Paraburkholderia youngii sp. nov. and '*Paraburkholderia atlantica*' – Brazilian and Mexican *Mimosa*-associated rhizobia that were previously known as *Paraburkholderia tuberum* sv. mimosae

Lazarus Mavima[a], Chrizelle W. Beukes[a], Marike Palmer[a,b], Sofie E. De Meyer[c,d], Euan K. James[e], Marta Maluk[e], Eduardo Gross[f], Fabio Bueno dos Reis Junior[g], Juanita R. Avontuur[a], Wai Y. Chan[a,h], Stephanus N. Venter[a,*], Emma T. Steenkamp[a]

[a]*Department of Biochemistry, Genetics and Microbiology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa*

[b]*School of Life Sciences, University of Nevada Las Vegas, Las Vegas, NV, United States of America*

[c]*MALDIID Pty Ltd, Murdoch, Western Australia, Australia*

[d]*Laboratory of Microbiology, Department Biochemistry and microbiology, Ghent University, K.L. Ledeganckstraat 35, B-9000 Ghent, Belgium*

[e]*The James Hutton Institute, Invergowrie, Dundee DD2 5DA, UK*

[f]*Universidade Estadual de Santa Cruz, km 16 Rodovia Ilhéus – Itabuna, CEP 45662-900 Ilhéus, BA, Brazil*

[g]*Embrapa Cerrados, Cx. Postal 08223, 73310-970, Planaltina, Distrito Federal, Brazil*

[h]*Biotechnology Platform, Agricultural Research Council Onderstepoort Veterinary Institute (ARC-OVI), Onderstepoort, South Africa*

* Corresponding Author:

S.N. Venter; Private Bag X20, Hatfield, 0028, South Africa;

e-mail: fanus.venter@up.ac.za; tel: + 27 (0) 12 420 4100

Declarations of interest: none

Abstract

Previous studies have recognized South and Central/Latin American mimosoid legumes in the genera *Mimosa, Piptadenia* and *Calliandra* as hosts for various nodulating *Paraburkholderia* species. Several of these species have been validly named in the last two decades, e.g., *P. nodosa*, *P. phymatum*, *P. diazotrophica, P. piptadeniae, P. ribeironis, P. sabiae* and *P. mimosarum*. There are still, however, a number of diverse *Paraburkholderia* strains associated with these legumes that have an unclear taxonomic status. In this study, we focus on 30 of these strains which originate from the root nodules of Brazilian and Mexican *Mimosa* species. They were initially identified as *P. tuberum* and subsequently placed into a symbiovar (sv. mimosae) based on their host preferences. A polyphasic approach for the delineation of these strains was used, consisting of genealogical concordance analysis (using *atpD*, *gyrB*, *acnA*, *pab* and 16S rRNA gene sequences), together with comparisons of Average Nucleotide Identity (ANI), DNA G+C content ratios and phenotypic characteristics with those of the type strains of validly named *Paraburkholderia* species. Accordingly, these 30 strains were delineated into two distinct groups, of which one is conspecific with '*P. atlantica*' CNPSo 3155^T and the other new to Science. We propose the name *Paraburkholderia youngii* sp. nov. with type strain JPY169^T (= LMG 31411^T; SARCC751^T) for this novel species.

Keywords: *Burkholderia*, legume, Central America, South America, genealogical concordance, Average Nucleotide Identity, rhizobia

Introduction

The genus *Paraburkholderia* was recently recognized as a distinct taxon in the broader *Burkholderia sensu lato* group and contains a range of diverse species [11,40,86]. Although a few *Paraburkholderia* species have been isolated from the clinical environment [24,33], they are mostly plant beneficial and/or environmental species [39,94]. These include species with antibiotic, bioremediation and plant growth promoting capabilities, as well as the ability to fix atmospheric nitrogen [11,27,32,50]. Nitrogen fixation can occur in the free-living form (e.g., *P. kururiensis*, *P. tropica* and *P. unamae* [17,82]), in symbiosis with legumes (e.g., *P. mimosarum, P. kirstenboschensis* and *P. nodosa* [20,21,92]) or both (e.g., *P. tuberum* and *P. phymatum* [38,39,96]). Those capable of symbiotic nitrogen fixation (referred to here as rhizobial *Paraburkholderia*), induce the formation of nodules on the roots of their respective plant hosts, and within these organs they produce the enzymatic machinery for converting molecular di-nitrogen to ammonia [47].

Paraburkholderia includes most of the rhizobia known from the Betaproteobacteria, and the genus currently contains 21 rhizobial species [12,16,30,59]. These are *P. tuberum*, *P. dilworthii*, *P. caribensis*, *P. fungorum*, *P. phymatum*, *P. mimosarum*, *P. sprentiae*, *P. sabiae*, *P. nodosa, P. phenoliruptrix*, *P. kirstenboschensis*, *P. rhynchosiae*, *P. dipogonis*, *P. piptadeniae*, *P. ribeironis*, *P. diazotrophica*, *P. strydomiana*, *P. steynii* and yet to be validated '*P. guartelaensis*', '*P. franconis*' and '*P. atlantica*' [12,16,30,43,59,66,77,78]. However, this list excludes *P. caballeronis*, which is considered not be rhizobial as it apparently lacks nodulation capabilities [84]. Nonetheless, this rhizobial group of species is capable of establishing a nitrogen-fixing symbiosis with a wide range of mimosoid and papilionoid legumes across the world [13,28,62,87,89,92], although their distribution seems to follow a geographic pattern. Those associating mostly with papilionoid hosts are generally documented from the Cape Floristic Region in South Africa (e.g., *P. tuberum*, *P. dilworthii* and *P. sprentiae*), while those that associate with mimosoid hosts have predominantly been found in South America (e.g., *P. nodosa* and *P. sabiae*) [10,29,30,36,50,62]. Among these, *P. tuberum* is a notable exception, as strains identified as this species have been reported, not only from the Cape Floristic Region where it was first recorded [37,50], but also from other parts of the world such as Brazil, Costa Rica, French Guiana, Panama and Mexico [6,7,13,14,25,68,73].

Based on legume host range, *P. tuberum* strains separate into two broad groups. Those originating from South Africa associate predominantly with legumes from the tribes Hypocalypteae, Podalyrieae, Indigofereae, Phaseoleae and Crotalarieae (subfamily Papilionoideae) [10,13,37,61,62,64]. Those from other parts of the world mostly associate with species of *Mimosa*, as well as species of *Piptadenia* and *Calliandra*, which are all in the mimosoid clade of the subfamily Caesalpinioideae [13,14,15,25,64,68,73,89]. A similar separation has also been observed in the phylogenies of genes encoding the nodulation and/or nitrogen-fixation apparatus of these rhizobia [40]. In these phylogenies, *P. tuberum* strains associating with mimosoid species share a more recent ancestry with other mimosoid-associated *Paraburkholderia* species and *Cupriavidus* (another beta-rhizobial genus) [68], than with *P. tuberum* from papilionoid legumes [68]. In fact, based on this and other phylogenies, *P. tuberum* associating with papilionoid legumes share a more recent ancestry with the alphaproteobacterial rhizobia *Bradyrhizobium* and *Methylobacterium* [10,18,19,68]. This separation among *P. tuberum* strains further correlates with the two centers of diversity suggested for rhizobial *Paraburkholderia* species [12,50,62], i.e., the Fynbos biome in the Cape Floristic Region [62,92] and the Caatinga and Cerrado biomes in South America [50]. Accordingly, Mishra and colleagues [68] proposed the biovars "mimosae" and "papilionoideae" to distinguish the two groups of *P. tuberum* strains. However, these authors questioned the conspecificity of non-South African *P. tuberum* strains to that of the type strain STM678^T [68] and suggested that the group could consist of several species.

In this study we investigated the taxonomic status of a collection of *P. tuberum* strains recovered from the root nodules of diverse *Mimosa* species native to Brazil and Mexico [13,14,73]. For this purpose, we utilized a polyphasic approach that incorporates genealogical concordance as suggested by Venter et al. [102] to objectively identify plausible species hypotheses. As outlined in their step-wise workflow, congruence among multiple gene genealogies were used to delineate putative species groups among the strains included, after which other corroborative evidence for the species hypotheses were evaluated [93,102]. The latter included various genotypic and phenotypic metrics. For those hypotheses receiving robust support based on the independent data sources examined, species names were proposed, and descriptions of the taxa are provided.

Table 1 Strains which were investigated in this study, their hosts, geographical origins and results of nodulation tests.

Strain[a]	Original host	Geographical locations[b]	*Mimosa pudica*[c]	*Lebeckia ambigua*[c]	Reference[d]
Cluster A (*Paraburkholderia youngii* sp. nov.)					
JPY161	*Mimosa foliolosa*	Brazil, GO	I	I	[13]
JPY162	*Mimosa melanocarpa*	Brazil, GO	ND	I	[13]
***JPY169^T**	*Mimosa xanthocentra* var. *subsericea*	Brazil, MG	E	I	[13]
JPY284	*Mimosa hirsutissima*	Brazil, GO	E	I	[13]
JPY396	*Mimosa setosa* var. *urbica*	Brazil, DF	E	I	[13]
JPY401	*Mimosa foliolosa*	Brazil, DF	E	I	[13]
JPY403	*Mimosa lanuginosa*	Brazil, DF	E	I	[13]
JPY407	*Mimosa somnians*	Brazil, GO	E	I	[13]
***JPY418**	*Mimosa gracilis*	Brazil, GO	E	I	[13]
JPY421	*Mimosa skinneri*	Brazil, GO	E	I	[13]
***JPY432**	*Mimosa pteridifolia*	Brazil, GO	E	I	[13]
***JPY454**	*Mimosa vestita*	Brazil, GO	I	I	[13]
JPY468	*Mimosa somnians*	Brazil, MT	E	I	[13]
JPY480	*Mimosa callithrix*	Brazil, MT	N	I	[13]
JPY485	*Mimosa nuda* var. *nuda*	Brazil, MT	N	I	[13]
JPY489	*Mimosa aff. xanthocentra*	Brazil, MT	N	I	[13]
JPY602	*Mimosa adenocarpa*	Brazil, GO	N	ND	[13]
JPY622	*Mimosa gracilis*	Brazil, DF	N	I	[13]
JPY623	*Mimosa radula*	Brazil, DF	N	I	[13]
Cluster B (‘*Paraburkholderia atlantica*’)					
***CCGE1002**	*Mimosa occidentalis*	Mexico, N	ND	ND	[73]
JPY156	*Mimosa setosa* var. *paludosa*	Brazil, DF	E	ND	[13]
JPY158	*Mimosa pigra*	Brazil, DF	ND	ND	[13]
JPY171	*Mimosa xanthocentra* var. *xanthocentra*	Brazil, MT	E	I	[13]
***JPY251**	*Mimosa velloziana*	Brazil, DF	E	I	[13]
***JPY303**	*Mimosa velloziana*	Brazil, GO	E	I	[13]
JPY306	*Mimosa pseudoradula*	Brazil, GO	E	I	[13]
JPY395	*Mimosa adenocarpa*	Brazil, DF	E	I	[13]
JPY422	*Mimosa caesalpiniifolia*	Brazil, GO	E	I	[13]
JPY425	*Mimosa nuda*	Brazil, GO	E	I	[13]
JPY681	*Mimosa somnians*	Mexico	N	I	[14]
CNPSo 3155^T	*Mimosa pudica*	Brazil, R	E	ND	[78]

[a] Strains indicated by * have whole genome sequences available.

[b] Provinces are indicated as follows: BA Bahia; DF Distrito Federal; GO Goias; MT Mato Grosso; N Nayarit; R Rio de Janeiro.

[c] Results of the various nodulation tests are indicated as follows: E Effective nodules; I Ineffective nodules; N No nodule formation; and ND No Data (these strains where not included in the nodulation tests). All results, except those of CNPSo 3155^T, are from tests done in this study.

[d] References listed are for strain isolation, except that of CNPSo 3155^T which is also for nodulation tests.

