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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).

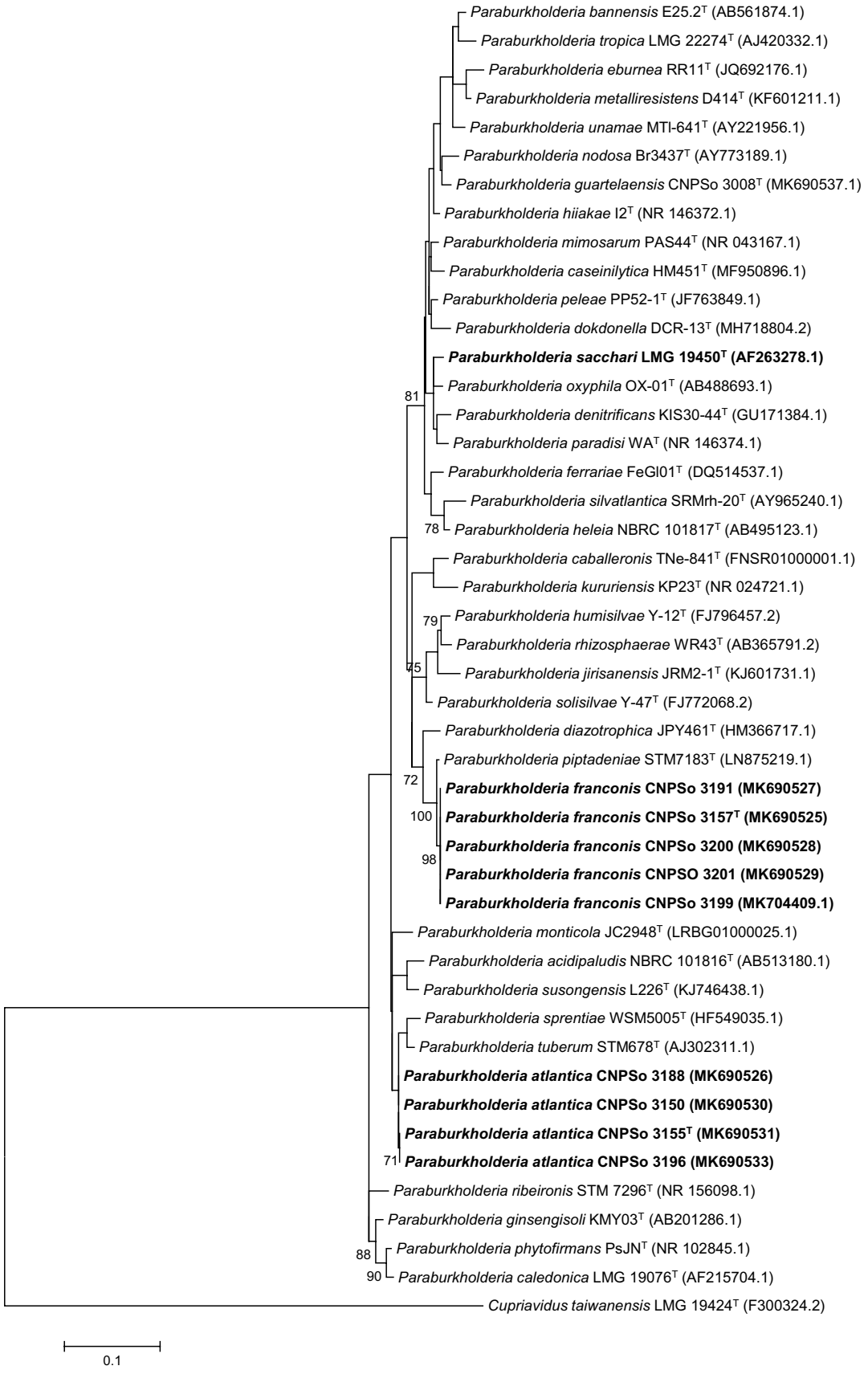
The DNA G + C contents of the CNPSo 3155 ^{T} and CNPSo 3157^T strains were calculated with QUAST (Gurevich et al. 2013).

