Whole Genome Analyses Suggests that Burkholderia sensu lato Contains Two Additional Novel Genera (Mycetohabitans gen. nov., and Trinickia gen. nov.): Implications for the Evolution of Diazotrophy and Nodulation in the Burkholderiaceae.
Article

Whole Genome Analyses Suggests that *Burkholderia* sensu lato Contains Two Additional Novel Genera (*Mycetohabitans* gen. nov., and *Trinickia* gen. nov.): Implications for the Evolution of Diazotrophy and Nodulation in the *Burkholderiaceae*

Paulina Estrada-de los Santos 1,*,†, Marike Palmer 2,†, Belén Chávez-Ramírez 1, Chrizelle Beukes 2, Emma T. Steenkamp 2, Leah Briscoe 3, Noor Khan 3,*, Marta Maluk 4, Marcel Lafos 4, Ethan Humm 3, Monique Arrabit 3, Matthew Crook 5, Eduardo Gross 6,*, Marcelo F. Simon 7, Fábio Bueno dos Reis Junior 8, William B. Whitman 9, Nicole Shapiro 10, Philip S. Poole 11, Ann M. Hirsch 3,*, Stephanus N. Venter 2,*, and Euan K. James 4,*

1 Instituto Politécnico Nacional, Escuela Nacional de Ciencias Biológicas, 11340 Cd. de Mexico, Mexico; belcha06@yahoo.com.mx
2 Department of Microbiology and Plant Pathology, Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria 0083, South Africa; marike.duplessis@fabi.up.ac.za (M.P.); chrizelle.beukes@fabi.up.ac.za (C.B.); emma.steenkamp@up.ac.za (E.T.S.)
3 Department of Molecular, Cell, and Developmental Biology and Molecular Biology Institute, University of California, Los Angeles, CA 90095, USA; lbriscoep81@ucla.edu (L.B.); noor.612@gmail.com (N.K.); ehumm@ucla.edu (E.H.); mnarrabit@ucla.edu (M.A.)
4 The James Hutton Institute, Dundee DD2 5DA, UK; Marta.Maluk@hutton.ac.uk (M.M.); marcel.lafos@hutton.ac.uk (M.L.)
5 450G Tracy Hall Science Building, Weber State University, Ogden, 84403 UT, USA; matthewcrook@weber.edu
6 Center for Electron Microscopy, Department of Agricultural and Environmental Sciences, Santa Cruz State University, 45662-900 Ilheus, BA, Brazil; egross@uesc.br
7 Embrapa CENARGEN, 70770-917 Brasilia, Distrito Federal, Brazil; marcelo.simon@embrapa.br
8 Embrapa Cerrados, 73310-970 Planaltina, Distrito Federal, Brazil; fabio.reis@embrapa.br
9 Department of Microbiology, University of Georgia, Athens, GA 30602, USA; whitman@uga.edu
10 DOE Joint Genome Institute, Walnut Creek, CA 94598, USA; nrshapiro@lbl.gov
11 Department of Plant Sciences, University of Oxford, South Parks Road, Oxford OX1 3RB, UK; philip.poole@plants.ox.ac.uk
* Correspondence: pestradadelossantos@gmail.com (P.E.-d.l.S.); ahirsch@ucla.edu (A.M.H.); fanus.venter@fabi.up.ac.za (S.N.V.); euan.james@hutton.ac.uk (E.K.J.); Tel.: +44-1382-568873 (E.K.J.)
† These authors made an equal contribution.

Received: 24 May 2018; Accepted: 24 July 2018; Published: 1 August 2018

**Abstract:** *Burkholderia* sensu lato is a large and complex group, containing pathogenic, phytopathogenic, symbiotic and non-symbiotic strains from a very wide range of environmental (soil, water, plants, fungi) and clinical (animal, human) habitats. Its taxonomy has been evaluated several times through the analysis of 16S rRNA sequences, concatenated 4–7 housekeeping gene sequences, and lately by genome sequences. Currently, the division of this group into *Burkholderia*, *Caballeronia*, *Paraburkholderia*, and *Robbsia* is strongly supported by genome analysis. These new genera broadly correspond to the various habitats/lifestyles of *Burkholderia* s.l., e.g., all the plant beneficial and environmental (PBE) strains are included in *Paraburkholderia* (which also includes all the N\textsubscript{2}-fixing legume symbionts) and *Caballeronia*, while most of the human and animal pathogens are retained in *Burkholderia* sensu stricto. However, none of these genera can accommodate two important groups of species. One of these includes the closely related *Paraburkholderia rhizoxinica* and *Paraburkholderia endofungorum*, which are both symbionts of the...
fungal phytopathogen *Rhizopus microsporus*. The second group comprises the *Mimosa*-nodulating bacterium *Paraburkholderia symbiotica*, the phytopathogen *Paraburkholderia caryophylli*, and the soil bacteria *Burkholderia dabaoshanensis* and *Paraburkholderia soli*. In order to clarify their positions within *Burkholderia* sensu lato, a phylogenomic approach based on a maximum likelihood analysis of conserved genes from more than 100 *Burkholderia* sensu lato species was carried out. Additionally, the average nucleotide identity (ANI) and amino acid identity (AAI) were calculated. The data strongly supported the existence of two distinct and unique clades, which in fact sustain the description of two novel genera *Mycetohabitans* gen. nov. and *Trinickia* gen. nov. The newly proposed combinations are *Mycetohabitans endofungorum* comb. nov., *Mycetohabitans rhizoxinica* comb. nov., *Trinickia caryophylli* comb. nov., *Trinickia dabaoshanensis* comb. nov., *Trinickia soli* comb. nov., and *Trinickia symbiotica* comb. nov. Given that the division between the genera that comprise *Burkholderia* s.l. in terms of their lifestyles is often complex, differential characteristics of the genomes of these new combinations were investigated. In addition, two important lifestyle-determining traits—diazotrophy and/or symbiotic nodulation, and pathogenesis—were analyzed in depth i.e., the phylogenetic positions of nitrogen fixation and nodulation genes in *Trinickia* via-à-vis other *Burkholderiaceae* were determined, and the possibility of pathogenesis in *Mycetohabitans* and *Trinickia* was tested by performing infection experiments on plants and the nematode *Caenorhabditis elegans*. It is concluded that (1) *T. symbiotica* *nif* and *nod* genes fit within the wider *Mimosa*-nodulating *Burkholderiaceae* but appear in separate clades and that *T. caryophylli* *nif* genes are basal to the free-living *Burkholderia* s.l. strains, while with regard to pathogenesis (2) none of the *Mycetohabitans* and *Trinickia* strains tested are likely to be pathogenic, except for the known phytopathogen *T. caryophylli.*

**Keywords:** Burkholderia; Paraburkholderia; Caballeronia; Robbsia; Mimosa; Rhizopus; symbionts; diazotrophy; root nodulation

1. Introduction

*Burkholderia* sensu lato (s.l.) comprise more than 100 species that thrive in several diverse environments [1]. Not long after the initial description of *Burkholderia* by Yabuuchi et al. [2], it was suggested that the genus be divided into several groups [3,4]. Since then, this notion has gathered considerable momentum, with many studies suggesting a formal split between the pathogenic and the plant beneficial environmental (PBE) species on the basis of their core genomes [5]. There has also been opposition to such a split, arguing that the two groups are not distinguished by sufficiently definable and clear phenotypes [6]. Currently, this large genus is divided into *Burkholderia* sensu stricto (s.s.), *Caballeronia*, *Paraburkholderia*, and *Robbsia andropogonis* [7–9].

The division of *Burkholderia* s.l. and the means by which the new genera were initially described has caused skepticism. This was also evident from the minutes of the International committee on systematics of prokaryotes subcommittee for the taxonomy of *Rhizobium* and *Agrobacterium*, which discussed this subject during the 12th Nitrogen fixation Conference held in Budapest, Hungary on 25 August 2016 [10]. The subcommittee stated their position as “Research efforts directed towards robust characterization and taxonomy of *Burkholderia* s.l. species can help in realizing this agricultural potential. Clearly, a large-scale phylogenomic study is required for resolving these taxa”. Therefore, in order to tackle the issue and to settle generic boundaries in *Burkholderia* s.l., a large phylogenomic analysis was carried out using the amino acid and nucleotide sequences of 106 genes from 92 species [11]. The analysis performed with maximum likelihood (ML) unambiguously supported five different lineages: *Burkholderia* s.s., *Caballeronia*, *Paraburkholderia*, *Robbsia andropogonis* and *Paraburkholderia rhizoxinica*.

In this study, an international effort was made to address the generic status of six important species of the *Burkholderia* s.l. assemblage. These are the fungal symbionts, *P. rhizoxinica* and
Paraburkholderia endofungorum [12], the Mimosa-nodulating bacterium Paraburkholderia symbiotica [13], the phytopathogen Paraburkholderia caryophylli [2], and the soil bacteria Burkholderia dabaoshanensis and Paraburkholderia soli [14,15]. In 2014, five of these species were transferred to Paraburkholderia [7], while the species name, B. dabaoshanensis, is still awaiting valid publication. Based on the analysis of the 16S rRNA sequence, all of these taxa formed part of the so-called Transition Group 1 of Estrada-de los Santos in 2016 [4]. The position of these species within the existing phylogenetic framework for Burkholderia s.l. was determined using the same phylogenomic approach previously employed by Beukes et al. [11]. Additionally, average nucleotide identity (ANI) [16–18] and average amino acid identity (AAI) [19–21] values were calculated, together with the analysis of some phenotypic features. Based on these findings, the above-named species belong to two novel genera for which we propose the names Mycetohabitans gen. nov. and Trinickia gen. nov. (see below).

Although an in-depth analysis of phenotypical differences was out of the scope of this study, in order to address concerns that genome differences alone do not justify the formation of new combinations within Burkholderia s.l. [6], the various genomes were consulted in depth for information about differential characteristics. Furthermore, as all of these strains were originally placed in Transition Group 1 between the PBE and the pathogenic Burkholderia species [4], two key lifestyles were investigated in more depth: (1) nitrogen fixation, both free-living and symbiotic in association with legumes, which is quite common in Burkholderia s.l., particularly in the PBE group [5,22], and (2) the possibility that they may include potential pathogens. In the case of diazotrophy and/or nodulation, we examined the occurrence and phylogeny of essential genes involved in these processes i.e., the nitrogenase enzyme-coding genes, nifD and nifH, as well as the nodulation genes, nodABCD, with particular emphasis on the phylogeny of Trinickia vis-à-vis members of the Burkholderiaceae. For pathogenesis, the genomes were searched for type III secretion system (T3SS) genes, which encode proteins produced by certain Gram-negative bacteria that are injected into their host, while also performing physiological assays to determine whether or not these strains can infect plants and/or have the ability to kill the nematode Caenorhabditis elegans. The presence or absence of sequences for the type IV secretion system (T4SS) used for the transfer of DNA or proteins into a host was also investigated.