Materials and methods

Rhizobial strains and culturing conditions

This study included 30 *Paraburkholderia* strains originally identified from previous studies [13,14,73] and collected from the root nodules of various *Mimosa* species indigenous to Brazil and Mexico (Table 1). These strains were preserved at -70 °C in glycerol stocks and were subsequently revived on Yeast Mannitol Agar (YMA) at 28 °C for 3 to 5 days [54]. This medium and growth conditions were also used for routine cultivation of the bacteria. The type strain for the new species proposed in this study has been submitted to the Belgian Coordinated Collections of Microorganisms (BCCM; Ghent University, Belgium) and the South African Rhizobium Culture Collection (SARCC; Agricultural Research Council, Plant Health and Protection Institute, South Africa).

DNA extraction, PCR and sequencing

DNA was extracted from 3 to 5-day old cultures of the respective strains using the Quick-gDNA™ MiniPrep kit (Zymo Research, USA), in accordance with the manufacturer's protocol. Following quantification with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA), 50 to 100 ng DNA was added to each 25 µl PCR mixture, which also contained 25 mM $MgCl_2$, 2.5 mM of each dNTP, 10 mM of each primer and 0.1 U μl^{-1} Super-Therm *Taq* DNA polymerase and reaction buffer (Separation Scientific, South Africa). These reaction mixtures were then subjected to PCR using a T100™ Thermal cycler (Bio-Rad Laboratories, USA) with the cycling conditions presented in Suppl. Table S1.

The sequences of five housekeeping genes were used in this study. These included the 16S ribosomal RNA (rRNA) gene, as well as portions of the genes *acnA* (encoding aconitate hydratase A, 715 base pairs [bp]), *atpD* (encoding ATP synthase subunit beta, 1150 bp), *gyrB* (encoding DNA gyrase subunit beta, 600 bp) and *pab* (encoding a protein with the anthranilate synthase/para-aminobenzoate synthase domain, 400 bp). These genes were specifically selected because of their variable nature and presence in the core genome of the broader *Burkholderia* sensu lato [11,40]. PCR primers for amplification of *acnA* and *pab* were designed during this study (Suppl. Table S1), whereas those for amplifying 16S rRNA, *atpD* and *gyrB* were available from previous studies [4,95,99,100]. The 16S rRNA gene was only sequenced for those

strains where the existing sequence available in the nucleotide database of the National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/genbank/) was shorter than 1000 bp.

All PCR products were purified using 20 U μl^{-1} Exonuclease 1 (Thermo Fisher Scientific) and 1 U μl^{-1} of Alkaline phosphatase (Thermo Fisher Scientific). The cleaned amplicons were then sequenced on an ABI 377 Automated Capillary DNA Sequencer (Applied Biosystems, USA) using the ABI PRISM Big Dye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems) and the original PCR primers. DNA chromatograms were manually curated using the software ChromasLite v2.6.2 (Technelysium, Australia) and BioEdit v7.2.5 [51]. All sequences resulting from this study have been submitted to the European Nucleotide Archive (ENA) (https://www.ebi.ac.uk/ena) and are publicly available with the following accession numbers: LR607185 [16S rRNA], LR607154-LR607184 [*atpD*], LR607061-LR607091 [*gyrB*], LR607007-LR607037 [*acnA*] and LR607123-LR607153 [*pab*].

Phylogenetic analyses

Single gene nucleotide datasets for the 16S rRNA, *atpD*, *gyrB*, *pab* and *acnA* genes were compiled using the sequences generated from this study and those previously determined. The latter was selected using BLASTN comparisons of 16S rRNA gene sequences of our strains to those in NCBI's nucleotide database (https://blast.ncbi.nlm.nih.gov/Blast.cgi [1,9]). Accordingly, our datasets included sequences for two previously identified *P. tuberum* strains (i.e., *P. tuberum* WSM4176 and *Paraburkholderia* sp. H160) [30,53,73], as well as those for the type strains of relevant reference species (i.e., *P. tuberum* STM678^T, '*P. atlantica*' CNPSo 3155^T [78], *P. ribeironis* STM 7296^T, *P. ginsengisoli* NBRC 100965^T, *P. susongensis* LMG 29540^T, *P. sprentiae* WSM5005^T and *P. monticola* JC2948^T) based on information obtained from the List of Prokaryotic Names with Standing in Nomenclature (LPSN; www.bacterio.net [41,75,76]). For outgroup purposes, we used the sequences for three distantly related *Paraburkholderia* species, i.e., *P. kururiensis* JCM 10599^T, *P. caballeronis* LMG 26416^T and *P. mimosarum* LMG 23256^T, and *Caballeronia calidae* LMG 29321^T. Several strains already had 16S rRNA sequences available from previous studies [13,14], and in instances where strains had whole genome sequences [73], the relevant gene sequences were extracted from these available resources. All

these sequences were obtained from the NCBI database and their accession numbers are listed in Suppl. Table S2.

Multiple sequence alignments for the protein-coding loci were manually generated with BioEdit [51] based upon the inferred amino acid sequences. The alignment for the 16S rRNA dataset was generated with MAFFT (Multiple Alignment using Fast Fourier Transformation; https://mafft.cbrc.jp/alignment/server/) by employing the Q-INS-I strategy, [56], which considers secondary structure. In addition to the five single gene alignments, we also constructed a partitioned five-gene concatenated nucleotide dataset, as well as a partitioned four-gene concatenated amino acid dataset. The partitioned concatenated datasets were generated from the single gene datasets with the aid of FASconCAT-G [58].

Maximum likelihood (ML) phylogenetic trees were inferred for the aligned nucleotide sequences for each single gene dataset and the concatenated datasets. For the single gene datasets, MEGA6 [98] was used to determine best-fit models of nucleotide substitution and for performing ML analyses. These analyses all included gamma correction (using five discrete gamma categories) to account for among-site rate variation [105], while the 16S rRNA also included a proportion of invariable sites. Also, all of the protein-coding datasets utilized the Tamura 3-parameter model (T92; [97]), while the Hasegawa-Kishino-Yano (HKY; [52]) model was used for the 16S rRNA dataset. The heuristic Nearest Neighbor Interchange (NNI) method with a strong 'branch swap filter' was used for tree searching based on an initial tree that was automatically created using the BioNJ neighbor-joining algorithm. Statistical support for the branches was estimated using bootstrap analysis of 1000 pseudoreplicates and the same parameter settings [42].

ML analyses of the partitioned five-gene concatenated nucleotide dataset and the partitioned four-gene concatenated amino acid dataset were performed using IQ-TREE (2.0.6) (http://www.iqtree.org). In these analyses, the best-fit substitution model for each gene partition was employed, with those in the concatenated nucleotide dataset being the same as described above. For the amino acid dataset, alignments, the Whelan and Goldman (WAG [104]) model was used for *acnA* and *gyrB*, the Jones, Taylor, and Thornton (JTT [55]) model was used for *atpD*, and the Le and Gascuel (LG [60]) model for *pab*. Branch support for the two

concatenated ML trees (i.e., multilocus sequence analysis [MLSA] trees) were also estimated using standard bootstrapping of using 1000 pseudoreplicates and the relevant model parameters [42]. All phylogenetic trees were visualized with Inkscape (0.92) (https://www.inkscape.org).

Whole genome sequence analyses

The genomes for CCGE1002, JPY251 and reference strains/species were obtained from NCBI ([9]; Suppl. Table S2), while those for JPY454, JPY418, JPY432, JPY303 and JPY169^T were sequenced during this study (Suppl. Table S2). The sequencing was done by MicrobesNG (University of Birmingham, UK) as per Estrada-de los Santos et al. [40], after which genomes were assembled using SPAdes v3.7 [5,71]. These whole genome assemblies have been deposited in the NCBI database under the accessions GCA_013372005.1 [JPY454], GCA_013371975.1 [JPY432], GCA_013367625.1 [JPY303], GCA_013367595.1 [JPY418] and GCA_013366925.1 [JPY169].

We used the whole genome sequences for strains CCGE1002, JPY251, JPY454, JPY418, JPY432, JPY303 and JPY169^T, in order to incorporate estimates of overall genome relatedness. These strains were selected to represent most of the distinct lineages observed within the phylogenies. For all pairs of genome sequences, average nucleotide identity (ANI; using the ANIb algorithm) [2,48] values and G+C content ratios were determined using JSpecies [83]. Digital DNA-DNA hybridization (dDDH) [67] values were calculated using a genome-to-genome distance calculator (https://ggdc.dsmz.de/), with JPY169^T against all other genome sequences interrogated. These analyses also included the genome sequences for the close relatives of the *P. tuberum* strains examined in this study, i.e., *Paraburkholderia* sp. H160, '*P. atlantica*' CNPSo 3155^T and *P. sprentiae* WSM5005^T as well as *P. tuberum* STM678^T and WSM4176.

Phenotypic characterization

By making use of 3-day old YMA cultures, bacterial cell morphology was determined using the Zeiss Crossbeam 540 FEG Scanning Electron Microscope (Zeiss, Germany) at the Laboratory for Microscopy and Microanalysis (University of Pretoria, South Africa). Motility was examined using wet mounts under 400× magnification with a light microscope [72] and with the soft agar stabbing technique using 0.4% (w/v) YMA and incubation at 28 °C for 3 days. Gram staining was

done according to O'Hara et al. [72]. Growth of all strains was also evaluated on YMA from 5 °C to 45 °C in increments of 5 °C, as well as at 37 °C. Growth was evaluated in the pH range of 3 to 10 in Yeast Mannitol broth according to Gerhardt et al. [45]. To investigate the effect of salinity, each isolate was grown on YMA enriched with varying NaCl concentrations ranging from 0 to 4% in increments of 1.0%.

For all strains, metabolic characteristics such as utilization of carbon and nitrogen sources were determined using Biolog GN2 micro-plate systems and API 20NE strips (BioMerieux). These tests were carried out according to the manufacturers' instructions, although incubation took place at 28 °C for 3 days. In addition, the catalase test was performed using 3% hydrogen peroxide (https://www.asm.org/Protocols/Catalase-Test-Protocol [81]) and the oxidase test was performed as described previously [65].

Nodulation tests

Many of the strains studied here were tested for their ability to nodulate the hosts *Mimosa pudica* and *Lebeckia ambigua* (Table 1). Tests for *Mimosa pudica* were performed at the James Hutton Institute using the growth system described by Bontemps et al. [13]. The tests performed on *Lebeckia ambigua* were performed at Murdoch University, using an axenic sand-culture system similar to the one described by Yates et al. [106].

Results

Phylogenetic analyses

Comparisons of 16S rRNA gene sequences to those in the NCBI database revealed that the strains from Brazil and Mexico shared high similarity to *Paraburkholderia* sp. H160 (≥98.2%), *P. tuberum* STM678^T (≥98.6%), *P. tuberum* WSM4176 (≥98.7%) and '*P. atlantica*' CNPSo 3155^T (≥99.5%). They shared 96.7 to 97.6% similarity with *P. ribeironis* STM 7296^T, *P. ginsengisoli* NBRC 100965^T, *P. susongensis* LMG 29540^T, *P. sprentiae* WSM5005^T and *P. monticola* JC2948^T. Accordingly, all our datasets also included the sequences for these additional taxa. The 16S rRNA dataset consisted of 1113 aligned nucleotide bases. Datasets for segments of the protein-coding genes *atpD*, *gyrB*, *acnA* and *pab* consisted of 1149, 603, 714 and 400 aligned nucleotides, respectively.