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 E25.2T (AB561874.1)
Paraburkholderia tropica LMG 22274T (AJ420332.1)
Paraburkholderia eburnea RR11T (JQ692176.1)
Paraburkholderia metalliresistens D414T (KF601211.1)
Paraburkholderia unamae MTI-641T (AY221956.1)
Paraburkholderia nodosa Br3437T (AY773189.1)
Paraburkholderia guartelaensis CNPSo 3008T (MK690537.1)
Paraburkholderia hiiakae I2T (NR 146372.1)
Paraburkholderia mimosarum PAS44T (NR 043167.1)
Paraburkholderia caseinilytica HM451T (MF950896.1)
Paraburkholderia peleae PP52-1T (JF763849.1)
Paraburkholderia dokdonella DCR-13T (MH718804.2)
Paraburkholderia sacchari LMG 19450T (AF263278.1)
Paraburkholderia oxyphila OX-01T (AB488693.1)
Paraburkholderia denitrificans KIS30-44T (GU171384.1)
Paraburkholderia paradisi WAT (NR 146374.1)
Paraburkholderia ferrariae FeGl01T (DQ514537.1)
Paraburkholderia silvatlantica SRMrh-20T (AY965240.1)
Paraburkholderia heleia NBRC 101817T (AB495123.1)
Paraburkholderia caballeronis TNe-841T (FNSR01000001.1)
Paraburkholderia kururiensis KP23T (NR 024721.1)
Paraburkholderia humisilvae Y-12T (FJ796457.2)
Paraburkholderia rhizosphaerae WR43T (AB365791.2)
Paraburkholderia jirisanensis JRM2-1T (KJ601731.1)
Paraburkholderia solisilvae Y-47T (FJ772068.2)
Paraburkholderia diazotrophica JPY461T (HM366717.1)
Paraburkholderia piptadeniae STM7183T (LN875219.1)
Paraburkholderia franconis CNPSo 3191 (MK690527)
Paraburkholderia franconis CNPSo 3157T (MK690525)
Paraburkholderia franconis CNPSo 3200 (MK690528)
Paraburkholderia franconis CNPSO 3201 (MK690529)
Paraburkholderia franconis CNPSo 3199 (MK704409.1)
Paraburkholderia monticola JC2948T (LRBG01000025.1)
Paraburkholderia acidipaludis NBRC 101816T (AB513180.1)
Paraburkholderia susongensis L226T (KJ746438.1)
Paraburkholderia sprentiae WSM5005T (HF549035.1)
Paraburkholderia tuberum STM678T (AJ302311.1)
Paraburkholderia atlantica CNPSo 3188 (MK690526)
Paraburkholderia atlantica CNPSo 3150 (MK690530)
Paraburkholderia atlantica CNPSo 3155T (MK690531)
Paraburkholderia atlantica CNPSo 3196 (MK690533)
Paraburkholderia ribeironis STM 7296T (NR 156098.1)
Paraburkholderia ginsengisoli KMY03T (AB201286.1)
Paraburkholderia phytofirmans PsJNT (NR 102845.1)
Paraburkholderia caledonica LMG 19076T (AF215704.1)
Cupriavidus taiwanensis LMG 19424T (F300324.2)
81
78
79
75
72
100
98
71
88
90
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