2. Materials and Methods

2.1. Bacterial Strains and Genomes

Of the six species considered in this study, only the genomes for P. rhizoxinica HKI 454T and P. symbiotica JPY347 were available in the public domain. The genomes for strains in the other five species were determined in this study (Table 1). For this purpose strains of the following species were obtained from various culture collections, P. symbiotica (JPY-345T, JPY-366 and JPY-581) from the JPY culture collection (housed at the University of York and the James Hutton Institute, Dundee, UK), B. dabaoshanensis CCTCC M 209109T (GIMN1.004T) from the Agricultural Research Service (ARS) (NRRL B-59553) U.S. Dept. of Agriculture culture collection, and strains of P. caryophylli (LMG 2155T = Ballard 720T), P. soli (GP25-8T), and P. endofungorum (HKI 454T) from the Belgian Coordinated Collection of Microorganisms (BCCM/LMG) culture collection. The type strain of P. eburnea (ICM 180703T), obtained from the latter collection, was also included, as was Paraburkholderia rhynchosiae WSM3937T from the WSM collection at the University of Murdoch, and Paraburkholderia caribensis TJ182 from the JPY collection.
### Table 1. The genome sequencing statistics for *Burkholderia* sensu lato strains sequenced in this study.

| Statistic                  | Paraburkholderia eburnea | Paraburkholderia rhynchosiae | Mycobacterium endofungorum | Trinickia caraphyllii | Trinickia caraphyllii | Trinickia dabaoshanensis | Trinickia soli | Trinickia symbiotica | Trinickia symbiotica | Trinickia symbiotica | Paraburkholderia caribensis |
|---------------------------|--------------------------|------------------------------|----------------------------|-----------------------|-----------------------|-------------------------|----------------|---------------------|---------------------|---------------------|----------------------------|
| Strain                    | JCM 18070<sup>7</sup>    | WSM3935<sup>2</sup>          | HKI 46<sup>7</sup>          | LMG 2153<sup>3</sup>  | Ballard 720<sup>2</sup> | LMG 2153<sup>3</sup> | GIMN1.004<sup>2</sup> | GP25-8<sup>7</sup> | JPY 345<sup>7</sup> | JPY 381              | JPY 366              | TJ182                      |
| Sequencing Centre         | DOE Joint Genome Institute | MicrobesNG                   | DOE Joint Genome Institute  | DOE Joint Genome Institute | MicrobesNG              | MicrobesNG              | DOE Joint Genome Institute | MicrobesNG              | MicrobesNG              | MicrobesNG              | MicrobesNG              |                           |
| Sequencing Platform       | Illumina                  | Illumina HiSeq-2000 1TB      | Illumina                  | Illumina HiSeq-2000 1TB | Illumina HiSeq-2000 1TB | Illumina HiSeq-2000 1TB | Illumina HiSeq-2000 1TB | Illumina HiSeq-2000 1TB | Illumina HiSeq-2000 1TB | Illumina HiSeq-2000 1TB | Illumina HiSeq-2000 1TB |                           |
| NCBI taxonomy ID          | 1,189,126                 | 487,049                      | 417,203                   | 28,094                | 28,094                | 564,714                 | 380,675              | 863,227              | 863,227              | 863,227              | 75,105                  |
| NCBI BioProject ID        | PRJNA369942               | PRJNA427925                  | PRJNA370785               | PRJNA369920           | PRJNA427926            | PRJNA427927            | PRJNA427928            | PRJNA369937           | PRJNA427929            | PRJNA445642            | PRJNA445638             |
| Number of reads           | 6,886,312                 | 1,204,873                    | 7,561,076                 | 7,357,578             | 962,962                | 828,393                 | 918,663              | 6,294,534             | 2,076,457             | 1,180,541              | 809,533                 |
| Assembly method           | SPAdes                    | SPAdes                       | SPAdes                    | SPAdes                | SPAdes                 | SPAdes                   | SPAdes                | SPAdes                | SPAdes                | SPAdes                | SPAdes                   |
| Sequencing coverage       | 149.1X                    | 58.8X                        | 348.7X                    | 169.1X                | 56.4X                  | 47.6X                    | 61.6X                 | 149.1X                | 131.3X                | 66.2X                  | 33.9X                    |
| N50                       | 294,829                   | 226,289                      | 213,816                   | 480,968               | 187,187                | 186,667                  | 231,363               | 252,951               | 255,942               | 387,494               | 89,490                  |
| L50                       | 7                         | 12                           | 6                         | 6                     | 13                    | 13                       | 10                    | 9                    | 9                     | 7                     | 31                       |
| Largest contig [bp]       | 983,800                   | 527,307                      | 365,500                   | 792,225               | 401,224                | 433,345                  | 514,473               | 819,300               | 663,178               | 786,277               | 294,652                 |
| Number of contigs         | 58                        | 181                          | 76                        | 49                    | 161                   | 104                      | 105                   | 61                   | 121                  | 57                    | 242                      |
| Genome size [bp]          | 6,947,977                 | 8,032,361                    | 3,288,408                 | 6,543,652             | 6,501,896              | 7,093,755               | 6,696,514             | 6,714,023             | 6,753,015             | 7,005,740             | 9,206,228               |
| G+C content              | 64.09%                    | 61.74%                       | 61.27%                    | 64.72%                | 64.72%                 | 63.28%                   | 62.98%                | 63.00%                | 63.00%                | 62.49%                |                           |
| Assembly Accession Number | GCA_002917095.1           | GCA_002879865.1              | GCA_002927045.1           | GCA_00200717465.1     | GCA_002879875.1        | GCA_002879885.1         | GCA_002879885.1        | GCA_00293455.1        | GCA_002879935.1        | GCA_002802865.1        | GCA_0028028645.1        |
The genomes of *P. caryophylli* Ballard 720T, *Paraburkholderia eburnea* JCM 18070T, *P. endofungorum* HKI 456T and *P. symbiotica* JPY-345T were sequenced by the DOE Joint Genome Institute (JGI) using Illumina technology [23]. An Illumina 300 bp insert standard shotgun library was constructed and sequenced using the Illumina HiSeq–2000 1TB platform (San Diego, CA, USA). All general aspects of library construction and sequencing performed at the JGI can be found at http://www.jgi.doe.gov. All raw Illumina sequence data were filtered using BBDuk [24], which removed known Illumina artifacts and PhiX. Reads with more than one N (flanking sequence-dependent N errors) or with quality scores (before trimming) averaging less than 8 or reads shorter than 51 bp (after trimming) were discarded. Remaining reads were mapped to masked versions of human, cat and dog references using BBMAP [24] and discarded if identity exceeded 95%. Sequence masking was performed with BBMask [24]. For assembly, artifact-filtered Illumina reads were assembled using SPAdes (version 3.6.2) [25] and assembly contigs were discarded if the length was < 1 kbp.

The genomes of *P. caribenensis* TJ182, *P. caryophylli* LMG 2155T, *B. dabaoshanensis* GIMN1.004T, *P. rhynchosiae* WSM3739T, *P. soli* GP25-8T, *P. symbiotica* JPY-366, and *P. symbiotica* JPY-581 were sequenced by MicrobesNG (Birmingham, UK) with the genomic DNA library prepared using the Nextera XT library prep kit (Illumina) following the manufacturer’s protocol with the following modifications: two ng of DNA were used as input and the PCR elongation time was increased to 1 min. DNA quantification and library preparation were carried out on a Microlab STAR automated liquid handling system (Hamilton Robotics, Chicago, IL, USA). Pooled libraries were quantified using the Kapa Biosystems Library Quantification Kit for Illumina on a Roche light cycler 96-qPCR machine (Roche, Geneva, Switzerland). Libraries were sequenced on the Illumina HiSeq using a 250 bp paired-end protocol. Reads were adapter trimmed using Trimmomatic 0.30 with a sliding window quality cutoff of Q15 [26]. De novo genome assembly was carried out with SPAdes (version 3.7) [25] and contigs were annotated using Prokka 1.11 [27]. The genome sizes (contigs) were determined by RAST [28].

### 2.2. Phylogenetic Analysis

The 106-gene amino acid dataset employed for phylogenetic analysis by Beukes et al. [11] was supplemented with the protein sequences for the additional taxa examined in this study (Table S1). Homologous protein sequences were identified and grouped using the Efficient Database framework for comparative Genome Analyses using BLAST score Ratios (EDGAR) server [29]. Individual sequence files were subsequently aligned with MUSCLE [30] as part of CLC Main Workbench 7.6 (CLC Bio, Cambridge, MA, USA). The aligned data sequences were subjected to evolutionary model testing in ProtTest 3.4 [31], followed by concatenation and partitioning in FASconCAT-G v. 1.02 [32]. The partitioned concatenated dataset was subjected to ML analysis with RAxML v. 8.2.1 [33], and branch support was inferred from 1000 bootstrap pseudo-replicates.

### 2.3. Average Nucleotide Identity and Average Amino Acid Identity

As an indication of the relatedness of the taxa investigated, pairwise ANI and AAI values were calculated for the full taxon set on the EDGAR server [29]. For ANI calculations, all shared genomic information was utilized to calculate a similarity value average across homologous regions, bringing into account the sequence similarity as well as the alignment length over homologous regions [34]. AAI calculations were conducted by averaging similarity values for all pairwise homologous protein sequences for each set of two genomes.

### 2.4. Genome-Informed Differential Characteristics

All isolates of *Burkholderia*, *Paraburkholderia* and the proposed genus, *Trinickia*, with available genome sequences included in the study, were compared to identify the potential differences between these genera at the genomic level. Rudimentary analyses were performed by comparing the functional annotations of each of the core genomic components of the respective genera to identify potentially characteristic traits. Core genomes for each genus were calculated with the EDGAR server [29] as
described above. This was followed by functional annotation using the Kyoto Encyclopedia of Genes and Genomes (KEGG) [35] following the approach of Palmer and colleagues [36]. Differences between the core genomes were subsequently subjected to the EDGAR server to confirm the presence or absence of genes within the respective members of the three genera.

2.5. Analysis of nif and nod Genes

The nif and nod genes were isolated from the indicated genome sequences with the National Center for Biotechnology Information (NCBI) stand-alone BLAST program by using the corresponding reference genes from *Paraburkholderia phymatum* STM815T (GCF000020045): nifD [WP_012406782.1]; nifH [WP_012406781.1]; nodA [WP_012406745.1]; nodB [WP_012406750.1]; nodC [WP_012406749.1] and nodD [WP_012406751.1]. The genome sequences with a GCF-reference were retrieved from NCBI (https://www.ncbi.nlm.nih.gov/assembly/), and those with an IMG-reference from JGI (https://genome.jgi.doe.gov/).

