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Burkholderia nodosa sp. nov., isolated from root nodules of the woody Brazilian legumes *Mimosa bimucronata* and *Mimosa scabrella*

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Three strains, Br3437^T, Br3461 and Br3470, were isolated from nitrogen-fixing nodules on the roots of *Mimosa scabrella* (Br3437^T) and *Mimosa bimucronata* (Br3461, Br3470), both of which are woody legumes native to Brazil. On the basis of 16S rRNA gene sequence similarities, all the strains were shown previously to belong to the genus *Burkholderia*. A polyphasic approach, including DNA–DNA hybridizations, PFGE of whole-genome DNA profiles, whole-cell protein analyses, fatty acid methyl ester analysis and extensive biochemical characterization, was used to clarify the taxonomic position of these strains further; the strains are here classified within a novel species, for which the name *Burkholderia nodosa* sp. nov. is proposed. The type strain, Br3437^T (=LMG 23741^T=BCRC 17575^T), was isolated from nodules of *M. scabrella*.

Recently, a considerable body of evidence has accumulated to show that legumes, particularly those in the genus *Mimosa* in the Mimosoideae, are not nodulated exclusively by members of the *Rhizobiaceae* in the *Alphaproteobacteria*, but may also be nodulated by members of the *Betaproteobacteria*. These so-called 'legume-nodulating β-proteobacteria' or 'β-rhizobia' include *Cupriavidus taiwanensis* (Chen *et al.*, 2001, 2003a, b; Vandamme & Coenye, 2004), which has been isolated from nodules of *Mimosa pudica*, *M. diplotricha* and *M. pigra* (syn. *M. pellita*) in Taiwan (Chen *et al.*, 2001, 2003a, b, 2005b), from *M. pudica* in India (Verma *et al.*, 2004) and from *M. pudica* and *M. pigra* in Costa Rica (Barrett & Parker, 2006). More recently, however, there has been a greater focus on β-rhizobia in the genus *Burkholderia*, as these are being isolated from *Mimosa* and related genera with much higher frequency than *C. taiwanensis*, particularly in South and Central America (Barrett & Parker, 2005, 2006; Chen *et al.*, 2005a), but also from the invasive legume *M. pigra* in Taiwan (Chen *et al.*, 2005b). However, with the exception of *Burkholderia caribensis* TJ182 and TJ183 (isolated from *M. pudica* and *M. diplotricha* in Taiwan; Chen *et al.*, 2003b; Vandamme *et al.*, 2002), *Burkholderia tuberum* STM678^T (isolated from *Aspalathus carnosa* in South Africa; Moulin *et al.*, 2001), *Burkholderia phymatum* STM815^T and NGR195A (isolated, respectively, from *Machaerium lunatum* in French Guiana and *Mimosa invisa* in New Guinea; Moulin *et al.*, 2001; Vandamme *et al.*, 2002; Elliott *et al.*, 2007) and various strains of *Burkholderia mimosarum* (isolated from *M. pigra* in Taiwan and Brazil; Chen *et al.*, 2006), the taxonomic positions of most *Burkholderia* legume symbionts have not yet been described. The aim of the present study is to clarify the taxonomic positions of three strains isolated from

The GenBank/EMBL/DDBJ accession numbers for 16S rRNA gene sequences of strains Br3437^T, Br3470 and Br3461 are respectively AY773189 and AM284971, AY773198 and AM284972, and AY533861 and AM284970 (two determinations for each strain).

An extended phylogenetic tree and details of genome sizes, DNA–DNA binding values and fatty acid compositions of the novel strains and related species are available as supplementary material in IJSEM Online.

Mimosa nodules in Brazil that have previously been shown by 16S rRNA gene sequence analyses to belong to the genus *Burkholderia* (Chen *et al.*, 2005a).

Strains Br3470 and Br3461 were isolated from root nodules on *Mimosa bimucronata* and strain Br3437^T was isolated from nodules on *Mimosa scabrella* (Chen *et al.*, 2005a). Both *Mimosa* species are woody legumes native to Brazil, and the geographical origins of the strains have been described previously (Chen *et al.*, 2005a). All were grown on yeast extract-mannitol agar plates (Vincent, 1970) and incubated at 28 °C unless indicated otherwise. *Burkholderia* reference strains have been described previously (Vandamme *et al.*, 2002).