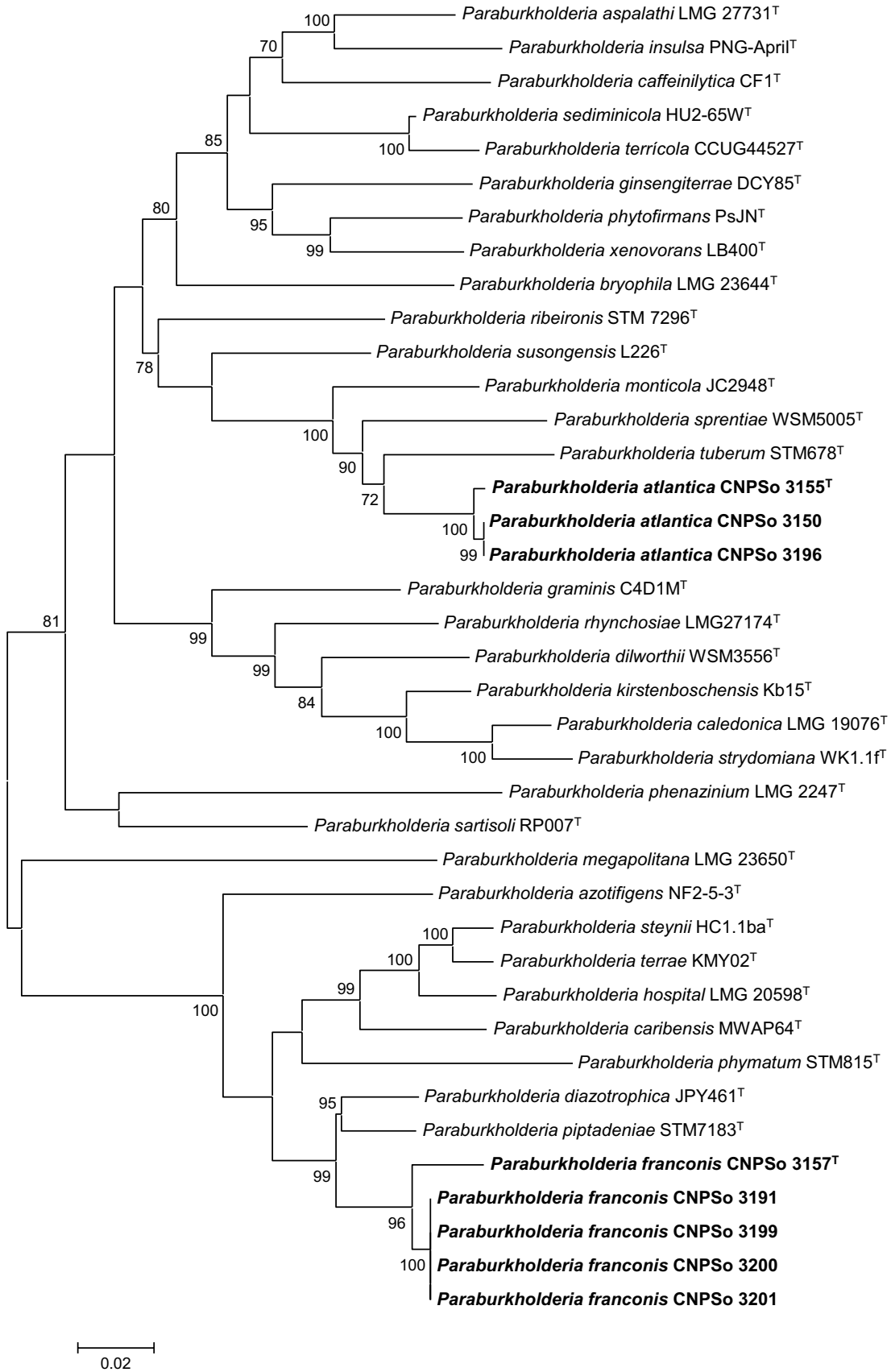
Phylogenetic analysis of the 16S rRNA and housekeeping genes

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


Paraburkholderia aspalathi LMG 27731T
Paraburkholderia insulsa PNG-AprilT
Paraburkholderia caffeinilytica CF1T
Paraburkholderia sediminicola HU2-65WT
Paraburkholderia terrícola CCUG44527T
Paraburkholderia ginsengiterrae DCY85T
Paraburkholderia phytofirmans PsJNT
Paraburkholderia xenovorans LB400T
Paraburkholderia bryophila LMG 23644T
Paraburkholderia ribeironis STM 7296T
Paraburkholderia susongensis L226T
Paraburkholderia monticola JC2948T
Paraburkholderia sprentiae WSM5005T
Paraburkholderia tuberum STM678T
Paraburkholderia atlantica CNPSo 3155T
Paraburkholderia atlantica CNPSo 3150
Paraburkholderia atlantica CNPSo 3196
Paraburkholderia graminis C4D1MT
Paraburkholderia rhynchosiae LMG27174T
Paraburkholderia dilworthii WSM3556T
Paraburkholderia kirstenboschensis Kb15T
Paraburkholderia caledonica LMG 19076T
Paraburkholderia strydomiana WK1.1fT
Paraburkholderia phenazinium LMG 2247T
Paraburkholderia sartisoli RP007T
Paraburkholderia megapolitana LMG 23650T
Paraburkholderia azotifigens NF2-5-3T
Paraburkholderia steynii HC1.1baT
Paraburkholderia terrae KMY02T
Paraburkholderia hospital LMG 20598T
Paraburkholderia caribensis MWAP64T
Paraburkholderia phymatum STM815T
Paraburkholderia diazotrophica JPY461T
Paraburkholderia piptadeniae STM7183T
Paraburkholderia franconis CNPSo 3157T
Paraburkholderia franconis CNPSo 3191
Paraburkholderia franconis CNPSo 3199
Paraburkholderia franconis CNPSo 3200
Paraburkholderia franconis CNPSo 3201
100
70
85
100
80
95
99
78
100
90
72
100
99
81
99
99
84
100
100
100
100
99
100
95
99
96
100
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,