The homology of the nif and nod genes to those in the *P. phymatum* STM815T (GCF000020045) reference genome was visualized as a heat map with the R-package ggplot2 v2.2.1 [37] and arranged with Inkscape v0.48. Protein sequences were aligned with MUSCLE v3.8.31 [30,38], and ML phylogenies were inferred with iqTREE v1.5.5 (http://www.iqtree.org) using an iqTREE model-selection [39] and a standard (b = 100) nonparametric bootstrap calculation [40]. The phylograms were edited with the R-packages ape v5.0 [41] and ggtree v1.8.2 [42] in R v3.4.1 and arranged with Inkscape v0.48.

2.6. Plant Growth Promotion Analysis

The strains were tested for their production of siderophores using Chrome Azurol S casaminoacid (CAS-CAA) medium, while National Botanical Research Institute Phosphate growth (NBRIP) medium was used to test for phosphate solubilization [43]. To determine their ability to fix nitrogen, the strains were grown in a semi-solid Burkholderia-malic acid-glucose-mannitol (BMGM) medium with 20 mg L\(^{-1}\) yeast extract added, and then tested for nitrogenase activity using the acetylene reduction assay [44]. The production of indole acetic acid (IAA) was assessed using the method of Jain and Patriquin [45]. The strains were tested on *Mimosa pudica* and siratro (*Macroptilium atropurpureum*) for their ability to nodulate legumes and/or to promote growth according to Elliott et al. [46].

2.7. Pathogenicity Tests

For the pathogenicity tests, three different organisms were employed: tobacco (*Nicotiana tabacum* L.), onion (*Allium cepa* L.), and nematodes (*C. elegans*). For the tobacco test, the strains were grown on R2A broth medium for two days at 30 °C with reciprocal shaking (120 rpm). The bacterial culture optical density (OD\(_{600}\)) was adjusted to 0.5 by dilution with a medium, and 500 µL was injected into the principal vein of a tobacco leaf. The leaf was checked for injury at 48 h. *Pseudomonas savastanoi* pv. *phaseolicola* PsFr-14 and PsFr-96 and *Xanthomonas axonopodis* pv. *phaseoli* XaFr-14 were used as the positive and negative controls, respectively.

In a second plant test, onions were first peeled to remove both the dry external covering and the most external layer without damaging the underlying tissues. The individual onions were quartered with a sterile knife and single onion scales were carefully removed, divided into two lengthwise sections and placed into 90-mm petri plates containing 2 discs of sterile Whatman filter paper no. 1 (Whatman, Los Angeles, CA, USA) using sterile forceps. The filter paper discs covered the entire surface of the petri plates and were pre-moistened with 25 mL of sterile distilled water. Overnight-grown cultures of the different bacterial strains were used in the assay. Individual onion scales were wounded on their inner surface with a sterile pipette tip, and 5 µL of a 10\(^7\) CFU mL\(^{-1}\) culture was inoculated into the wound. The scales were incubated at 30 °C for 72 h. Maceration was rated on a scale described by Jacobs et al. [47]. Each strain was tested three times and an average rating was tabulated. A known
onion pathogen strain, *Burkholderia cepacia* 68P128, served as the positive control, while the culture medium alone served as the negative control.

The activity of *C. elegans* fed with different bacterial strains under slow killing conditions was assayed as described by Vilchez et al. [48,49]. Briefly, bacterial strains were spread on two nematode growth media (NGM) plates and incubated at 30 °C for 24 h. Each plate was then seeded with a known number of nematodes from the original control plate (*Escherichia coli* OP50), which was determined using a Zeiss microscope at 10× magnification (Carl Zeiss, Oberkochen, Germany). This number served as a zero-h reading. After counting, the plates were incubated at 24 °C and scored for nematode death every 24 h for 5 days. In all cases, the *E. coli* strain OP50 was a control to estimate the natural death rate of the nematodes, and *Paraburkholderia aeruginosa* PA14 was the positive control for pathogenicity. The experiment was conducted three times with two replicates for each strain.

The evaluation of the effect of bacteria on *C. elegans* was conducted based on the pathogenicity score given by Cardona et al. [50]. The authors established that a given strain could be designated pathogenic for the nematode if one of the following criteria were met: (i) a diseased appearance at day 2, which included reduced locomotive capacity and the presence of a distended intestine; (ii) percentage of live nematodes at day 2 ≤ 50%; and (iii) total number of nematodes at day 5 ≤ 50%. The presence of any one, two, or three of these criteria was scored to differentiate mild from severe infections. A pathogenic score (PS 1, 2, or 3) was given based on the number of criteria met. A strain was considered non-pathogenic when no symptoms of disease were observed (pathogenicity score, PS 0). Additionally, the influence of the bacteria on movement and propagation of the nematodes was monitored for 120 h.

The data are presented as mean ± standard deviation (SD). The statistical analysis was performed using GraphPad Prism software version 5.01 (GraphPad Software, San Diego, CA, USA).

### 2.8. Bioinformatics Analysis of the T3SS

Amino acid sequences for 21 T3SS genes in the *P. rhizoxinica* HKI454T genome were obtained from the DOE-JGI website. This gene set included *sct*, *hpa*, *hrp*, and *araC*-type regulator genes, which were queried using the command line blastp tool from NCBI against a custom database of 10 genomes: *Paraburkholderia caballeronis* LMG 26416T, *P. caryophylli* Ballard720T, *Paraburkholderia dabaoshanensis* GIMN1.004T, *P. endofungorum* HKI456T, *P. phymatum* STM815T, *P. soli* GP25-8T, *P. symbiotica* JPY-345T, *P. symbiotica* JPY-347, *P. symmetrica* JPY-581, and *Paraburkholderia tuberum* STM678T. With the filtering of blastp hits for sequences with less than 1e-2 e-value and at least 30% identity with *P. rhizoxinica* HKI454T, the highest scoring amino acid sequence, if any, from each genome, was used to build a gene tree. Using the MEGA7 (Tokyo Metropolitan University, Tokyo, Japan) ML phylogenetic tree-building algorithm with the Jones–Taylor–Thornton (JTT) model of amino acid substitution, a tree was built for each gene. Using the MEGA7 (Tokyo Metropolitan University, Tokyo, Japan) ML phylogenetic tree-building algorithm with the Jones–Taylor–Thornton (JTT) model of amino acid substitution, a tree was built for each gene with 1000 bootstrap replications, selecting the tree with the highest log-likelihood [51]. As *hrpB1* and *sctF* were found in fewer than four strains, these genes were excluded from the analysis.

The 21 gene trees with their respective bootstrap values and branch lengths were used to build a single consensus tree using the multi-species coalescent model implemented by ASTRAL-II [52]. For the coalesced tree, the final quartet score was 0.80, representing the percent of quartet trees induced by the 21 input gene trees in the final species trees. The local posterior probabilities displayed on the branches represent the percent of quartets in gene trees that agree with a branch [53].

The analysis for the T4SS was performed using the DOE-JGI, MicrobesNG, and NCBI websites.

### 3. Results and Discussion

#### 3.1. Whole-Genome Sequences

The genome features of *P. caribensis* TJ182, *P. eburnea* JCM 18070T, *P. rhynchosiae* WSM3937T, *Mycetohabitans endofungorum* HKI 456T, *Trinickia caryophylli* LMG 2155T and Ballard 720T,
Trinickia dabaoshanensis GIMN1.004T, T. soli GP25-8T, and Trinickia symbiotica JPY-345T, JPY-366 and JPY-581 are shown in Table 1.

The genome sequences for the type strains of both M. endofungorum and Myctohabitans rhizoxinica were markedly smaller than what can be expected for members of Burkholderia s.l. Typically, the genome sequences of species in Burkholderia s.l. range from 6.0 Mb to 11.0 Mb, with the smallest being 5.8 Mb for Burkholderia mallei and the largest being 11.2 Mb for Paraburkholderia hospita. In contrast, M. rhizoxinica has a genome of 3.8 Mb, while M. endofungorum has a genome of 3.3 Mb. This vast difference in genome size can be attributed to the endosymbiotic nature of these species as genome streamlining often occurs in endosymbiotic bacteria [47,54,55].

Overall, the genome sequences for T. caryophylli, T. dabaoshanensis, Trinickia soli, and T. symbiotica are comparable in terms of size and G+C content to the remaining members of Burkholderia s.l. Amongst the four species, T. soli had the smallest genome at 6.1 Mb, while the largest genome was that of T. dabaoshanensis at 7.1 Mb. The G+C content of these four species was more similar to that of Paraburkholderia and Caballeronia (61.9% for T. soli to 65.1% for T. caryophylli) than that of the higher G+C content reported for Burkholderia s.s. [11].

3.2. Phylogenetic Analysis

The concatenated 106-gene dataset of 122 taxa consisted of 27,138 amino acids. ML analysis of the dataset separated the ingroup taxa into five distinct monophyletic groups (Figures 1 and S1). Each of the distinct groups was highly supported with bootstrap values ≥ 95%. These groups corresponded to the genera Burkholderia s.s., Caballeronia and Paraburkholderia as described previously [11], with a further two distinct groups with members currently assigned to Paraburkholderia. The first group contained four species (T. caryophylli, T. dabaoshanensis, T. soli and T. symbiotica). This group corresponds to Trinickia gen. nov., proposed in this study. The second group was sister to all other genera of Burkholderia s.l. (except for Robbsia) and contained M. endofungorum and M. rhizoxinica. This group corresponds to Myctohabitans gen. nov., as proposed in this study. Although the distinctness of these groups from one another was highly supported (reflected by the high branch support values), the relationships between these groups remain unclear, as intergeneric relationships were not supported (collapsed branches had support values of <80%).
vis-à-vis the previously established genera *Burkholderia*, *Caballeronia*, and *Paraburkholderia*. The scale bar indicates the number of changes per site. All branches with support values below 80% were collapsed to indicate polytomies, as intergeneric relationships depicted by unsupported branching patterns were uncertain.

### 3.3. Average Nucleotide and Average Amino Acid Identity

Based on ANI and AAI calculations (Table S2), the generic groups as recovered in the phylogenetic tree were in overall supported (Figure 2). Intragenic AAI values were generally comparable, with AAI values for *Paraburkholderia* greater than 74.34%, *Trinickia* greater than 76.74%, *Burkholderia* s.s. greater than 76.88%, and *Caballeronia* greater than 75.8%. For *Mycetohabitans*, only two species are known at this time, and AAI values of 93.35% were obtained for interspecies comparisons. Similarly, intragenic ANI values for *Paraburkholderia* were greater than 75.18%, the *Trinickia* values were greater than 75.97%, values for *Burkholderia* s.s. were greater than 77.33%, and for *Caballeronia*, values were greater than 75.16%. For *Mycetohabitans*, the interspecies comparison between the two species in this genus resulted in an ANI value of 91.29%.