The 16S rRNA gene sequences of strains Br3437^T, Br3470 and Br3461 have been reported previously by Chen *et al.* (2005a) (GenBank accession numbers AY773189, AY773198 and AY533861). However, whereas the sequences for strains Br3470 and Br3461 are >99 % similar, there is a difference of 1.5–2 % between them and that of strain Br3437^T. As subsequent analyses revealed all three strains to represent a single species (see below), we repeated the 16S rRNA gene sequence analyses for all three strains. The latter sequences were deposited as GenBank accession numbers AM284971 (Br3437^T), AM284972 (Br3470) and AM284970 (Br3461). All repeat analyses revealed virtually identical sequences (data not shown).

A phylogenetic analysis of the 16S rRNA gene sequences showed that strains Br3437^T, Br3461 and Br3470 formed a single cluster with 99.7–98.1 % similarity and that they belonged to the genus *Burkholderia* within the *Betaproteobacteria* (Fig. 1 and Supplementary Fig. S1 available in IJSEM Online). 16S rRNA gene sequence comparison of strain Br3437^T and its closest neighbours, *Burkholderia unamae*, *B. mimosarum*, *B. silvatlantica*, *B. sacchari* and *B. tropica*, showed it to have 97.9, 97.1, 96.8, 96.5 and 96.4 % similarity, respectively, to the type strains of these species. The similarity levels of strains Br3461, Br3470 and Br3437^T to other *Burkholderia* species were less than 96.0 %.

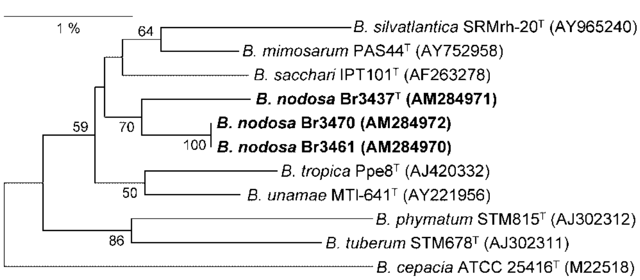

Fig. 1. Phylogenetic tree of strains Br3437^T, Br3470 and Br3461 (*Burkholderia nodosa* sp. nov.) and related *Burkholderia* type strains based on 16S rRNA gene sequence comparisons. Distances were calculated and clustering with the neighbour-joining method was performed by using the software package BioEdit. Numbers at nodes are percentage bootstrap values based on 1000 resampled datasets; only values ⩾50 % are given. Bar, 1 % sequence dissimilarity. The sequence of *Burkholderia cepacia* ATCC 25416^T was used as an outgroup. A tree including a wider selection of reference sequences is available as Supplementary Fig. S1 in IJSEM Online.

DNA samples were prepared from strains Br3437^T, Br3470 and Br3461 as described by Pitcher *et al.* (1989). The DNA base composition was determined as described by Mesbah *et al.* (1989). DNA–DNA hybridizations were performed with photobiotin-labelled probes as described by Ezaki *et al.* (1989). The hybridization temperature was 50 °C and the reaction was carried out in 30 % formamide. The DNA G+C content of strains Br3437^T, Br3470 and Br3461 was between 62 and 63 mol% (Supplementary Table S1). The DNA–DNA binding values among strains Br3437^T, Br3470 and Br3461 were between 73 and 100 % (Supplementary Table S1), whereas mean binding values of strains Br3437^T and Br3461 of 15–54 % were calculated towards their closest phylogenetic neighbours, the type strains of *B. mimosarum*, *B. unamae*, *B. sacchari* and *B. tropica* (Supplementary Table S1).

The finding that these three strains represent a single species was unexpected, given the considerable divergence in 16S rRNA gene sequences. However, the high DNA–DNA binding value was further supported by the high similarity in whole-cell protein content (see below), and a repeat analysis of the sequences indeed confirmed the initial sequences. Although not unique in prokaryotic taxonomy, such a large intraspecies divergence in 16S rRNA gene sequences has, so far, not been documented in the genus *Burkholderia*.

For PFGE genome organization analysis as described by Chen *et al.* (2003b), intact genomic DNA in agarose plugs was electrophoresed on a 0.8 % agarose gel in TAE for 41 h with a pulse time of 500 s at 100 V (CHEF-III system; Bio-Rad). Br3437^T contained four replicons with a total genome size of 9.0 Mb (Supplementary Table S1 and Fig. 2).