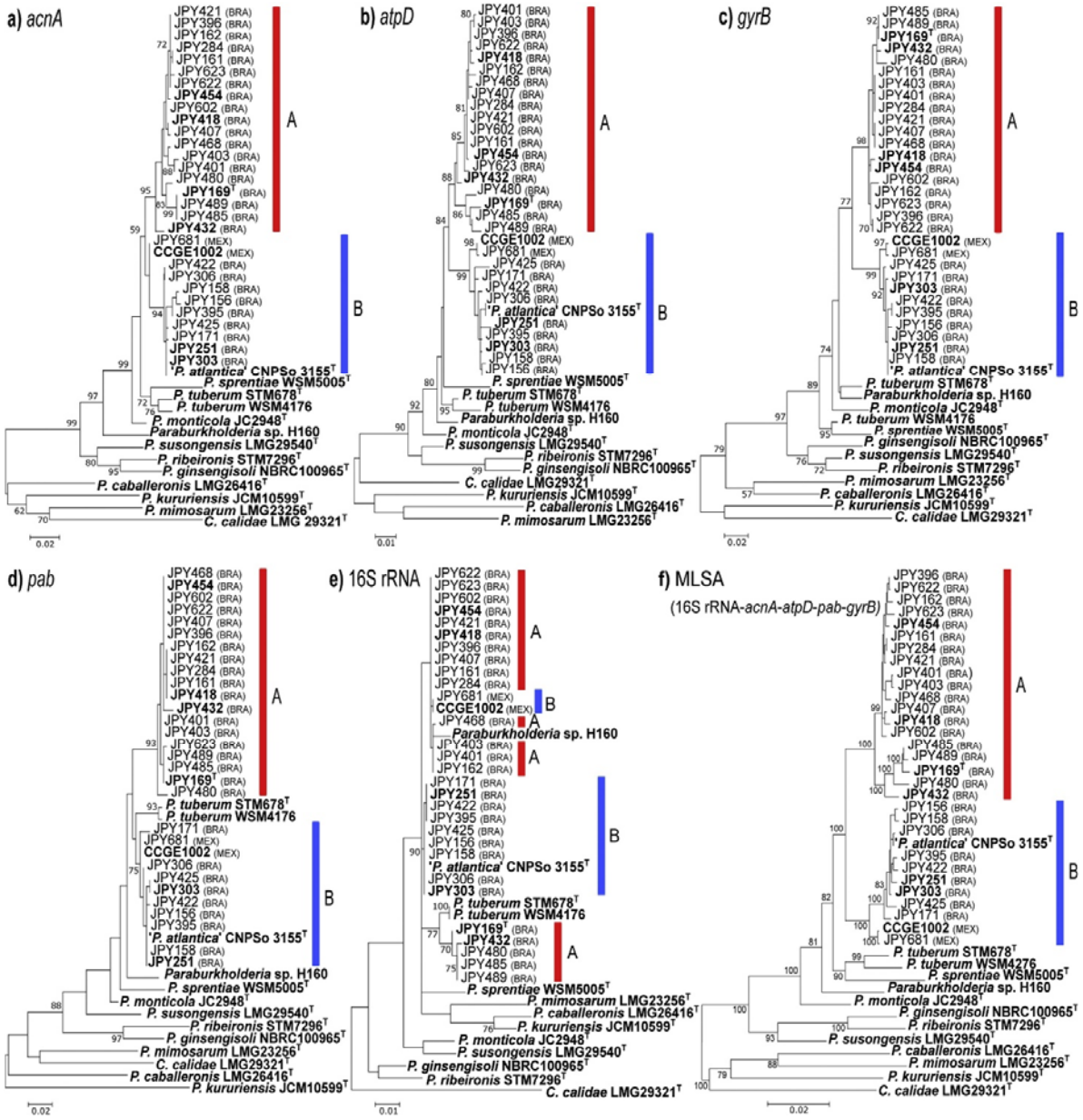

Fig. 1. Maximum likelihood phylogenies inferred from nucleotide sequence data for the 30 strains investigated and associated reference sequences. These included sequences for the four protein-coding genes *acnA* (a), *atpD* (b), *gyrB* (c) and *pab* (d), the 16S rRNA gene (e), and the concatenated 16S rRNA-*acnA*-*atpD*-*pab*-*gyrB* dataset (f), which also represents the MLSA tree. The scale bars indicate the number of nucleotide changes per site for each phylogeny, and bootstrap support values of >70% are indicated at the nodes. Isolates indicated in bold represent those with genome sequence data available. The DNA sequences for *C. calidae* LMG 29321^T [35,79], *P. kururiensis* JCM 10599^T [107], *P. caballeronis* LMG 26416^T [66] and *P. mimosarum* LMG 23256^T [20] were used for outgroup purposes. While those for *P. ribeironis* STM 7296^T [16], *P. ginsengisoli* NBRC 100965^T [57], *P. susongensis* LMG 29540^T [49], *P. sprentiae* WSM5005^T [28], *P. tuberum* STM678^T [69], *P. tuberum* WSM4176 [30,53], '*P. atlantica*' CNPSo 3155^T [78] and *Paraburkholderia* sp. H 160 [103] were used for reference purposes. GenBank accession numbers are listed in (Suppl. Table S2).

The phylogenies inferred from the protein-coding gene datasets, with the exception of the *pab* dataset, separated the 30 strains examined in this study, into a distinct cluster (Fig. 1a-d). This cluster included the type strain of '*P. atlantica*' CNPSo 3155^T, but excluded that of *P. tuberum* STM678^T, as well as *P. tuberum* WSM4176, *Paraburkholderia* sp. H160, and all other known species included in the analysis. In the *pab* phylogeny, the cluster containing all strains examined in this study did not only include '*P. atlantica*' CNPSo 3155^T but also *P. tuberum* STM678^T and WSM4176. The 16S rRNA data, however, were generally uninformative and the inferred phylogeny lacked resolution (with most branches receiving no to limited bootstrap support) (Fig. 1e), which is similar to what has previously been observed for *Paraburkholderia* [92].

An analysis determining genealogical concordance among the loci revealed that the 30 *P. tuberum* strains represented two unique and consistent evolutionary lineages that likely represent distinct species [102]. This is because the strains separated into two well-supported groups (Clusters A and B) across the four single-gene genealogies (Fig. 1a to d). Cluster A contained 19 strains (JPY622, JPY162, JPY421, JPY396, JPY284, JPY161, JPY623, JPY454, JPY602, JPY418, JPY407, JPY468, JPY403, JPY401, JPY480, JPY489, JPY485, JPY169^T and JPY432), while Cluster B consistently contained the remaining 11 strains (CCGE1002, JPY681, JPY158, JPY156, JPY395, JPY422, JPY306, JPY425, JPY171, JPY251 and JPY303) as well as '*P. atlantica*' CNPSo 3155^T.

Phylogenetic analysis of the five-gene concatenated dataset (i.e., MLSA of the combined *atpD*, *gyrB*, *acnA* and *pab* gene sequences [46]) revealed that the two putative species (presented by Clusters A and B) are most closely related to one another with 99% bootstrap support (Fig. 1f). Together they appeared to have a sister-group relationship with a lineage of South African taxa (containing *P. tuberum* STM678^T and *P. tuberum* WSM4176), albeit with no statistical support. Furthermore, Cluster B appeared to be conspecific with the type stain of the recently described mimosoid-associated '*P. atlantica*' CNPSo 3155^T. Neither of our proposed species grouped closely with other mimosoid-associated *Paraburkholderia* species (i.e., *P. mimosarum* and *P. ribeironis*) included in the analysis (Fig. 1f). Additionally, the grouping of isolates on the trees inferred from the concatenated nucleotide dataset (Fig. 1f and Fig. 3) and the concatenated amino acid dataset (Suppl. Fig. S1) was the same.

Table 2 Statistics for whole genomes of *Paraburkholderia* strains sequenced or used in this study

Statistics	*Paraburkholderia youngii* sp. nov.				'*Paraburkholderia atlantica*'			
	JPY169^{T}	JPY432	JPY454	JPY418	*CNPSo 3155^{T}	JPY303	*JPY251	*CCGE1002
Sequencing coverage	50.0	136.9	74.2	117.8	97.0	43.9	ND	ND
No. of contigs	4	249	326	198	246	249	124	4
Largest contig (bases)	6,823,646	721,168	399,214	569,127	ND	703,144	ND	ND
N50**	6823644	199,826	155,393	231,050	188,677	148,388	333,797	ND
GC content (%)	61.22	63.04	62.93	63.01	62.90	63.16	63.05	63.27
Genome size (Mb)	9.49	8.82	9.68	8.96	8.95	8.42	8.61	7.88
NCBI BioProject ID	PRJNA607730	PRJNA558401	PRJNA558402	PRJNA558409	PRJNA578139	PRJNA563793	PRJNA169692	PRJNA37719
Assembly accession number	GCA_013366925.1	GCA_013371975.1	GCA_013372005.1	GCA_013367595.1	GCA_009362785.1	GCA_013367625.1	GCA_000372985.1	GCA_000092885.1

* Strains whose genomes were downloaded from GenBank

** Length of the shortest contig that accumulatively show ≥50% the genome size [23].

ND No data

Whole genome sequence analyses

The statistics for the final genome assemblies of the five strains sequenced in this study are shown in Table 2. The sequencing coverage for isolates JPY432, JPY454, JPY418 and JPY169^T conform to the most recently proposed minimal standard (≥50X) for taxonomic purposes [23,26]. The remaining strain (JPY303) had sequencing coverage of less than 50X but greater than 43X. However, as this strain does not represent a type strain, its genome assembly is sufficient for use in pair-wise similarity estimations as reliable ANI values can be obtained by using as little as 20% of the complete genome [83].

Comparison of ANI values supported recognition of the two putative species, represented by Clusters A and B, as distinct and unique taxa (Fig. 2). ANI values among representatives of Cluster A (i.e., JPY418, JPY454, JPY432 and JPY169^T) were all more than 96%, and the same was also true for those within Cluster B (i.e., CCGE1002, JPY303, JPY251 and '*P. atlantica*' CNPSo 3155^T). ANI values between strains of the two clusters were all less than 95% (Fig. 2), which is in line with the suggested inter-species ANI range [2,48,83]. Additionally, the highest ANI values obtained for comparisons of *Paraburkholderia* sp. JPY169^T from Cluster A with '*P. atlantica*' CNPSo 3155^T, *P. tuberum* STM678^T, *P. tuberum* WSM4176, *P. sprentiae* WSM5005^T and *Paraburkholderia* sp. H160 were 93.36%, 89.94%, 89.78%, 89.19% and 89.59%, respectively. Similarly, the highest ANI values obtained for comparisons of '*P. atlantica*' *CNPSo* 3155^T (Cluster B) with and *P. tuberum* STM678^T, *P. tuberum* WSM4176, *P. sprentiae* WSM5005^T and *Paraburkholderia* sp. H160 were 89.96%, 89.9%, 89.15% and 89.07%, respectively. Furthermore, the dDDH values obtained from comparing representatives of Clusters A and B with JPY169^T (Fig. 2) supported the recognition of the two putative species according to the established dDDH inter-species cutoff [23]. Digital DDH values for the representatives of Cluster A (i.e., JPY418, JPY454 and JPY432) all exceeded 70%, while those for the representatives of Cluster B (i.e., CCGE1002, JPY303, JPY251 and '*P. atlantica*' CNPSo 3155^T) were all less than 70% compared to JPY169^T (Fig. 2).

