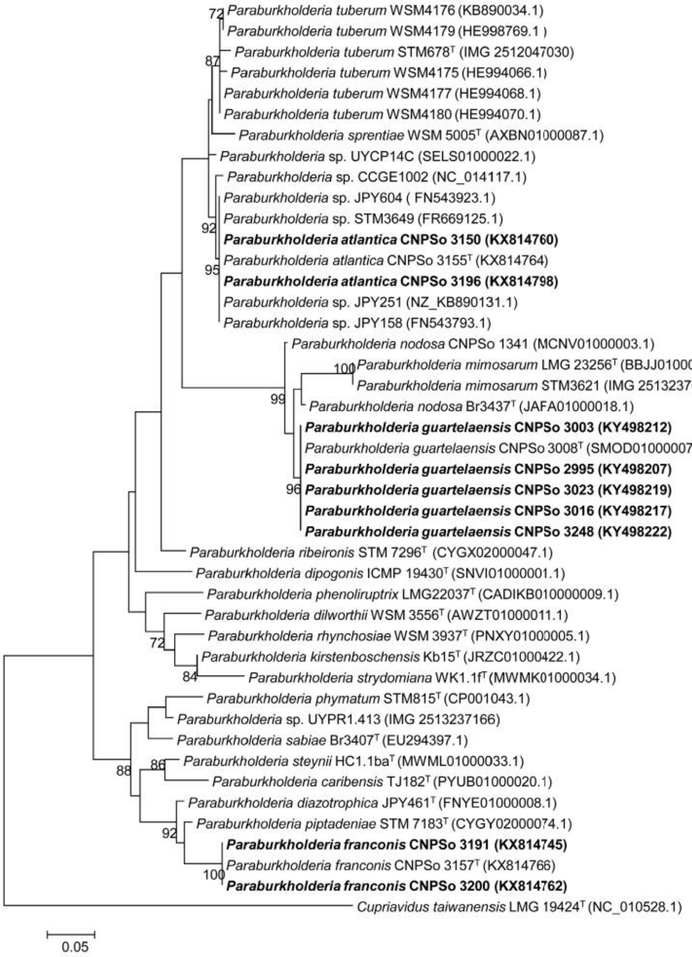

Fig. 2. Maximum-likelihood (ML) phylogeny based on parcial *recA* gene sequences (414 bp) showing the relationships between nodulating species of *Paraburkholderia*. Accession numbers are indicated in parentheses and in Table S2. Strains under study are shown in boldface. Bootstrap values >70% are indicated at the nodes. Scale bar indicates five substitutions per 100 nucleotide positions.

nis STM7296^T and *Paraburkholderia* sp. UYPR1.413 showed unique positions (Fig. 3). Single phylogenies of *nodA* (257 bp) (Fig. S1) and *nodC* (442 bp) (Fig. S2) confirmed the phylogenetic positions of the strains. Unfortunately, the *nodC* sequence of *P. sabiae* Br3407^T was not available in the databanks, therefore, we used the sequences of the strains *P. sabiae* STM7315 and *P sabiae* BRUESC861 in the *nodC* phylogeny (Fig. S2). The aligned sequences were shorter because of the inclusion of *P. sabia* STM 7315, with lower number of bp. Also, in the *nodC* phylogeny sequences of *P. ribeironis* STM7168 and STM7217 were used to verify the phylogenetic position of the *P. ribeironis* STM7296^T and all strains grouped together with 99% of bootstrap support. In congruence with the *nodAC* phylogenetic analysis (Fig. 3), five distinct clusters were observed in the *nifH* phylogram, all with high bootstrap support (Fig. 4). Based on the *nodAC* and *nifH* phylogenies, we propose five symbiovar clusters for nitrogen-fixing symbiotic *Paraburkholderia*. Each symbiovar cluster received the name of the first location or host plant of isolation. Other information as plant host, country of origin of the strains belonging to each symbiovar are shown in Table 1 and nucleotide identity information is presented in Table 2.

The first cluster of *nodAC* (Fig. 3) and *nifH* (Fig. 4) phylogenies composed the sv. mimosae (mi.mo'sae. N.L. gen. n. mimosae, first isolated from a plant of the genus *Mimosa*), grouping *P. mimosarum* strains STM3621 and LMG 23256^T, both isolated from Taiwan, all *P. guartelaensis* strains including CNPSo 3008^T, and *P. nodosa* strains Br3437^T and CNPSo 1341, all isolated from Brazilian soils. Strains of sv. mimosae shared among them 92–100% and 94–100% of NI in the *nodAC* and *nifH* phylogenies, respectively (Table 2).

The second cluster of sv. Africana (a.fri.ca'na. L. fem. adj. africana, first isolated from a legume in South Africa) grouped several strains isolated from South Africa, including *P. kirstenboschensis* KB15^T, *P. dilworthii* WSM3556^T, *P. rhynchosiae* WSM 3937^T, *P. spren-*

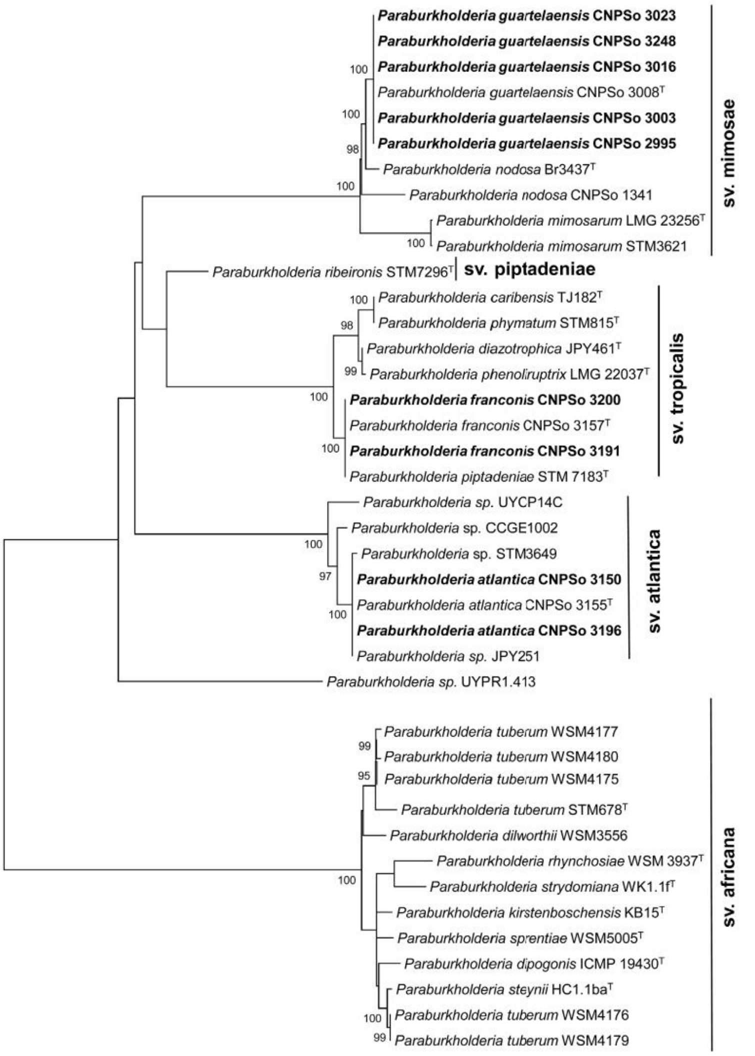

Fig. 3. Maximum-likelihood (ML) phylogeny based on the concatenated gene sequences (876 bp) of *nodA* (410 bp) + *nodC* (466 bp) showing the relationships between nodulating species of *Paraburkholderia*. Accession numbers are indicated in Table S2. Strains under study are shown in boldface. Bootstrap values >70% are indicated at the nodes. Scale bar indicates five substitutions per 100 nucleotide positions.

tiae WSM5005^{T}, *P. strydomiana* WK1.1ft^{T}, *P. steynii* HC1.1ba^{T}, and *P. tuberum* strains WSM4175, WSM4176, WSM4177, WSM4179, WSM4180 and STM678^{T}. *P. dipogonis* ICMP 19430^{T}, isolated from New Zealand, was also included in this cluster (Figs. 3 and 4). The NI among sv. africana strains ranged from 93 to 100% in the *nodAC* phylogeny and from 95.3 to 100 % in the *nifH* phylogeny (Table 2).

The third cluster, of sv. tropicalis (tro.pi.ca'lis. N.L. adj. tropicalis, first isolated from a legume in French Guiana, in the tropics), was named after *P. phymatum* STM815^{T}, isolated from *Machaerium lunatum* in French Guiana [78] and included *P. caribensis* TJ182 isolated in Taiwan. All other strains of this group were isolated in Brazil, and included *P. diazotrophica* JPY461^{T}, *P. franconis* strains CNPSo 3157^{T}, CNPSo 3191 and CNPSo 3200, *P. piptadeniae* STM 7183^{T}, *P. phenoliruptrix* BR3459a^{T} and *P. sabiae* Br3407^{T} (Figs. 3 and 4). The NI among the strains ranged from 95.8 to 100% and from 95.3 to 100% in the *nodAC* and *nifH* phylogenies, respectively (Table 2).

The fourth symbiovar, sv. atlantica (at.lan'ti.ca. L. fem. adj. atlantica, first isolated, from the Atlantic Forest) was named after *P. atlantica* CNPSo 3155^{T} and clustered *P. atlantica* strains CNPSo 3150, CNPSo 3196, JPY251, JPY158 and JPY604, isolated in Brazil and *Paraburkholderia* sp. CCGE1002 isolated in Mexico.

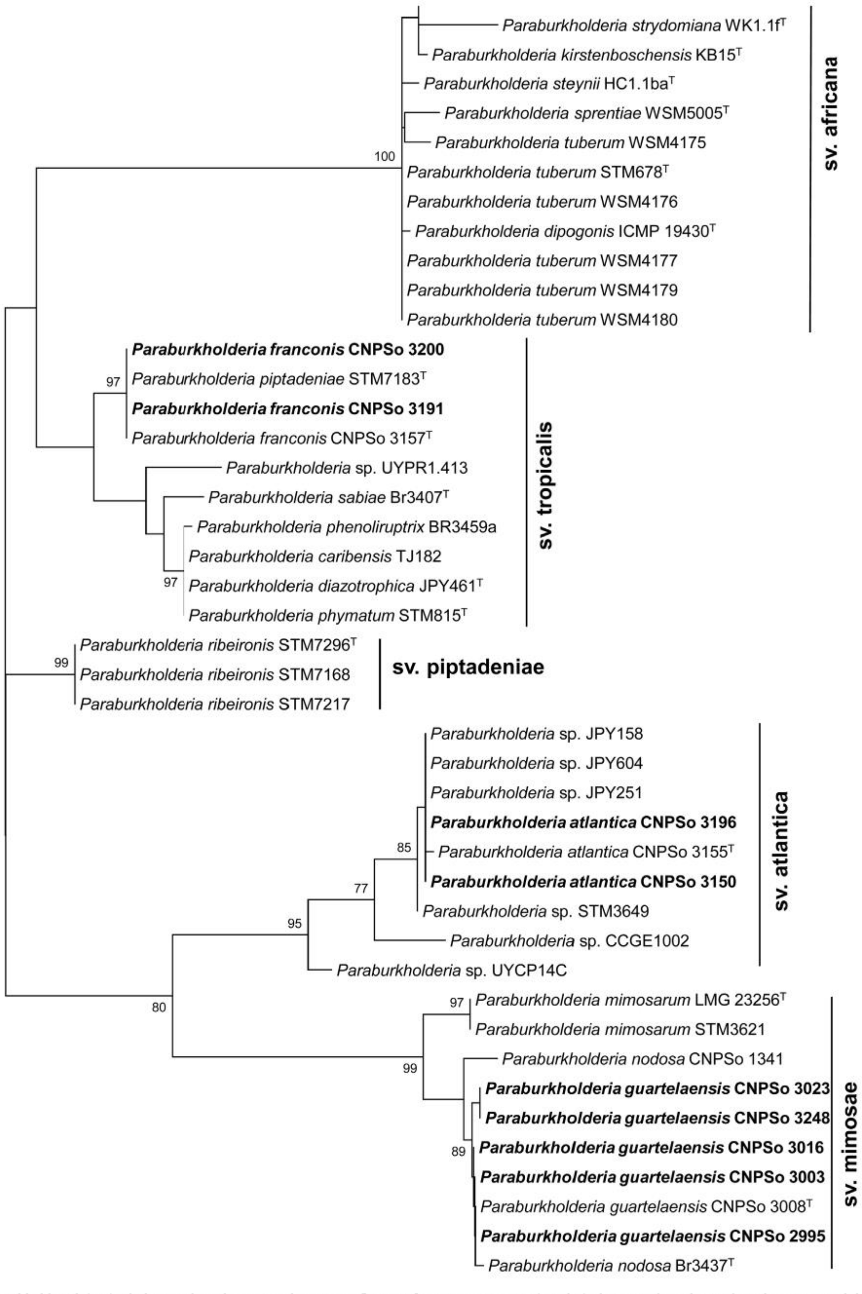

Fig. 4. Maximum-likelihood (ML) phylogeny based on partial nitrogen fixing *nifH* gene sequences (326 bp) showing the relationships between nodulating species of *Paraburkholderia*. Accession numbers are indicated in Table S2. Strains under study are shown in boldface. Bootstrap values >70% are indicated at the nodes. Scale bar indicates five substitutions per 100 nucleotide positions.

Table 1
Symbiovar clusters of nodulating Paraburkholderia.

Symbiovar cluster	Strain	Host	Country of origin	Also nodulate	References
sv. mimosae	*P. guartelaensis* CNPSo 3008^T	*Mimosa gymnas*	Brazil	*Mimosa pudica, Phaseolus vulgaris, Macroptilium atropurpureum*	Paulitsch et al. [60]
	P. guartelaensis CNPSo 2995				
	P. guartelaensis CNPSo 3003				
	P. guartelaensis CNPSo 3016				
	P. guartelaensis CNPSo 3023				
	P. guartelaensis CNPSo 3248				
	Paraburkholderia nodosa DSM 1341^T	*Mimosa scabrella*	Brazil	Mimosa spp/*Piptadenia, Parapiptadenia, Anadenanthera and Phaseolus vulgaris*	Chen et al. [10]; Bontemps et al. [4]; Bournaud et al. [7]; Dall'agnol et al. [14]
	Paraburkholderia nodosa CNPSo 1341	*Phaseolus vulgaris*	Brazil	ND	Dall'agnol et al. [15]
	Paraburkholderia mimosarum LMG 23256^T	*Mimosa pigra*	Taiwan	*Mimosa* spp., *Phaseolus vulgaris*, *Vigna unguiculata, Macroptilium atropurpureum*	Chen et al. [11,12]; Bontemps et al. [4]; Liu et al. [51]; Gehlot et al. [34]; Lardi et al. [46]
	Paraburkholderia mimosarum STM3621	*Mimosa pudica*	French Guiana	ND	Mishra et al. [54]
sv. africana	*Paraburkholderia tuberum* STM678^T	*Aspalathus carnosa*	South Africa	*Cyclopia* spp., *Lebeckia ambigua*, *Macroptilium, Atropurpureum, Crotalarieae*, *Hypocalypteae, Indigofereae*, and *Podalyrieae*	Moulin et al. [56]; Taulé et al. [78]; Elliot et al. [28]; Howieson et al. [39]; Lemaire et al. [47]; De Meyer et al. [22];
	Paraburkholderia kirstenboschensis KB15^T	*Virgilia oroboides*	South Africa	*Hypocalyptus sophoroides, Hypocalyptus oxalidifolius*	Beukes et al. [3]; Steenkamp et al. [74]
	Paraburkholderia dilworthii WSM3556^T	*Lebeckia ambigua*	South Africa	*Lebeckia sepiaria*	Howieson et al.[39]; De Meyer et al. [21]
	Paraburkholderia dipogonis ICMP 19430^T	*Dipogon lignosus*	New Zealand	ND	Liu et al. [50]; Sheu et al. [70]
	Paraburkholderia rhynchosiae WSM 3937^T	*Rhynchosia ferulifolia*	South African Western Cape	ND	De Meyer et al. [20]
	Paraburkholderia sprentiae WSM5005^T	*Lebeckia ambigua*	South Africa	ND	De Meyer et al. [19]
	Paraburkholderia strydomiana WK1.1f^T	*Hypocalyptus sophoroides*	South Africa	*Macroptilium atropurpureum, Vigna unguiculata*	Beukes et al. [2,3]
	Paraburkholderia steynii HC1.1ba^T				
	Paraburkholderia tuberum WSM4175	*Lebeckia ambigua*	South Africa	ND	De Meyer et al. [22]
	Paraburkholderia tuberum WSM4176			ND	De Meyer et al. [23]
	Paraburkholderia tuberum WSM4177			*Lebeckia sepiaria*	Howieson et al. [39]; De Meyer et al. [22]
	Paraburkholderia tuberum WSM4179			ND	De Meyer et al. [22]
	Paraburkholderia tuberum WSM4180				
sv. tropicalis	*Paraburkholderia caribensis* TJ182	*Mimosa diplotricha*	Taiwan	*Dalbergia louveli*	Taulé et al. [78], Chen et al. [13]; Rasolomampianina et al. [66]
	Paraburkholderia diazotrophica JPY461^T	*Mimosa candollei*	Brazil	Mimosa spp., *Anadenanthera, Parapiptadenia blanchetti, Piptadenia viridiflora, P. gonoacantha,Calliandra spp.*	Sheu et al. [71]; Bontemps et al. [4]; Mishra et al. [54]; Bournaud et al. [7]; Silva et al. [72]
	Paraburkholderia franconis CNPSo 3157^T	*Mimosa pudica*	Brazil	*Phaseolus vulgaris*	Paulitsch et al. [61]
	Paraburkholderia franconis CNPSo 3191				
	Paraburkholderia franconis CNPSo 3200				
	Paraburkholderia piptadeniae STM 7183^T	*Piptadenia gonoacantha*	Brazil	*Mimosa pudica*	Bournaud et al. [8]
	Paraburkholderia phenoliruptrix BR3459a	*Mimosa flocculosa*	Brazil	*Mimosa pudica, Macroptilium atropurpureum, Piptadenia gonoacantha, Microlobius foetidus Anadenanthera peregrina, Parapiptadenia rígida, Pityrocarpa monoliformis*	Zuleta et al. [81]; Bournaud et al. [7]

Table 1 (*Continued*)

Symbiovar cluster	Strain	Host	Country of origin	Also nodulate	References
	Parabukholderia phymatum STM815^T	*Machaerium lunatum*	French Guiana	Mimosa spp., *Phaseolus vulgaris, Parapiptadenia. Pterosperma, Cyclopia* spp., *Virgilia oroboides,*	Taulé et al. [78]; Elliott et al. [29]; dos Reis et al. [25]; Talbi et al. [75]; Mishra et al. [54]; Liu et al. [51]; Bournaud et al. [7]
	Paraburkholderia sabiae Br3407^T	*Mimosa caesalpiniifolia*	Brazil	Pitadeniae, Parapiptadeniae, Anadenanthera	Chen et al. [9,11]; Taulé et al. [77]; Bournaud et al. [7]; Silva et al. [72]
sv. atlantica	*Paraburkholderia atlantica* CNPSo 3155^T	*Mimosa pudica*	Brazil	*Phaseolus vulgaris, Macroptilium atropurpureum*	Paulitsch et al. [61]
	Paraburkholderia atlantica CNPSo 3150				
	Paraburkholderia atlantica CNPSo 3196	*Phaseolus vulgaris*		*Mimosa pudica, Macroptilium atropurpureum*	
	Paraburkholderia sp. CCGE1002	*Mimosa occidentalis*	Mexico	ND	Ormeño-Orrillo et al. [59]
	Paraburkholderia sp. JPY251	*Mimosa velloziana*	Brazil	ND	Gyaneshwar et al. [36]
	Paraburkholderia sp. UYCP14C	*Calliandra parvifolia*	Uruguay	ND	Langleib et al. [45]
	Paraburkholderia tuberum JPY158	*Mimosa pigra*	Brazil	ND	Bontemps et al. [4]
	Paraburkholderia tuberum JPY604	*M. xanthocentra var. xanthocentra*			
	Paraburkholderia tuberum STM3649	*Mimosa pudica*	French Guiana	ND	Mishra et al. [54]
sv. piptadeniae	*Paraburkholderia ribeironis* STM7296^T	*Piptadenia gonoacantha*	Brazil	*Mimosa pudica*	Bournaud et al. [8]
	Paraburkholderia sp. UYPR1.413	*Parapiptadenia rigida*	Uruguay	ND	Tamura et al. [77]

The other two strains within this symbiovar were isolated from neighbor countries, *Paraburkholderia* sp. UYCP14C (Uruguay) and *Paraburkholderia* sp. STM3649 (French Guiana) (Figs. 3 and 4). The NI in the *nodAC* and *nifH* ranged from 95.5 to 100% and from 94.7 to 100% respectively (Table 2).