Based on these analyses, it appears that numerous species are potentially conspecifics, such as *P. sediminicola* and *P. terricola*, although some high values for well-differentiated species such as *B. mallei* and *B. pseudomallei* were also obtained. There was, however, a clear separation between the monophyletic groups where individuals within a genus were generally more closely related to each other than to individuals outside of each genus. Both *M. endofungorum* and *M. rhizoxinica* are assigned to the novel genus *Mycetohabitans* gen. nov. since they share a 91% ANI but less than 80% ANI to members of any other genus.

![Figure 2](image-url) **Figure 2.** A heat map depicting the average amino acid and nucleotide identity values of the 122 *Burkholderia* sensu lato strains for which whole genomes are available. The cladogram indicating the various intra- and inter-generic relationships were inferred from the amino acid-based ML topology. Average nucleotide identity (ANI) values are indicated in the upper triangle of the map, with average amino acid identity (AVI) values indicated in the lower triangle of the map. For specific values, refer to Table S2.
3.4. Genome-Informed Differential Characteristics

As a means to investigate the distinctness of the proposed genus *Trinickia* from *Burkholderia* and *Paraburkholderia*, genome comparisons were conducted to identify potential biologically informative differences between the gene content of members of these genera (Table S3). These genomic comparisons consisted of basic functional comparisons between the respective core genomes to identify metabolically important differences. Based on these genomic comparisons, some genes were found to be present in the majority of members of a genus as opposed to absent in the members of another genus or vice versa. Examples of these differences were genes for benzoate degradation (present in all members of *Paraburkholderia*), starch and sucrose metabolism (present in all members of *Paraburkholderia* and *Trinickia*), glycerolipid metabolism (present in all members of *Trinickia*), cysteine and methionine metabolism (absent in all members of *Trinickia* and present in all members of *Burkholderia*), and D-arginine and D-ornithine metabolism (present in all members of *Burkholderia*). Based on these initial analyses, it appeared that all members of *Paraburkholderia* possess the ability to metabolize 4-hydroxybenzoate, whereas *Paraburkholderia* and *Trinickia* can metabolize starch to amylose, while *Burkholderia* have the ability to utilize additional amino acids. The main differential phenotypic features of the type species all the genera in the family Burkholderiaceae, including *Mycetohabitans* and *Trinickia*, are given in Table 2.

3.5. nif and nod Gene Analysis

Phylogenies were constructed from full-length sequences of the *nifH* (Figure 3A), *nifD* (Figure S2A), *nodA* (Figure 3B), *nodB* (Figure S2B), *nodC* and *nodD* (Figure S3A,B) genes obtained from the genomes of the strains featured in the present study. First, it should be noted that *nif* was not detected in any of the *Caballeronia*, *Mycetohabitans* or Robbsia species. It was widely present in *Paraburkholderia* and in free-living/plant-associated species, such as *P. tropica* and *P. xenovorans*, and in legume symbionts (e.g., *P. phyumatum*). It was also present in *Burkholderia contaminans*, *B. lata* and *B. vietnamiensis*, [56,57], but it was absent in most *Burkholderia s.s.* species. In terms of phylogeny, *nif* genes were highly conserved across the *Burkholderiaceae*, including *Cupriavidus*, and both genes analyzed had a similar topology (Figures 3A and S2A). This showed that the *nif*-containing *Burkholderiaceae* strains in the genera *Burkholderia*, *Cupriavidus*, *Paraburkholderia* and *Trinickia* constituted a large and separate cluster from other diazotrophs in the β-proteobacteria, such as *Azoarcus* and *Herbaspirillum*, and were separate from plant-associated and symbiotic diazotrophs in the α-proteobacteria, such as *Azospirillum* and *Rhizobium s.l.* This further suggests that *nif* in the *Burkholderiaceae* has a different evolutionary origin from other symbionts/plant-associated bacteria, including those in the β-proteobacteria, which we might have assumed had a similar origin. However, this may simply reflect the likelihood that *nif* in the β-proteobacteria has been acquired from a number of sources via horizontal gene transfer; e.g., this has clearly happened with *Azoarcus*, both branches of which (represented in Figures 3A and S2A by *A. olearius* BH72 and *Azoarcus* sp. CIB) have *nif* from separate origins [58].
Table 2. The differential phenotypic features among the type species of all the genera in the family Burkholderiaceae.

| Species | Feature | Plant Pathogen | N-Fixation | Chitinolytic Activity * | Predatory Bacterium | Cell Type | Fungus Endosymbiont | Legume Nodulation | Obligately Endosymbiont | NO$_3$ to NO$_2$ | Growth at >60 °C | OL-1 | OL-2 |
|---------|---------|----------------|------------|------------------------|---------------------|-----------|---------------------|---------------------|----------------------|-----------------|----------------|------|------|
| Burkholderia cepacia J2315$^T$ | + | − | ** | − | − | Rods | − | − | − | − | − | − | + | + |
| Caballeronia glathei ATCC 29195$^T$ | − | + | − | − | − | Rods | − | − | − | − | nf | − | nd | nd |
| Chitinimonas taiwanensis cf$^T$ | nd | − | + | − | − | Rods | − | − | − | + | − | nd | nd |
| Cupriavidus necator N-1$^T$ | − | − | ** | − | − | Short rods | − | − | − | + | − | nd | nd |
| Lautropia mirabilis AB2188$^T$ | nd | − | − | − | Coccoid | − | − | − | + | − | nd | nd |
| Limnobacter thiooxidans CS-K2$^T$ | nd | − | − | − | Rods | − | − | − | − | − | − | nd | nd |
| Myceliobacter rhizoxinica HKI 454$^T$ | + | − | − | − | Coccoid rods | + | − | − | − | nd | − | nd | nd |
| Pandorana apioae LMG 16407$^T$ | nd | − | − | − | Rods | − | − | − | − | − | − | nd | nd |
| Paraburkholderia graminis C4D1M$^T$ | − | − | − | − | Rods | − | − | − | + | − | nd | nd |
| Paucimonas lemoignei A62$^T$ | nd | + | − | − | Rods | − | − | − | − | − | − | nd | nd |
| Polynucleobacter necessarius ATCC 30859$^T$ | nd | − | − | − | Rods | − | − | + | − | − | nd | nd |
| Rasotonia pickettii ATCC 27511$^T$ | + | − | − | − | Rods | − | − | − | + | − | − | − | − |
| Roberta andropogonis LMG 2129$^T$ | + | − | − | − | Rods | − | − | − | − | − | − | − | − |
| Thermothrix thiopara ATCC 29244$^T$ | nd | nd | − | nd | Rods | nd | nd | − | + | + | nd | nd |
| Trinickia symbiotica JPY345$^T$ | − | + | − | − | Rods | − | − | + | − | − | − | − | − |

* Use of chitin as the exclusive carbon, nitrogen, and energy source for growth, both under aerobic and anaerobic conditions. **, The type strain does not have the activity but other strains have the feature. nd: data not determined. nf: data not found. OL: Ornithine lipid. The feature information was taken from the original description. ATCC, American Type Culture Collection.
Within the *Burkholderiaceae*, it was previously noted from a smaller number of complete genome sequences [59] that the *nif* genes were divided into three clades: (1) free-living diazotrophs (*Burkholderia, Paraburkholderia*), (2) *Paraburkholderia* strains, which nodulate diverse papilionoid legumes native to the Fynbos biome of the South African Cape region (this group is derived from the free-living diazotrophs) and (3) strains of *Paraburkholderia* and *Cupriavidus*, which nodulate legumes within the mimosoid clade native to the Americas (*Mimosa* and its close relatives in the *Piptadenia* Group). The present study using several more genomes confirms this division, but also shows that the *T. symbiotica* strains are clearly in a sub-clade of the mimosoid-nodulating clade. However, the *nif* genes of *T. caryophylli* are more closely related to those of the free-living clade and occupy a basal position within this group (Figure 3A).

The *nod* gene phylogenies also exhibit a similar organization to that described by de Meyer et al. [59], i.e., the papilionoid-nodulating *Paraburkholderia* strains are in a separate clade to the mimosoid-nodulating *Paraburkholderia* and *Cupriavidus* strains. Although both β-proteobacterial clades are distinct from nodulating α-proteobacteria, the *nod* genes of the papilionoid-nodulating strains appear to be derived from the α-rhizobia, particularly *Methyllobacterium nodulans* and *Bradyrhizobium* (Figures 3B, S2B and S3A,B). However, this is clearly not the case with the mimosoid-nodulating *Paraburkholderia* strains and related genera, whose *nod* genes are highly divergent from α-rhizobia, suggesting a very different evolutionary origin. The *nod* genes of the newly-sequenced strains *P. diazotrophic*, *P. piptadeniae* and *P. ribeironis*, isolated from *Mimosa* and *Piptadenia* species in South America [60–62], are all in the mimosoid-nodulating clade, as expected, as is *P. caribensis* TJ182 (isolated from invasive *Mimosa* in Taiwan), which is actually identical to *P. phymatum* in both *nif* and *nod* genes (Figures 3A,B, S2A,B and S3A,B). However, within the mimosoid-nodulators, the *T. symbiotica* strains isolated from *Mimosa* species in Brazil [13] occupy a separate lineage that is comparable to that occupied by *Cupriavidus* (Figure 3B). This suggests that *T. symbiotica* is not so closely related to other *Mimosa*-nodulating β-rhizobia in terms of its *nod* genes, and this may be connected to their host, *M. cordistipula*, which is a rare endemic species whose habitat is confined to the highland (>900 m) *campos rupestres* environments of the Chapada Diamantina in northeast Brazil [13,63]. The only other reported host of *T. symbiotica* is *M. misera* [13], which is a widespread species in North East Brazil and occurs at a wider range of altitudes than *M. cordistipula*. It remains to be seen if rhizobial strains isolated from *M. misera* are similar to JPY345T and JPY-581, in terms of their *nod* genes. With further regard to other potential hosts of *T. symbiotica*, a recent study of symbionts from another genus in the mimosoid clade, *Calliandra*, which is native to the same sites as *M. cordistipula* and *M. misera*, was only nodulated by *Paraburkholderia*. This suggests that *Calliandra* may not nodulate with *Trinickia* [64].