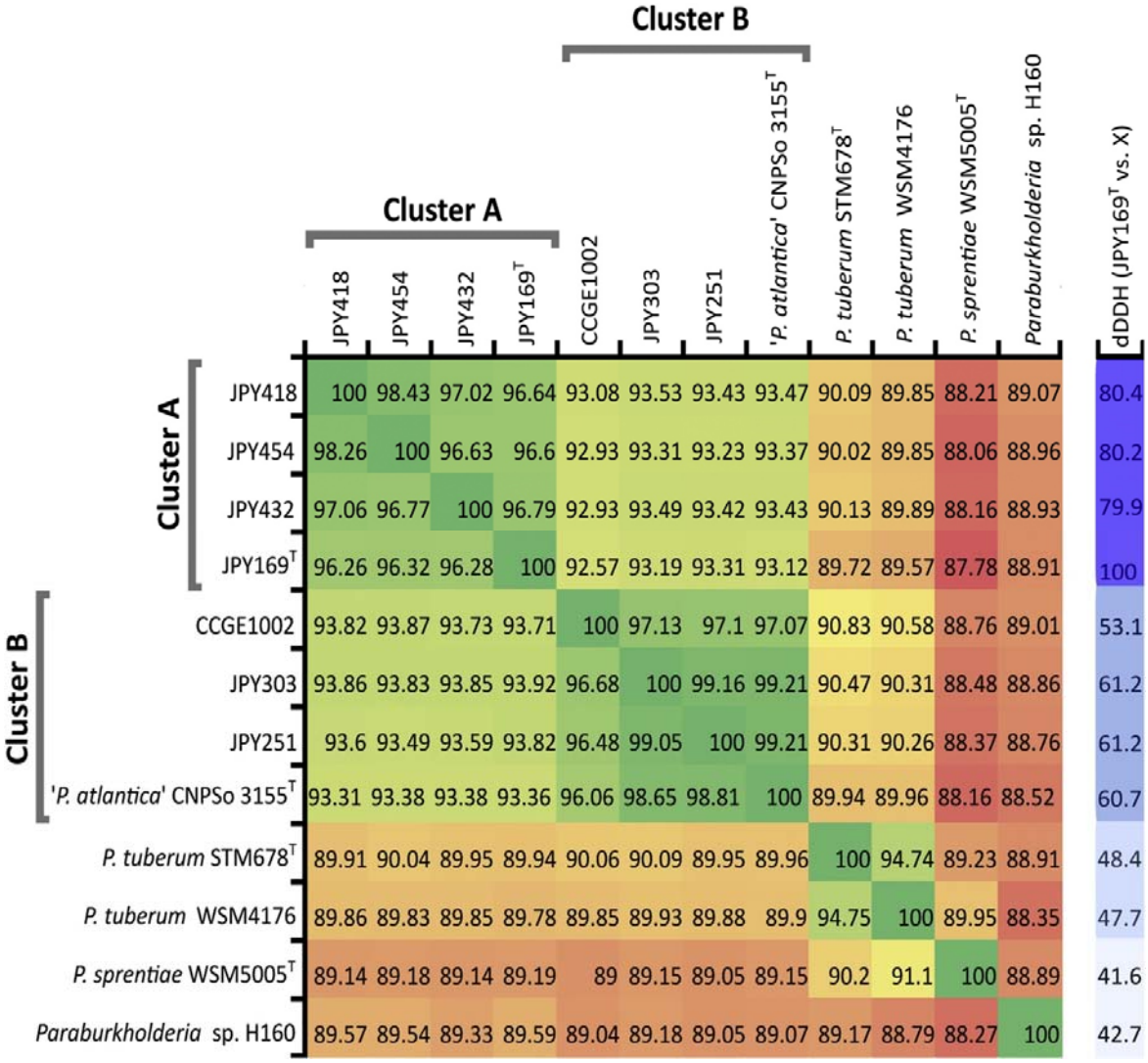

Fig. 2. ANI values between representatives of our delineated clusters of strains (i.e., Cluster A with JPY418, JPY454, JPY432 and JPY169^T and Cluster B with CCGE 1002, JPY303, JPY251 and '*P. atlantica*' CNPSo 3155^T), reference strains (i.e., *Paraburkholderia* sp. H160 and *P. tuberum* WSM4176) and type strains of the phylogenetically closest species (i.e., *P. tuberum* STM678^T and *P. sprentiae* WSM5005^T). The dDDH values listed are from the comparisons of JPY169^T with representatives of Clusters A and B and reference strains.

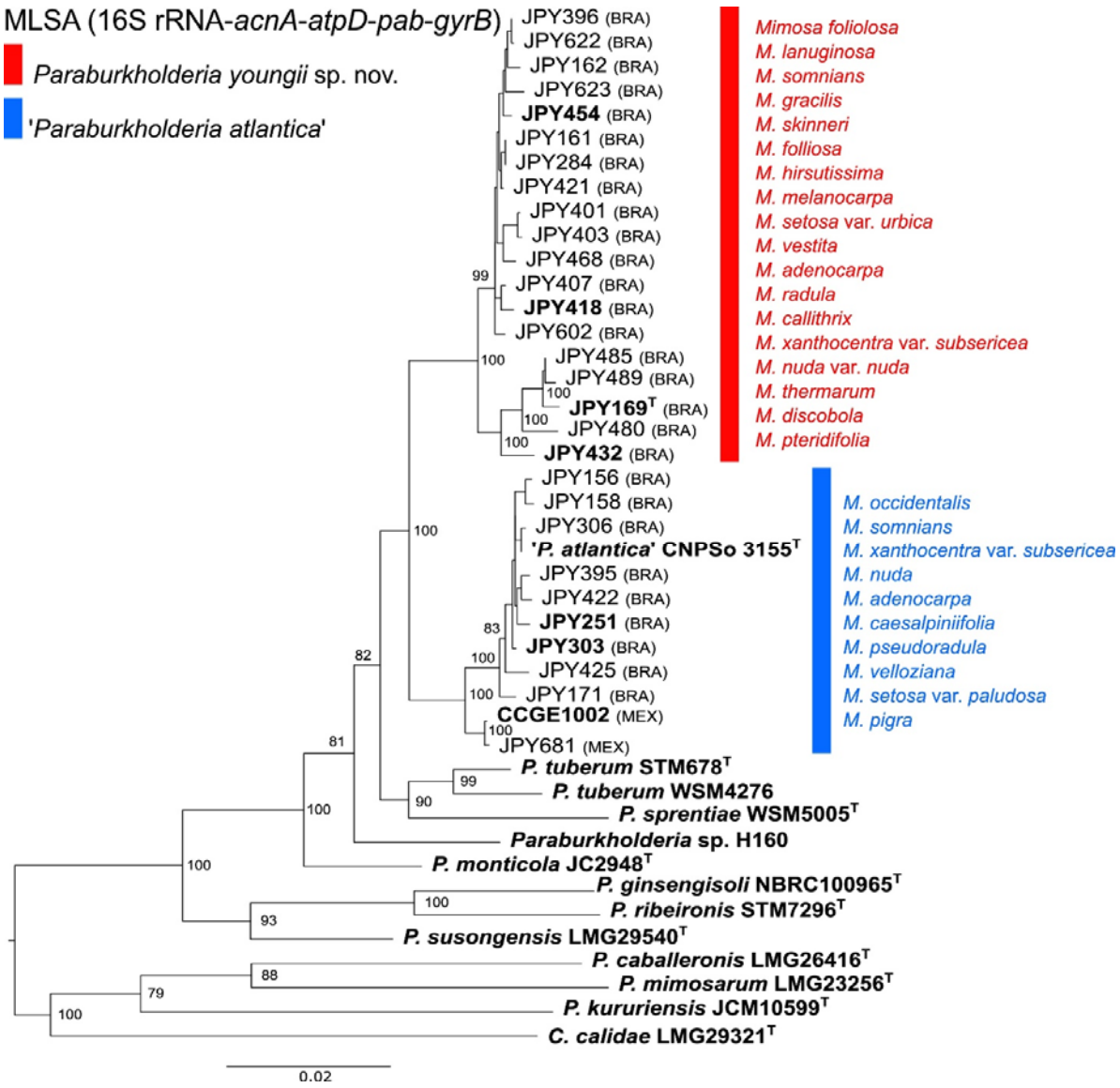

Fig. 3. An MLSA phylogeny based upon the concatenated 16S rRNA-*acnA-atpD-pab-gyrB* dataset indicating legume hosts associated with each proposed species. A tree with similar clustering patterns was recovered from the concatenated *acnA-atpD-pab-gyrB* amino acid sequence dataset (see Suppl. Fig. S1). The legume hosts presented in red and blue text associate with *P. youngii* sp. nov. and '*P. atlantica*', respectively. Bootstrap support values higher than 60% are indicated at the nodes, and the scale bar indicates the number of nucleotide changes per site. Strains in bold have whole genome sequences available. The geographical origin of the strains is indicated as: BRA (Brazil) and MEX (Mexico).

Phenotypic characteristics

All 30 strains were Gram-negative, motile, rod-shaped, and able to grow at 10 °C to 45 °C, across a pH range of 4 to 8 and within a 0-1% range of NaCl (Table 3). Based on the rate of colony or cell-biomass formation on YMA, the optimum conditions for growth of these strains

Table 3 Phenotypic characteristics of *P. youngii* sp. nov. when compared to closely related *Paraburkholderia* species.

Phenotypic characteristics [a]	***Paraburkholderia* species** [b]									
	1*	2* [Θ]	3 [#, ά, Θ]	4 [β, ψ, Θ]	5 [#, ϒ, Θ]	6 [#, Θ]	7 [#, ϒ, Θ]	8 [#, Θ]	9 [β, Θ]	10 [#, Θ]
Isolation source	RN	RN	RN	RN	RN	RN	RN	RN	RN	RN
Nitrate reduction	+	+	-	-	V	+	+	+	+	+
Motility	+	+	+	+	ND	ND	ND	ND	+	ND
Growth conditions:										
Temperature (°C)	10-40	10-40	28	10-40	28,30,37	ND	ND	28	15-40	28,30,37
pH	4-8	4-8	ND	4.5-9	ND	ND	ND	ND	4-8	ND
Salinity (NaCl%)	0-1	0-1	ND	0-10	ND	ND	ND	ND	0-1	ND
Activity of:										
Arginine dihydrolase	-	-	-	+	-	-	ND	-	-	-
Tryptophan deaminase	-	-	-	w+	ND	+	ND	-	ND	ND
Urease	V	V	-	-	V	V	+	-	+	+
β-Galactosidase	+	+	+	+	-	V	+	+	+	+
β-Glucosidase	-	-	ND	ND	ND	ND	ND	ND	-	ND
Catalase	+	+	ND	+	+	-	ND	ND	+	+
Oxidase	+	+	+	+	+	+	ND	+	+	+
Assimilation of:										
D-Glucose	+	+	+	+	+	+	+	+	+	+
L-Arabinose	+	+	+	+	+	+	+	+	+	+
D-Mannose	+	+	+	+	V	+	+	+	+	+
D-Mannitol	+	+	+	+	+	+	+	+	+	+
N-Acetyl-Glucosamine	+	+	+	+	+	+	+	+	+	+
D-Maltose	-	-	-	ND	-	-	+	-	-	-
Potassium gluconate	+	+	+	+	V	+	-	+	ND	+
Capric	-	-	+	-	-	V	+	+	-	+
Adipic acid	V	V	-	-	-	+	+	-	-	-
Malic acid	-	V	-	+	+	+	+	-	+	+
Trisodium citrate	-	V	-	+	-	V	+	-	+	+
Phenylacetic acid	V	+	-	+	V	+	+	-	-	+
Fermentation of:										
Adonitol	w+	+	+	ND	-	+	+	+	+	+
L-Arabinose	+	+	+	-	+	+	+	+	+	+

D-Arabitol	+	+	+	ND	+	+	+	+	+	+
D-Cellobiose	w+	+	-	ND	-	V	+	-	+	+
D-Fructose	+	+	+	ND	+	+	+	+	+	+
L-Fucose	+	+	+	ND	+	+	+	+	ND	+
α-D-lactose	-	V	-	ND	-	-	-	-	-	-
Maltose	-	-	-	ND	-	-	-	-	ND	-
D-Melibiose	-	-	-	-	-	-	-	+	-	-
D-Raffinose	-	-	-	ND	-	-	-	-	+	-
L-Rhamnose	+	+	+	-	-	+	+	+	+	+
D-Sorbitol	+	+	+	-	+	+	+	+	+	+
Sucrose	-	-	-	-	-	V	-	+	+	-
D-Ttrehalose	-	w+	-	ND	-	+	+	-	+	+
Xylitol	-	+	+	ND	-	-	+	+	ND	-
D-Glucose	+	+	-	-	-	-	-	-	-	-
D-Mannitol	+	w+	-	-	+	-	+	+	+	+
Inositol	+	+	-	-	+	-	+	ND	ND	w+
Whole genome data										
G+C content (mol%)	54.6-63	63-63.3	62.8	61.6	64.8	61.8	62.8	62.1	63-65	64.5

[a] Origins of the phenotypic characteristics used for comparison: [Θ] Data from the description of the species; * (this study); [#] Mthombeni [70]; [α] De Meyer et al. [28]; [β] Steenkamp et al. [92] [Ψ] Baek et al. [3]; [Υ] Weber et al. [103]; RN, root nodule; RH, rhizosphere; +, all strains positive; w+, weakly positive; -, all strains negative; ND, no data available.