The fifth and last symbiovar, sv. piptadeniae (pip.ta.de'ni.ae. N.L. gen. n. piptadeniae, first isolated from a plant of the genus *Piptadenia*) included only *P. ribeironis* species, isolated from soils of the Brazilian Atlantic Forest. *P. ribeironis* STM7296^T presented a unique position in the *nodAC* phylogeny (Fig. 3), and the phylogenetic position was confirmed with two other *P. ribeironis* strains, STM7168 and STM7217 in the analysis of *nifH* (Fig. 4), as well as on the single *nodC* phylogeny (Fig. S2). The NIs among the strains in the *nodAC*, *nodC* and *nifH* were of 100% (Table 2).

Paraburkholderia sp. strain UYPR1.413 presented unique positions in the *nodAC* (Fig. 3) and *nifH* (Fig. 4) phylograms, not grouping with any of the proposed symbiovars.

To confirm the phylogenetic position of the symbiovars, phylogenetic analyses of the concatenated sequences of *nifHDK* (3065 bp) (Fig. S3) and *fixABC* (2986 bp) (Fig. S4) genes were performed, including only nodulating *Paraburkholderia* strains with available genomes. All strains maintained the same clusters observed in the *nodAC* (Fig. 3) and *nifH* (Fig. 4) phylogenies. The exception was *Paraburkholderia* sp. strain UYPR1.413, that clustered with the sv. caribensis in the *nifHDK* phylogeny but confirmed a unique position in the *fixABC* phylogram, not allowing a clear classification of the symbiotic genes.

Discussion

Phylogenetic studies using core and symbiotic genes suggest that nodulating *Paraburkholderia* can be split into two major groups: (i) strains from Central and South America that nodulate *Mimosa* and other members of the Mimosoideae, also called Mimosoid-nodulating *Paraburkholderia* [7,8,36,60,73], and (ii) South African strains that nodulate papilionoid species native to the Cape Floristic Region (CFR) [18,33,47,49], also called Papilionoid-nodulating *Paraburkholderia* (Fig. 5).

Rogel et al. [69] suggested that one symbiovar represents a subset of strains, at the intra- or interspecific levels, differentiated on the bases of symbiotic abilities concerning the host plants and the variability of the symbiotic genes. Since this definition, as the phylogenies of *nodA* and *nodC* genes are intimately associated with host specificity, they have been used for symbiovar definition, with an emphasis on *nodC* [35,63,68,69,79].

Mishra et al. [54] were the first to suggest that symbiotic *Paraburkholderia* could present different symbiovars. In their study, the sv. mimosae comprised the American strains considered as *P. tuberum* and the sv. papilionoid comprised South African strains such as *P. tuberum* STM678^T. However, our results have clearly shown that symbiotic genes of nodulating *Paraburkholderia* are far more complex and diverse than previously thought, and the results do not agree with the two symbiovars proposed by Mishra et al. [54]. Our results indicate that the previously suggested "sv. mimosae" should be split into four different and well-defined symbiovars that are now proposed.

Based on the analysis of nodulation and nitrogen-fixation genes, we were able to identify five well-defined symbiovars in nodulating *Paraburkholderia*. Interestingly, the results confirm previous analyses of core and symbiotic genes by De Meyer et al. [18] and Estrada de los Santos et al. [31]. Based on our analysis of NI values (Table 2), we were also able to suggest a 92% threshold for symbiovar delimitation in the analysis of *nodA*, *nodC*, *nodAC*, *nifH*, *nifHDK* and *fixABC* genes of *Paraburkholderia*. This cut-off value has also been suggested for symbiotic genes of alpha-rhizobia [6,24].

The strains belonging to the sv. mimosae presented the same phylogenetic pattern in the analyses of core and symbiotic genes. Despite grouping few strains of a unique species, *P. ribeironis*, indications are that core and symbiotic genes are also congruent in sv. piptadeniae. However, many strains belonging to the three other symbiovars (sv. africana, sv. tropicalis and sv. atantica) showed different phylogenetic positions in the comparison of core and symbiotic genes phylogenies. This finding supports the hypothe-

Table 2
Nucleotide identity (NI, %) of core and symbiotic genes of nodulating *Paraburkholderia* belonging to four different symbiovars. Length of aligned regions were as follows: 16S rRNA (929 bp), recA (414 bp), nodAC (876 bp), nifH (326 bp), nifHDK (3065 bp) and fixABC (2986 bp).

16S rDNA

between:	sv. mimosae	sv. africana	sv. tropicalis	sv. atlantica	sv. piptadeniae
sv. mimosae	97.4–100	95.5–98.1	95.3–98.1	96.6–98.8	96.1–96.3
sv. africana	95.5–98.1	96–100	95.4 –98.9	96.4–99.1	96.2–97.1
sv. tropicalis	95.3–98.1	95.4–98.9	96.1–100	96–98.3	96–97
sv. atlantica	96.6–98.8	96.4–99.1	96–98.3	96.1–100	96.1–96.6
sv. piptadeniae	96.1–96.3	96.2–97.1	96–97	96.1–96.6	100

recA

between:	sv mimosarum	sv. africana	sv. tropicalis	sv. atlantica	sv. piptadeniae
sv. mimosae	95.4–100	85.7–92.7	86.9–92.9	89.8–92.9	89.3–91.3
sv. africana	85.7–92.7	89.3–100	87.9–98.5	89.8–99	91.3–95.1
sv. tropicalis	86.9–92.9	87.9–98.5	89.3–100	89.1–99.7	89.6–92.5
sv. atlantica	89.8–92.9	89.8–99	89.1–99.7	98.5–100	94.6–95.1
sv. piptadeniae	89.3–91.3	91.3–95.1	89.6–92.5	94.6–95.1	100

nodAC

between:	sv. mimosae	sv. africana	sv. tropicalis	sv. atlantica	sv. piptadeniae
sv. mimosae	92.4–100	69.8–71.6	76.9–78.8	78.3–80	81.6–83.7
sv. africana	69.8–71.6	93–100	71.9–73.6	71.4–73.1	72.6–73.9
sv. tropicalis	76.9–78.8	71.9–73.6	95.8–100	77.6 –78.6	84.4–86.2
sv. atlantica	78.3–80	71.4–73.1	77.6–78.6	95.5–100	83.3–83.7
sv. piptadeniae	81.6–83.7	72.6–73.9	84.4–86.2	83.3–83.7	100

nifH

between:	sv. mimosae	sv. africana	sv. tropicalis	sv. atlantica	sv. piptadeniae
sv. mimosae	94.7–100	81.9–83.7	83.7–87.4	86.1–88.3	86.1–87.4
sv. africana	81.9–83.7	95.3–100	85.8–88.6	81- 84.9	85.2–86.8
sv. tropicalis	83.7–87.4	85.8–88.6	95.3–100	85.2–89.2	92–94.4
sv. atlantica	86.1–88.3	81–84.9	85.2–89.2	94.7–100	86.8–87.1
sv. piptadeniae	86.1–87.4	85.2–86.8	92–94.4	86.8–87.1	100

nifHDK

between:	sv. mimosae	sv. africana	sv. tropicalis	sv. atlantica	sv. piptadeniae
sv. mimosae	94.4–99.9	78.1–80.1	85.4–86	83–84.3	86.3–86.5
sv. africana	78.1–80.1	95.9–99.4	82.4–83.2	78.9–80.4	82.2–83.4
sv. tropicalis	85.4–86	82.4–83.2	96.6–100	83.9–85.5	90.9–91.4
sv. atlantica	83–84.3	78.9–80.4	83.9–85.5	92.8–99.9	86–87
sv. piptadeniae	86.3–86.5	82.2–83.4	90.9–91.4	86–87	100

fixABC

between:	sv. mimosae	sv. africana	sv. tropicalis	sv. atlantica	sv. piptadeniae
sv. mimosae	92.9–98.6	75.8–77.3	80.4–81.5	78.8–80.2	82.5–83.6
sv. africana	75.8–77.3	92–99.4	79.2–80.8	77–78.4	80.2–81.8
sv. tropicalis	80.4–81.5	79.2–80.8	94.1–100	81.8–83	88.1–89.6
sv. atlantica	78.8–80.2	77–78.4	81.8–83	94.7–99.9	84.7–85.5
sv. piptadeniae	82.5–83.6	80.2–81.8	88.1–89.6	84.7–85.5	100

sis that horizontal gene transfer of symbiotic genes occurred after the divergence of *Paraburkholderia* lineages [18]. The greater division in clades of Mimosa-nodulating and Papilionoid-nodulating *Paraburkholderia* is clear in the symbiotic gene phylogenies (*nodAC*, *nifH*, *nifHDK* and *fixABC*) (Figs. 3 and 4, S3 and S4). Strains belonging to the sv. mimosae are considered as *Mimosa*-nodulating *Paraburkholderia.* This correlation between core (16S rRNA, *recA*) and symbiotic genes (specially *nodC)* phylogenies has been reported in most *Mimosa*-nodulating *Paraburkholderia*, suggesting an ancestral acquisition of those genes, with rare events of horizontal gene transfer [4,7,18,60,61].

It is important to highlight that, in both 16S rRNA and *recA* phylogenies, some strains classified as *P. tuberum*, including JPY604 and JPY158 isolated from *Mimosa xanthocentra* and *M. pigra*, respectively, in Brazil [4], *Paraburkholderia* sp. STM3649 isolated from *M. pudica* in French Guiana [54], and *Paraburkholderia* sp. CCGE1002 isolated from *M. occidentalis* in México [59] are more phylogenetic related to the newly described *P. atlantica* species [61] than with *P. tuberum* STM678^T [78], and should be reclassified. Furthermore, *P. atlantica* CNPSo 3155^T was isolated from the Brazilian Atlantic forest, while *P. tuberum* STM678^T was isolated from South Africa, an additional endorsement that *Paraburkholderia* sp. JPY604, JPY158 and STM3649 are more closely related to *P. atlantica* species. As discussed before our results also do not agree with the suggestion [55] that *P. tuberum* species can include both the previously called "sv. mimosae" and "sv. papilionoideae". We clearly show that Center-South American and South African strains differ drastically regarding their symbiotic genes, demonstrating a different source of acquisition of these genes.

The third symbiovar tropicalis was named after *P. phymatum* STM815^T, isolated in French Guiana, in the tropics. This strain was first isolated from *Machaerium lunatum* but failed to re-nodulate

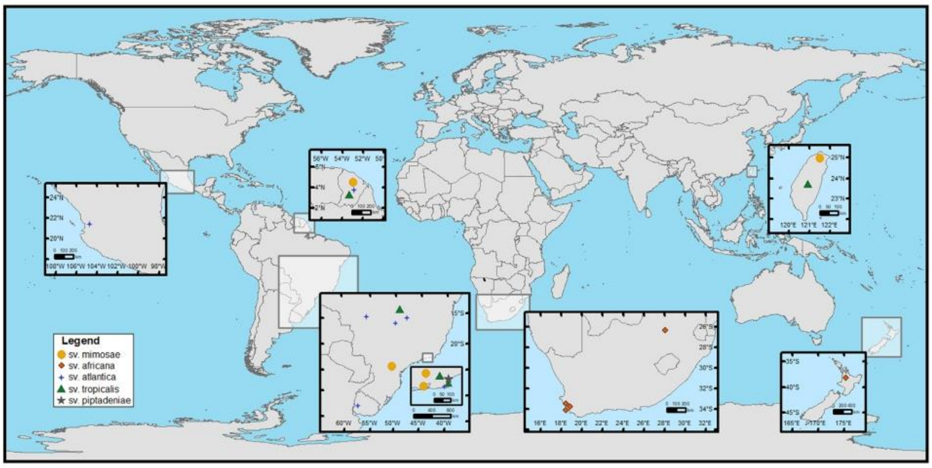

Fig. 5. World distribution of symbiovars of *Paraburkholderia*.

its original host or any *Machaerium* species [29]; however, it has the remarkable ability to nodulate many species of *Mimosa, Parapiptadenia, Pterosperma, Cyclopia* spp., *Virgilia oroboide* and *Phaseolus vulgaris* [7,25,28,29,49,51,54,75]. In our results, *P. phymatum* STM815^T and *P. caribensis* TJ182 presented almost identical patterns in all symbiotic genes, as also observed by Estrada-de los Santos et al. [31], implying that they share a close evolutionary history of symbiotic gene acquisition and that both are probably Mimosa-nodulating *Paraburkholderia*. Still in the sv. tropicalis, *P. sabiae* and *P. phenoliruptrix* are reported to carry *nodC* variants. In the study conducted by Bournaud et al. [7], *P. sabiae* strains showed two different *nodC* genes, exhibiting different nodulation phenotypes, indicating that the variability in the *nodC* affects both conditions of host specificity and effectiveness of nodules [7]. Recently, *P. sabiae* strains were also isolated from *Calliandra* spp. in Brazil, but all strains showed high similarity in the *nodC* gene [72]. As the *nodC* sequence of the type strain of *P. sabiae* is not available, we used two other strains. *P. sabiae* STM7315 came from the study of Bournaud et al. [7], representing the most frequent *nodC* variant (*nodC3*), while the other strain BRUESC861 was isolated by Silva et al. [72], and both shared high similarity (Fig. S2).

Bournaud et al. [7] also isolated *P. phenoliruptrix* strains and were the first to report nodulation in the *Piptadeniae* group. The *nodC* variants within this species were also observed and many strains harbor *nodC3*, as some of the *P. sabiae* strains, suggesting horizontal gene transfer between the species [7]. A comparative genomic analysis revealed that several *Mimosa*-nodulating *Paraburkholderia* present a unique copy of nodulation genes, whereas those of the papilionoid-nodulating clade present two copies of *nodC* and *nodB* genes, of different sizes, and located at a considerable distance in the chromosome [18]. Interestingly, the shorter *nodC* copy may also play a role in host range.

The sv. africana clustered South African CFR (Cape Floristic Region) strains isolated from nodules of native and endemic Papilionoid legumes, except for *P. dipogonis* ICMP 19430^T, isolated from nodules of *Dipogon lignosus* in New Zealand (Fig. 5). It is worth mentioning that both *P. tuberum* STM678^T and *P. dipogonis* ICMP 19430^T failed to nodulate mimosoids [28,50]. This reflects the highly divergent *nod* genes (and probably Nod factors) of the papilionoid and mimosa nodulating *Paraburkholderia*.

Finally, the sv. piptadeniae is for now represented only by one species, *P. ribeironis*, exhibiting the same phylogenetic pattern in all phylograms. *P. ribeironis* STM7296^T was isolated from the same geographic location and same plant host as *P. piptadeniae* STM 7183^T but presented different phylogenetic positions in the symbiotic genes analysis.

The *nodAC*, *nifH* and *nifHDK* (Figs. 3 and 4, S3) phylogenies revealed that the *nodABC* and *nifHDK* of the svs. mimosae, tropicalis, atlantica and piptadenia have probably been acquired simultaneously, given the monophyletic character [18]. Oppositely, *nod* and *nif* genes from papilionoid-nodulating *Paraburkholderia* belonging to sv. africana appear to have a different origin. Previous comparative genomic analysis [18,31] revealed that South-African strains have their symbiotic genes in chromosomal islands, while in strains from Central and South-American they are plasmid-borne. In the Papilionoid-nodulating strains, *nod* and *nif* genes are very closely related to alpha-rhizobia, particularly *Methylobacterium nodulans* and *Bradyrhizobium*, from which those genes have been probably acquired [18,31]. Altogether, this suggests a different evolutionary origin of these genes, similar to the observed in the sv. africana. Differences in nodulation genes should affect *Paraburkholderia* host range, explaining why South-African and American strains fail in nodulating each other hosts [36,47].

The phylogram of *fixABC* (Fig. S4) indicates the same evolutionary pattern of *nod* and *nif* phylogenies, reinforcing the proposed symbiovar clusters. Results obtained by De Meyer et al. [18] suggest that *fix* genes were obtained from a free-living diazotrophic ancestor in all beta-rhizobia, which could explain the ability of *P. phymatum* and *P. tuberum* to fix nitrogen *ex planta* [18,29]. However, in the study by De Meyer et al. [18] several differences in *nod*, *nif* and *fix* genes were detected between *Mimosa*-nodulating and *Papilionoid Paraburkholderia*, with an emphasis on *nifA*, indicating different evolutionary stories. Other phylogenetic studies with symbiotic *Paraburkholderia* are necessary to truly clarify the origin of symbiotic genes.