Differentiation of the proposed novel taxon from its closest phylogenetic neighbours was examined by several approaches. For the analysis of protein electrophoretic patterns, strains were grown on nutrient agar (Oxoid CM3) supplemented with 0.04 % (w/v) KH_2PO_4 and 0.24 % (w/v) $Na_2HPO_4.12H_2O$ (pH 6.8) and incubated for 48 h at 28 °C. Preparation of whole-cell proteins and SDS-PAGE were performed as described by Pot *et al.* (1994). Densitometric analysis, normalization and interpolation of the protein profiles and numerical analysis using Pearson's product-moment correlation coefficient were performed using the GelCompar 4.2 software package (Applied Maths). Whole-cell protein extracts were prepared from strains Br3437^T, Br3470 and Br3461 and compared with others present in our database. Strains Br3437^T, Br3470 and Br3461 formed a single cluster with similarities of >92 %, in comparison with similarities of less than 85 % to other *Burkholderia* species (Fig. 3).

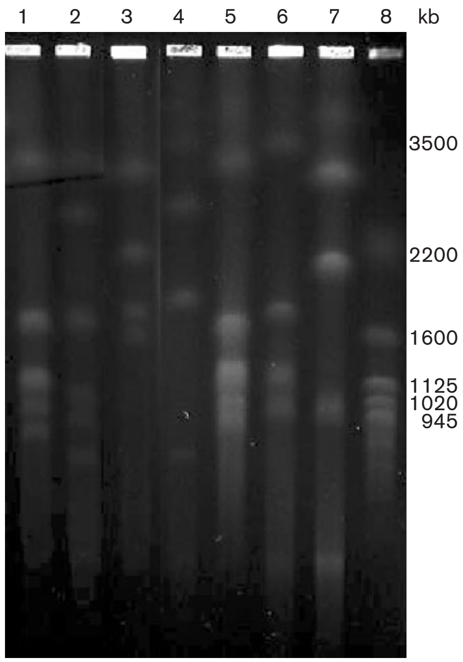

Fig. 2. PFGE of undigested whole-genome DNA profiles. Lanes: 1 and 5, *B. mimosarum* PAS44^{T}; 2, *B. tropica* LMG 22274^{T}; 3, strain Br3437^{T}; 4, *B. phymatum* STM815^{T}; 6, *B. unamae* LMG 22722^{T}; 7, *B. sacchari* LMG 19450^{T}. Molecular markers were *Saccharomyces cerevisiae* Marker (Bio-Rad) (lane 8) and *B. phymatum* STM815^{T} (3.5, 2.8, 2.1 and 0.5 Mb; Chen *et al.*, 2003b) (lane 4).

For fatty acid methyl ester analysis, cells were harvested after an incubation period of 48 h at 28 °C; fatty acid methyl esters were then prepared, separated and identified using the Microbial Identification System (Microbial ID) as described previously (Vandamme *et al.*, 2002). Fatty acid profiles of strains Br3437^{T}, Br3461 and Br3470 were determined and compared with those of other *Burkholderia* species. Fatty acid profiles of strains Br3437^{T}, Br3461, Br3470 and other reference strains were similar, and were predominated by 16 : 0, 18 : 1ω7*c*, summed feature 2 (comprising 14 : 0 3-OH, 16 : 1 iso I, an unidentified fatty acid with an equivalent chain-length of 10.928 or 12 : 0 ALDE, or any combination of these fatty acids) and summed feature 3 (comprising 16 : 1ω7*c* and/or 15 : 0 iso 2-OH). Details of the cellular fatty acid compositions and those of closely related *Burkholderia* species are shown in Supplementary Table S2. In general, all these organisms had very similar whole-cell fatty acid profiles, which were therefore not useful for species discrimination.

For biochemical characterization, the API 20NE and API ZYM microtest systems were used according to the recommendations of the manufacturer (bioMérieux). For carbon substrate assimilation tests, Biolog GN2 microtitre test plates were used.

When using the API 20NE microtest gallery, the following characteristics were present in all strains: nitrate reduction, activity of oxidase, catalase, urease and β-galactosidase and assimilation of glucose, arabinose, mannose, mannitol, *N*-acetylglucosamine, gluconate, caprate, adipate, citrate, malate and phenylacetate. The following characteristics were uniformly absent: indole production, glucose fermentation, aesculin hydrolysis, gelatin hydrolysis and assimilation of maltose.

When using the API ZYM microtest gallery, activities of alkaline phosphatase, C4 esterase, leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase were present in all strains. Activities of C14 lipase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase were uniformly absent.