The *nif* and *nod* gene comparisons within the *Burkholderiaceae* are summarized in the heatmap in Figure 3 which uses *P. phymatum* STM815T as a reference genome. It is hypothesized that geographical separation of the two nodulating β-rhizobial clades (American vs. African) have led to the separate evolution of their *nod* genes. Therefore, they have very different host ranges and do not have an ability to nodulate each other’s hosts [5,65]. However, this is not strictly true. An interesting feature of β-rhizobial strains in the mimosoid clade, such as *P. phymatum* and *P. nodosa*, is that they also often nodulate promiscuous papilionoid legumes in the tribe *Phaseoleae*, including common bean (*Phaseolus vulgaris*), cowpea (*Vigna unguiculata*), siratro (*Macroptilium atropurpureum*) and *Dipogon lignosus* [66–68]. With specific regard to *Trinickia*, Lardi et al. [69] have recently shown that *T. symbiotica* JPY-345T was unable to nodulate any of these promiscuous legumes. This reinforces the idea that *nod* genes in *T. symbiotica* are functionally as well as genetically different from other mimosoid-nodulating β-rhizobia, and hence have a greatly restricted host range, being confined to *Mimosa* species [69,70]. Host range studies like those undertaken with *P. phymatum* STM815T [71] and *P. tuberum* STM678T [65] are needed to establish if this is indeed the case.
Figure 3. The ML phylogenies of Burkholderiaceae species using sequences of nifH (A) and nodA genes (B) inferred with iqTREE and using 100 nonparametric bootstrap calculations. Only bootstrap values greater than 50 are shown. α-Proteobacteria are labeled in black, Paraburkholderia in blue, Burkholderia in magenta, Cupriavidus in brown and Trinickia in green. In the nifH phylogram, non-nodulating species of bacteria harboring nitrogen-fixing genes, but no nodulation genes, are labeled as free-living non-nodulators (this group is absent in the nodA-gene phylogeny). In both phylograms, the group of bacteria specifically nodulating papilionoid legumes is indicated with green shading, and the group specifically nodulating mimosoids is indicated with yellow shading; note that both the nifH and nodA gene phylogenies reveal similar grouping of nodulating strains in accordance with their indicated host specificity. Colors in the heatmap correspond to the percent identity of protein sequences to the nif and nod genes of Paraburkholderia phymatum STM815, which was used as the reference genome; color gradient from blue (0%) to green (25%), yellow (50%), orange (75%) and red (100%).
3.6. Nodulation and Plant Growth Promotion Features

In nodulation tests with *Mimosa pudica*, plants inoculated with the *T. symbiotica* strains JPY-345T, JPY-366, and JPY-581 formed nodules, but not those with JPY-347. No nodules were formed on plants infected by *M. endofungorum*, *M. rhizoxinica*, *T. caryophylli*, *T. dabaoshanensis* or *T. soli*. In the case of *T. caryophylli*, it rapidly killed the inoculated *Mimosa* plants, but the other non-nodulating strains either had no effect on *Mimosa* growth or slightly enhanced it, suggesting that they might be plant growth-promoting rhizobacteria (PGPR).

*Mycetohabitans endofungorum* HKI 456T, *M. rhizoxinica* HKI 454T, *T. caryophylli* LMG 2155T, *T. dabaoshanensis* GIMN1.004T, *T. soli* GP25-8T, *T. symbiotica* JPY-345T and *T. symbiotica* JPY-581, were accordingly tested for PGPR activities. All strains produced siderophores, except for *M. rhizoxinica*. Only *T. dabaoshanensis* was able to solubilize phosphates. Synthesis of IAA was carried out by each strain, but the level of production was lower than the control strain *Azospirillum brasilense* SP7T. *Trinickia caryophylli* was able to fix nitrogen under free-living conditions, which was previously shown [4], but the other strains were unable to (Table S4). This is in contrast to the ability of *T. symbiotica* to fix nitrogen symbiotically in nodules (Reference [13] and this study).

3.7. Virulence Tests

*Mycetohabitans rhizoxinica* HKI 454T, *M. endofungorum* HKI 456T, *T. symbiotica* JPY-345T and JPY-581, *T. caryophylli* LMG 2155T, *T. dabaoshanensis* GIMN1.004T, and *T. soli* GP25-8T were tested for their effects on tobacco leaves. *T. caryophylli* caused water-soaked lesions and a loss of leaf tissue integrity (Figure S4), and *T. soli* and *T. dabaoshanensis* elicited small water-soaked lesions, a response not recorded previously for these bacteria. The other tested strains, except for the positive controls in H-I, caused no ill effects on tobacco leaves (Figure S4).

In trial experiments using entire pearl onion bulbs, *B. gladioli* BSR3 resulted in reduced biomass accumulation and increased tissue browning and maceration compared to *P. tuberum* STM678T (not shown). To determine whether or not the *Trinickia* strains exhibited any pathogenic potential, we used a bona fide onion pathogen, *B. cepacia* 68P128, on a detached onion bulb scale assay [45]. Table 3 and Figure S5 show that the *B. cepacia* strain induced the greatest amount of tissue damage (score of 3) whereas *B. caryophylli* Ballard720T was less virulent (score of 2). In contrast, none of the other tested strains were pathogenic, including *M. rhizoxinica* HKI 454T.

| Strains                     | Rating for the Degree of Tissue Maceration after 72 h |
|-----------------------------|------------------------------------------------------|
| Control                     | 0                                                   |
| *B. cepacia* 68P128         | 3 (67–100% macerated tissue area)                    |
| *M. rhizoxinica* HKI 454T   | 0                                                   |
| *T. symbiotica* JPY 581     | 0                                                   |
| *T. symbiotica* JPY 366     | 0                                                   |
| *T. symbiotica* JPY 347     | 0                                                   |
| *T. caryophylli* Ballard 720T | 2 (34–66% macerated tissue area)                     |
| *T. soli* GP25-8T           | 0                                                   |
| *T. dabaoshanensis* GIMN1004T | 0                                               |
| *P. caballeronis* LMG 26416T | 0                                               |

Onions treated with culture medium alone served as negative controls. Individual onion scales were wounded on their inner surface with a sterile pipette tip, and 5 µL of a 10⁷ CFU mL⁻¹ culture was inoculated into the wound. The scales were incubated at 30 °C for 72 h. Maceration was rated on a scale described by Jacobs et al. [45]. Data are means ± SD of three replicates.

*Caenorhabditis elegans* tests are frequently used to analyze broad-host range microbial pathogenicity, with *Pseudomonas aeruginosa* PA14 included as a positive control as it is an effective killer of nematodes. On NGM, *C. elegans* exposed to PA14 were motile, but avoided the bacteria, which remained...
unconsumed by the nematodes leading to their death (Table S5) in contrast to the normal food source *E. coli* OP50. None of the tested strains exhibited an inhibitory effect on *C. elegans* motility, except for *M. rhizoxinica* HKI 454T, *T. caryophylli* Ballard 720T, and *T. dabaoshanensis* GIMN1.004T, where decreased motility was observed after 24–48 h (Table S5). Additionally, feeding with *M. rhizoxinica* HKI 454T and *T. caryophylli* Ballard 720T resulted in significantly lower numbers of adult worms. The worm populations in these treatments were similar to that of the *P. aeruginosa* PA14 (positive control) treatment (Figure S6). When grown on *M. rhizoxinica*, the nematodes did not digest the bacteria and eventually starved (Table S4). *Mycetohabitans* species synthesize rhizonin, a cyclopeptide important for plant diseases caused by its fungal host *Rhizopus microsporus* [72], but the mechanism by which it kills *C. elegans* is not known. In NGM medium, in which *P. caballeronis* LMG 26416T and the *Trinickia* species (with the exception of *T. caryophylli* Ballard 720T) grew, *C. elegans* nematodes were motile and digested the bacterial lawn by 72 h, although the feeding behavior was altered. In the case of *T. caryophylli* Ballard 720T, worm motility slowed after 24 h. In comparison to the *C. elegans* population fed with *E. coli* OP50, the presence of *T. symbiotica* JPY581, *T. symbiotica* JPY347, *T. dabaoshanensis* GIMN1.004T, and *P. caballeronis* LMG 26416T resulted in a reduction of 40%, 28%, 31% and 36% of the nematode population, respectively (Figure S6).

Earlier it was noted that 12 different environmental and symbiotic *Burkholderia* species completely lacked the virulence-associated T3SS-3, which is essential in pathogenic species for infecting mammals [73]. Although the T3SS influences host range in the rhizobium-legume symbiosis, to our knowledge, no evidence exists so far that this secretion system affects host range in the nodulating β-rhizobia.

We analyzed the T3SS in several *Burkholderia* species and strains using *M. rhizoxinica* T3SS genes to query the other species. *Paraburkholderia* species are very different from the two pathogenic *Mycetohabitans* species, and the T3SS genes are not well conserved between the species (Figure 4). In *P. caballeronis* LMG 26416T, *P. phymatum* STM815T, and *P. tuberum* STM678T, the sctNVURTS genes are, in most cases, more similar to flagellar biosynthesis proteins. SctN most significantly aligned to a flagellum-specific ATPase, whereas SctV [74] aligned with the flagellar biosynthesis protein FlhA. SctR aligned to the flagellar biosynthesis protein FliP, and SctU to the flagellar biosynthesis protein FlhB.

The *T. symbiotica* strains lacked most of the T3SS genes, and those that were present lacked similarity to the genes of *Mycetohabitans* species (Figure 4). *Trinickia caryophylli*, *T. dabaoshanensis*, and *T. soli* possessed a more complete T3SS, but were missing the sctF and hrpB1 genes, which are also not found in rhizobial species [74]. For these species, additional T3SSs may exist based on the presence of multiple gene copies. As described above, a gene encoding SctU is homologous to genes in *M. rhizoxinica* (46%) and *Xanthomonas campestris* (57%), with a second gene in *X. campestris* (55%) and has homologs in *Yersinia enterocolitica* (35%) and *Escherichia albertii* (30%). In contrast, *Mycetohabitans* species appear to have all the components of a functional T3SS, including sctF, which encodes the needle monomer, and hrpB1/hrpK, which is required for Hrp pilus formation. Both are found in phytopathogens such as *X. campestris pv. vesicatoria* strains (Figure 4). Although *T. dabaoshanensis* and *T. soli* did not kill *C. elegans* or affect onion leaves, *T. caryophylli* killed *C. elegans*, onion tissue, and *Mimosa pudica*, and elicited water-soaked lesions on tobacco leaves. It is unclear which mechanism(s) *T. caryophylli* employs for pathogenesis in these organisms, but it is likely to be independent of the T3SS. Interestingly, *T. soli* and *T. dabaoshanensis* induced the formation of water-soaked lesions on tobacco leaves. It is possible that other pathogenic strategies or alternate secretion systems are involved in this response.
Characteristics for this genus were derived from the literature [12]. Cells are Gram-negative, short, motile rods. Oxidase and catalase positive. Colonies are very small, flat, circular, and cream colored. The growth on media is very poor. It grows in an aerobic or microaerophilic atmosphere, but not under
anaerobic conditions. Growth is observed between 16–45 °C. β-Galactosidase negative. Positive for the utilization of glycerol, but glucose is not metabolized. The type species of the genus is *Mycetohabitans rhizoxinica*.