Differences in the phylogenetic position of core and symbiotic genes are also reported in alpha-rhizobia. For example, a study conducted with symbionts of *Phaseolus dumosus* shows that *Rhizobium* strains belonging to the sv. tropici present different position in the 16S rRNA and MLSA phylogenies. However, they share a very similar symbiotic plasmid, apparently indicating horizontal gene transfer [65].

In the sv. mimosae, *P. mimosarum* LMG 23256^{T} was isolated from the invasive *M. pigra* in Taiwan [12]. Interestingly, *M. pigra* is originally from tropical America, but is now widespread throughout the tropics (e.g. Australia, Africa, Southeast Asia, Florida) [38]. *M. pigra* was possibly introduced in these countries together with its symbionts, explaining the phylogenetic pattern with the other strains of the sv. mimosae. Furthermore, *P. mimosarum* STM3621 was isolated from French Guiana soils using *M. pudica* as trap plant [54], reaffirming that *P. mimosarum* strains have a South American origin.

Strains belonging to the sv. tropicalis, *P. piptadeniae* STM 7183^{T} and *P. franconis* strains CNPSo 3157^{T}, CNPSo 3191 and CNPSo 3200 are phylogenetic related and they have all been isolated from the Brazilian Atlantic Forest biome. Bournaud et al. [8] isolated *P. piptadeniae* using *Piptadenia gonoacantha* as trap plant and reported differences in the rhizobia diversity between site samples, indicating that soil conditions influence the survival and biogeography location of the symbionts, modulating rhizobia diversity [7].

Finally, *Paraburkholderia* sp. UYPR1.413 isolated from *Parapiptadenia rigida* in Uruguay did not group with any of the proposed symbiovars, indicating that others symbiovars should soon be described.

Altogether, our results confirm Brazil as a major center of nodulating nitrogen-fixing *Paraburkholderia* diversity [4,7,14,62,72], as well as South Africa [3,18,39,48] (Fig. 5), with potential influence of biogeographical [7,25,17,72] and abiotic [7,30,33,51,54] conditions, and of co-evolution with the host plants, that resulted in differences in the evolution of the symbiotic genes, clearly defining symbiovars. The delineation of symbiovars and other bacterial ecotypes contributes to the understanding of functional diversity and evolutionary ecology. We also highlight the importance of studies conducted with native and endemic species of legumes and their *Paraburkholderia* symbionts, as they are key to define the evolutionary relationships among the symbiotic partners.

The following new sequences have been deposited in GenBank database:

nodA of *P. guartelaensis* CNPSo 2995 (MT465921), *P. guartelaensis* CNPSo 3003 (MT465922), *P. guartelaensis* CNPSo 3016 (MT465923), *P. guartelaensis* CNPSo 3023 (MT465924), *P. guartelaensis* CNPSo 3248 (MT465925), *P. atlantica* CNPSo 3150 (MT465909), *P. atlantica* CNPSo 3196 (MT465910), *P. franconis* CNPSo 3191 (MT465907) and *P. franconis* CNPSo 3200 (MT465908).

nifH of *P. guartelaensis* CNPSo 2995 (MT465902), *P. guartelaensis* CNPSo 3003 (MT465903), *P. guartelaensis* CNPSo 3016 (MT465904), *P. guartelaensis* CNPSo 3023 (MT465905), *P. guartelaensis* CNPSo 3248 (MT465906), *P. atlantica* CNPSo 3150 (MT465890), *P. atlantica* CNPSo 3196 (MT465891), *P. franconis* CNPSo 3191 (MT465888) and *P. franconis* CNPSo 3200 (MT465889).

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Ethics approval and consent to participate

The authors declare no ethical conflicts; authors declare that they have consented to participate in the manuscript and publish it.

Competing interests

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.syapm.2020.126151.

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SUPPLEMENTARY MATERIAL

Phylogeny of symbiotic genes reveals symbiovars within legume-nodulating *Paraburkholderia* species

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Table S1

Strains used in this study.

Species/strains	Host plant	Geographical origin	Reference
Paraburkholderia guartelaensis CNPSo 2995	*Mimosa gymnas*	Paraná, Brazil	Paulitsch et al. (2019)
Paraburkholderia guartelaensis CNPSo 3003	*Mimosa gymnas*	Paraná, Brazil	
Paraburkholderia guartelaensis CNPSo 3016	*Mimosa gymnas*	Paraná, Brazil	
Paraburkholderia guartelaensis CNPSo 3023	*Mimosa gymnas*	Paraná, Brazil	
Paraburkholderia guartelaensis CNPSo 3248	*Mimosa gymnas*	Paraná, Brazil	
Paraburkholderia franconis CNPSo 3191	*Mimosa pudica*	Rio de Janeiro, Brazil	Paulitsch et al. (2020)
Paraburkholderia franconis CNPSo 3200	*Mimosa pudica*	Rio de Janeiro, Brazil	
Paraburkholderia atlantica CNPSo 3150	*Mimosa pudica*	Rio de Janeiro, Brazil	
Paraburkholderia atlantica CNPSo 3196	*Phaseolus vulgaris*	Rio de Janeiro, Brazil	

Paulitsch, F., Dall'Agnol, R.F., Delamuta, J.R.M., Ribeiro, R.A., da Silva Batista, J.S., Hungria, M. (2019) *Paraburkholderia guartelaensis sp.* nov., a nitrogen-fixing species isolated from nodules of *Mimosa gymnas* in an ecotone considered as a hotspot of biodiversity in Brazil. Arch. Microbiol. 201 1435-1446.

Paulitsch, F., Dall'Agnol, R.F., Delamuta, J.R.M., Ribeiro, R.A., da Silva Batista, J.S., Hungria, M. (2020) *Paraburkholderia atlantica* sp. nov. and *Paraburkholderia franconis* sp. nov., two new nitrogen-fixing nodulating species isolated from Atlantic forest soils in Brazil. Arch. Microbiol. 202, 1369–1380.

Table S2

Accession numbers of the sequences used for the phylogenetic analysis.

Strain	16S rRNA	*recA*	*nifH*	*nifD*	*nifK*	*fixA*	*fixB*	*fixC*	*nodA*	*nodC*
P. caribensis TJ182^T	PYUB01000193.1	PYUB01000020.1	PYUB01000189.1	PYUB01000100.1	NZ_PYUB01000100.1	PYUB01000048.1	PYUB01000048.1	PYUB01000048.1	NZ_PYUB01000048.1	NZ_PYUB01000048.1
P. diazotrophica JPY461^T	HM366717.1	NZ_FNYE01000008.1	NZ_FNYE01000099.1	NZ_FNYE01000062.1	NZ_FNYE01000062.1	NZ_FNYE01000044.1	NZ_FNYE01000044.1	NZ_FNYE01000044.1	NZ_FNYE01000044.1	NZ_FNYE01000044.1
P. dilworthii WSM3556^T	HQ698908.1	NZ_AWZT01000011.1	NZ_AWZT01000045.1	NZ_AWZT01000045.1	NZ_AWZT01000045.1	NZ_AWZT01000052.1	NZ_AWZT01000001.1	AWZT01000045.1	NZ_AWZT01000097.1	NZ_SNVI01000008.1
P. dipogonis ICMP 19430^T	SNVI01000001.1	SNVI01000001.1	NZ_SNVI01000008.1	NZ_SNVI01000008.1	SNVI01000008.1	SNVI01000008.1	NZ_SNVI01000008.1	SNVI01000008.1	NZ_SNVI01000008.1	NZ_SNVI01000008.1
P. guartelaensis CNPSo 3008^T	MK690537.1	SMOD01000007.1	NZ_SMOD01000089.1	NZ_SMOD01000089.1	NZ_SMOD01000089.1	SMOD01000010.1	SMOD01000010.1	SMOD01000010.1	NZ_SMOD01000053.1	NZ_SMOD01000053.1
P. kirstenboschensis KB15^T	NR 146352.1	NZ_JRZC01000422.1	NZ_JRZC01000819.1	NZ_JRZC01000819.1	NZ_JRZC01000819.1	NZ_JRZC01000824.1	JRZC01000815.1	JRZC01000815.1	NZ_JRZC01000824.1	NZ_JRZC01000835.1
P. mimosarum LMG 23256^T	FN908408.1	NZ_BBJJ01000016.1	AXAN01000068.1	AXAN01000068.1	NZ_AXAN01000068.1	AXAN01000044.1	AXAN01000044.1	AXAN01000044.1	NZ_BBJJ01000051.1	NZ_BBJJ01000051.1
P. mimosarum STM 3621	FN908408.1	IMG 2513237082	IMG 2513237082	IMG 2513237082	IMG 2513237082		no fix genes found		IMG 2513237082	IMG 2513237082
P. nodosa CNPSo 1341	KU097045.1	NZ_MCNV01000003.1	NZ_MCNV01000028.1	NZ_MCNV01000028.1	NZ_MCNV01000028.1	NZ_MCNV01000028.1	NZ_MCNV01000028.1	MCNV01000028.1	NZ_MCNV01000028.1	NZ_MCNV01000028.1
P. nodosa Br3437^T	AY773189.1	JAFA01000018.1	NZ_JAFA01000069.1	NZ_JAFA01000069.1	NZ_JAFA01000069.1	NZ_JAFA01000063.1	JAFA01000063.1	JAFA01000063.1	JAFA01000055.1	NZ_JAFA01000055.1
P. phymatum STM815^T	AJ302312.1	CP001043.1	NC_010627.1	NC_010627.1	NC_010627.1	CP001046.1	CP001046.1	NC_010627.1	CP001046.1	NC_010627.1
P. phenoliruptrix LMG 22037^T	AY435213.1	CADIKB010000009.1	CP003865.1	CP003865.1	NC_018696.1	CP003865.1	CP003865.1	NC_018696.1	NC_018696.1	NC_018696.1
P. piptadeniae STM 7183^T	LN875219.1	CYGY02000074.1	NZ_CYGY02000216.1	NZ_CYGY02000212.1	NZ_CYGY02000212.1	CYGY02000180.1	CYGY02000180.1	CYGY02000180.1	NZ_CYGY02000180.1	NZ_CYGY02000180.1
P. rhynchosiae WSM 3937^T	EU219865.1	PNXY01000005.1	PNXY01000002.1	PNXY01000002.1	PNXY01000002.1	PNXY01000002.1	PNXY01000002.1	PNXY01000002.1	NZ_PNXY01000002.1	NZ_PNXY01000002.1
P. ribeironis STM 7296^T	NR_156098.1	CYGX02000047.1	NZ_CYGX02000044.1	NZ_CYGX02000044.1	NZ_CYGX02000044.1	CYGX02000044.1	CYGX02000044.1	CYGX02000044.1	NZ_CYGX02000044.1	NZ_CYGX02000044.1
P. ribeironis STM 7217		ND	LN875252.1			ND				LN875245.1
P. ribeironis STM 7168		ND	LN875251.1			ND				LN875244.1
P. sabiae Br3407^T	NR_115261.1	EU294397.1	AY533867.1			ND			AY533872.1	ND
P. sabiae BRUESC861					ND					KT390825.1
P. sabiae STM7315					ND					HE983795.1
Paraburkholderia sp. UYPR1.413	(IMG 2513237166)	(IMG 2513237166)	(IMG 2513237166)	(IMG 2513237166)	(IMG 2513237166)	(IMG 2513237166)	(IMG 2513237166)	(IMG 2513237166)	(IMG 2513237166)	(IMG 2513237166)
Paraburkholderia sp. JPY251	FN543663.1	NZ_KB890131.1	NZ_KB890083.1	NZ_KB890083.1	NZ_KB890083.1	ARDA01000027.1	ARDA01000027.1	ARDA01000027.1	NZ_KB890083.1	NZ_KB890083.1
Paraburkholderia sp. UYCP14C	NZ_SELS00000000.1	SELS01000022.1	NZ_SELS01000114.1	NZ_SELS01000114.1	NZ_SELS01000114.1	NZ_SELS01000112.1	NZ_SELS01000112.1	NZ_SELS01000112.1	NZ_SELS01000107.1	NZ_SELS01000107.1
Paraburkholderia sp. CCGE1002	NC_014117.1	NC_014117.1	NC_014120.1	NC_014120.1	NC_014120.1	NC_014120.1	NC_014120.1	NC_014120.1	NC_014120.1	NC_014120.1
P. sprentiae WSM5005^T	HF549035.1	AXBN01000087.1	AXBN01000051.1	AXBN01000051.1	NZ_KI421529.1	CP017562.1	CP017562.1	CP017562.1	NZ_KI421529.1	NZ_KI421529.1
P. steynii HC1.1ba^T	HF674712.1	NZ_MWML01000033.1	NZ_MWML01000063.1	NZ_MWML01000063.1	NZ_MWML01000063.1	MWML01000114.1	MWML01000114.1	MWML01000114.1	NZ_MWML01000518.1	NZ_MWML01000063.1
P. strydomiana WK1.1f^T	HF674688.1	NZ_MWMK01000034.1	NZ_MWMK01000060.1	NZ_MWMK01000060.1	NZ_MWMK01000060.1	MWMK01000060.1	MWMK01000060.1	MWMK01000060.1	NZ_MWMK01000113.1	NZ_MWMK01000078.1
Paraburkholderia sp. STM3649	FN908403.1	FR669125.1	FN908425.1			ND			FN908415.1	FR853110.1
P. tuberum WSM4177	HE862275.1	HE994068.1	HG422559.1			ND			HG934325.1	HG425190.1
P. tuberum WSM4180	HE862274.1	HE994070.1	HG422561.1			ND			HG934327.1	HG425192.1
P. tuberum WSM4175	HE962574.1	HE994066.1	HG422557.1			ND			HG934323.1	HG425188.1
P. tuberum WSM4179	HQ698905.1	HE998769.1	HG422560.1			ND			HG934326.1	HG425191.1
P. tuberum STM678^T	AJ302311.1				IMG 2512047030				AJ302321.1	IMG 2512047036
P. tuberum WSM4176	HQ698909.1	NZ_KB890034.1	NZ_KB890037.1	NZ_KB890037.1	NZ_KB890037.1	NZ_KB890037.1	ARCY01000043.1	NZ_KB890037.1	NZ_KB890037.1	NZ_KB890037.1
Paraburkholderia sp. JPY604	FN543780.1	FN543923.1	FN544057.1			ND				FN543637.1
Paraburkholderia sp. JPY158	FN543650.1	FN543793.1	FN543934.1			ND				FN543512.1
P. atlantica CNPSo 3155^T	MK690531	KX814764	NZ_WHNQ01000029.1	NZ_WHNQ01000029.1	NZ_WHNQ01000029.1		genome		WHNQ01000029.1	NZ_WHNQ01000029.1
P. franconis CNPSo 3157^T	MK690525	KX814766	NZ_WHNP01000186.1	NZ_WHNP01000072.1	NZ_WHNP01000072.1	NZ_WHNP01000015.1	NZ_WHNP01000015.1	NZ_WHNP01000015.1	NZ_WHNP01000015.1	NZ_WHNP01000015.1
Cupriavidus taiwanensis LMG 19424^T	NR_074823.1	NC_010528.1				ND				
P. guartelaensis CNPSo 2995	KY498127	KY498207	MT465902			ND			MT465921	KY498167.1
P. guartelaensis CNPSo 3003	KY498137	KY498212	MT465903			ND			MT465922	KY498171.1
P. guartelaensis CNPSo 3016	KY498141	KY498217	MT465904			ND			MT465923	KY498179.1
P. guartelaensis CNPSo 3023	KY498146	KY498219	MT465905			ND			MT465924	KY498183.1
P. guartelaensis CNPSo 3248	KY498150	KY498222	MT465906			ND			MT465925	KY498190.1
P. atlantica CNPSo 3150	MK690530	KX814760	MT465890			ND			MT465909	KX814639
P. atlantica CNPSo 3196	MK690533	KX814798	MT465891			ND			MT465910	KX814677

P. franconis CNPSo 3191	MK690527	KX814745	MT465888	ND	MT465907	KX814626
P. franconis CNPSo 3200	MK690528	KX814762	MT465889	ND	MT465908	KX814641

*ND: not determined

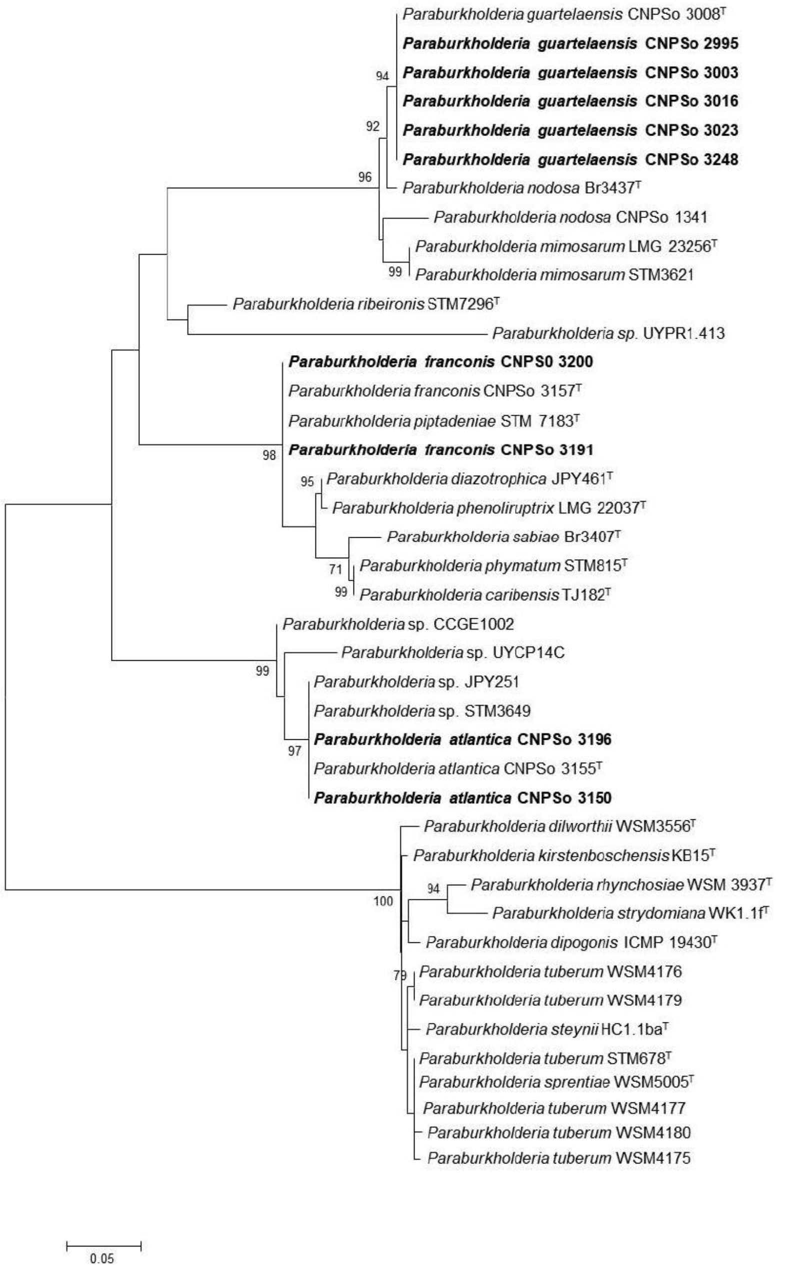

Fig. S1 - Maximum-likelihood (ML) phylogeny based on parcial *nodA* gene sequences (257 bp) showing the relationships between nodulating species of *Paraburkholderia*. Accession numbers are indicated in Table S2. Strains under study are shown in boldface. Bootstrap values > 70% are indicated at the nodes. Scale bar indicates five substitutions per 100 nucleotide positions.