[b] Isolates and strains were presented as follows: 1, *P. youngii* sp. nov. (n=19; this study); 2, '*P. atlantica*' CNPSo 3155^T [78]; 3, *P. tuberum* STM678^T [69]; 4, *P. sprentiae* WSM 5005^T [28]; 5, *P. mimosarum* LMG 23256^T [20]; 6, *P. kirstenboschensis* Kb15^T [92]; 7, *P. nodosa* LMG 23741^T [21]; 8, *P. phymatum* LMG 21445^T [101]; 9, *P. diazotrophica* LMG 26031^T [88]; 10, *P. sabiae* LMG 24235^T [22].

were 25 °C to 37 °C, pH in the range of 5 to 7 and 0.1 to 0.5% of NaCl. Additional morphological and biochemical features specific to our novel species are presented in the protologue and Table 3 and Suppl. Fig. S2. Although the commonly employed phenotypic characteristics were generally uninformative, the isolates in Clusters A and B differed in their ability to ferment xylitol. Our data showed that all strains belonging to Cluster A were negative for this trait, while strains belonging to Cluster B (including '*P. atlantica*' CNPSo 3155^T) were capable of fermenting this compound (Table 3). Additionally, their ability to ferment D-glucose differentiated the two clusters from their closest relatives, including *P. tuberum* STM678^T that cannot ferment this compound (Table 3).

Nodulation tests

Of the 18 strains belonging to Cluster A that were tested for nodulation on *M. pudica*, 10 formed effective nitrogen-fixing nodules, two (i.e., JPY161, JPY454) formed ineffective nodules and six failed to nodulate (Table 1). For the nodulation tests with the nine strains from Cluster B, eight strains effectively nodulated *M. pudica*, while JPY681 failed to nodulate (Table 1). For the nodulation tests on *Lebeckia*, all strains of both Clusters induced only the formation of ineffective nodules (Table 1). In other words, isolates from both Clusters seem to have the capacity to effectively nodulate *Mimosa* species, but not the South African native papilionoid legume tested here.

Discussion

This study showed that the collection of 30 strains, initially identified as *P. tuberum* and originating from Brazil and Mexico, represent two well-supported and unique groups. The genealogical concordance approach employed [8,102], consistently separated the strains into two distinct clusters across four independent housekeeping gene phylogenies. One of the groups was exclusive with its members being one another's closest relatives, and did not contain the strains of any other species [91]. The other group, however, included the type strain of '*P. atlantica*' (CNPSo 3155^T) [78] suggesting that the strains are members of this species. Although these groupings were not supported in the 16S rRNA phylogeny, the high conservation and limited resolving power of this marker at the species-level is well recognized in bacterial taxonomy [34,80,85]. This is also true for *Paraburkholderia* where different species

may share more than 98% 16S rRNA sequence similarity [12,92]. Nevertheless, delineation of the two groups were also mirrored in the results of the other metrics investigated. For example, each group displayed a unique set of biochemical properties that were common within the group, and each also contained strains sharing >96% ANI with one another, but <95% with other taxa. In other words, the polyphasic approach showed that the strains from the two groups represent distinct taxa, one containing 12 strains that is conspecific with '*P. atlantica*' and another, containing 19 strains, that is new to Science. For this new taxon we accordingly propose to name *Paraburkholderia youngii* sp. nov. with type strain JPY169^T (=LMG 31411^T =SARCC751^T).

The evolution of rhizobial *Paraburkholderia* taxa likely involves allopatric speciation. All of the strains examined here were recovered from nodules of indigenous Brazilian and Mexican *Mimosa* hosts [13,14,73], while *P. tuberum* STM678^T is the type strain of a species apparently indigenous to South Africa where it associates with papilionoid hosts [37,61]. The fact that all these strains were initially identified as *P. tuberum* using 16S rRNA and *recA* phylogenies [13,73] created the impression that a variant of *P. tuberum* is also present in South and Central/Latin America. Also, depending on which type of *nod* genes these strains harbor [68], they were thought to have a wide legume host range with the ability to nodulate legumes in both the Papilionoideae and Caesalpinioideae subfamilies. However, our findings counter the idea that non-South African *P. tuberum* strains occur in indigenous settings in Brazil and Mexico. Although *P. youngii* sp. nov. and '*P. atlantica*' might be closely related to taxa/strains of South African origin (particularly *P. tuberum* STM678^T, *P. tuberum* WSM4176 and *P. sprentiae* WSM5005^T), their divergence is likely to be the result of geographic isolation and adaptation to particular sets of environmental conditions [13,14,25,44,63,68]. Such processes typically provide the spatial and temporal separation among populations to ultimately halt the homogenizing effects of interpopulation gene flow, thereby allowing, and even driving, the speciation of bacterial taxa in allopatry [61,74].

Another factor that could have influenced the observed distribution of the rhizobial *Paraburkholderia* species *P. youngii* sp. nov. and '*P. atlantica*' is host phylogeny and/or coevolution with the plant host. The evolution of *Mimosa* species, particularly those endemic to Brazil and Mexico, might have influenced the selection and establishment of their rhizobial

symbionts [14]. This group of plants is thought to have diverged into the Brazilian and Mexican lineages, following separation of the South and North American continents [14,90]. Subsequently, these lineages seem to have evolved independently in concert with the rhizobial symbionts inhabiting local soils [14]. The available data suggest that Brazilian *Mimosa* species preferably associate with *Paraburkholderia* symbionts, while the Mexican species apparently prefer symbionts from the Alphaproteobacteria [14]. Therefore, based on our findings and those from previous studies [13,14], *P. youngii* sp. nov., '*P. atlantica*' and other rhizobial *Paraburkholderia* are unlikely to be common symbionts of endemic Mexican *Mimosa* species.

It would be interesting to investigate the diversity and abundance of rhizobial *Paraburkholderia* species in Mexican and Brazilian soils and how it correlates with the observed *Mimosa*-*Paraburkholderia* distribution. With regard to this, edaphic factors such as soil pH are clearly of importance. For example, it was recently shown that *Mimosa* species in acidic soils in central Brazil mostly associate with *Paraburkholderia* symbionts, but that these same *Mimosa* species growing in neutral-alkaline soils in the region are nodulated by *Rhizobium* [25]. Furthermore, the sv. mimosae symbiotic genotype, which Mishra et al. [68] devised to describe *P. tuberum*-like strains nodulating *Mimosa* in South America, is clearly mobile, as several *P. nodosa* strains harboring "*P. tuberum* sv. mimosae" *nodC* genes were recently isolated from *Calliandra* species in the Northeast of Brazil [89].

Paraburkholderia youngii sp. nov. and '*P. atlantica*' are probably native to Brazil and, to some extent, Mexico, where they represent rhizobial symbionts of diverse *Mimosa* species [13,14,25,73]. The type strain of *P. youngii* sp. nov. (JPY169^T) was originally isolated from the root nodules of *Mimosa xanthocentra var. subsericea* located in Mato Grosso, Brazil [13], and the other 18 strains were recovered from the root nodules of numerous Brazilian *Mimosa* species. The type strain of '*P. atlantica*' (CNPSo 3155^T) was originally isolated from the root nodules of *M. pudica* following trapping experiments with this host in soil from the Brazilian Atlantic Forest situated in Rio de Janeiro, Brazil [78]. Most of the examined strains in the current study also originated from Brazilian *Mimosa* species, particularly in the central Cerrado region where this legume genus has radiated most widely [13,78]. Only two of the examined strains of '*P. atlantica*' were isolated from Mexican hosts, i.e., CCGE1002 and JPY681 that originate from the root nodules of *M. occidentalis* [73] and *M. somnians* [14], respectively.

These two closely-related strains are also known to effectively nodulate several other *Mimosa* species found in Mexico, such as *M*. *affinis* and the pantropical species *M. pudica* [14]. Similarly, the majority of *P. youngii* sp. nov. and '*P. atlantica*' strains examined here were capable of effectively nodulating *M. pudica*, native to Central and South America, but not the South African legume *Lebeckia*. Future research should seek to determine whether *P. youngii* sp. nov. and '*P. atlantica*' is compatible with papilionoid hosts or other legume hosts native to South Africa.

This study has expanded the number of rhizobial *Paraburkholderia* species known from South America. Including *P*. *youngii* sp. nov., there are now 13 rhizobial *Paraburkholderia* species that have their origins in South and Central America. These include *P. caribensis* [15], *P*. *mimosarum* [20], *P*. *sabiae* [22], *P. nodosa* [21], *P. phenoliruptrix* [31], *P*. *piptadeniae* [16], *P*. *ribeironis* [16], *P. diazotrophica* [88], '*P*. *guartelaensis*' [77], '*P*. *franconis*' [78], '*P. atlantica*' [78] and possibly *P*. *fungorum* [43]. They are all associated with mimosoid legumes, particularly native *Mimosa* and *Piptadenia* species from Brazil, French Guiana and Venezuela [15,16,20,21,22,50,77,78], and invasive *Mimosa* species in Taiwan [15,16,20,50]. Also, the *nodC* gene of these species are closely related and members of the same cluster, [13,68,77,78] and previous work has also shown that their *nod* locus is prone to horizontal gene transfer [11,12]. This study thus confirms that a wealth of *Paraburkholderia* species, including novel ones awaiting discovery, associate with mimosoid legumes, particularly *Mimosa* species, from South and Central/Latin America. The description of *Paraburkholderia youngii* sp. nov. is listed in Table 4.

Table 4 Description of *Paraburkholderia youngii* sp. nov.

Genus name	*Paraburkholderia*
Species name	*Paraburkholderia youngii*
Specific epithet	*youngii*
Species status	sp. nov.
Species etymology	young'i.i. N.L. gen. n. *youngii*, of Young, named after J. Peter W. Young for his contributions to rhizobial systematics
Description of the new taxon and	Cells are Gram-negative, motile and rod-shaped with an average length and width of 1.57 µm and 0.63 µm, respectively. Growth occurs on YMA with 0 to

diagnostic traits	1% NaCl, at temperatures of 10 °C to 45 °C (optimum 25 °C to 37 °C), and at pH 4 to 8 (optimum pH 5 to 7). Colony morphology for strains in this species is quite diverse, they can be white to creamy (except for JPY161 which is yellow), with circular or irregular form, convex or raised elevation, and entire or curled margins. Strains are positive for the activity of catalase, oxidase and β-galactosidase as well as for the reduction of nitrate to nitrite. Strains test positive for the assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, potassium gluconate and N-acetyl-glucosamine and for the hydrolysis of Tween 40, Tween 80, D-glucose, D-mannitol, D-sorbitol, inositol, N-acetyl-D glucosamine, adonitol, L-arabinose, D-arabitol, D-cellobiose, D-fructose, L-fucose, D-galactose, D-mannose, L-rhamnose, D-sorbitol, pyruvic acid methyl ester, succinic acid mono-methyl ester, acetic acid, cis-aconitic acid, citric acid, D-galactonic acid, D-gluconic acid, glucosaminic acid, β-hydroxybutyric acid, D-L-lactic acid, propionic acid, quinic acid, D-saccharic acid, succinic acid, bromosuccinic acid, succinamic acid, D-alanine, L-alanine, asparagine, L-aspartic acid, L-glutamic acid, glycyl-L glutamic acid, L-histidine, hydroxy-L-proline, L-phenylalanine, L-proline, L-pyroglutamic acid, L-serine, L-threonine, urocanic acid and glycerol. Negative reactions are observed for the assimilation of D-maltose and capric acid. Additionally, strains are negative for the hydrolysis of xylitol, α-cyclodextrin, dextrin, glycogen, i-erythritol, gentiobiose, maltose, D-melibiose, β-methyl-D-glucoside, D-raffinose, sucrose, D-trehalose, turanose, α-hydroxybutyric acid, γ-hydroxybutyric acid, α-ketobutyric acid, α-ketovaleric acid, glycyl-L-aspartic acid, L-leucine L-ornithine, D-serine, D-L-carnitine, γ-aminobutyric acid, uridine, thymidine, phenylethyl-amine, 2-aminoethanol, 2-3-butanediol and α-D-glucose-1-phosphate.
Country of origin	Brazil
Region of origin	Mato Grosso
Source of isolation	Legume root nodules
Sampling date (dd/mm/yyyy)	01/09/2005
Latitude	15° 18' 13.2" S
Longitude	55° 49' 50.3" W
Altitude (meters above sea level)	689m
16S rRNA gene accession nr.	FN543659