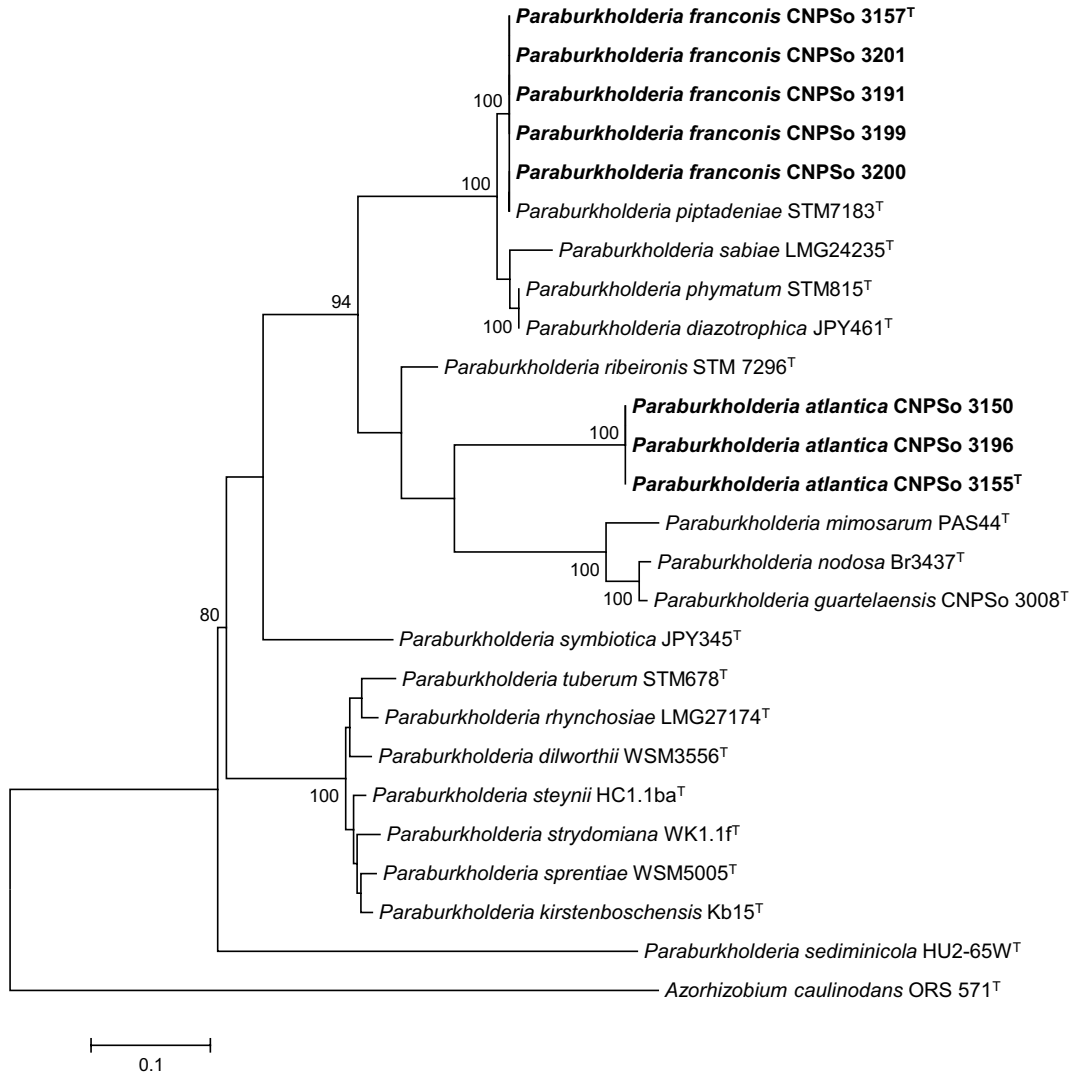


Fig. 3 Maximum-likelihood phylogeny based on sequences of *nodC* gene (347 bp) among novel *Paraburkholderia* species (in bold) and other nodulating members of the genus. Bootstrap values > 70% are indicated at the nodes. Accession numbers are indicated in Table S3. *Azorhizobium caulinodans* ORS 571 ^{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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