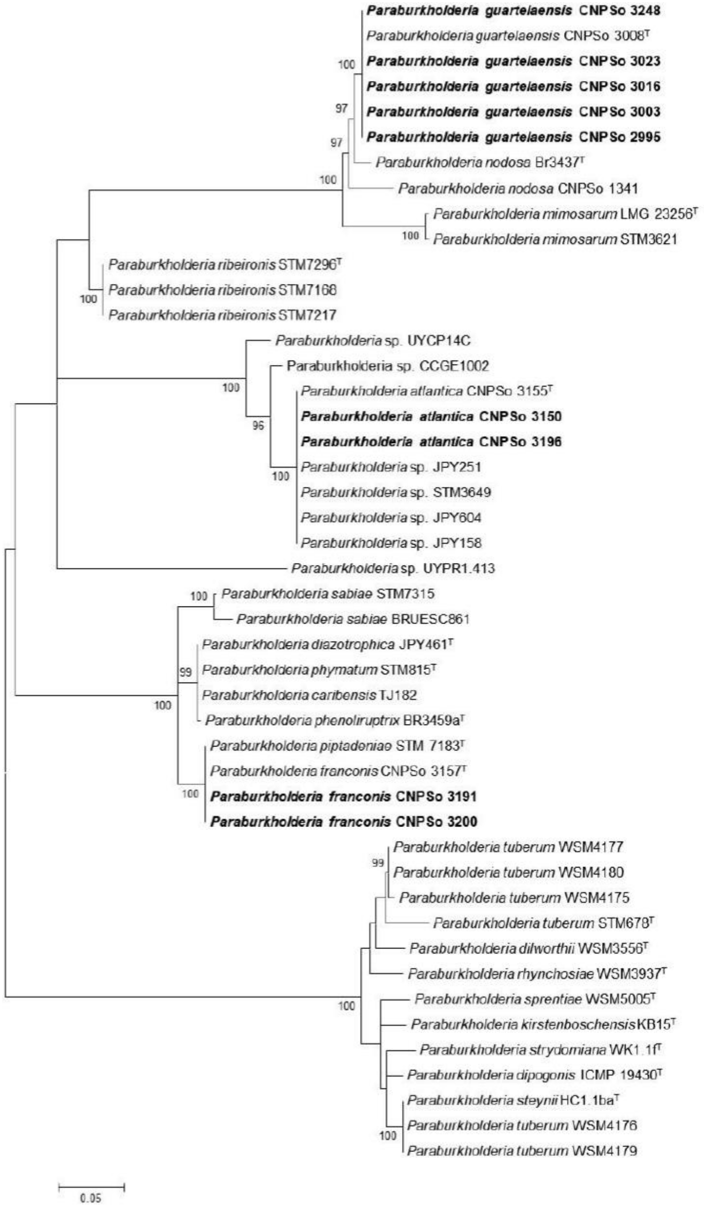

Fig. S2 - Maximum-likelihood (ML) phylogeny based on parcial *nodC* gene sequences (442 bp) showing the relationships between nodulating species of *Paraburkholderia*. Accession numbers are indicated in Table S2. Strains under study are shown in boldface. Bootstrap values > 70% are indicated at the nodes. Scale bar indicates five substitutions per 100 nucleotide positions.

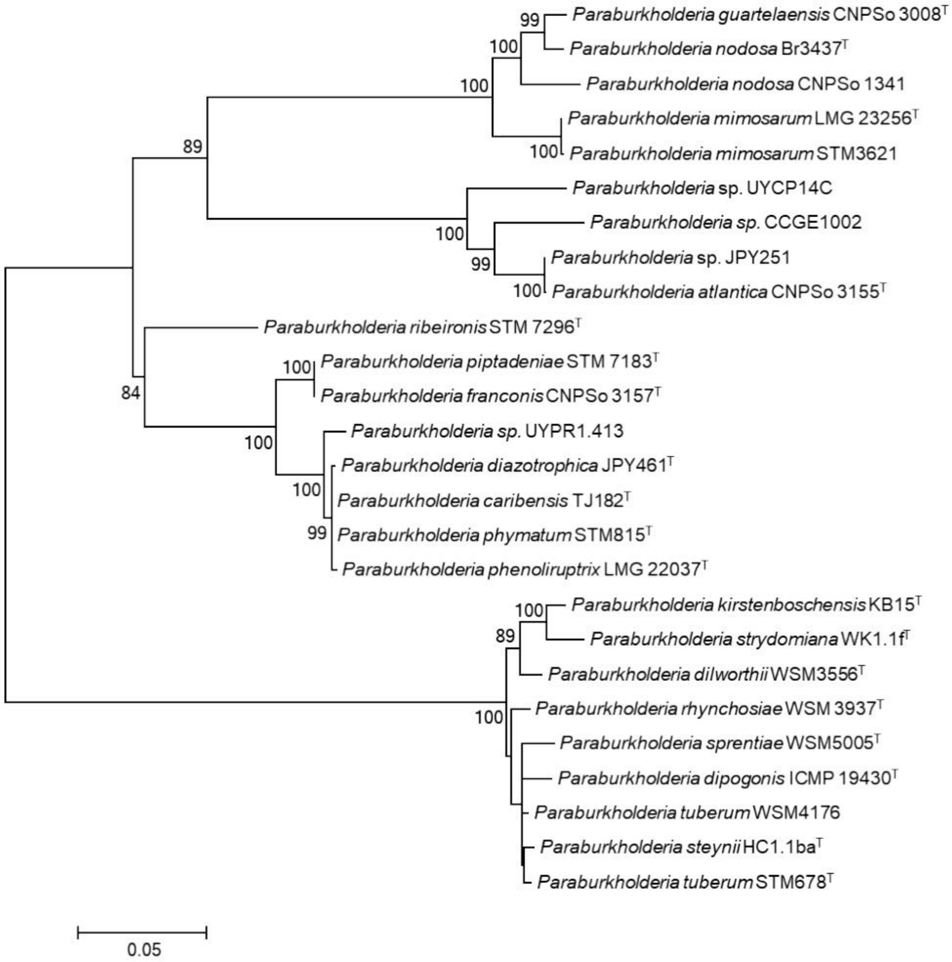

Fig. S3 Maximum-likelihood (ML) phylogeny based on the concatenated gene sequences (3,065 bp) of *nifH* (762 bp) + *nifD* (890 bp) + *nifK (*1413 bp*)* showing the relationships between nodulating species of *Paraburkholderia*. Accession numbers are indicated in Table S2. Bootstrap values > 70% are indicated at the nodes. Scale bar indicates five substitutions per 100 nucleotide positions.

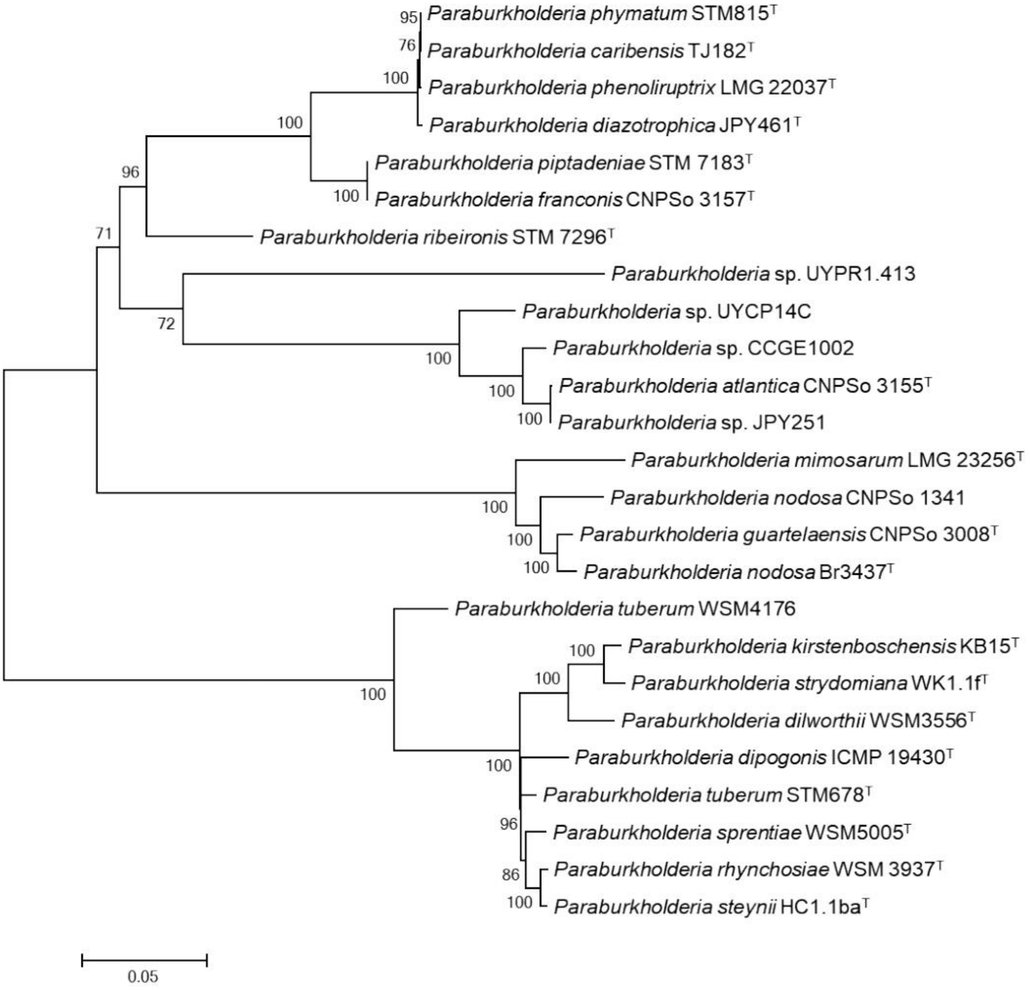

Fig S4 Maximum-likelihood (ML) phylogeny based on the concatenated gene sequences (2, 986 bp) of *fixA* (827 bp) + *fixB* (983 bp) + *fixC (*1,176 bp*)* showing the relationships between nodulating species of *Paraburkholderia*. Accession numbers are indicated in Table S2. Bootstrap values > 70% are indicated at the nodes. Scale bar indicates five substitutions per 100 nucleotide positions.

9. ESTUDO 4

Archives of Microbiology

Twenty years of paradigm-breaking studies of taxonomy and symbiotic nitrogen fixation by beta-rhizobia, and indication of Brazil as a hotspot of *Paraburkholderia* diversity

Short title: Symbiotic nitrogen-fixing *Paraburkholderia*

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Authors' contribution

F.P.; F.B.R.Jr, M.H. - participated in all stages of study, read and approved the final manuscript

Availability of Data and Materials: Data and materials cited in the manuscript are freely available for the scientific community.

Twenty years of paradigm-breaking studies of taxonomy and symbiotic nitrogen fixation by beta-rhizobia, and indication of Brazil as a hotspot of *Paraburkholderia* diversity

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Abstract

Twenty years ago, the first members of the genus *Burkholderia* capable of nodulating and fixing N_2 during symbiosis with leguminous plants were reported. The discovery that β-proteobacteria could nodulate legumes represented a breakthrough event because, for over 100 years, it was thought that all rhizobia belonged exclusively to the α-Proteobacteria class. Over the past 20 years, efforts toward robust characterization of these bacteria with large-scale phylogenomic and taxonomic studies have led to the separation of clinically important and phytopathogenic members of *Burkholderia* from environmental ones, and the symbiotic nodulating species are now included in the genera *Paraburkholderia* and *Trinickia*. *Paraburkholderia* encompasses the vast majority of β-rhizobia and has been mostly found in South America and South Africa, presenting greater symbiotic affinity with native members of the families Mimosoideae and Papilionoideae, respectively. Being the main center of *Mimosa* spp. diversity, Brazil is also known as the center of symbiotic *Paraburkholderia* diversity. Of the 21 symbiotic *Paraburkholderia* species described to date, 11 have been isolated in Brazil, and others first isolated in different countries have also been found in this country. Additionally, besides the symbiotic N_2-fixation capacity of some of its members, *Paraburkholderia* is considered rich in other beneficial interactions with plants and can promote growth through several direct and indirect mechanisms. Therefore, these bacteria can be considered biological resources employed as environmentally friendly alternatives that could reduce the agricultural dependence on agrochemical inputs.

Keywords: Biological nitrogen fixation; BNF; Nodulation; Betarhizobia; *Burkholderia*

Legumes, alpha-rhizobia, and "classical" nitrogen-fixing symbiosis

Nitrogen (N) participates in the composition of key life components, such as DNA, RNA, chlorophyll, amino acids, and proteins. Therefore, the nutrient is required in large amounts by plants and is almost always the limiting factor in croplands. Some prokaryotes called diazotrophs (*di* = two; *azote* = nitrogen; *trophs* = eaters) evolved with the enzyme nitrogenase, capable of capturing the nitrogenous gas from the atmosphere (N_2), primarily reducing it to ammonia. Ammonia is then transformed into nitrogenous compounds that plants can assimilate. This process is called biological nitrogen fixation (BNF) and greatly contributes to the N supply of natural ecosystems and cropped lands (Ormeño-Orrillo et al. 2013; de Bruijn 2015).

In the evolution process, the highest and most successful level of BNF occurred in a group of diazotrophs capable of establishing symbiotic partnerships with specific host plants. The symbioses between bacteria classified in the Alphaproteobacteria class and collectively known as "rhizobia" and plants of the family Fabaceae (Leguminosae) have been the most studied and widely spread in agriculture and the environment, contributing to the highest N inputs (Ormeño-Orrillo et al. 2013). A typical symbiosis is recognized by forming specific structures in the roots of the host legume species, called nodules, where the BNF process occurs (Ormeño-Orrillo et al. 2013; de Bruijn 2015).

Fabaceae is one of the largest families of plants consisting of over 18,000 species classified into approximately 650 genera occupying all terrestrial biomes and comprising important grain, pasture, and forestry species (Polhill and Raven 1981). Previously, Fabaceae was composed of three subfamilies: Papilionoideae, Mimosoideae, and Caesalpinioideae. More recently, the last subfamily was subdivided into four subfamilies, including Duparquetioideae, Cercidoideae, Detarioideae, and

Dialioideae, all consisting of non-nodulating species (Sprent et al. 2017). However, the majority of symbioses occurs in the Papilionoideae, including important grain legumes, such as soybean (*Glycine max* (L.) Merr), chickpea (*Cicer arietinum* L.), and pea (*Pisum sativum* L.). Historically, the benefits of BNF with legumes were used far before the principles of the biological processes were understood. Approximately 9,500 to 8,000 BC, lentils (*Lens esculenta* L.) were domesticated in Iran, whereas domestication of soybean occurred later in China (1,700-1,100 BC). There are also reports that Romans took advantage of the benefits of crop rotation of non-legumes with legumes (Hungria and Campo 2004). An interesting report dates from 1813, by Sir Humphrey Davy, indicating that the legumes "seemed to prepare the ground for wheat" and he speculated that the N came from the atmosphere. Two decades later, in 1838, the French chemistry Boussingault reported on experiments that indicated that legumes had more N than cereals and suggested that the source could be the atmosphere. Unfortunately, he was discredited by the chemistry Liebig in 1848, who argued that the source of N was ammonia, not the atmosphere. Finally, in 1886, two German scientists, Hermann Hellriegel and Hermann Wilfarth, demonstrated that the ability of legumes to convert N_2 from the atmosphere into compounds that the plant could use was caused by the presence of swellings or nodules on the legume root and to the presence of particular bacteria within these nodules. The first rhizobia were isolated from nodules of legumes soon after. In 1888, the Dutch microbiologist Martinus Beijerinck demonstrated the ability to re-infect legume hosts with bacteria, which were nominated *Bacillus radicicola* (Fred et al. 1932; Hungria and Campo 2004, 2005). The reclassification and description of the genus *Rhizobium* came later, in 1896 by Kirchner, and in 1889, Frank defined the name *Rhizobium leguminosarum.* It was only four decades later, in 1932, that Fred et al. described six species of the genus, *R. japonicum, R. leguminosarum, R.*

lupini, R. meliloti, R. phaseoli, and *R. trifolii*, based mainly on the concept of cross-inoculation groups, demonstrating the nodulation capacity of the host plant by a specific symbiont (Fred et al. 1932). Several physiological, biochemical, and genetic properties have been evaluated for the next five decades, but the unique genus *Rhizobium* has been confirmed for all rhizobia (Jordan & Allen 1974; Buchanan 1980). It was only in 1982 that the genus was split to contemplate a group of rhizobia with different properties, having slow growth in culture medium containing mannitol as the carbon source. Because of this property, the genus was nominated *Bradyrhizobium,* from *bradus,* from the Greek slow (Jordan, 1982, 1984). The 1980s were revolutionary in terms of new molecular methodologies, which resulted in the definition of the 16S rRNA gene as the backbone of the taxonomy of prokaryotes (Woese 1987). The analyses of nitrogen-fixing rhizobia confirmed them as Alphaproteobacteria, belonging to the order Rhizobiales, but split from the unique Rhizobiaceae family to new families and genera: Rhizobiaceae [genera *Rhizobium* and (*Ensifer/Sinorhizobium*], Phillobacteriaceae (*Allorhizobium* and *Mesorhizobium*), Bradyrhizobiaceae (*Bradyrhizobium*), and Hiphomicrobiaceae (*Azorhizobium*) (Garrity and Holt 2001). Nevertheless, it was at this time that the dogma of nodulation exclusively by alpha-rhizobia was broken, with the first reports of the ability of some Betaproteobacteria to establish symbiosis with legumes, as will be described in the next section.