- **Description of *Mycetohabitans rhizoxinica***

*Mycetohabitans rhizoxinica* (rhi.zo.xi’ni.ca. N.L. n. rhizoxinum rhizoxin; L. f. suff. -ica suffix used with various meanings; N.L. fem. adj. rhizoxinica referring to the ability of this organism to produce the antimitotic agent rhizonin).

Basonym: *Paraburkholderia rhizoxinica* [7].

The description for the species is provided in Partida-Martinez et al. [12]. Based on phylogenetic analysis of 106 conserved protein sequences, high support is obtained for the placement of this species into the novel genus *Mycetohabitans*.

The type strain of the species is HKI 454^T (= DSM 19002^T = CIP 109453^T).

- **Description of *Mycetohabitans endofungorum***

*Mycetohabitans endofungorum* (en.do.fun.go’rum. N.L. pref. endo- from Gr. endon within; L. gen. pl. n. fungorum of fungi; N.L. gen. n. endofungorum referring to the endosymbiotic nature of this organism with fungi).

Basonym: *Paraburkholderia endofungorum* [7].

The species description is provided in Partida-Martinez et al. [12]. The phylogenetic placement of this species into the novel genus *Mycetohabitans* is highly supported based on the concatenation of 106 conserved protein sequences.

The type strain of the species is HKI 456^T (=DSM 19003^T = CIP 109454^T).

### 3.8.2. Description of *Trinickia* gen. nov.

- **Trinickia** (Tri.nick’i.a. N.L. fem. n. *Trinickia* formed after M.J. Trinick, an Australian microbiologist who was the first to isolate β-rhizobia from *Mimosa*).

All characteristics for this genus were derived from the literature [2,13–15]. Cells are Gram-negative, aerobic, non-spore-forming rods. Growth occurs between 10–40 °C for all members of this genus. Most members are catalase positive with the exception of *T. dabaoshanensis*. Positive for the hydrolysis of Tween 40 and 80. Positive for the utilization of N-acetyl-D-glucosamine, L-arabinose, D-fructose, L-fucose, α-D-glucose, D-mannitol, D-sorbitol, pyruvic acid methyl ester, succinic acid, bromosuccinic acid, L-alanine, L-alanylglycine, and L-asparagine. Compounds utilized by most members within the genus are D-arabitol, adonitol, D-galactose, myo-inositol, D-mannose, D-raffinose, L-rhamnose, succinic acid mono-methyl-ester, cis-aconitic acid, citric acid and formic acid.

The type species for the genus is *Trinickia symbiotica*.

- **Description of *Trinickia symbiotica* comb. nov.**

*Trinickia symbiotica* (sym.bio’ti.ca. N.L. fem. adj. symbiotica from Gr. n. symbios, a companion, partner, living together, symbiotic).

Basonym: *Paraburkholderia symbiotica* [7].

The species description is provided in Sheu et al. [13]. Phylogenetic analysis based on 106 conserved protein sequences provided high support for the placement of this species in the novel genus *Trinickia*.

The type strain of this species is JPY345^T (=LMG 26032^T = BCRC 80258^T).

- **Description of *Trinickia caryophylli* comb. nov.**

*Trinickia caryophylli* (ca.ry.o.phyl’li. N.L. masc. n. caryophyllus, specific epithet of *Dianthus caryophyllus*, carnation; N.L. gen. n. caryophylli, of the carnation).
Genes 2018, 9, 389

Basonym: Paraburkholderia caryophylli [7].

The description of this species is provided in Yabuuchi et al. [2]. Phylogenetic analysis based on the concatenation of 106 conserved protein sequences indicates a high support for the inclusion of this species in the new genus Trinickia.

The type strain of this species is LMG 2155T (=ATCC 25418T = CFBP 2429T = JCM 10488T).

- **Description of Trinickia dabaoshanensis** comb. nov.

Trinickia dabaoshanensis (d’a.bao.shan.en’sis. N.L. dabaoshanensis, fem. adj. pertaining to Dabaoshan, South China, where the type strain was isolated).

Basonym: Burkholderia dabaoshanensis [14].

The description of the species is provided in Zhu et al. [14]. Concatenated phylogenetic analysis of 106 conserved protein sequences places this species into the novel genus Trinickia with high support.

The type strain of the species is GIMN-1.004T (= CCTCC M 209109T = NRRL B-59553T = LMG 30479T).

- **Description of Trinickia soli** comb. nov.

Trinickia soli (so’li. L. gen. n. soli, of soil, the source of the type strain).

Basonym: Paraburkholderia soli [7].

The description of this species is provided in Yoo et al. [15]. Phylogenetic analysis of 106 conserved protein sequences indicates the placement of this species into the new genus Trinickia with high support.

The type strain of the species is GP25-8T (=KACC 11589T = DSM 18235T).

4. Conclusions

The present study revealed the existence of two new genera within Burkholderia s.l.: Mycetohabitans as a genus containing fungal symbionts with small genomes, and Trinickia as a diverse genus containing plant-associated and soil bacteria. The analyses of genes and activities involved in N₂-fixation, legume symbiosis, and pathogenicity did not reveal any particular patterns in the new combinations vis-à-vis Burkholderia s.l., except that T. symbiotica was clearly divergent from other nodulating (para)burkholderias in terms of its nif and nod genes, and T. caryophylli has nif genes, which are basal to free-living diazotrophic burkholderias. Moreover, uniquely among the Trinickia species, T. caryophylli is a phytopathogen. Neither of the other Trinickia species (B. dabaoshanensis and B. soli) is diazotrophic, symbiotic or pathogenic, and this perhaps reflects the “mosaic” nature of such lifestyles, which in itself is such a common and intriguing feature across the Burkholderiaceae [22].

We believe that Mycetohabitans and Trinickia are robust genera based upon the genome comparisons and on the analysis of genes specific for metabolism and/or certain lifestyles, but we also accept that Burkholderia s.l. continues to experience taxonomical changes due to the analysis of more sequenced genomes, and the present study may undergo revision as more becomes known about Burkholderia s.l. taxonomy. Indeed, the frequent release of genomic information provides unparalleled breakthroughs for evaluating taxonomic relationships among microorganisms. A cursory examination of ANI and AAI values suggests that based on the lower similarity values typically associated with existing genera, Burkholderia s.s., Paraburkholderia, and Trinickia gen. nov. in particular, could theoretically be further divided into several other new genera. However, we would caution against proceeding too rapidly (if at all) in this direction without first establishing that any new combinations also have some functional biology differentiating them.

**Supplementary Materials:** The following are available online at http://www.mdpi.com/2073-4425/9/8/389/s1, Table S1: Sources and accession numbers for all the Burkholderia (s.l.) genomes used in this study. Table S2: Average nucleotide identities (ANI) and average amino acid identities (AAI) calculated using all the Burkholderia (s.l.) and Cupriavibius genomes available for this study. Table S3: Genome-informed differential characteristics of Burkholderia s.s., Paraburkholderia Mycetohabitans and Trinickia strains with particular focus on genes involved in metabolism. Table S4: Activities of plant growth promotion (PGP) traits of Mycetohabitans and Trinickia strains. Table S5: Pathogenicity score and behavioral response of Caenorhabditis elegans to Mycetohabitans and Trinickia strains. Figure S1: A maximum-likelihood phylogeny of the amino acid sequences of 106 concatenated genes
for the 122 strains used in this study of available Burkholderia (s.l.) genomes. Figure S2: Maximum-likelihood phylogenies of Burkholderiaceae species using sequences of nifD (A) and nifB genes (B) inferred with iqTREE and using 100 nonparametric bootstrap calculations. Figure S3: Maximum-likelihood phylogenies of Burkholderiaceae species using sequences of nodC (A) and nodD genes (B) inferred with iqTREE and using 100 nonparametric bootstrap calculations. Figure S4: Hypersensitivity test on tobacco leaves. Figure S5: Pathogenicity of Mycetohabitans and Trinickia strains on onion bulb scales (Allium cepa L.) compared with bacteria known to be pathogenic (Burkholderia cepacia 68P128). Figure S6: Survival and growth of Caenorhabditis elegans on culture plates of Mycetohabitans and Trinickia strains compared with bacteria known to be harmless (E. coli OP50) and pathogenic (Pseudomonas aeruginosa PA14) to the worms.

Author Contributions: P.E.-d.l.S., P.S.P., A.M.H., E.T.S., S.N.V. and E.K.J. conceived and designed the experiments; M.P., C.B., M.M., M.L., P.S.P., L.B., N.K., E.H., A.M.H., S.N.V. and E.K.J. wrote the paper.

Funding: E.K.J. was funded by the BBiSR Newton Fund (UK) and the CAPES/CNPq Ciencias sem Fronteiras programme (Brazil). We also thank the South African National Research Foundation and the Department of Science and Technology for the funding received via the Centre of Excellence Programme. The work conducted by the United States Department of Energy Joint Genome Institute, a DOE Office of Science User Facility, is supported by the Office of Science of the United States Department of Energy under Contract No. DE-AC02-05CH11231. PES was partially funded by project from IPN SIP 20170492. Research in the Hirsch lab on Burkholderia is funded by the Shanbrom Family Foundation.

Acknowledgments: The authors would like to thank Stefano Ventura for assistance with nomenclature, and G. Herrera, S. Jaweed, and A. Navas for help with the T4SS analysis. Unless noted otherwise, genome sequencing was provided by MicrobesNG (http://www.microbesng.uk), which is supported by the BBiSR (grant number BB/L024209/1).

Conflicts of Interest: The authors declare no conflict of interest.