Genome accession number	NCBI = GCA_013366925.1
Genome status	Complete
Genome size	9,492 Kbp
GC mol%	61.22
Number of strains in study	30
Source of isolation of non-type strains	Legume root nodules
Designation of the Type Strain	JPY169^T
Strain Collection Numbers	JPY169^T = LMG 31411^T = SARCC751^T

Acknowledgements

The authors would like to acknowledge the National Research Foundation (NRF) and the Department of Science and Innovation (DSI) of South Africa for financial support. The authors also extend their gratitude to the Laboratory for Microscopy and Microanalysis Unit of the University of Pretoria for providing electron microscopy infrastructure and guidance. Whole genome sequencing was provided by MicrobesNG (http://www.microbesng.uk), which is supported by the BBSRC (grant number BB/L024209/1).

References

1. Altschul, S.F., Gish, W., Miller, W., Meyers, E.W., Lipman, D.J. (1990) Basic local alignment search tool. J. Mol. Biol. 215, 403-410.
2. Arahal, D. (2014) Whole-genome analyses: average nucleotide identity. Method Microbiol. 41, 103-122.
3. Baek, I., Seo, B., Lee, I., Yi, H., Chun, J. (2015) *Burkholderia monticola* sp. nov., isolated from mountain soil. Int. J. Syst. Evol. Microbiol. 65, 504-509.
4. Baldwin, A., Mahenthiralingam, E., Thickett, K.M., Honeybourne, D., Maiden, M.C., Govan, J.R., Speert, D.P., LiPuma, J.J., Vandamme, P., Dowson, C.G. (2005) Multilocus sequence typing scheme that provides both species and strain differentiation for the *Burkholderia cepacia* complex. J. Clin. Microbiol. 43, 4665-4673.

5. Bankevich, A., Nurk, S., Antipov, D., Gurevich, A.A., Dvorkin, M., Kulikov, A.S., Lesin, V.M., Nikolenko, S.I., Pham, S., Prjibelski, A.D., Pyshkin, A.V., Sirotkin, A.V., Vyahhi, N., Tesler, G., Alekseyev, M.A., Pevzner, P.A. (2012) SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J. Comput. Biol. 19, 455-477.
6. Barrett, C.F., Parker, M.A. (2005) Prevalence of *Burkholderia* sp. nodule symbionts on four mimosoid legumes from Barro Colorado Island, Panama. Syst. Appl. Microbiol. 28, 57-65.
7. Barrett, C.F., Parker, M.A. (2006) Coexistence of *Burkholderia*, *Cupriavidus*, and *Rhizobium* sp. nodule bacteria on two *Mimosa* spp. in Costa Rica. Appl. Environ. Microbiol. 72, 1198-1206.
8. Baum, D.A., Shaw, K.L. (1995) Genealogical perspectives on the species problem. In: Hoch, P.C., Stephenson, A.G. (Eds.), Experimental and molecular approaches to plant biosystematics. vol 53. Missouri Botanical Garden, St Louis, pp 289-303.
9. Benson, D.A., Cavanaugh, M., Clark, K., Karsch-Mizrachi, I., Lipman, D.J., Ostell, J., Sayers, E.W. (2017) GenBank. Nucleic Acids Res. 45, D37-D42.
10. Beukes, C.W., Venter, S.N., Law, I.J., Phalane, F.L., Steenkamp, E.T. (2013) South African papilionoid legumes are nodulated by diverse *Burkholderia* with unique nodulation and nitrogen-fixation loci. PLoS One 8(7): e68406.
11. Beukes, C.W., Palmer, M., Manyaka, P., Chan, W.Y., Avontuur, J.R., van Zyl, E., Huntemann, M., Clum, A., Pillay, M., Palaniappan, K., Varghese, N., Mikhailova, N., Stamatis, D., Reddy, T.B.K., Daum, C., Shapiro, N., Markowitz, V., Ivanova, N., Kyrpides, N., Woyke, T., Blom, J., Whitman, W.B., Venter, S.N., Steenkamp, E.T. (2017) Genome data provides high support for generic boundaries in *Burkholderia* sensu lato. Front. Microbiol. 8, 1154.
12. Beukes, C.W., Steenkamp, E.T., van Zyl, E., Avontuur, J., Chan, W.Y., Hassen, A.I., Palmer, M., Mthombeni, L.S., Phalane, F.L., Sereme, T.K., Venter, S.N. (2019) *Paraburkholderia strydomiana* sp. nov. and *Paraburkholderia steynii* sp. nov.: rhizobial symbionts of the fynbos legume *Hypocalyptus sophoroides*. Anton. Leeuw. Int. J. G. 112, 1369-1385.
13. Bontemps, C., Elliott, G.N., Simon, M.F., dos Reis, F.B. Jr., Gross, E., Lawton, R.C., Neto N.E., de Fátima Loureiro, M., de Faria, S.M., Sprent, J.I., James, E.K., Young, J.P.W. (2010) *Burkholderia* species are ancient symbionts of legumes. Mol. Ecol. 19, 44-52.
14. Bontemps, C., Rogel, M.A., Wiechmann, A., Mussabekova, A., Moody, S., Simon, M.F., Moulin, L., Elliott, G.N., Lacercat-Didier, L., Dasilva, C., Grether, R., Camargo-Ricalde, S.L., Chen, W., Sprent, J.I., Martínez-Romero, E., Young, J.P.W., James, E.K. (2016) Endemic

Mimosa species from Mexico prefer alphaproteobacterial rhizobial symbionts. New Phytol. 209, 319-333.

15. Bournaud, C., de Faria, S.M., Ferreira dos Santos, J.M., Tisseyre, P., Silva, M., Chaintreuil, C., Gross, E., James, E.K., Prin, Y., Moulin, L. (2013) *Burkholderia* species are the most common and preferred nodulating symbionts of the Piptadenia Group (tribe Mimoseae). PLoS ONE 8: e63478.
16. Bournaud, C., Moulin, L., Cnockaert, M., Faria, S., Prin, Y., Severac, D., Vandamme, P. (2017) *Paraburkholderia piptadeniae* sp. nov. and *Paraburkholderia ribeironis* sp. nov., two root-nodulating symbiotic species of *Piptadenia gonoacantha* in Brazil. Int. J. Syst. Evol. Microbiol. 67, 432-440.
17. Caballero-Mellado, J., Martínez-Aguilar, L., Paredes-Valdez, G. (2004) *Burkholderia unamae* sp. nov., an N_2-fixing rhizospheric and endophytic species. Int. J. Syst. Evol. Microbiol. 54, 1165-72.
18. Chen, W-M., de Faria, S.M., Straliotto, R., Pitard, R.M., Simões-Araùjo, J.L., Chou, J-H., Chou, Y-J., Barrios, E., Prescott, A.R., Elliott, G.N., Sprent, J.I., Young, J.P.W., James, E.K. (2005a) Proof that *Burkholderia* strains form effective symbioses with legumes: a study of novel *Mimosa*-nodulating strains from South America. Appl. Environ. Microbiol. 71, 7461-7471.
19. Chen, W-M., James, E.K., Chou, J-H., Sheu, S-Y., Yang S-Z., Sprent, J.I. (2005b) β-Rhizobia from *Mimosa pigra*, a newly discovered invasive plant in Taiwan. New Phytol. 168, 661-675.
20. Chen, W-M., James, E.K., Coenye, T., Chou, J-H., Barrios, E., de Faria, S.M., Elliott, G.N., Sheu, S-Y., Sprent, J.I., Vandamme, P. (2006) *Burkholderia mimosarum* sp. nov., isolated from root nodules of *Mimosa* spp. from Taiwan and South America. Int. J. Syst. Evol. Microbiol. 56, 1847-51.
21. Chen, W-M., de Faria, S.M., James, E.K., Elliott, G.N., Lin, K-Y., Chou, J-H., Sheu, S-Y., Cnockaert, M., Sprent, J.I., Vandamme, P. (2007) *Burkholderia nodosa* sp. nov., isolated from root nodules of the woody Brazilian legumes *Mimosa bimucronata* and *Mimosa scabrella*. Int. J. Syst. Evol. Microbiol. 57, 1055-1059.
22. Chen, W-M., de Faria, S.M., Chou, J-H., James, E.K., Elliott, G.N., Sprent, J.I., Bontemps, C., Young, J.P.W., Vandamme, P. (2008) *Burkholderia sabiae* sp. nov., isolated from root nodules of *Mimosa caesalpiniifolia*. Int. J. Syst. Evol. Microbiol. 58, 2174-2179.

23. Chun, J., Oren, A., Ventosa, A., Christensen, H., Arahal, R.D., da Costa, M.S., Rooney, A.P., Yi, H., Xu, X-W., De Meyer, S., Trujillo, M.E. (2018) Proposed minimal standards for the use of genome data for the taxonomy of prokaryotes. Int. J. Syst. Evol. Microbiol. 68, 461–466.
24. Coenye, T., Vandamme, P. (2003) Diversity and significance of *Burkholderia* species occupying diverse ecological niches. Environ. Microbiol. 5, 719-729.
25. de Castro Pires, R., dos Reis Júnior, F.B., Zilli, J.E., Fischer, D., Hofmann, A., James, E.K., Simon, M.F. (2018) Soil characteristics determine the rhizobia in association with different species of *Mimosa* in central Brazil. Plant Soil 423, 411-428.
26. de Lajudie, P.M., Andrews, A., Ardley, J., Eardly, B., Jumas-Bilak, E., Kuzmanović, N., Lassalle, F., Lindström, K., Mhamdi, R., Martínez-Romero, E., Moulin, L., Mousavi, S.A., Nesme, X., Peix, A., Puławska, J., Steenkamp, E., Stępkowski, T., Tian, C-F., Vinuesa, P., Wei, G., Willems, A., Zilli, J., Young, P. (2019) Minimal standards for the description of new genera and species of rhizobia and agrobacteria. Int. J. Syst. Evol. Microbiol. 69, 1852-1863.
27. De León-Martínez, A.J., Yanez-Ocampob, G., Wong-Villarrealc, A. (2017) *Burkholderia* species associated with legumes of Chiapas, Mexico, exhibit stress tolerance and growth in aromatic compounds. Rev. Argent. Microbiol. 49, 394-401.
28. De Meyer, S.E., Cnockaert, M., Ardley, J.K., Maker, G., Yates, R., Howieson, J.G., Vandamme, P. (2013) *Burkholderia sprentiae* sp. nov., isolated from *Lebeckia ambigua* root nodules. Int. J. Syst. Evol. Microbiol. 63, 3950-3957.
29. De Meyer, S.E., Briscoe, L., Martínez-Hidalgo, P., Agapakis, C.M., Estrada de-los Santos, P., Seshadri, R., Reeve, W., Weinstock, G., O'Hara, G., Howieson, J.G., Hirsch, A.M. (2016) Symbiotic *Burkholderia* species show diverse arrangements of *nif/fix* and *nod* genes and lack typical high-affinity cytochrome *cbb3* oxidase genes. Mol. Plant Microbe Interact. 29, 609-619.
30. De Meyer, S.E., Cnockaert, M., Moulin, L., Howieson, J.G., Vandamme, P. (2018) Symbiotic and non-symbiotic *Paraburkholderia* isolated from South African *Lebeckia ambigua* root nodules and the description of *Paraburkholderia fynbosensis* sp. nov. Int. J. Syst. Evol. Microbiol. 68, 2607-2614.
31. de Oliveira Cunha, C., Goda Zuleta, L.F., Paula de Almeida, L.G., Prioli Ciapina, L., Lustrino Borges, W. Maria Pitard, R., Ivo Baldani, J., Straliotto, R., Miana de Faria, S., Hungria, M., Sousa Cavada, B., Martins Mercante, F., Ribeiro de Vasconcelos, A.T. (2012) Complete

genome sequence of *Burkholderia phenoliruptrix* BR3459a (CLA1), a heat-tolerant, nitrogen-fixing symbiont of *Mimosa flocculosa*. J. Bacteriol. 194, 6675-6676.