Over the past two decades, intensive studies on alpha-rhizobia have led to the description of several new genera, including *Agrobacterium, Aminobacter, Blastobacter, Devosia, Methylobacterium, Microvirga, Neorhizobium, Ochrobactrum, Pararhizobium, Phyllobacterium,* and *Shinella*, encompassing more than 200 species, and the list is enriched every year (Velázquez et al. 2017; LPSN 2021). It is noteworthy that Brazil is known in studies for the application of the benefits of symbiotic nitrogen fixation in agriculture, e.g., in soybean crops (Hungria and Mendes 2015). However, we must also highlight the successful efforts to improve human capacity in taxonomy in the country, particularly in the past 15 years, resulting in hundreds of relevant studies on alpha- and beta-rhizobia diversity and the description of more than 30 rhizobial species. Estimates are that Brazil shelters 15%–20% of the biodiversity of the world (United Nations 2021), and it was not expected to be different for rhizobia.

Redemption of beta-rhizobia with the creation of a new genus

Burkholderia sensu lato is a well-known bacterial genus with a cosmopolitan distribution characterized by a remarkable ability to occupy a great diversity of niches, such as water (Lim et al. 2008; Lopes et al. 2018), soil (Yang et al. 2006; Farh et al. 2015; Martina et al. 2018), and hospital environments (Vial et al. 2011; da Costa Capizzani et al. 2017; Martina et al. 2018). The genus has been studied as an important agent in plant and animal diseases, but special emphasis has been placed on its role as a human pathogen (Vandamme et al. 2000; Vermis et al. 2004; Balder et al. 2010; Chewapreecha et al. 2017; Tavares et al. 2020). However, the genus has also been long cited, including beneficial microorganisms with roles as xenobiotic and plant growth-promoting bacteria (Coenye and Vandamme, 2003; Compant et al. 2001, Trân Van et al. 2000; da Silva et al. 2012; Lin et al. 2012; Esmaeel et al. 2017; Beukes et al. 2017; Estrada de los Santos et al. 2018).

Pseudomonas cepacia was originally described by William Burkholder in 1950 as the causative agent of bacterial rot in onion (*Allium cepa*) bulbs (Burkholder 1950). In 1992, Yabuuchi *et al*. (1992) conducted a study with the 16S rRNA gene and DNA-DNA hybridization analyses with five groups of *Pseudomonas*. This study described the genus *Burkholderia* to accommodate and reclassify seven *Pseudomonas* strains in the rRNA homology group II (*B. cepacia*, *B. pseudomallei, B. mallei, B. gladioli, B. caryophylli, B. pickettii,* and *B. solanacearum*) (Yabuuchi et al. 1992).

Six out of the seven species of *Burkholderia* described in 1992 are primary pathogens of plants and animals, except for *B. pickettii*, an opportunistic human pathogen (Eberl & Vandamme, 2016). Additionally, the occurrence of *Burkholderia* spp. in different geographic locations and environments established the genus as a reference in terms of genetic variability, and metabolic and ecological versatility (Compant et al. 2008).

As an example, the type species of the genus, *B. cepacia* (basynom *P. cepacia)* (Yabuuchi et al. 1992), was originally identified as an onion (*Allium cepa*) root pathogen (Burkholder 1950). In the 1980s, the species emerged associated with opportunistic nosocomial infections in immunocompromised patients, intimately associated with cystic fibrosis (Isles et al. 1984; Holmes 1986; Vandamme et al. 2000; Coenye and Vandamme, 2003; Rojas-Rojas et al. 2019). In 1997, Vandamme et al. showed that *B. cepacia* was composed of at least five genomic species, designed as genomovars, which were collectively designated as the *B. cepacia* complex (Bcc) (Vandamme et al. 1997, Coenye and Vandamme 2003). In 2021, 22 valid named species had been included in the Bcc (Jin et al. 2020; Martina et al. 2018; Rojas-Rojas et al. 2019), which are broadly distributed in natural environments. In addition to their plant, animal, and human pathogenic properties, *Burkholderia* species have been studied because they also encompass strains with important biotechnological applications, such as biocontrol of phytopathogens, plant growth promotion, and bioremediation (Trân Van et al. 2000; Coenye and Vandamme 2003; Compant et al. 2008; da Silva et al. 2012; Lin et al. 2012; Depoorter et al. 2016; Esmaeel et al. 2017).

Since the first studies based on 16S rDNA phylogeny and DNA-DNA hybridization, the existence of subgroups within *Burkholderia sensu lato* (s.l.) has been determined (Gillis et al. 1995; Vandamme et al. 1997; Vandamme et al. 2000; Estrada de los Santos et al. 2001). Additionally, based on the analysis of the 16S rDNA gene, in the last decade a division was suggested, which included group A, containing environmental species and group B, including pathogenic species (Gyaneshwar et al. 2011).

Regarding environmental species, Moulin et al. (2001) reported that two members of the *Burkholderia* s.l. were able to form nodules in legumes and presented *nod* genes establishing rhizobial-similar symbiosis (Moulin et al. 2001). This discovery was remarkable, because for over 100 years of study, it was supposed that all rhizobia belonged to the Alphaproteobacteria class. Therefore, a revolutionary change in the phylogenetic, evolutionary, and ecological view of *Burkholderia* began to take place. It should be mentioned that in the same year, Chen et al. (2001) reported another Betaproteobacteria that was isolated from nodules, identified as *Ralstonia taiwanensis*, and later reclassified as *Cupriavidus taiwanensis* (Chen et al. 2001). Following *Burkholderia*, more robust studies using the phylogeny of housekeeping genes and multilocus sequence analysis (MLSA) were enlightening. Payne et al. (2005) conducted a phylogenetic analysis of 188 *Burkholderia* strains using the *recA* recombinase gene. The results clearly showed two different clades within the species of *Burkholderia*, one mostly comprised of strains of the Bcc, along with *B. pseudomallei, B. glathei,* and other pathogenic species, and a second clade consisting of environmental species (Payne et al. 2005). MLSA is a powerful phylogenetic tool to obtain a higher resolution of phylogenetic relations within a genus usually defined with previous 16S rDNA analysis. Both are accurate but with different resolutions. Therefore, several researchers have adopted this analysis in their studies. Tayeb et al. (2008) analyzed *Burkholderia* strains using *rpoB* and *gyrB* genes, whereas Spilker et al. (2009) used seven housekeeping genes (*atpD*, *gltB*, *gyrB*, *lepA*, *phaC*, *recA*, and *trpB*), leading to the same conclusion that the genus is comprised of at least two distinct lineages. Estrada de los Santos conducted a study with 77 *Burkholderia* (s.l.) strains, using *recA*, *gyrB*, *rpoB*, and *acdS* genes, as well as whole-genome sequences (Estrada de los Santos et al. 2013) and confirmed the initial proposal of Gyaneshwar et al. (2011). They were sufficiently different in terms of evolutionary and ecological properties, confirming the applicability of a division into two groups: group A, containing the environmental species, and group B comprising the pathogenic strains, which should correspond to two different genera (Estrada de los Santos et al. 2013).

Along with the evidence of two subgroups within *Burkholderia* supported by consistent phylogenetic analysis, the need to define a new genus gained support. First, Gyaneshwar et al. (2011) proposed that bacteria from group A should be reclassified into a new genus *Caballeronia,* which was supported in the genomic analyses of Zuleta et al. (2014). However, the proposal that resulted in the definition of the new genus *Paraburkhloderia* came from a phylogenetic study based on 21 conserved proteins, the 16S rRNA gene, and the identification of 42 highly specific molecular markers comprising conserved sequence indels (CSIs) (Sawana et al. 2014). This study clearly separated the genus into two major clades, with clinically important and phytopathogenic members belonging to Clade I and environmental strains allocated to Clade II, redefined as *Paraburkholderia* (Sawana et al. 2014).

The proposal of the new genus was criticized by some scientists because the analysis of horizontal gene transfer (HGT) between beneficial and pathogenic *Burkholderia* species was not clear enough, implying safety concerns regarding human infections (Vandamme and Peeters 2014). Additionally, intermediate groups of strains, mostly from environmental sources, were identified through the 16S rRNA phylogeny, sharing high similarity (97.4%) with Group B (Clade I) (Estrada de los Santos et al. 2016). Despite this debate, strains clearly positioned in the new clade were confirmed as *Paraburkholderia*, and a new genus *Caballeronia* was created to accommodate strains belonging to a distinct clade positioned between *Burkholderia* and *Paraburkholderia* (Oren and Garrity 2015; Dobritsa et al. 2016). Efforts to clarify the phylogeny of *Burkholderia* and beta-rhizobia continued. Beukes et al. (2017) conducted a study based on 106 conserved proteins from 92 species, and the results supported five distinct lineages, *Burkholderia stricto sensu* (s.s.), *Paraburkholderia*, *Caballeronia, Robbsia,* and the fifth lineage represented by *P. rhizoxinica*. The study also proposed the transfer of 13 *Burkholderia* s.l. strains to the *Caballeronia* genus (Beukes et al. 2017). The genus *Burkholderia* (s.s.) together with *Robbsia* encompasses non-nodulating pathogenic species, whereas *Caballeronia* and *Paraburkholderia* are environmental ones, but only *Paraburkholderia* includes nodulating species.

The phytopathogen *Burkholderia andropogonis* was reassessed based on 16S rRNA and MLSA, and was accommodated as *Robbsia andropogonis* gen. nov., comb. nov. (Lopes-Santos et al. 2017). However, some groups of species still presented different positions and properties regarding *Burkholderia* (s.s.), *Paraburkholderia, Caballeronia,* and *Robbsia* (Estrada de los Santos et al. 2018). Based on whole genome analyses, two new genera were identified, and the strains were reclassified as *Mycetohabitans* gen. nov. and *Trinickia* gen. nov. (Estrada de los Santos et al. 2018). Interestingly, in addition to *T. caryophylli,* none of the *Mycetohabitans* and

Trinickia strains tested in this study are likely to be pathogenic (Estrada de los Santos et al. 2018). Finally, in early 2020, the genus *Pararobbsia* was proposed to have two species, *Pararobbsia silviterrae* and *Pararobbsia alpina* (Basynom: *Burkholderia alpina*) (Lin et al. 2020). In the *Pararobbsia* genus*,* even though the species is composed of environmental isolates, the type strain *P. silviterrae* DHC34^T contains genes coding virulence factors (Lin et al. 2020). The nodulating species previously classified as *Burkholderia* (s. l.) are now included in the genera *Paraburkholderia* and *Trinickia*.

We should highlight the importance of creating new genera to accommodate environmental *Burkholderia* in an attempt to minimize the stigma of bacteria highly related to human pathogenicity. This facilitates studies searching for the biotechnological potential of these bacteria. However, as mentioned by Kaur et al. (2017), caution should be taken to eliminate any possibility of mammalian pathogenicity and the possible transfer of virulence genes from the members of the genus *Burkholderia*, a challenge in all studies.

Regarding the *Paraburkholderia* genus, according to the list of prokaryotic names with standing in nomenclature, currently there are approximately 85 validly named species and 12 waiting to be validated (Parte et al. 2020; LPSN 2021). In this review, we will treat all species published in peer-reviewed journals as described species, not only those validated by the IJSEM. From all *Paraburkholderia* described to date, 21 are considered beta-rhizobia.

Evolutionary hypothesis and distribution of symbiotic nitrogen-fixing *Paraburkholderia* in the world

Nodulating *Paraburkholderia* have mostly been found in South America and South Africa. The South American species present great symbiotic affinity with *Mimosa* spp. and are considered their preferred symbiont. *Paraburkholderia* species have been isolated in Brazil, French Guiana, and Mexico from native and endemic species, as well as by trapping methods using species, such as *Mimosa pudica* (Chen et al. 2008; dos Reis Jr. et al. 2010; Bontemps et al. 2010; Ormeño-Orrillo et al. 2012; Sheu et al. 2013; Paulitsch et al. 2019). Because many *Paraburkholderia* species have been isolated in Brazil, the country is considered a center of genetic diversity; consequently, many studies refer to the South American *Paraburkholderia* as *Mimosa*-nodulating species (Bontemps et al. 2010; dos Reis Jr. et al 2010; Moulin et al., 2015; Paulitsch et al 2020b). However, many studies performed with the Brazilian *Paraburkholderia* strains indicated that they are preferred symbionts not only of *Mimosa* spp., but also of the *Piptadeniae* group plants and *Calliandra* spp., especially in acidic soils (Bontemps et al. 2010; dos Reis Jr. et al. 2010; Moulin et al. 2015; de Castro Pires et al. 2018; Paulitsch et al. 2019a, 2019b).

Brazilian nodulating *Paraburkholderia* appear to be affected by some abiotic conditions. A study conducted by de Castro Pires and collaborators (2018) revealed that acidic soils influenced the symbiosis between *Paraburkholderia* and *Mimosa* (de Castro Pires et al. 2018); however, this "preference" is perhaps caused by the fact that *Paraburkholderia* has mechanisms to tolerate acidity, reflecting competitive advantages in relation to rhizobial groups that are not tolerant to low pH (Stopnisek et al. 2014). In addition to soil pH, the prevalence of *P. nodosa* strains may also be related to high altitudes. Strains of *P. nodosa* were isolated in the Caatinga and Cerrado biomes, predominantly at altitudes above 800 m (Bontemps et al. 2010, dos Reis Jr. et al. 2010). *P. nodosa* was also isolated from an undisturbed area of the Brazilian Cerrado collected at high geographical altitudes (1,175 m), once again showing a preference for this condition (Dall'Agnol et al. 2016).

Paraburkholderia strains have also been isolated from soils of the Amazon biome (Chen et al. 2005; Lima et al. 2009; da Silva et al. 2012; Guimarães et al. 2012; Oliveira-Longati et al. 2013) and a few from the Pantanal biome, perhaps because this biome is found in low-lying regions (Bontemps et al. 2010). Although there are still no reports of nodulating *Paraburkholderia* in the Brazilian Pampa biome, there are studies describing the presence of diazotroph plant growth-promoting strains that have not been properly identified but show properties indicating they might be *Paraburkholderia* (Arruda et al. 2013; Vargas et al. 2015; Granada et al. 2019).

Another center of nodulating-*Paraburkholderia* diversity is the South African Cape Floristic Region (CFR), which is included in the Fynbos biome, in which the genus is associated with endemic papilionoid legumes, known as papilionoid-nodulating *Paraburkholderia* (Elliot et al. 2007b; Garau et al. 2009; de Meyer et al. 2013, 2014; Lemaire et al. 2016; De Meyer et al. 2018).

Evolutionary events lead to the divergence of *Paraburkholderia* lineages, involving both core and symbiotic genes. *Paraburkholderia* from South America and South Africa differ considerably in the symbiotic genes, including that the South African symbionts possess symbiotic genes in chromosomal islands, whereas the South American species present a symbiotic plasmid-borne (de Meyer et al. 2016). Studies based on the phylogenetic analysis of *nif* and *nod* genes revealed the monophyletic character for the South American species, indicating that these genes might have been acquired simultaneously and from a different origin than those acquired by the South African species (de Meyer et al. 2016; Paulitsch et al. 2020b). Interestingly, both South African and South American species have failed to nodulate hosts of the other, suggesting the influence of geographical factors and co-evolution between symbiont and host plant, resulting in host range specificity (de Meyer et al. 2016; Paulitsch et al. 2020b). Regarding *fix* genes, De Meyer et al. (2016) suggested that the acquisition occurred by HGT from a free-living diazotrophic ancestor to all beta-rhizobia, explaining the ability of *P. phymatum* and *P. tuberum* to fix nitrogen *ex planta* (Elliot et al. 2007a).

Phylogenetic analysis using concatenated sequences of *recA* and *gyrB* genes was conducted with most of the *Paraburkholderia* species described, with *Cupriavidus taiwanensis* LMG19424^T considered an outgroup (Fig. 1). Some species were not included in the analysis because their *recA* and *gyrB* sequences were not available in the databases at the time of analysis. The nodulating species are highlighted in boldface. Many nodulating *Paraburkholderia* grouped together with non-symbiotic diazotrophic bacteria, including *P. nodosa* Br3437^T and *P. guartelaensis* CNPSo 3008^T cluster together with *P. silvatlantica* SRMrh-85 and *P. heleia* NBRC 101817^T, and *P. sabiae* LMG 24235^T with *P. azotifigens* NF 2-5-3^T. Moreover, some nodulating *Paraburkholderia* grouped with non-diazotrophic species, such as *P. ribeironis* STM 7296^T, with the mineral-weathering *P. susongensis* LMG 29540^T as the closest neighbor. However, many nitrogen-fixing symbiotic *Paraburkholderia* grouped together in a unique cluster, such as *P. diazotrophica* LMG 26031^T*, P. franconis* CNPSo 3157^T*,* and *P. piptadeniae* STM7183^T, as well as *P. atlantica* CNPSo 3155^T and *P. youngii* JPY 169. The phylogenetic analysis of core genes within nodulating *Paraburkholderia* and other species in the genus has been previously performed (de Meyer et al. 2016; Estrada de los Santos et al. 2018), and comparisons between core and symbiotic genes of nodulating *Paraburkholderia* revealed different phylogenetic patterns (de Meyer et al. 2016; Paulitsch et al. 2020b). The phylogenetic analysis of the concatenated tree of *recA* and *gyrB* (Fig. 1), together with the fact that symbiotic genes were probably acquired through HGT from a diazotrophic donor, lead de Meyer (2016) to hypothesize that the HGT of symbiotic genes occurred after the divergence of *Paraburkholderia*; an assumption supported by the analyses in other studies (Estrada de los Santos et al. 2018; Paulitsch et al. 2020b).

Many factors can affect the symbiosis between nodulating *Paraburkholderia* and host plants. Therefore, with the isolation and description of new *Paraburkholderia* species, the abiotic and biotic factors that influence symbiosis can be further explored to elucidate its evolutionary history.

***Paraburkholderia* symbiovars**

The term symbiovar (sv.) was proposed by Rogel et al. (2011) as equivalent to "biovar," but used for pathogenic bacteria to identify strains with variant biochemical and enzymatic characteristics within a species. First, the term refers to a group of symbiotic variant strains within a species, defined by the capacity to nodulate a specific host plant (legume) (Rogel et al. 2011; Peix et al. 2015). However, host specificity can cross the border of species and even genera, because the symbiotic genes are often located in mobile genetic elements and can be easily transferred to another rhizobial species by HGT (Rogel et al. 2011; Peix et al. 2015; Remigi et al. 2016; Wang et al. 2019). Therefore, symbiovars are now not only defined by the rhizobia host range and symbiotic capabilities, but they are also defined by the phylogenetic analysis of symbiotic genes, especially *nod* and *nif* genes (Rogel et al. 2011; Peix et al. 2015).