References
1. Euzéby, J.P. List of bacterial names with standing in nomenclature: A folder available on the internet. Int. J. Syst. Evol. Microbiol. 1997, 47, 590–592. [CrossRef] [PubMed]
2. Yabuuchi, E.; Kosako, Y.; Oyaizu, H.; Yano, I.; Hotta, H.; Hashimoto, Y.; Ezaki, T.; Arakawa, M. Proposal of Burkholderia gen. nov. and transfer of seven species of the genus Pseudomonas homology group ii to the new genus, with the type species Burkholderia cepacia (Palleroni and Holmes 1981) comb. nov. Microbiol. Immunol. 1992, 36, 1251–1275. [CrossRef] [PubMed]
3. Estrada-de los Santos, P.; Vinuesa, P.; Martínez-Aguilar, L.; Hirsch, A.M.; Caballero-Mellado, J. Phylogenetic analysis of Burkholderia species by multilocus sequence analysis. Curr. Microbiol. 2013, 67, 51–60. [CrossRef] [PubMed]
4. Estrada-de los Santos, P.; Rojas-Rojas, F.U.; Tapia-García, E.Y.; Vásquez-Murrieta, M.S.; Hirsch, A.M. To split or not to split: An opinion on dividing the genus Burkholderia. Ann. Microbiol. 2016, 66, 1303–1314. [CrossRef]
5. 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. Legume-nodulating β-proteobacteria: Diversity, host range, and future prospects. Mol. Plant Microbe Int. 2011, 24, 1276–1288. [CrossRef] [PubMed]
6. Eberl, L.; Vandumme, P. Members of the genus Burkholderia: Good and bad guys. F1000Research 2016, 5. [CrossRef] [PubMed]
7. Sawana, A.; Adeolu, M.; Gupta, R.S. Molecular signatures and phylogenomic analysis of the genus Burkholderia: Proposal for division of this genus into the emended genus Burkholderia containing pathogenic organisms and a new genus Paraburkholderia gen. nov. harboring environmental species. Front. Genet. 2014, 5, 429. [CrossRef] [PubMed]
8. Dobritsa, A.P.; Samadpour, M. Transfer of eleven Burkholderia species to the genus Paraburkholderia and proposal of Caballeronia gen. nov., a new genus to accommodate twelve species of Burkholderia and Paraburkholderia. Int. J. Syst. Evol. Microbiol. 2016, 66, 2836–2846. [CrossRef] [PubMed]
9. Lopes-Santos, L.; Castro, D.B.A.; Ferreira-Tonin, M.; Corrêa, D.B.A.; Weir, B.S.; Park, D.; Ottoboni, L.M.M.; Neto, J.R.; Destefano, S.A.L. Reassessment of the taxonomic position of Burkholderia andropogonis and description of Robbsia andropogonis gen. nov., comb. nov. Anton. Leeuw. Int. J. Gen. 2017, 110, 727–736. [CrossRef] [PubMed]
10. De Lajudie, P.M.; Young, J.P.W. International committee on systematics of prokaryotes subcommittee for the taxonomy of Rhizobium and Agrobacterium minutes of the meeting, Budapest, 25 August 2016. *Int. J. Syst. Evol. Microbiol.* 2017, 67, 2485–2494. [CrossRef] [PubMed]

11. Beukes, C.; Palmer, M.; Manyaka, P.; Chan, W.Y.; Avontuur, J.; van Zyl, E.; Huntemann, M.; Clum, A.; Pillay, M.; Palaniappan, K. Genome data provides high support for generic boundaries in *Burkholderia* sensu lato. *Front. Microbiol.* 2017, 8, 1154. [CrossRef] [PubMed]

12. Partida-Martinez, L.P.; Groth, L.; Schmitt, L.; Richter, W.; Roth, M.; Hertweck, C. *Burkholderia rhizoxinica* sp. nov. and *Burkholderia endofungorum* sp. nov., bacterial endosymbionts of the plant-pathogenic fungus *Rhizopus microsporus*. *Int. J. Syst. Evol. Microbiol.* 2007, 57, 2583–2590. [CrossRef] [PubMed]

13. Sheu, S.Y.; Chou, J.H.; Bontemps, C.; Elliott, G.N.; Gross, E.; James, E.K.; Sprent, J.I.; Young, J.P.W.; Chen, W.M. *Burkholderia symbiotica* sp. nov., isolated from root nodules of *Mimosid* spp. native to north-east Brazil. *Int. J. Syst. Evol. Microbiol.* 2012, 62, 2272–2278. [CrossRef] [PubMed]

14. Zhu, H.; Guo, J.; Chen, M.; Feng, G.; Yao, Q. *Burkholderia dabaoshanensis* sp. nov., a heavy-metal-tolerant bacteria isolated from Dabaoshan mining area soil in China. *PLoS ONE* 2012, 7, e50225. [CrossRef] [PubMed]

15. Yoo, S.-H.; Kim, B.-Y.; Weon, H.-Y.; Kwon, S.-W.; Go, S.-J.; Stackebrandt, E. *Burkholderia soli* sp. nov., isolated from soil cultivated with Korean ginseng. *Int. J. Syst. Evol. Microbiol.* 2007, 57, 122–125. [CrossRef] [PubMed]

16. Goris, J.; Konstantinidis, K.T.; Klappenbach, J.A.; Coenye, T.; Vandamme, P.; Tiedje, J.M. DNA–DNA hybridization values and their relationship to whole-genome sequence similarities. *Int. J. Syst. Evol. Microbiol.* 2007, 57, 81–91. [CrossRef] [PubMed]

17. Konstantinidis, K.T.; Tiedje, J.M. Genomic insights that advance the species definition for prokaryotes. *Proc. Natl. Am. Sci. USA* 2005, 102, 2567–2572. [CrossRef] [PubMed]

18. Richter, M.; Rosselló-Móra, R. Shifting the genomic gold standard for the prokaryotic species definition. *Proc. Natl. Am. Sci. USA* 2009, 106, 19126–19131. [CrossRef] [PubMed]

19. Konstantinidis, K.T.; Tiedje, J.M. Towards a genome-based taxonomy for prokaryotes. *J. Bacteriol.* 2005, 187, 6258–6264. [CrossRef] [PubMed]

20. Konstantinidis, K.T.; Tiedje, J.M. Prokaryotic taxonomy and phylogeny in the genomic era: Advancements and challenges ahead. *Curr. Opin. Microbiol.* 2007, 10, 504–509. [CrossRef] [PubMed]

21. Rodriguez-R, L.M.; Konstantinidis, K.T. Bypassing cultivation to identify bacterial species. *Microbe Mag.* 2014, 9, 111–118. [CrossRef]

22. Suárez-Moreno, Z.R.; Caballero-Mellado, J.; Coutinho, B.G.; Mendonça-Previato, L.; James, E.K.; Venturi, V. Common features of environmental and potentially beneficial plant-associated *Burkholderia* spp. native to north-east Brazil. *Curr. Opin Microbiol.* 2016, 44, 2114–2120. [CrossRef] [PubMed]

23. Bennett, S. Solexa Ltd. *Pharmacogenomics* 2004, 5, 433–438. [CrossRef] [PubMed]

24. Bushnell, B. Bbtools Software Package. Available online: http://sourceforge.net/projects/bbmap (accessed on 23 May 2018).

25. Bankevich, A.; Nurk, S.; Antipov, D.; Gurevich, A.A.; Dvorkin, M.; Kulikov, A.S.; Lesin, V.M.; Nikolenko, S.I.; Pham, S.; Prijibelski, A.D. Spades: A new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol.* 2012, 19, 455–477. [CrossRef] [PubMed]

26. Bolger, A.M.; Lohse, M.; Usadel, B. Trimmomatic: A flexible trimmer for illumina sequence data. *Bioinformatics* 2014, 30, 2114–2120. [CrossRef] [PubMed]

27. Seemann, T. Prokka: Rapid prokaryotic genome annotation. *Bioinformatics* 2014, 30, 2068–2069. [CrossRef] [PubMed]

28. Aziz, R.K.; Bartels, D.; Best, A.A.; DeJongh, M.; Disz, T.; Edwards, R.A.; Formsma, K.; Gerdes, S.; Glass, E.M.; Kubal, M.; et al. The rast server: Rapid annotations using subsystems technology. *BMC Genom.* 2008, 9, 75. [CrossRef] [PubMed]

29. Blom, J.; Kreis, J.; Spänig, S.; Juhre, T.; Bertelli, C.; Ernst, C.; Goesmann, A. Edgar 2.0: An enhanced software platform for comparative gene content analyses. *Nucleic Acids Res.* 2016, 44, W22–W28. [CrossRef] [PubMed]

30. Edgar, R.C. Muscle: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 2004, 32, 1792–1797. [CrossRef] [PubMed]

31. Abascal, F.; Zardoya, R.; Posada, D. Prottest: Selection of best-fit models of protein evolution. *Bioinformatics* 2005, 21, 2104–2105. [CrossRef] [PubMed]