32. Depoorter, E., Bull, M.J., Peeters, C., Coenye, T., Vandamme, P., Mahenthiralingam, E. (2016) *Burkholderia*: an update on taxonomy and biotechnological potential as antibiotic producers. Appl. Microbiol. Biot. 100, 5215-5229.
33. Deris, Z.Z., Van Rostenberghe, H., Habsah, H., Noraida, R., Tan, G.C., Chan, Y.Y., Rosliza, A.R., Ravichandran, M. (2010) First isolation of *Burkholderia tropica* from a neonatal patient successfully treated with imipenem. Int. J. Infect. Dis. 14, e73-e74.
34. Dobritsa, A.P., Samadpour, M. (2016) Transfer of eleven species of the genus *Burkholderia* to the genus *Paraburkholderia* and proposal of *Caballeronia* gen. nov. to accommodate twelve species of the genera *Burkholderia* and *Paraburkholderia*. Int. J. Syst. Evol. Microbiol. 66, 2836-2846.
35. Dobritsa, A.P., Linardopoulou, E.V., Samadpour, M. (2017) Transfer of 13 species of the genus *Burkholderia* to the genus *Caballeronia* and reclassification of *Burkholderia jirisanensis* as *Paraburkholderia jirisanensis* comb. nov. Int. J. Syst. Evol. Microbiol. 67, 3846-3853.
36. dos Reis, F.B. Jr., Simon, M.F., Gross, E., Boddey, R.M., Elliott, G.N., Neto, N., de Fatima Loureiro, M., de Queiroz, L.P., Scotti, M.R., Chen, W-M., Norén, A., Rubio, M.C., de Faria, S.M., Bontemps, C., Goi, S.R., Young, J.P.W., Sprent, J.I., James, E.K. (2010) Nodulation and nitrogen fixation by *Mimosa* spp. in the Cerrado and Caatinga biomes of Brazil. New Phytol. 186, 934-946.
37. Elliott, G.N., Chen, W-M., Bontemps, C., Chou, J-H., Young, J.P.W., Sprent, J.I., James, E.K. (2007) Nodulation of *Cyclopia* spp. (Leguminosae, Papilionoideae) by *Burkholderia tuberum*. Ann. Bot. 100, 1403-1411.
38. Elliott, G.N., Chen, W-M., Chou, J-H., Wang, H-C., Sheu, S-Y., Perin, L., Reis, V.M., Moulin, L., Simon, M.F., Bontemps, C., Sutherland, J.M., Bessi, R., de Faria, S.M., Trinick, M.J., Prescott, A.R., Sprent, J.I., James, E.K. (2007) *Burkholderia phymatum* is a highly effective nitrogen-fixing symbiont of *Mimosa* spp. and fixes nitrogen *ex planta*. New Phytol. 173, 168-180.
39. Estrada-de los Santos, P., Rojas-Rojas, F.U., Tapia-García, E.Y., Vásquez-Murrieta, M.S., Hirsch, A.M. (2016) To split or not to split: an opinion on dividing the genus *Burkholderia*. Ann. Microbiol. 66, 1202-1314.

40. Estrada-de los Santos, P., Palmer, M., Chavez-Ramirez, B., Beukes, C., Steenkamp, E.T., Briscoe, L., Khan, N., Maluk, M., Lafos, M., Humm, E., Arrabit, M., Crook, M., Gross, E., Simon, M.F., dos Reis, F.B. Jr., Whitman, W.B., Shapiro, N., Poole, P.S., Hirsh, A.M., Venter, S.N., James, E.K. (2018) Whole genome analysis suggests that *Burkholderia* sensu lato contains two additional novel genera (*Mycetohabitans* gen. nov., and *Trinickia* gen. nov.): implications for the evolution of diazotrophy and nodulation in the *Burkholderiaceae.* Genes. 9, 389.
41. Euzéby, J.P. (1997) List of bacterial names with standing in nomenclature: a folder available on the internet. Int. J. Syst. Bacteriol. 47, 591-591.
42. Felsenstein, J. (1985) Confidence-limits on phylogenies—an approach using the bootstrap. Evol. 39, 783-791.
43. Ferreira, P.A.A., Bomfeti, C.A., Soares, B.L., de Souza Moreira, F.M. (2012) Efficient nitrogen-fixing *Rhizobium* strains isolated from Amazonian soils are highly tolerant to acidity and aluminium. World J. Microbiol. Biotechnol. 28, 1947-1959.
44. Garau, G., Yates, R.J., Deiana, P., Howieson, J.G. (2009) Novel strains of nodulating *Burkholderia* have a role in nitrogen fixation with papilionoid herbaceous legumes adapted to acid, infertile soils. Soil Biol. Biochem. 41, 125-134.
45. Gerhardt, P., Murray, R.G.E., Wood, A.W., Krieg, N.R. (1994) Methods for general and molecular bacteriology. Society for Microbiology, Washington D.C., USA.
46. Gevers, D., Cohan, F.M., Lawrence, J.G., Spratt, B.G., Coenye, T., Feil, E.J., Stackebrandt, E., De Peer, Y.V., Vandamme, P., Thompson, F.L., Swings, J. (2005) Re-evaluating prokaryotic species. Nat. Rev. Microbiol. 3, 733-739.
47. Giller, K.E., Herridge, D.F., Sprent, J.I. (2016) The legume-rhizobia symbiosis and assessing the need to inoculate. In: Howieson, J.G., Dilworth, M.J. (Eds.), Working with rhizobia, Australian Centre for International Agricultural Research (ACIAR), Australia, pp 15-17.
48. Goris, J., Konstantinidis, K.T., Klappenbach, J.A., Coenye, T., Vandamme, P., Tiedje, J.M. (2007) DNA-DNA hybridization values and their relationship to whole-genome sequence similarities. Int. J. Syst. Evol. Microbiol. 57, 81-91.
49. Gu, J.Y., Zang, S.G., Sheng, X.F., He, L.Y., Huang, Z., Wang, Q. (2015) *Burkholderia susongensis* sp. nov., a mineral-weathering bacterium isolated from weathered rock surface. Int. J. Syst. Evol. Microbiol. 65, 1031-1037.

50. Gyaneshwar, P., Hirsch, A.M., Moulin, L., Chen, W-M., Elliott, G.N., Bontemps, C., Estrada-de los Santos, P., Gross, E., dos Reis, F.B., Jr., Sprent, J.I., Young, J.P.W., James, E.K. (2011) Legume-nodulating beta proteobacteria: diversity, host range, and future prospects. Mol. Plant Microbe Interact. 24, 1276-1288.
51. Hall, T.A. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser.41, 95-98.
52. Hasegawa, M., Kishino, K., Yano, T. (1985) Dating the human-ape splitting by a molecular clock of mitochondrial DNA. J. Mol. Evol. 22, 160-174.
53. Howieson, J.G., De Meyer, S.E., Vivas-Marfisi, A., Ratnayake, S., Ardley, J.K., Yates, R.J. (2013) Novel *Burkholderia* bacteria isolated from *Lebeckia ambigua* – a perennial suffrutescent legume of the fynbos. Soil. Biol. Biochem. 60, 55-64.
54. Hungria, M., O'Hara, G.W., Zilli, J.E., Araujo, R.S., Deaker, R., Howieson, J.G. (2016) Isolation and growth of rhizobia. In: Howieson, J.G., Dilworth, M.J. (Eds.), Working with rhizobia, Australian Centre for International Agricultural Research (ACIAR), Australia, pp. 50.
55. Jones, D.T., Taylor, W.R., Thornton, J.M. (1992) The rapid generation of mutation data matrices from protein sequences. Bioinformatics. 8, 275-282.
56. Katoh, K., Toh, H. (2008) Improved accuracy of multiple ncRNA alignment by incorporating structural information into a MAFFT-based framework. BMC Bioinform. 9, 212.
57. Kim, H.B., Park, M.J., Yang, H.C., An, D.S., Jin, H.Z., Yang, D.C. (2006) *Burkholderia ginsengisoli* sp. nov., a β-glucosidase-producing bacterium isolated from soil of a ginseng field. Int. J. Syst. Evol. Microbiol. 56, 2529-2533.
58. Kück, P., Meusemann, K. (2010) FASconCAT: Convenient handling of data matrices. Mol. Phylogen. Evol. 56, 1115-1118.
59. Lardi, M., Liu, Y., Purtschert, G., de C¸pos, B.S., Pessi, G. (2017) Transcriptome Analysis of *Paraburkholderia phymatum* under nitrogen starvation and during symbiosis with *Phaseolus vulgaris*. Genes. 8, 389.
60. Le, S.Q., Gascuel, O. (2008) An Improved General Amino Acid Replacement Matrix. Mol. Biol. Evol. 25, 1307-1320.
61. Lemaire, B., Dlodlo, O., Chimphango, S., Stirton, C., Schrire, B., Boatwright, J. S., Honnay, O., Smets, E., Sprent, J., James, E.K., Muasya, A.M. (2015) Symbiotic diversity, specificity and distribution of rhizobia in native legumes of the Core Cape Subregion (South Africa). FEMS Microbiol. Ecol. 91, 1-17.

62. Lemaire, B., Chimphango, S.B.M., Stirton, C., Rafudeen, S., Honnay, O., Smets, E., Chen, W-M., Sprent, J., James, E.K., Muasya, A.M. (2016) Biogeographical patterns of legume-nodulating *Burkholderia* spp.: from African fynbos to continental scales. Appl. Environ. Microbiol. 82, 5099-5115.
63. Liu, X.Y., Wei, S., Wang, F., James, E.K., Guo, X.Y., Zagar, C., Xia, L.G., Dong, X., Wang, Y.P. (2012) *Burkholderia* and *Cupriavidus* spp. are the preferred symbionts of *Mimosa* spp. in southern China. FEMS Microbiol. Ecol. 80, 417-426.
64. LPWG (2017) A new subfamily classification of the Leguminosae based on a taxonomically comprehensive phylogeny. Taxon 66, 44-77.
65. MacFaddin, J.F. (2000) Gram-negative bacteria, Biochemical tests for identification of medical bacteria. 3rd edition. Philadelphia: Lippincott Williams and Wilkins, pp 363-367.
66. Martínez-Aguilar, L., Salazar-Salazar, C., Méndez, R.D., Caballero-Mellado, J., Hirsch, A.M., Vásquez-Murrieta, M.S., Estrada-de los Santos, P. (2013) *Burkholderia caballeronis* sp. nov., a nitrogen fixing species isolated from tomato (*Lycopersicon esculentum*) with the ability to effectively nodulate *Phaseolus vulgaris*. Anton. Leeuw. Int. J. G. 104, 1063–1071.
67. Meier-Kolthoff, J.P., Auch, A.F., Klenk, H.P., Göker, M. (2013) Genome sequence-based species delimitation with confidence intervals and improved distance functions. BMC Bionformatics. 14, 60.
68. Mishra, R.P., Tisseyre, P., Melkonian, R., Chaintreuil, C., Miché, L., Klonowska, A., Gonzalez, S., Bena, G., Laguerre, G., Moulin, L. (2012) Genetic diversity of *Mimosa pudica* rhizobial symbionts in soils of French Guiana: investigating the origin and diversity of *Burkholderia phymatum* and other beta-rhizobia. FEMS Microbiol. Ecol. 79, 487-503.
69. Moulin, L., Munive, A., Dreyfus, B., Boivin-Masson, C. (2001) Nodulation of legumes by members of the beta-subclass of *Proteobacteria*. Nature. 411, 948-950.
70. Mthombeni, L.S. (2012) Characterization of *Burkholderia* species associated with root nodules of legumes indigenous to South Africa. MSc dissertation, Pretoria: University of Pretoria.
71. Nurk, S., Bankevich, A., Antipov, D., Gurevich, A., Korobeynikov, A., Lapidus, A., Prjibelsky, A., Pyshkin, A., Sirotkin, A., Sirotkin, Y., Stepanauskas, R., McLean, J., Lasken, R., Clingenpeel, S.r., Woyke, T., Tesler, G., Alekseyev, M.A., Pevzner, P.A. (2013) Assembling genomes and mini-metagenomes from highly chimeric reads. In: Deng, M., Jiang, R., Sun, F.,

Zhang, X. (Eds) Research in Computational Molecular Biology. RECOMB 2013. Lecture Notes in Computer Science, vol 7821. Springer, Berlin, Heidelberg.