Symbiovars have been mostly described for alpha-rhizobia, especially in the genera *Rhizobium*, *Ensifer, Mesorhizobium*, and *Bradyrhizobium* (Velázquez et al. 2017). In contrast, symbiovars in beta-rhizobia have been poorly studied. In 2012, Mishra et al. (2012) suggested that *Paraburkholderia* presented at least two symbiovars, one harboring South African strains capable of establishing symbiosis with Papilionoid plants (sv. papilionoid), and the second one comprising the South American strains that nodulate *Mimosa* (sv. mimosa) (Mishra et al. 2012). Recently, the description of *Paraburkholderia* symbiovars occurred in a study by Paulitsch et al. (2020) based on the phylogeny of *nod*, *nif*, and *fix* genes, and the study revealed at least five symbiovars within the genus. The previous "sv. mimosae" was split into four different well-defined symbiovars: mimosae, tropicalis, atlantica, and piptadeniae, whereas sv. papilionoid was renamed africana (Paulitsch et al. 2020b). Table 1 displays the plant host, isolation site, and symbiovar cluster of *Paraburkholderia* in Brazil.

The sv. mimosae comprises *P. mimosarum*, *P. nodosa*, and *P. guartelaensis* strains and shows the same phylogenetic pattern in the analyses of core and symbiotic genes (Paulitsch et al. 2020b). In the sv. atlantica, strains were isolated from four different South American countries, including *P. phymatum*, *P. atlantica*, and *P. sabiae* (Paulitsch et al. 2020b). In a study conducted by Bournaud et al. (2013), two different *nodC* variants were observed, reflecting differences in the host specificity and effectiveness of nodules; therefore, the most common and frequent *nodC* sequence was used to infer nodulation phylogeny, including *P. sabiae* strain sv. tropicalis (Paulitsch et al., 2020b). The sv. tropicalis includes *P. caribensis* (isolated in Taiwan) and the Brazilian species *P. diazotrophica*, *P. franconis*, *P. piptadeniae*, and *P. phenoliruptrix* (Paulitsch et al. 2020b). Concerning the sv. africana, strains were isolated from native and endemic Papilionoid legumes in the Cape Floristic Region in South Africa, with the exception of *P. dipogonis* isolated in New Zealand (Paulitsch et al. 2020b). The last symbiovar has only one representative species, Piptadeniae, with a unique species, *P. ribeironis* (Paulitsch et al. 2020b).

The Brazilian nitrogen-fixing *Paraburkholderia*

Brazil has a great diversity of nodulating nitrogen-fixing *Paraburkholderia*, occupying many Brazilian biomes (Fig 2). From 21 symbiotic *Paraburkholderia,* 11 have been isolated in Brazil (Chen et al. 2005, 2006, 2007, 2008; Sheu et al. 2013; Bournaud et al. 2017; Paulitsch et al. 2019, 2020a). Other strains belonging to the species first isolated in other countries have also been identified in the country. Here, it is important to describe the biomes where *Paraburkholderia* are found. Brazil has six terrestrial biomes: (*i*) Amazon, occupying 49.5% of the territory, representing the largest forest on the planet and very high biodiversity; (*ii*) Cerrado, covering 23.3% of the territory in the highlands, mostly in the Central-Western region, with different types of vegetation, from grasslands to dry forests, representing the most important area for agriculture in the country; (*iii*) Atlantic Forest, covering 13% of the territory and present in 15 coastal states, with humid weather; (*iv*) Caatinga, covering 10.1% of the territory mostly in the Northeastern region, with dry weather with xeric vegetation; (*v*) Pampa, with 2.3% of the territory in the Southern region covered mostly with natural grasslands; (*vi*) Pantanal, in the Central-Western region, and although being the smallest, constituting 1.8% of the territory, is the largest tropical wetland in the world, subject to seasonal inundation and desiccation (Kaschuk et al. 2011; IBGE 2019).

Paraburkholderia mimosarum

In a study conducted by Chen et al. (2005), several *Paraburkholderia* strains were isolated from nodules of *Mimosa* spp. in different regions of Brazil, especially in the southeastern region of Atlantic Forest (Mata Atlântica), but also in the Amazon and the Cerrado biomes, resulting in the description of three new species of nodulating *Paraburkholderia* (Chen et al. 2006, 2007, 2008).

The species was the first one described in Brazil, contemplating two strains, Br3454 and Br3467, isolated from *Mimosa scabrella* and *M. pigra*, respectively. The type strain PAS44^T, along with other strains used to describe the species, were isolated from *M. pigra* nodules in Taiwan and Venezuela (Chen et al. 2006). Phylogenetically, in the analysis of core genes, *P. mimosarum* is highly similar to other nodulating *Paraburkholderia* of South America, especially *P. nodosa* (Chen et al. 2007), and has even higher similarity in the analysis of symbiotic genes (Paulitsch et al. 2020b). Another *P. mimosarum* strain (JPY 321) was isolated from the nodules of *M. pigra* in Mato Grosso State, Central-Western Brazil (Bontemps et al. 2010). Although *M. pigra* is a widespread species, its origin is tropical America (Harley et al. 1995); therefore, it is possible that the introduction in different countries occurred together with the symbionts, explaining the phylogenetic pattern along with other South American strains.

Paraburkholderia nodosa

The first strains of *P. nodosa* were isolated from root nodules of native woody legumes in Brazil, *Mimosa bimucronata* (strains Br3470 and Br3461), and *M. scabrella* (type strain Br3437^T) (Chen et al. 2005; 2007). Currently, *P. nodosa* has only been isolated in Brazil and is a frequent symbiont of *Mimosa* spp. in the Caatinga and Cerrado biomes (Bontemps et al. 2010, dos Reis Jr. et al. 2010). An extensive study showed that *P. nodosa* has an affinity for plants of the *Piptadenia* group, isolated from four different Brazilian states in the Atlantic Forest biome (Bournaud et al. 2013). Strains were also isolated from soils of the Atlantic Forest in São Paulo and Santa Catarina states, where *M. scabrella* abundantly occurs (Lammel et al. 2015; Primieri et al. 2016) and in Rio de Janeiro soils, trapped with *Mimosa pudica* and common beans (*Phaseolus vulgaris*) (Dall'Agnol et al. 2017). *P. nodosa* was also the main symbiont trapped in *P. vulgaris* nodules grown in soils from an undisturbed area of the Brazilian Cerrado (Dall'Agnol et al. 2016). *P. nodosa* strains were also isolated from the native *Mimosa gymnas* in an ecotone area of the Atlantic Forest and Cerrado biomes in South Brazil (Paulitsch et al. 2019a). Finally, a study conducted in Central-Western Brazil compared the rhizobia association with *Mimosa* spp. in relation to soil properties (especially pH), and the results showed that the most acidic and less fertile soils favored the association with *Paraburkholderia*, with the isolation of many *P. nodosa* strains (de Castro Pires et al. 2018). Regarding symbiotic genes, *P. nodosa* strains were included in the sv. mimosae (Paulitsch et al., 2020b).

P. nodosa is the symbiont with a broad distribution in Brazil, reported in eight Brazilian States located in the Cerrado, Caatinga, and Atlantic Forest biomes (Table 1). It is also worth mentioning that *P. nodosa* CNPSo 1341 was as effective as the elite *Rhizobium tropici* strain in fixing nitrogen with common beans (Dall'Agnol et al. 2016).

Paraburkholderia sabiae

The two strains used for species description (Br3405 and Br3407^T) were isolated from *Mimosa caesalpiniifolia*, a legume tree native to Brazil (Chen et al. 2005; Chen et al. 2008). In addition to *M. caesalpiniifolia*, the species is also capable of nodulating plants of the *Piptadeniae* group from different Brazilian states (Bournaud et al. 2013) including *Calliandra luetzelburgii* in Northeastern Brazil (Silva et al. 2018), and has also been isolated from *Parapiptadenia rigida* in Uruguay (Taulé et al. 2012).

Interestingly, Bournaud et al. (2013) reported an HGT event in *P. sabiae* strains. The strains under study presented two different *nodC* genes (C1 and C3), reflecting distinct phenotypes that lead to variance in host specificity and effectiveness of nodules (Bournaud et al. 2013). These findings were later confirmed by Moulin et al. (2015), who revealed that some *P. piptadeniae* strains isolated from Brazil (including Br3407^T) and the Uruguayan strain UYPR3.611, presented *nodC1*, whereas two other Brazilian strains presented *nodC3* (Moulin et al. 2015). *P. sabiae* strains were included in sv. tropicalis (Paulitsch et al., 2020b).

Paraburkholderia phenoliruptrix

The species description took place with a single strain (AC1100) isolated from a chemostat mixed culture containing a potent herbicide, and the strain is well known for its ability to degrade various halogenated phenol compounds (Coenye et al. 2004). However, Chen et al. (2005) isolated the strain Br3462 (Br3459) from a root nodule of *Mimosa flocculosa* in Brazil (Chen et al. 2005). The complete genome sequence of strain BR3459a (a mucoidy colony variant of strain Br3459) was released, and the genes coding for symbiotic ability, including nodulation and nitrogen fixation ability, were confirmed (de Oliveira Cunha et al. 2012; Zuleta et al. 2014).

Paraburkholderia phenoliruptrix strains are also diazotrophic symbionts of the *Piptadenia* group (Bournaud et al. 2013). Similar to *P. sabiae* strains isolated in the same study, *P. phenoliruptrix* strains also presented two *nodC* variants (C3 and C4), and both alleles were effective in nodulation (Bournaud et al. 2013; Moulin et al. 2015). Curiously, the genome of the *P. phenoliruptrix* type strain (AC1100; =LMG 22037) is available in the NCBI database, but it was not possible to locate symbiotic genes. The lack of symbiotic genes in the type strain and the fact that *P. phenoliruptrix* strains from the study of Bournaud et al. (2013) presented *nodC* variants led to the hypothesis that these genes might have been acquired by HGT from another symbiotic diazotrophic strain. Symbiotic genes retrieved from the Br3459a strain showed that this strain belongs to the sv. tropicalis (Paulitsch et al., 2020b).

Paraburkholderia diazotrophica

The type strain of *P. diazotrophica* JPY 461^T was isolated from root nodules of *Mimosa candollei* in Central-Western Brazil by Bontemps et al. (2010). The other strains used in the species description were isolated from *Mimosa* spp. in Brazil (JPY 359, JPY 389), Taiwan (DPU-3), and French Guiana, trapped by *M. pudica* (STM 4206) (Bontemps et al. 2010; Mishra et al. 2012; Sheu et al. 2013). In Brazil, *P. diazotrophica* strains were able to nodulate *Piptadeniae* and *Calliandra* spp. (Bournaud et al. 2013; Silva et al. 2018). The species is also the main symbiont of *M. caesalpiniifolia* in the soils of the Caatinga biome (de Oliveira et al. 2019). Regarding symbiotic genes, the species belongs to the sv. tropicalis (Paulitsch et al., 2020b).

Paraburkholderia piptadeniae

Five strains isolated from *Piptadenia gonoacantha* in Rio de Janeiro state (RJ) were used in the species description, defining STM 7183^T as the type strain (Bournaud et al. 2017). *P. piptadeniae* strains were also isolated from soils of Búzios (RJ), using *M. pudica* as a trap host (Dall'Agnol et al. 2017). Cabo Frio, Macaé, and Búzios, the cities from which the strains were collected, are included in the Atlantic Forest biome. The type strain is capable of nodulating and fixing nitrogen with *M. pudica*, *M. scabrella*, and *Acacia nilotica* legumes (Bournaud et al. 2017). The strains present symbiotic genes closely related to "*P. franconis*" (more details in the "*P. franconis*" section) and are included in the sv. tropicalis (Paulitsch et al. 2020b).

Paraburkholderia ribeironis

The type strain STM 7296^T together with the other two strains (STM 7168 and STM 7217) used in the species description share the same isolation site and plant host as *P.*

piptadeniae (Bournaud et al. 2017). However, they present many phylogenetical (core and symbiotic genes) and phenotypical differences, highlighting the diversity of beta-rhizobia in the Atlantic Forest. *P. ribeironis* STM 7296^T can nodulate and fix nitrogen in *M. pudica, Leucaena leucocephala,* and *Acacia nilotica* (Bournaud et al. 2017). This species has a distinctive characteristic regarding its symbiotic genes because it presents a unique position in the *nodA*, *nodC*, *nifH*, and in the concatenated *fixABC*, *nodAC*, and *nifHDK* phylogenies (Paulitsch et al. 2020b). Therefore, *P. ribeironis* was the only species included in sv. piptadeniae (Paulitsch et al. 2020b).

Paraburkholderia guartelaensis

The species was isolated from *M. gymnas* nodules grown in Guartelá State Park, South Brazil. The Park represents an ecotone between the Atlantic Forest and Cerrado biomes, where soils are sandy, acidic (pH 3.6–3.8), and poor in nutrients and organic matter. In addition to *P. guartelaensis*, many strains of *P. nodosa* were also isolated in the Park (Paulitsch et al. 2019a, 2019b). The species has thus far six identified strains, with CNPSo 3008^T defined as the type strain, and can nodulate *P. vulgaris* and *Macroptilium atropurpureum,* and nodulate and fix nitrogen in *M. gymnas* and *M. pudica* (Paulitsch et al. 2019). All six strains of this species were included in sv. mimosae and present the same phylogenetic pattern in the core and are symbiotic with the other species of the symbiovar (Paulitsch et al. 2020b).

Paraburkholderia atlantica and *Paraburkholderia franconis*

A study conducted by Dall'Agnol et al. (2017) isolated many *Paraburkholderia* strains from the Atlantic Forest using *P. vulgaris* and *Mimosa pudica* as trapping hosts and reported substantial diversity. The author also pointed out two clades that might represent new species, which were then studied in greater detail by Paulitsch et al. (2020a)and resulted in the description of *P. atlantica* and *P. franconis* (Dall'Agnol et al. 2017; Paulitsch et al. 2020a).

The strains of *P. atlantica* were retrieved from soils of Rio de Janeiro collected near *P. gonoacantha* plants, and CNPSo 3155^T and CNPSo 3150 were isolated from *M. pudica* nodules, and strain CNPSo 3196 from *P. vulgaris* nodules (Dall'Agnol et al. 2017; Paulitsch et al. 2020a).

All three strains were able to nodulate *P. vulgaris*, *M. pudica,* and *M. atropurpureum*, but nodules were ineffective in the latter (Paulitsch et al. 2020a). Interestingly, in both core and symbiotic genes, *P. atlantica* presented great similarity with some strains previously classified as *P. tuberum*, *Paraburkholderia* sp. JPY 604 and JPY 158 in Brazil (Bontemps et al. 2010), *Paraburkholderia* sp. STM3649 from French Guiana (Mishra et al. 2012), and *Paraburkholderia* sp. CCGE1002 isolated from Mexico (Ormeño-Orillo et al. 2012), indicating that they might belong to *P. atlantica* (Paulitsch et al. 2020b). In the symbiotic gene phylogenies, the strains aforementioned grouped together with *P. atlantica* CNPSo 3155^T*,* forming the sv. atlantica (Paulitsch et al., 2020b). Mavima et al. (2020) recently conducted a study with 30 strains, initially identified as *P. tuberum* isolated from *Mimosa* spp. nodules. The authors used a polyphasic approach (MLSA, ANI, C+G DNA content, phenotypic characteristics) and concluded that 12 strains under study were conspecific with *P. atlantica* CNPSo 3155^T, as suggested by Paulitsch et al. (2020b), and the other strains represented the new species "*Paraburkholderia youngii,"* as will be discussed (Mavima et al. 2020).

Regarding *P. piptadeniae* and *P. atlantica*, *P. franconis* strains were also isolated from soils of the Brazilian Atlantic Forest (RJ), isolated from the trap plant *M. pudica*, with CNPSo 3157^T defined as the type strain (Dall'Agnol et al. 2017; Paulitsch et al. 2020a). "*P. franconis*" and *P. piptadeniae* species shared high similarity in the core genes (16S rDNA, *recA + gyrB* + *gltB* + *trpB*) and genome (ANI values = 94.4%), yet they represented different species (Paulitsch et al. 2020a). The richness of *Paraburkholderia* in the Atlantic Forest biome is again highlighted, because although "*P. atlantica"* CNPSo 3155^T and *P. franconis* CNPSo 3157^T have been isolated from the same geographic region, their symbiotic gene phylogeny revealed distinct evolutionary histories (Paulitsch et al. 2020a; 2020b). In contrast, *P. franconis* CNPSo 3157^T and *P. piptadeniae* STM183^T share almost identical DNA sequences in nodulation and nitrogen fixation genes, revealing very close evolutionary events. *P. franconis* strains were able to nodulate *P. vulgaris* and *M. pudica* forming effective nodules, but were unable to nodulate *Macroptilium atropurpureum* (Paulitsch et al. 2020a); in the analysis of nitrogen fixation genes, the species was positioned in the sv. tropicalis (Paulitsch et al. 2020b).

Paraburkholderia youngii

In a study conducted by Mavima et al. (2020), strains previously classified as *P. tuberum* in the provisional sv. mimosae were identified as belonging to *P. atlantica* and *P. youngii*. *P. youngii* was described considering 19 strains, all isolated from *Mimosa* spp. in three different Brazilian states (Goiás, Distrito Federal, and Mato Grosso), and all included in the Cerrado and Pantanal biomes (Bontemps et al. 2010), with JPY 169^T defined as the type strain. It is noteworthy that some strains of *P. youngii* have been isolated from the Pantanal biome, outnumbered in comparison to the Caatinga and Cerrado biomes, probably because of the low altitude in the Pantanal region (Bontemps et al. 2010). Ten strains were able to nodulate and fix nitrogen with *M. pudica.* Furthermore, with the exception of the strain JPY 602 isolated from *Mimosa adenocarpa,* all other strains were able to nodulate *Lebeckia ambigua*, but the nodules were ineffective. Symbiotic gene phylogenies were not analyzed in the study; however, BLAST comparisons with the JPY 169^T partial nucleotide sequences of *nodC* and *nifH* genes indicated that they were probably included in the sv. atlantica (data not shown)

In conclusion, the majority of the *Paraburkholderia* described thus far have been isolated from soils of the Atlantic Forest, Cerrado, and Caatinga, revealing that these three biomes are not only rich in flora and fauna (Myers et al. 2000), but they are also host spots of biodiversity of microorganisms, including the genus *Paraburkholderia* (Fig 2). Additionally, some strains of nodulating *Paraburkholderia* have been isolated from the Pantanal (Bontemps et al. 2010) and Amazon biomes (Lima et al. 2009; da Silva et al. 2012; Oliveira-Longatti et al. 2013). Currently, a low diversity of the genus has been found in the Pantanal, and there are still no reports of nodulating *Paraburkholderia* in the Brazilian Pampa biome. However, diazotrophic *Paraburkholderia*-resembling strains reported in the Pampa biome indicate the plausible presence of beta-rhizobia. Therefore, more studies are necessary to truly assess the *Paraburkholderia* diversity in these biomes, especially in the Pampa and Pantanal biomesStudies of native and endemic plants and their natural symbionts have led and will lead to a better understanding of the evolutionary history of Brazilian *Paraburkholderia* and their host plants.