32. Kück, P.; Longo, G.C. FASconCAT-G: Extensive functions for multiple sequence alignment preparations concerning phylogenetic studies. *Front. Zool.* 2014, 11, 81. [CrossRef] [PubMed]
33. Stamatakis, A. RAxML version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 2014, 30, 1312–1313. [CrossRef] [PubMed]
34. Gevers, D.; Cohan, F.M.; Lawrence, J.G.; Spratt, B.G.; Coenye, T.; Feil, E.J.; Stackebrandt, E.; Van de Peer, Y.; Vandamme, P.; Thompson, F.L. Opinion: Re-examining prokaryotic species. *Nat. Rev. Microbiol.* 2005, 3, 733–739. [CrossRef] [PubMed]
35. Kanehisa, M.; Goto, S.; Kawashima, S.; Nakaya, A. The KEGG databases at GenomeNet. *Nucleic Acids Res.* 2002, 30, 42–46. [CrossRef] [PubMed]
36. Palmer, M.; Steenkamp, E.T.; Coetsee, M.P.A.; Blom, J.; Venter, S.N. Genome based characterization of biological processes that differentiate closely related bacteria. *Front. Microbiol.* 2018. [CrossRef] [PubMed]
37. Wickham, H. *Ggplot2: Elegant Graphics for Data Analysis*; Springer: New York, NY, USA, 2016. [CrossRef]
38. Edgar, R.C. Muscle: A multiple sequence alignment method with reduced time and space complexity. *BMC Bioinformatics* 2004, 5, 113. [CrossRef] [PubMed]
39. Kalyaanamoorthy, S.; Minh, B.Q.; Wong, T.K.F.; von Haeseler, A.; Jermiin, L.S. Modelfinder: Fast model selection for accurate phylogenetic estimates. *Nat. Methods* 2017, 14, 587–589. [CrossRef] [PubMed]
40. Nguyen, L.-T.; Schmidt, H.A.; von Haeseler, A.; Minh, B.Q. IQ-TREE: A fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Mol. Biol. Evol.* 2014, 32, 268–274. [CrossRef] [PubMed]
41. Paradis, E.; Claude, J.; Strimmer, K. Ape: Analyses of phylogenetics and evolution in R language. *Bioinformatics* 2004, 20, 289–290. [CrossRef] [PubMed]
42. Yu, G.; Smith, D.K.; Zhu, H.; Guan, Y.; Lam, T.T.Y. Ggtree: An R package for visualization and annotation of phylogenetic trees with their covariates and other associated data. *Methods Ecol. Evol.* 2017, 8, 28–36. [CrossRef]
43. Caballero-Mellado, J.; Onofre-Lemus, J.; Estrada-de Los Santos, P.; Martinez-Aguilar, L. The tomato rhizosphere, an environment rich in nitrogen-fixing *Burkholderia* species with capabilities of interest for agriculture and bioremediation. *Appl. Environ. Microbiol.* 2007, 73, 5308–5319. [CrossRef] [PubMed]
44. Estrada-de los Santos, P.; Bustillos-Cristales, R.; Caballero-Mellado, J. *Burkholderia*, a genus rich in plant-associated nitrogen fixers with wide environmental and geographic distribution. *Appl. Environ. Microbiol.* 2001, 67, 2790–2798. [CrossRef] [PubMed]
45. Jain, D.K.; Patriquin, D.G. Characterization of a substance produced by *Azospirillum* which causes branching of wheat root hairs. *Can. J. Microbiol.* 1985, 31, 206–210. [CrossRef]
46. 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. *Burkholderia phymatum* is a highly effective nitrogen-fixing symbiont of *Mimosa* spp. and fixes nitrogen ex planta. *New Phytol.* 2007, 173, 168–180. [CrossRef] [PubMed]
47. Jacobs, J.L.; Fasi, A.C.; Ramette, A.; Smith, J.J.; Hammerschmidt, R.; Sundin, G.W. Identification and onion pathogenicity of *Burkholderia cepacia* complex isolates from the onion rhizosphere and onion field soil. *Appl. Environ. Microbiol.* 2008, 74, 3121–3129. [CrossRef] [PubMed]
48. Vilchez, J.I.; Navas, A.; González-López, J.; Arcos, S.C.; Manzanera, M. Biosafety test for plant growth-promoting bacteria: Proposed environmental and human safety index (EHSI) protocol. *Front. Microbiol.* 2016, 6, 1514. [CrossRef] [PubMed]
49. Moran, N.A. Accelerated evolution and Muller’s ratchet in endosymbiotic bacteria. *Proc. Natl. Am. Sci. USA* 1996, 93, 2873–2878. [CrossRef]
50. Cardona, S.T.; Wopperer, J.; Eberl, L.; Valvano, M.A. Diverse pathogenicity of *Burkholderia cepacia* complex strains in the *Caenorhabditis elegans* host model. *FEMS Microbiol. Lett.* 2005, 250, 97–104. [CrossRef] [PubMed]
51. Kumar, S.; Stecher, G.; Tamura, K. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* 2016, 33, 1870–1874. [CrossRef] [PubMed]
52. Mirarab, S.; Warnow, T. ASTRAL-II: Coalescent-based species tree estimation with many hundreds of taxa and thousands of genes. *Bioinformatics* 2015, 31, 144–152. [CrossRef] [PubMed]
53. Sayyari, E.; Mirarab, S. Fast coalescent-based computation of local branch support from quartet frequencies. *Mol. Biol. Evol.* 2016, 33, 1654–1668. [CrossRef] [PubMed]
54. McCutcheon, J.P.; Moran, N.A. Extreme genome reduction in symbiotic bacteria. *Nat. Rev. Microbiol.* 2012, 10, 13–26. [CrossRef] [PubMed]
55. Moran, N.A.; Wernegreen, J.J. Lifestyle evolution in symbiotic bacteria: Insights from genomics. *Trends Ecol. Evol.* 2000, 15, 321–326. [CrossRef]
56. Gillis, M.; Van, T.V.; Bardin, R.; Goor, M.; Heulin, T.; Fernandez, M.P. Polyphasic taxonomy in the genus *Burkholderia* leading to an emended description of the genus and proposition of *Burkholderia vietnamiensis* sp. nov. for N2-fixing isolates from rice in Vietnam. *Int. J. Syst. Evol. Microbiol.* 1995, 45, 274–289. [CrossRef]

57. Da Silva, K.; de Souza Cassetari, A.; Lima, A.S.; De Brandt, E.; Pinnock, E.; Vandamme, P.; de Souza Moreira, F.M. Diazotrophic *Burkholderia* species isolated from the amazon region exhibit phenotypical, functional and genetic diversity. *Syst. Appl. Microbiol.* 2012, 35, 253–262. [CrossRef] [PubMed]

58. Faoro, H.; Rene Menegazzo, R.; Battistoni, F.; Gyaneshtwar, P.; dos Reis Junior, F.B.; Taulé, C.; Rausch, S.; Gonçalves Galvão, P.; de los Santos, C.; Mitra, S. The oil-contaminated soil diazotroph *Azarcus olearius* DQS-4T is genetically and phenotypically similar to the model grass endophyte *Azarcus* sp. BH72. *Environ. Microbiol. Rep.* 2017, 9, 223–238. [CrossRef] [PubMed]

59. De Meyer, S.E.; Briscoe, L.; Martinez-Hidalgo, P.; Agapakis, C.M.; Estrada-de-los Santos, P.; Seshadri, R.; Reeve, W.; Weinstock, G.; O’Hara, G.; Howieson, J.G. Symbiotic *Burkholderia* species show diverse arrangements of *nif/fix* and *nod* genes and lack typical high-affinity cytochrome cbb3 oxidase genes. *Mol. Plant Microbe Int.* 2016, 29, 609–619. [CrossRef] [PubMed]

60. Sheu, S.-Y.; Chou, J.-H.; Bontemps, C.; Elliott, G.N.; Gross, E.; dos Reis Junior, F.B.; Melkonian, R.; Moulin, L.; James, E.K.; Sprent, J.I. *Burkholderia diazotrophica* sp. nov., isolated from root nodules of *Mimosa* spp. *Int. J. Syst. Evol. Microbiol.* 2013, 63, 435–441. [CrossRef] [PubMed]

61. Bournaud, C.; de Faria, S.M.; dos Santos, J.M.F.; Tisseyre, P.; Silva, M.; Chaintreuil, C.; Gross, E.; James, E.K.; Prin, Y.; Moulin, L. *Burkholderia* species are the most common and preferred nodulating symbionts of the piptadenia group (Tribe mimoseae). *PLoS ONE* 2013, 8, e63478. [CrossRef] [PubMed]

62. Bontemps, C.; Elliott, G.N.; Gross, E.; dos Reis Junior, F.B.; Melkonian, R.; Moulin, L.; James, E.K.; Sprent, J.I. *Burkholderia diazotrophica* sp. nov., isolated from root nodules of *Mimosa* spp. *Int. J. Syst. Evol. Microbiol.* 2013, 63, 435–441. [CrossRef] [PubMed]

63. Dougherty, M.; Ryan, K.; van der Meijden, J.; Male, A.; Keough, M.; James, E.K.; Andrews, M. competition experiments for legume infection *Paraburkholderia nodosa* is the main N2-fixing species trapped by promiscuous common bean *Phaseolus vulgaris* (L.) in the Brazilian cerrado. *New Phytol.* 2010, 186, 934–946. [CrossRef] [PubMed]

64. Silva, V.C.; Alves, P.C.; Rhem, M.F.K.; dos Santos, J.M.F.; James, E.K.; Gross, E. Brazilian species of *Calliandra* Benth. (Tribe ingeae) are nodulated by diverse strains of *Paraburkholderia*. *Syst. Appl. Microbiol.* 2018, 41, 114–123. [CrossRef] [PubMed]

65. Lemaire, B.; Chimphango, S.B.; Stinton, C.; Rafudeen, S.; Honnay, O.; Smets, A.; Chen, W.M.; Sprent, J.; James, E.K.; Musasya, A.M. Biogeographical patterns of legume-nodulating *Burkholderia* spp.: From African fynbos to continental scales. *Appl. Environ. Microbiol.* 2016, 82, 5099–5115. [CrossRef] [PubMed]

66. Talbi, C.; Delgado, M.; Girard, L.; Ramirez-Trujillo, A.; Caballero-Mellado, J.; Bedmar, E. *Burkholderia phymatum* strains capable of nodulating *Phaseolus vulgaris* are present in Moroccan soils. *Appl. Environ. Microbiol.* 2010, 76, 4587–4591. [CrossRef] [PubMed]

67. Liu, W.Y.; Ridgway, H.J.; James, T.K.; James, E.K.; Chen, W.-M.; Sprent, J.I.; Young, J.P.W.; Andrews, M. *Burkholderia* sp. induces functional nodules on the south african invasive legume *Dipogon lignosus* (Phaseoleae) in New Zealand soils. *Microb. Ecol.* 2014, 68, 542–555. [CrossRef] [PubMed]

68. Dall’Agnol, R.F.; Plotegher, F.; Souza, R.C.; Mendes, I.C.; dos Reis Junior, F.B.; Bena, G.; Moulin, L.; Hungria, M. *Paraburkholderia nodosa* is the main N2-fixing species trapped by promiscuous common bean *Phaseolus vulgaris* (L.) in the Brazilian ‘cerradão’. *FEMS Microbiol. Ecol.* 2016, 92, fis108. [CrossRef] [PubMed]

69. Lardi, M.; de Campos, S.B.; Purtschert, G.; Eberl, L.; Pessi, G. Competition experiments for legume infection identify *Burkholderia phymatum* as a highly competitive β-rhizobium. *Front. Microbiol.* 2017, 8, 1527. [CrossRef] [PubMed]

70. Bontemps, C.; Elliott, G.N.; Simon, M.F.; Reis Júnior, F.B.; Gross, E.; Lawton, R.C.; Neto, N.E.; Fatima Loureiro, M.; Faria, S.M.; Sprent, J.I.; et al. *Burkholderia* species are ancient symbionts of legumes. *Mol. Ecol.* 2010, 19, 44–52. [CrossRef] [PubMed]

71. Moulin, L.; Klonowska, A.; Caroline, B.; Booth, K.; Vriezen, J.A.; Melkonian, R.; James, E.K.; Young, J.P.W.; Bena, G.; Hauser, L. Complete genome sequence of *Burkholderia phymatum* STM815T, a broad host range and efficient nitrogen-fixing symbiont of *Mimosa* species. *Stand. Genom. Sci.* 2014, 9, 763–774. [CrossRef] [PubMed]
72. Partida-Martinez, L.P.; Hertweck, C. Pathogenic fungus harbours endosymbiotic bacteria for toxin production. *Nature* **2005**, *437*, 884–888. [CrossRef] [PubMed]

73. Angus, A.A.; Agapakis, C.M.; Fong, S.; Yerrapragada, S.; Estrada-de Los Santos, P.; Yang, P.; Song, N.; Kano, S.; Caballero-Mellado, J.; De Faria, S.M. Plant-associated symbiotic *Burkholderia* species lack hallmark strategies required in mammalian pathogenesis. *PLoS ONE* **2014**, *9*, e83779. [CrossRef] [PubMed]

74. Hueck, C.J. Type III protein secretion systems in bacterial pathogens of animals and plants. *Microbiol. Mol. Biol. Rev.* **1998**, *62*, 379–433. [PubMed]

75. Lackner, G.; Moebius, N.; Partida-Martinez, L.P.; Boland, S.; Hertweck, C. Evolution of an endofungal lifestyle: Deductions from the *Burkholderia rhizoxinica* genome. *BMC Genom.* **2011**, *12*, 210. [CrossRef] [PubMed]

© 2018 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).