72. O'Hara, G.W., Zilli, J.E., Poole, P.S., Hungria, M. (2016) Taxonomy and physiology of rhizobia. In: Howieson, J.G., Dilworth, M.J. (Eds.), Working with rhizobia, Australian Centre for International Agricultural Research (ACIAR), Australia, pp. 133-134.
73. Ormeño-Orrillo, E., Rogel, M.A., Chueire, L.M., Tiedje, J.M., Martínez-Romero, E., Hungria, M. (2012) Genome sequences of *Burkholderia* sp. strains CCGE1002 and H160, isolated from legume nodules in Mexico and Brazil. J. Bacteriol. 194, 6927.
74. Palmer, M., Venter, S.N., Coetzee, M.P.A., Steenkamp, E.T. (2018) Prokaryotic species are *sui generis* evolutionary units. Syst. Appl. Microbiol. 42, 145-158.
75. Parte, A.C. (2013) LPSN – list of prokaryotic names with standing in nomenclature. Nucleic Acids Res. 42, D613-D616.
76. Parte, A.C. (2018) LPSN – List of prokaryotic names with standing in nomenclature (bacterio.net), 20 years on. Int. J. Syst. Evol. Microbiol. 68, 1825-1829.
77. Paulitsch, F., Dall'Agnol, R.F., Marçon Delamuta, J.R., Augosto Ribeiro, R., 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.
78. Paulitsch, F., Dall'Agnol, R.F., Marçon Delamuta, J.R., Augosto Ribeiro, R., 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.
79. Peeters, C., Meier-Kolthoff, J.P., Verheyde, B., De Brandt, E., Cooper, V.S., Vandamme, P. (2016) Phylogenomic study of *Burkholderia glathei*-like organisms, proposal of 13 novel *Burkholderia* species and emended descriptions of *Burkholderia sordidicola*, *Burkholderia zhejiangensis*, and *Burkholderia grimmiae*. Front. Microbiol. 7, 877.
80. Ramasamy, D., Mishra, A.K., Lagier, J-C., Padhmanabhan, R., Rossi, M., Sentausa, E., Raoult, D., Fournier, P-E., (2014) A polyphasic strategy incorporating genomic data for the taxonomic description of novel bacterial species. Int. J. Syst. Evol. Microbiol. 64, 384–391.
81. Reiner, K. (2010) Catalase test protocol. American Society for Microbiology.
82. Reis, V.M., Estrada-de los Santos, P., Tenorio-Salgado, S., Vogel, J., Stoffels, M., Guyon, S., Mavingui, P., Baldani, V.L.D., Schmid, M., Baldani, J.I., Balandreau, J., Hartmann, A.,

Caballero-Mellado, J. (2004) *Burkholderia tropica* sp. nov., a novel nitrogen-fixing, plant associated bacterium. Int. J. Syst. Evol. Microbiol. 54, 2155-2162.

83. Richter, M., Rosselló-Móra, R. (2009) Shifting the genomic gold standard for the prokaryotic species definition. Proc. Natl. Acad. Sci. U. S. A. 106, 19126-19131.
84. Rojas-Rojas, F.U., Tapia-García, E.Y., Maymon, M., Humm, E., Huntemann, M., Clum, A., Pillay, M., Palaniappan, K., Varghese, N., Mikhailova, N., Stamatis, D., Reddy, T.B.K., Markowitz, V., Ivanova, I., Kyrpides, N., Woyke, T., Shapiro, N., Hirsch, A.M., Estrada-de los Santos, P. (2017) Draft genome of *Paraburkholderia caballeronis* TNe-841^{T}, a free-living, nitrogen-fixing, tomato plant-associated bacterium. Stand. Genomic. Sci. 12, 80
85. Rosselló-Mora, R.R., Amann, R. (2001) The species concept for prokaryotes. FEMS Microbiol. Rev. 25, 39-67.
86. Sawana, A., Adeolu, M., Gupta, R.S. (2014) Molecular signatures and phylogenomic analysis of the genus *Burkholderia*: proposal for division of this genus into the emended genus *Burkholderia* containing pathogenic organisms and a new genus *Paraburkholderia* gen. nov. harbouring environmental species. Front. Genet. 5, 429.
87. Sheu, S.Y., Chen, M.H., Liu, W.Y.Y., Andrews, M., James, E.K., Ardley, J.K., De Meyer, S.E., James, T.K., Howieson, J.G., Coutinho, B.G., Chen, W-M. (2015) *Burkholderia dipogonis* sp. nov., isolated from root nodules of *Dipogon lignosus* in New Zealand and Western Australia. Int. J. Syst. Evol. Microbiol. 65, 4716-4723.
88. Sheu, S-Y., Chou, J-H., Bontemps, C., Elliott, G.N., Gross, E., dos Reis, F.B. Jr., Melkonian, R., Moulin, L., James, E.K., Sprent, J.I., Young, J.P.W., Chen, W-M. (2013) *Burkholderia diazotrophica* sp. nov., isolated from root nodules of *Mimosa* spp. Int. J. Syst. Evol. Microbiol. 63, 435-441.
89. Silva, V. C., Alves, P.A.C., Rhem, M.F.K., dos Santos, J.M.F., James, E.K., Gross, E. (2018) Brazilian species of *Calliandra* Benth. (tribe Ingeae) are nodulated by diverse strains of *Paraburkholderia*. Syst. Appl. Microbiol. 41, 241–250.
90. Simon, M.F., Grether, R., Queiroz, L.P., Sarkinen, T.E., Dutra, V.F., Hughes, C.E. (2011) The evolutionary history of *Mimosa* (Leguminosae): towards a phylogeny of the sensitive plants. Am. J. Bot. 98, 1201-1221.
91. Sites, J.W., Marshall, J.C. (2004) Operational criteria for delimiting species. Annu. Rev. Ecol. Evol. Syst. 35, 199-227.

92. Steenkamp, E.T., van Zyl, E., Beukes, C.W., Avontuur, J.R., Chan, W.Y., Palmer, M., Mthombeni, L.S., Phalane, F.L., Sereme, K.T., Venter, S.N. (2015) *Burkholderia kirstenboschensis* sp. nov. nodulates papilionoid legumes indigenous to South Africa. Syst. Appl. Microbiol. 38, 545-54.
93. Steenkamp, E.T., Wingfield, M.J., Mctaggart, A.R., Wingfield, B.D. (2017) Fungal species and their boundaries matter—definitions, mechanisms and practical implications. British Mycological Society. 32, 104-116.
94. Suárez-Moreno, Z.R., Caballero-Mellado, J., Coutinho, B.G., Mendonça-Previato, L., James, E.K., Venturi, V. (2012) Common features of environmental and potentially beneficial plant-associated *Burkholderia*. Microb. Ecol. 63, 249-266.
95. Suau, A., Bonnet, R., Sutren, M., Godon, J.J., Gibson, G.R., Collins, M.D., Doré, J. (1999) Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. Appl. Environ. Microbiol. 65, 4799-807.
96. Talbi, C., Delgado, M.J., Girard, L., Ramírez-Trujillo, A., Caballero-Mellado, J., Bedmar, E.J. (2010) *Burkholderia phymatum* strains capable of nodulating *Phaseolus vulgaris* are present in Moroccan soils. Appl. Environ. Microbiol. 76, 4587-91.
97. Tamura, K. (1992) "Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G+C content biases". Mol. Biol. Evol. 9, 678-687.
98. Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S. (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725-2729.
99. Tayeb, L.A., Lefevre, M., Passet, V., Diancourt, L., Brisse, S., Grimont, P.A.D. (2008) Comparative phylogenies of *Burkholderia*, *Ralstonia*, *Comamonas*, *Brevundimonas* and related organisms derived from *rpoB*, *gyrB* and *rrs* gene sequences. Res. Microbiol. 159, 169-177.
100. Vaneechoutte, M., De Beenhouwer, H., Claeys, G., Verschraegen, G., De Rouck, A., Paepe, N., Elaichouni, A., Portaels, F., Vaneechoutte, M., De Beenhouwer, H., Claeys, G., Verschraegen, G., De Rouck, A., Paepe, N., Elaichouni, A., Portaels, F. (1993) Identification of *Mycobacterium* species by using amplified ribosomal DNA restriction analysis. J. Clin. Microbiol. 1, 2061-2065.
101. Vandamme, P., Goris, J., Chen, W-M., de Vos, P., Willems, A. (2002) *Burkholderia tuberum* sp. nov. and *Burkholderia phymatum* sp. nov., nodulate the roots of tropical legumes. Syst. Appl. Microbiol. 25, 507-512.

102. Venter, S.N., Palmer, M., Beukes, C.W., Chan, W-Y., Shin, G., Zyl, E., Seale, T., Coutinho, T.A., Steenkamp, E.T. (2017) Practically delineating bacterial species with genealogical concordance. Anton. Leeuw. Int. J. G.110, 1311-1325.

103. Weber, C.F., King, G.M. (2017) Volcanic soils as sources of novel CO-oxidizing *Paraburkholderia* and *Burkholderia*: *Paraburkholderia hiiakae* sp. nov., *Paraburkholderia metrosideri* sp. nov., *Paraburkholderia paradisi* sp. nov., *Paraburkholderia peleae* sp. nov., and *Burkholderia alpina* sp. nov. a member of the *Burkholderia cepacia* complex. Front. Microbiol. 8, 207.

104. Whelan, S., Goldman, N. (2001) A General Empirical Model of Protein Evolution Derived from Multiple Protein Families Using a Maximum-Likelihood Approach, Mol. Biol. Evol. 18, 691-699.

105. Yang, Z. (1994) Maximum likelihood phylogenetic estimation from DNA sequences with variable rates over sites: Approximate methods. J. Mol. Evol. 39, 306-314.

106. Yates, R.J., Howieson, J.G., Reeve, W.G., Nandasena, K.G., Law, I.J., Bräu, L., Ardley, J.K., Nistelberger, H.M., Real, D., O'Hara, G.W. (2007) *Lotononis angolensis* forms nitrogen fixing, lupinoid nodules with phylogenetically unique, fast-growing, pink-pigmented bacteria, which do not nodulate *L. bainesii* or *L. listii*. Soil Biol. Biochem. 39, 1680-1688.

107. Zhang, H., Hanada, S., Shigematsu, T., Shibuya, K., Kamagata, Y., Kanagawa, T., Kurane, R. (2000) *Burkholderia kururiensis* sp. nov., a trichloroethylene (TCE)-degrading bacterium isolated from an aquifer polluted with TCE. Int. J. Syst. Evol. Microbiol. 50, 743-749.