Agro-biotechnological potential

A high agro-biotechnological potential is expected in such a versatile genus that contains species with outstanding metabolic and physiological adaptability (Coenye and Vandamme 2003; Estrada-de los Santos et al. 2013). The ecological versatility of *Paraburkholderia* species could be attributed to their large genome sizes and insertion sequences, which promote genomic plasticity and general adaptability (O'Sullivan and Mahenthiralingam 2005). This explains why *Paraburkholderia* is considered a genus rich in beneficial interactions with plants and capable of promoting growth through several direct or indirect mechanisms (Estrada-de Los Santos et al. 2001; Alves et al. 2016).

Among the plant growth-promoting features of these bacteria are phosphate solubilization capacity, ACC deaminase activity, phytohormone production, siderophore synthesis, acyl-homoserine-lactones (AHL) production, antifungal activity, and BNF (Mitter et al. 2013; Kaur et al. 2017; Vio et al. 2020). These benefits exerted on plants and the impressive capacity of *Paraburkholderia* to adapt to different environments have increased the interest in its use in agriculture (Castanheira et al. 2015).

In the 1990s, several *Burkholderia*-based products were developed. For example, Deny®, Blue Circle®, and Intercept®, based on BCC strains, were registered in the USA as biopesticides (Parke and Gurian-Sherman 2001). Nevertheless, their registries had to be canceled after risk assessment studies showed that there was a threat to humans because of the opportunistic pathogenic potential of the bacteria used in their formulation (Kaur et al. 2017).

Before the separation of *Paraburkholderia* from the clinically important phytopathogenic members of *Burkholderia*, the agro-biotechnological use of these bacteria was highly restricted, and their beneficial features were overshadowed by their pathogenic potential (Kaur et al. 2017). In August of 2016, during the "12th Nitrogen Fixation Conference" held in Budapest, Hungary, discussions regarding the use of *Paraburkholderia* in agriculture, highlighted the importance of the efforts toward robust characterization (large-scale phylogenomic studies) and taxonomy of these bacteria (de Lajudie and Young 2017). Recent studies that led to the taxonomical realignments of the genus *Burkholderia* and the origin of *Paraburkholderia* have reopened the opportunity to explore the potential of these bacteria for use in agriculture.

Among the non-symbiotic *Paraburkholderia* species, *P. phytofirmans* is probably the most studied (Mitter et al. 2013). *P. phytofirmans* colonizes the rhizosphere and endosphere of several crops and vegetables, promoting growth and enhancing tolerance to abiotic and biotic stresses (Mitter et al. 2013). Issa et al. (2018) evaluated the effect of *P. phytofirmans* PsJN on tomatoes (*Lycopersicon esculentum* Mill.) in response to heat stress at 32 °C. Their results showed that inoculation with this bacterium improved photosynthetic features and led to a higher accumulation of sugars, total amino acids, proline, and malate, enhancing heat tolerance and promoting tomato growth. The authors concluded that *P. phytofirmans* PsJN could alleviate heat stress in agriculture, opening up new and emerging microbial applications (Issa et al. 2018). Naveed et al. (2014) studied the response of wheat under different drought stress conditions with inoculation of *P. phytofirmans* PsJN under field conditions. Physiology and growth parameters were improved, and the adverse effects of drought were reduced, resulting in grain yield up to 21% higher in inoculated plants than in the non-inoculated control (Naveed et al. 2014). In view of the potential of this species in several studies, different initiatives have already been conducted in the search for the most suitable carrier that can be used in formulations of an inoculant product containing *P. phytofirmans* (Bejarano et al. 2017; Berninger et al. 2017; Aziz et al. 2020).

Another non-symbiotic *Paraburkholderia* species, among the most studied species, is *P. tropica*. This endophytic nitrogen-fixing bacterium, with other plant growth-promoting abilities, such as phosphate solubilization and antifungal activity, was isolated from sugarcane and corn in different geographical regions (Bernabeu et al. 2018). Rahman et al. (2018) showed that field-grown strawberry plants inoculated with *P. tropica* yielded 48% more fruit than the non-inoculated control. *P. tropica* Ppe8 is part of the sugarcane consortium inoculant, developed by the Brazilian Agricultural Research Corporation (Embrapa), which promotes sugarcane yield increments in the field (Silva et al. 2018). Schultz et al. (2017) conducted two field experiments with two sugarcane varieties (RB867515 and RB72454) using this consortium inoculant and showed that inoculation increased yield up to 38.0 Mg of stems ha^{-1} compared to the non-inoculated control. Pereira et al. (2019) claimed that this consortium inoculant allowed decreased use of nitrogenous fertilizers normally used in sugarcane crops.

The vast majority of studies on the symbiotic *Paraburkholderia* are still focused on elucidating their phylogeny and ecology, generally conducted with native and non-commercial leguminous species. These bacteria are usually associated with acidic and low-fertility soils that are poorly suited for agriculture; therefore, initial studies have been conducted to assess its agro-biotechnological potential, mainly performed in gnotobiotic systems or in greenhouses with non-sterile soil, with the goal of determining the efficiency of plant nodulation and its symbiotic capacity.

Araujo et al. (2017) used several *Paraburkholderia* spp. strains isolated from *Mimosa tenuiflora* and *Macroptilium atropurpureum* to evaluate their symbiotic capacity when inoculated in *Mimosa bimucronata* and *M. foliolosa* in greenhouse experiments under gnotobiotic conditions. According to their results, the authors considered as potential inoculants for *M. bimucronata* the *Paraburkholderia* spp. strains UFLA 01-739, 01-748, and 04-405, and for *M. foliolosa* strains UFLA 01-751 and UFLA 04-260. Souza et al. (2020) also selected *Paraburkholderia* spp. strains that showed high symbiotic efficiency with *M. foliolosa* in a greenhouse experiment under gnotobiotic conditions; their strains were also capable of solubilizing calcium phosphate and producing siderophores. The possibility of inoculation of *M. bimucronata* and *M. foliolosa* with efficient symbiotic diazotrophic bacteria is interesting, because they are recommended for ecological recovery of degraded areas because of their high growth rate and adaptation to low-fertility soils (Negreiros et al. 2009; Silva et al. 2011).

The Papilionoidae species *Erythrina velutina* is a native tree from the Brazilian Caatinga semi-arid region (Menezes et al. 2016). Its use as a medicinal herb is noteworthy for its anticonvulsant and anxiolytic effects (Ribeiro et al. 2006; Ozawa et al. 2008). Menezes et al. (2016) assessed the symbiotic efficiency of *Paraburkholderia* sp*.* strain ESA 71 associated with *E. velutina* in two greenhouse experiments conducted under gnotobiotic conditions and non-sterile soil. The authors found that this strain induced increases in the shoot N concentration and total N accumulation, indicating that *E. velutina* is able to establish efficient associations with β-rhizobia in Brazilian semi-arid soils (Menezes et al. 2016).

Regarding agricultural crops of high economic and nutritional importance, Gopalakrishnan et al. (2018) evaluated the potential of inoculation of the *Paraburkholderia* sp. strain IC-76A in chickpea, the second most important grain legume crop for human nutrition after common bean (*Phaseolus vulgaris* L.). In laboratory tests, this strain was able to produce cellulase, protease, β-1,3-glucanase, indole acetic acid, siderophore, hydrocyanic acid, and ACC deaminase (Gopalakrishnan et al. 2018). Under greenhouse conditions using a pot mixture of black soil and sand (3:2), IC-76A increased shoot weight by 40%, pod number by 69%, pod weight by 45%, seed number by 31%, and seed weight by 56%, compared to the uninoculated control. The authors concluded that this strain could potentially be exploited for improving nodulation, nitrogen fixation, plant growth promotion, and chickpea yield (Gopalakrishnan et al. 2018).

Dall'Agnol et al. (2016) used common bean (*Phaseolus vulgaris* L.) plants to trap rhizobia from an undisturbed soil of the Brazilian Cerrado under the vegetation type Cerradão. In a greenhouse experiment under gnotobiotic conditions, all isolates identified as *P. nodosa* were capable of nodulating common beans, generally, with low N_2-fixing capacity. Nevertheless, strains CNPSo 1258 and CNPSo 1341 were as efficient as the *Rhizobium* strain used in commercial inoculants in Brazil (Dall'Agnol et al. 2016). *P. nodosa* is associated with the edaphic properties of the Cerrado biome, which is characterized by acidic and low-fertility soils. The inoculation of these bacteria may be especially important for smallholder farmers who cultivate common beans, but are unable to properly manage their soils.

Artiga-Ramirez et al. (2019) obtained several *Paraburkholderia* spp. isolates from soybean nodules from different topographical regions of Venezuela. The nodulating capacity of these isolates was confirmed in plants grown aseptically in 300 mL glass jars containing sterilized vermiculite in a growth chamber. Five *Paraburkholderia* isolates presented a higher number of nodules than the α-rhizobia included in the test. Furthermore, among the 44 isolates tested, including *Rhizobium* and *Bradyrhizobium* members, one *Paraburkholderia* isolate exhibited the highest nitrogen fixation activity in root nodules, four weeks after planting. The authors suggested that *Paraburkholderia* isolates could be used as inoculants for soybean, but further studies are necessary to confirm their effectiveness under field conditions (Artiga-Ramirez et al. 2019).

Considering the ecological importance of plant-associated *Paraburkholderia* and their potential for applications in agriculture and biotechnology, it is evident that the number of studies involving these bacteria will grow continuously. *Paraburkholderia* species are important biological resources to be employed in environmentally friendly alternatives that could reduce the agricultural dependence of agrochemical inputs.

Concluding remarks

The agricultural advantages of the biological nitrogen fixation process with legumes are known for almost 10,000 years, but revealing the role of symbiotic partners occurred only 130 years ago. Since then, symbiosis with legumes has been attributed to alpha-rhizobia, and it was only 20 years ago that the ability of some Betaproteobacteria to establish nitrogen-fixing nodules with legumes was reported. The major beta-rhizobia were classified as *Burkholderia*, and in 2014, they were re-classified into the new genus *Paraburkholderia*, created to encompass environmental non-pathogenic species. Scientific and ecological progress has been achieved, with the description of new species and symbiovars, with Brazil being the major center of diversity of the genus, followed by South Africa. Despite the progress in beta-rhizobia achieved in the past two decades, we are far from knowing the diversity of species in the Brazilian territory, their relationship with edaphoclimatic conditions, and their biotechnological potential for agriculture, indicating the need for future research.

Abbreviations

All abbreviations have been cited in their complete forms when mentioned for the first time in the manuscript.

Ethics Approval and consent of participation

The study has not involved any human or animal participation or data

Authors' contribution

F.P.; F.B.R.Jr, M.H. - participated in all stages of study, read and approved the final manuscript

Consent for publication

All authors gave the consent for publication

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Authors declare no competing of conflict of interests regarding the data or the manuscript

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Authors declare no ethical problems.

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Figure Legends

Fig. 1 Maximum-likelihood (ML) phylogeny based on the concatenated gene sequences (3,453 bp) of *recA* (1,025 bp) + *gyrB* (2,428 bp) showing the relationships between *Paraburkholderia* species. Nodulating species are in boldface. Accession numbers are indicated in parentheses and in Table S1. Bootstrap values >70% are indicated at the nodes. *Cupriavidus taiwanensis* LMG 19424^T was used as outgroup. Bar indicates one substitution per 100 nucleotide positions.

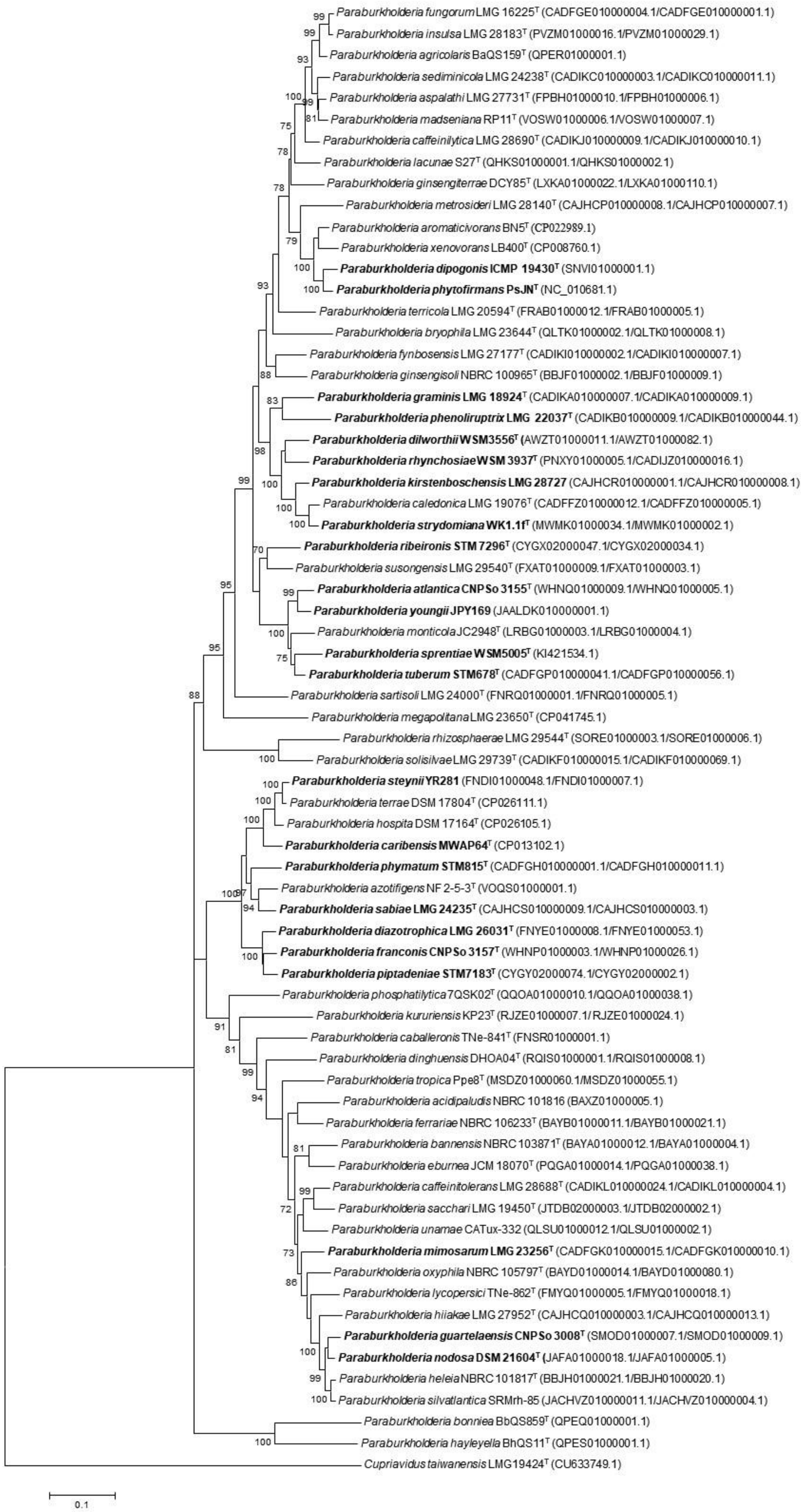
Fig. 2 Distribution of Brazilian nodulating *Paraburkholderia* type strains

Table 1. Host plants, isolation site and symbiovar cluster of Brazilian *Paraburkholderia*

Species	Plant Host	Brazilian State	Symbiovar cluster (Paulitsch et al. (2020b)	References
P. mimosarum	*M. scabrella, M. pigra*	MG	sv. mimosae	Chen et al. (2006); Bontemps et al. (2010)
P. nodosa	*Mimosa spp., Piptadenia, Parapiptadenia, Anadenanthera and Phaseolus vulgaris,*	DF, GO, BA, SP, RJ, ES, SC, PR	sv. mimosae	Chen et al. (2005, 2007); Bontemps et al. (2010); dos Reis Jr. et al. (2010); Bournaud et al. (2013); Lammel et al. (2015); Primieri et al. (2016); Dall'agnol et al. (2016); Dall'agnol et al. (2017); de Castro Pires et al. (2018); Paulitsch et al. (2019a)
P. sabiae	*Mimosa caesalpiniifolia, Piptadeniae* group, *Calliandra luetzelburgii*	RJ, SP, BA	sv. tropicalis	Chen et al. (2005); Chen et al. (2008); Bournaud et al. (2013); Silva et al. (2018)
P. phenoliruptrix	*Mimosa flocculosa, Mimosa pudica, Macroptilium atropurpureum, Piptadenia gonoacantha, Microlobius foetidus Anadenanthera peregrina, Parapiptadenia rígida, Pityrocarpa monoliformis*	SP, RJ	sv. tropicalis	Chen et al. (2005); Bournaud et al. (2013)
P. diazotrophica	Mimosa spp, *Anadenanthera*, *Parapiptadenia blanchetti*, *Piptadenia viridiflora*, *P. gonoacantha, Calliandra spp.*	GO, BA, RJ, RN, PE, BA	sv. tropicalis	Bontemps et al. (2010); Sheu et al. (2013); Bournaud et al. (2013); Silva et al. (2018); de Oliveira et al. (2019)
P. piptadeniae	*Piptadenia gonoacantha, Mimosa púdica, Mimosa scabrella, Acacia nilotica*	RJ	sv. tropicalis	Bournaud et al. (2017); Dall'agnol et al. (2017)

P. ribeironis	*Piptadenia gonoacantha, Mimosa scabrella, Acacia nilotica*	RJ	sv. piptadeaniae	Bournaud et al. (2017)
P. guartelaensis	*M. gymnas*	PR	sv. mimosae	Paulitsch et al. (2019b)
"*P. atlantica*"	*M. púdica, Macroptilium atropurpureum, Phaseolus vulgaris*	RJ	sv. atlantica	Paulitsch et al. (2020a)
"*P. franconis*"	*M. pudica, Phaseolus vulgaris*	RJ	sv. tropicalis	Paulitsch et al. (2020a)
"*P. youngii*"	*Mimosa xanthocentra var. subsericea, Mimosa spp., Lebeckia ambigua*	MG, GO, DF, MT	sv. atlantica*	Mavima et al. (2020)

* Based on a preliminary BLAST analysis of *nodC* and *nifH* genes.


Paraburkholderia fungorum LMG 16225^{T} (CADFGE010000004.1/CADFGE010000001.1)
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Paraburkholderia agricolaris BaQS159^{T} (QPER01000001.1)
Paraburkholderia sediminicola LMG 24238^{T} (CADIKC010000003.1/CADIKC010000011.1)
Paraburkholderia aspalathi LMG 27731^{T} (FPBH01000010.1/FPBH01000006.1)
Paraburkholderia madseniana RP11^{T} (VOSW01000006.1/VOSW01000007.1)
Paraburkholderia caffeinilytica LMG 28690^{T} (CADIKJ010000009.1/CADIKJ010000010.1)
Paraburkholderia lacunae S27^{T} (QHKS01000001.1/QHKS01000002.1)
Paraburkholderia ginsengiterrae DCY85^{T} (LXKA01000022.1/LXKA01000110.1)
Paraburkholderia metrosideri LMG 28140^{T} (CAJHCP010000008.1/CAJHCP010000007.1)
Paraburkholderia aromaticivorans BN5^{T} (CP022989.1)
Paraburkholderia xenovorans LB400^{T} (CP008760.1)
Paraburkholderia dipogonis ICMP 19430^{T} (SNVI01000001.1)
Paraburkholderia phytofirmans PsJN^{T} (NC_010681.1)
Paraburkholderia terricola LMG 20594^{T} (FRAB01000012.1/FRAB01000005.1)
Paraburkholderia bryophila LMG 23644^{T} (QLTK01000002.1/QLTK01000008.1)
Paraburkholderia fynbosensis LMG 27177^{T} (CADIKI010000002.1/CADIKI010000007.1)
Paraburkholderia ginsengisoli NBRC 100965^{T} (BBJF01000002.1/BBJF01000009.1)
Paraburkholderia graminis LMG 18924^{T} (CADIKA010000007.1/CADIKA010000009.1)
Paraburkholderia phenoliruptrix LMG 22037^{T} (CADIKB010000009.1/CADIKB010000044.1)
Paraburkholderia dilworthii WSM3556^{T} (AWZT01000011.1/AWZT01000082.1)
Paraburkholderia rhynchosiae WSM 3937^{T} (PNXY01000005.1/CADIJZ010000016.1)
Paraburkholderia kirstenboschensis LMG 28727 (CAJHCR010000001.1/CAJHCR010000008.1)
Paraburkholderia caledonica LMG 19076^{T} (CADFFZ010000012.1/CADFFZ010000005.1)
Paraburkholderia strydomiana WK1.1f^{T} (MWMK01000034.1/MWMK01000002.1)
Paraburkholderia ribeironis STM 7296^{T} (CYGX02000047.1/CYGX02000034.1)
Paraburkholderia susongensis LMG 29540^{T} (FXAT01000009.1/FXAT01000003.1)
Paraburkholderia atlantica CNPSo 3155^{T} (WHNQ01000009.1/WHNQ01000005.1)
Paraburkholderia youngii JPY169 (JAALDK010000001.1)
Paraburkholderia monticola JC2948^{T} (LRBG01000003.1/LRBG01000004.1)
Paraburkholderia sprentiae WSM5005^{T} (KI421534.1)
Paraburkholderia tuberum STM678^{T} (CADFGP010000041.1/CADFGP010000056.1)
Paraburkholderia sartisoli LMG 24000^{T} (FNRQ01000001.1/FNRQ01000005.1)
Paraburkholderia megapolitana LMG 23650^{T} (CP041745.1)
Paraburkholderia rhizosphaerae LMG 29544^{T} (SORE01000003.1/SORE01000006.1)
Paraburkholderia solisilvae LMG 29739^{T} (CADIKF010000015.1/CADIKF010000069.1)
Paraburkholderia steynii YR281 (FNDI01000048.1/FNDI01000007.1)
Paraburkholderia terrae DSM 17804^{T} (CP026111.1)
Paraburkholderia hospita DSM 17164^{T} (CP026105.1)
Paraburkholderia caribensis MWAP64^{T} (CP013102.1)
Paraburkholderia phymatum STM815^{T} (CADFGH010000001.1/CADFGH010000011.1)
Paraburkholderia azotifigens NF 2-5-3^{T} (VOQS01000001.1)
Paraburkholderia sabiae LMG 24235^{T} (CAJHCS010000009.1/CAJHCS010000003.1)
Paraburkholderia diazotrophica LMG 26031^{T} (FNYE01000008.1/FNYE01000053.1)
Paraburkholderia franconis CNPSo 3157^{T} (WHNP01000003.1/WHNP01000026.1)
Paraburkholderia piptadeniae STM7183^{T} (CYGY02000074.1/CYGY02000002.1)
Paraburkholderia phosphatilytica 7QSK02^{T} (QQOA01000010.1/QQOA01000038.1)
Paraburkholderia kururiensis KP23^{T} (RJZE01000007.1/ RJZE01000024.1)
Paraburkholderia caballeronis TNe-841^{T} (FNSR01000001.1)
Paraburkholderia dinghuensis DHOA04^{T} (RQIS01000001.1/RQIS01000008.1)
Paraburkholderia tropica Ppe8^{T} (MSDZ01000060.1/MSDZ01000055.1)
Paraburkholderia acidipaludis NBRC 101816 (BAXZ01000005.1)
Paraburkholderia ferrariae NBRC 106233^{T} (BAYB01000011.1/BAYB01000021.1)
Paraburkholderia bannensis NBRC 103871^{T} (BAYA01000012.1/BAYA01000004.1)
Paraburkholderia eburnea JCM 18070^{T} (PQGA01000014.1/PQGA01000038.1)
Paraburkholderia caffeinitolerans LMG 28688^{T} (CADIKL010000024.1/CADIKL010000004.1)
Paraburkholderia sacchari LMG 19450^{T} (JTDB02000003.1/JTDB02000002.1)
Paraburkholderia unamae CATux-332 (QLSU01000012.1/QLSU01000002.1)
Paraburkholderia mimosarum LMG 23256^{T} (CADFGK010000015.1/CADFGK010000010.1)
Paraburkholderia oxyphila NBRC 105797^{T} (BAYD01000014.1/BAYD01000080.1)
Paraburkholderia lycopersici TNe-862^{T} (FMYQ01000005.1/FMYQ01000018.1)
Paraburkholderia hiiakae LMG 27952^{T} (CAJHCQ010000003.1/CAJHCQ010000013.1)
Paraburkholderia guartelaensis CNPSo 3008^{T} (SMOD01000007.1/SMOD01000009.1)
Paraburkholderia nodosa DSM 21604^{T} (JAFA01000018.1/JAFA01000005.1)
Paraburkholderia heleia NBRC 101817^{T} (BBJH01000021.1/BBJH01000020.1)
Paraburkholderia silvatlantica SRMrh-85 (JACHVZ010000011.1/JACHVZ010000004.1)
Paraburkholderia bonniea BbQS859^{T} (QPEQ01000001.1)
Paraburkholderia hayleyella BhQS11^{T} (QPES01000001.1)
Cupriavidus taiwanensis LMG19424^{T} (CU633749.1)
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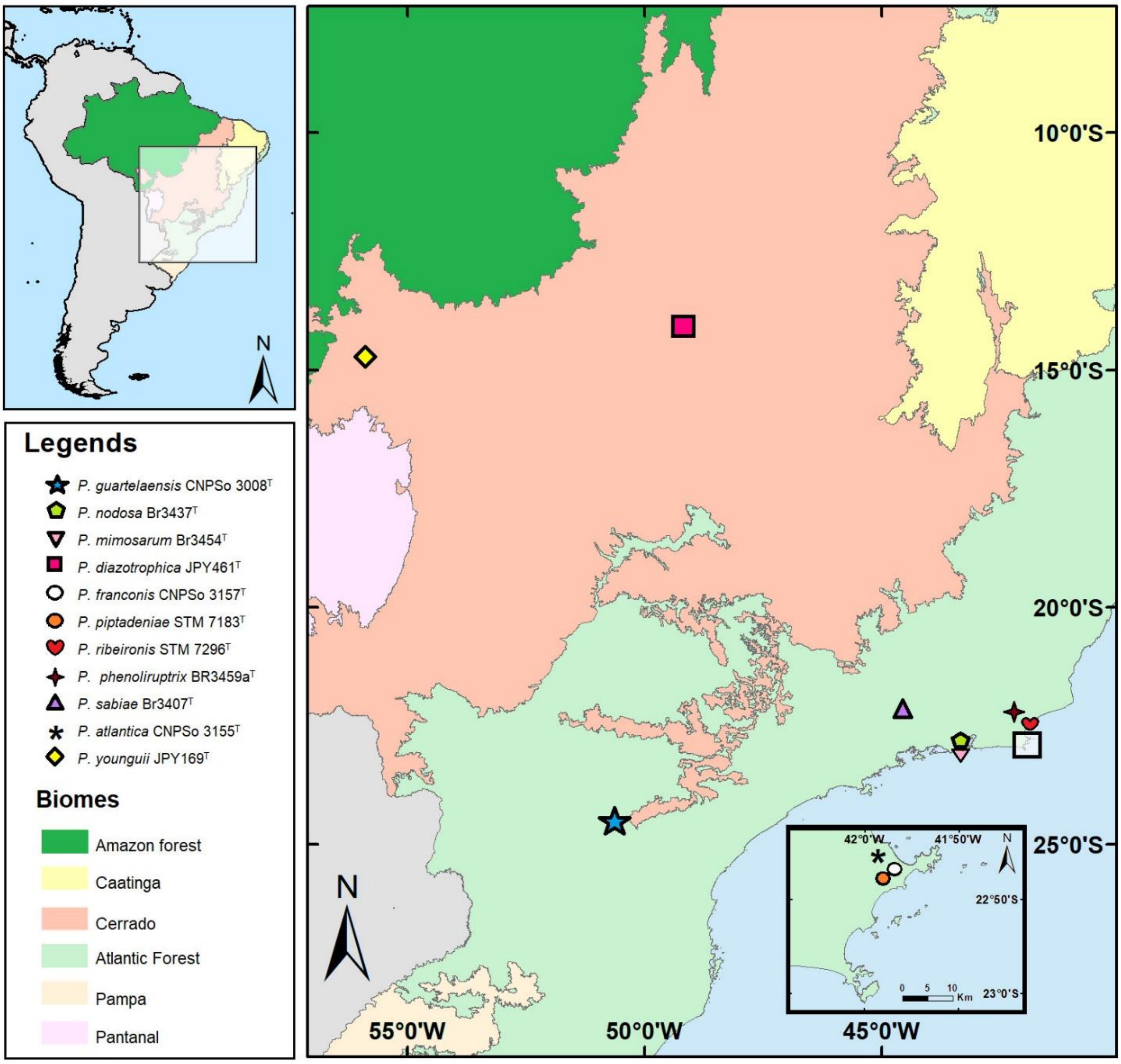
Fig. 1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P. ribeironis STM 7296^T
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P. sabiae Br3407^T
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P. younguii JPY169^T
Biomes
Amazon forest
Caatinga
Cerrado
Atlantic Forest
Pampa
Pantanal
10°0'S
15°0'S
20°0'S
25°0'S
55°0'W
50°0'W
45°0'W
N
42°0'W
41°50'W
N
22°50'S
23°0'S
0 5 10 Km

Fig. 2

Supplementary table

Table S1. Acession numbers of *Paraburkholderia* species used in the *recA*+*gyrB* phylogenetic analysis

Strain	*recA*	*gyrB*
P. acidipaludis NBRC 101816^{T}	BAXZ01000005.1	BAXZ01000002.1
P. agricolaris BaQS159^{T}	QPER01000001.1	QPER01000001.1
P. aromaticivorans BN5^{T}	CP022989.1	CP022989.1
P. aspalathi LMG27731^{T}	FPBH01000010.1	FPBH01000006.1
P. atlantica CNPSo 3155^{T}	WHNQ01000009.1	WHNQ01000005.1
P. azotifigens NF2-5-3^{T}	VOQS01000001.1	VOQS01000001.1
P. bannensis NBRC 103871^{T}	BAYA01000012.1	BAYA01000004.1
P. bonniea BbQS859^{T}	QPEQ01000001.1	QPEQ01000001.1
P. bryophila LMG 23644^{T}	QLTK01000002.1	QLTK01000008.1
P. caballeronis TNe-841^{T}	FNSR01000001.1	FNSR01000001.1
P. caffeinilytica LMG 28690^{T}	CADIKJ010000009.1	CADIKJ010000010.1
P. caffeinitolerans LMG 28688^{T}	CADIKL010000024.1	CADIKL010000004.1
P. caledonica LMG 19076^{T}	CADFFZ010000012.1	CADFFZ010000005.1
P. caribensis MWAP64^{T}	CP013102.1	CP013102.1
P. diazotrophica LMG 26031^{T}	FNYE01000008.1	FNYE01000053.1
P. dilworthii WSM3556^{T}	AWZT01000011.1	AWZT01000082.1
P. dinghuensis DHOA04^{T}	RQIS01000001.1	RQIS01000008.1
P. dipogonis ICMP 19430^{T}	SNVI01000001.1	SNVI01000001.1
P. eburnea JCM 18070^{T}	PQGA01000014.1	PQGA01000038.1
P. ferrariae NBRC 106233^{T}	BAYB01000011.1	BAYB01000021.1
P. franconis CNPSo 3150^{T}	WHNP01000003.1	WHNP01000026.1
P. fungorum LMG 16225^{T}	CADFGE010000004.1	CADFGE010000001.1
P. fynbosensis LMG 27177^{T}	CADIKI010000002.1	CADIKI010000007.1
P. ginsengisoli NBRC 100965^{T}	BBJF01000002.1	BBJF01000009.1
P. ginsengiterrae DCY85^{T}	LXKA01000022.1	LXKA01000110.1
P. graminis LMG 18924^{T}	CADIKA010000007.1	CADIKA010000009.1
P. guartelaensis CNPSo 3008^{T}	SMOD01000007.1	SMOD01000009.1
P. hayleyella BhQS11^{T}	QPES01000001.1	QPES01000001.1
P. heleia NBRC 101817^{T}	BBJH01000021.1	BBJH01000020.1
P. hiiakae LMG 27952^{T}	CAJHCQ010000003.1	CAJHCQ010000013.1
P. hospita DSM 17164^{T}	CP026105.1	CP026105.1
P. insulsa LMG 28183^{T}	PVZM01000016.1	PVZM01000029.1
P. kirstenboschensis LMG 28727^{T}	CAJHCR010000001.1	CAJHCR010000008.1
P. kururiensis KP23^{T}	RJZE01000007.1	RJZE01000024.1
P. lacunae S27^{T}	QHKS01000001.1	QHKS01000002.1
P. lycopersici TNe-862^{T}	FMYQ01000005.1	FMYQ01000018.1
P. madseniana RP11^{T}	VOSW01000006.1	VOSW01000007.1
P. megapolitana LMG 23650^{T}	CP041745.1	CP041745.1
P. metrosideri LMG 28140^{T}	CAJHCP010000008.1	CAJHCP010000007.1
P. mimosarum LMG 23256^{T}	CADFGK010000015.1	CADFGK010000010.1
P. monticola JC2948^{T}	LRBG01000003.1	LRBG01000004.1

P. nodosa DSM 21604^{T}	JAFA01000018.1	JAFA01000005.1
P. oxyphila NBRC 105797^{T}	BAYD01000014.1	BAYD01000080.1
P. phenoliruptrix LMG 22037^{T}	CADIKB010000009.1	CADIKB010000044.1
P. phosphatilytica 7QSK02^{T}	QQOA01000010.1	QQOA01000038.1
P. phymatum STM815^{T}	CADFGH010000001.1	CADFGH010000011.1
P. phytofirmans PsJN^{T}	NC_010681.1	NC_010681.1
P. piptadeniae STM7183^{T}	CYGY02000074.1	CYGY02000002.1
P. rhizosphaerae LMG 29544^{T}	SORE01000003.1	SORE01000006.1
P. rhynchosiae WSM 3937^{T}	PNXY01000005.1	CADIJZ010000016.1
P. ribeironis STM 7296^{T}	CYGX02000047.1	CYGX02000034.1
P. sabiae LMG 24235^{T}	CAJHCS010000009.1	CAJHCS010000003.1
P. sacchari LMG 19450^{T}	JTDB02000003.1	JTDB02000002.1
P. sartisoli LMG 24000^{T}	FNRQ01000001.1	FNRQ01000005.1
P. sediminicola LMG 24238^{T}	CADIKC010000003.1	CADIKC010000011.1
P. silvatlantica SRMrh-85	JACHVZ010000011.1	JACHVZ010000004.1
P. solisilvae LMG 29739^{T}	CADIKF010000015.1	CADIKF010000069.1
P. sprentiae WSM5005^{T}	KI421534.1	KI421534.1
P. steynii YR281	FNDI01000048.1	FNDI01000007.1
P. strydomiana WK1.1f^{T}	MWMK01000034.1	MWMK01000002.1
P. susongensis LMG 29540^{T}	FXAT01000009.1	FXAT01000003.1
P. terrae DSM 17804^{T}	CP026111.1	CP026111.1
P. terricola LMG 20594^{T}	FRAB01000012.1	FRAB01000005.1
P. tropica Ppe8^{T}	MSDZ01000060.1	MSDZ01000055.1
P. tuberum STM678^{T}	CADFGP010000041.1	CADFGP010000056.1
P. unamae CATux-332	QLSU01000012.1	QLSU01000002.1
P. xenovorans LB400^{T}	CP008760.1	CP008760.1
P. youngii JPY169^{T}	JAALDK010000001.1	JAALDK010000001.1
Cupriavidus taiwanensis LMG 19424^{T}	CU633749.1	CU633749.1

10. CONCLUSÕES

Os rizóbios desempenham um papel de grande relevância no desenvolvimento vegetal ao disponibilizarem o nitrogênio em forma biologicamente assimilável pelas plantas, mas até duas décadas atrás somente alfa-rizóbios eram conhecidos e estudados. Neste estudo, por meio da abordagem polifásica, que reúne informações fenotípicas, genotípicas e filogenéticas das estirpes, três novas espécies nodulíferas do gênero *Paraburkholderia,* isoladas do Brasil, foram descritas*. guartelaensis*, *P. atlantica* e *P. franconis*. Além disso, pela análise de genes simbióticos, foram propostos cinco biovares, mimosae, atlantica, tropicalis, piptadeniae e africana.

Em termos evolutivos os resultados indicaram, também, que os genes simbióticos de estirpes da América do Sul e da África do Sul possuem origens distintas e que a habilidade de nodular um hospedeiro específico pode estar relacionada com fatores biogeográficos e abióticos.

Os resultados confirmam que o Brasil é um dos principais centros de diversidade de *Paraburkholderia* nodulíferas, sendo considerado um reservatório de potenciais novas espécies. Desse modo, estudos com espécies nativas e endêmicas de plantas brasileiras e seus microssimbiontes devem ser encorajados.