When using the Biolog GN2 microtitre test system, the following substrates were oxidized: glycogen, Tween 40, Tween 80, *N*-acetyl-D-glucosamine, adonitol, arabinose, arabitol, cellobiose, i-erythritol, D-fructose, L-fucose, D-galactose, α-D-glucose, *myo*-inositol, D-mannitol, D-mannose, D-psicose, L-rhamnose, D-sorbitol, D-trehalose, xylitol, methyl pyruvate, acetic acid, citrate, formic acid,

Fig. 3. Dendrogram based on numerical analysis of the whole-cell protein profiles of *Mimosa* isolates and type strains of closely related *Burkholderia* species.

D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, β-hydroxybutyric acid, *p*-hydroxyphenylacetic acid, α-ketoglutaric acid, DL-lactate, quinic acid, D-saccharic acid, sebacic acid, succinic acid, bromosuccinic acid, succinamic acid, alaninamide, D-alanine, L-alanine, L-alanyl glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, L-histidine, L-leucine, L-phenylalanine, L-proline, L-pyroglutamic acid, L-serine, γ-aminobutyric acid and urocanic acid. None of the strains oxidized α-cyclodextrin, dextrin, *N*-acetyl-D-galactosamine, gentiobiose, α-D-lactose, lactulose, maltose, D-melibiose, methyl β-D-glucoside, D-raffinose, sucrose, turanose, D-glucuronic acid, γ-hydroxybutyric acid, itaconic acid, α-hydroxybutyric acid, α-ketovaleric acid, malonic acid, glucuronamide, L-ornithine, inosine, uridine, thymidine, 2-aminoethanol, 2,3-butanediol, DL-α-glycerol phosphate or glucose 1-phosphate. Oxidation of the remaining substrates (monomethyl succinate, *cis*-aconitic acid, α-ketobutyric acid, propionic acid, hydroxy-L-proline, D-serine, L-threonine, DL-carnitine, phenylethylamine, putrescine, glycerol and glucose 6-phosphate) was strain dependent.

A comparison of the phenotypic characteristics of the type strain of the novel taxon with those of the type strains of related *Burkholderia* species is shown in Table 1. Strain Br3437^T can be differentiated from *B. mimosarum* by the activity of β-galactosidase and oxidation of adipate, adonitol, caprate, rhamnose, trehalose and xylitol; from *B. sacchari* by the activity of urease, β-galactosidase and oxidation of caprate, rhamnose, sucrose, trehalose and xylitol; and from *B. unamae* and *B. tropica* by the activity of urease and oxidation of trehalose and xylitol. Only strains Br3437^T, Br3461 and Br3470 and *B. mimosarum* can produce N_2-fixing nodules on *Mimosa* species (Chen *et al.*, 2005a).

Table 1. Comparison of phenotypic characters of strain Br3437^T and the type strains of related *Burkholderia* species

Strains: 1, strain Br3437^T; 2, *B. sacchari* LMG 19450^T; 3, *B. tropica* LMG 22274^T; 4, *B. unamae* LMG 22722^T; 5, *B. mimosarum* PAS44^T. Data for reference strains were obtained in this study with the exception of the G+C contents, which were taken from Reis *et al.* (2004) and Chen *et al.* (2006).

Character	1	2	3	4	5
Urease	+	−	−	−	+
β-Galactosidase	+	−	+	+	−
Assimilation of:					
Adipate	+	+	+	+	−
Adonitol	+	+	+	+	−
Caprate	+	−	+	+	−
Rhamnose	+	−	+	+	−
Sucrose	−	+	−	−	−
Trehalose	+	−	−	+	−
Xylitol	+	−	−	−	−
Nodulation on:					
M. pudica	+	−	−	−	+
M. diplotricha	+	−	−	−	+
M. pigra	+	−	−	−	+
G+C content (mol%)	62.8	63.7	63.5	63.5	64.8

In conclusion, the present study demonstrated that three isolates from root nodules of *M. bimucronata* and *M. scabrella* from Brazil represent a single species that is readily distinguished from its nearest phylogenetic neighbours by whole-genome PFGE patterns (Fig. 2), whole-cell protein profiles (Fig. 3), DNA–DNA reassociation experiments (Supplementary Table S1), nodulation ability on *Mimosa* species (Table 1) and biochemical characterization (Table 1). We propose to name this organism *Burkholderia nodosa* sp. nov. Moreover, isolates Br3437^T, Br3461 and Br3470 produced N_2-fixing nodules on *Mimosa* species. These results strongly confirm that these *Burkholderia* strains can form effective symbioses with legumes of *Mimosa* species (Chen *et al.*, 2005a, b).

Description of *Burkholderia nodosa* sp. nov.

Burkholderia nodosa (no.do′sa. L. fem. adj. *nodosa* knotty or swollen, indicating that the type strain was isolated from root nodules).

Cells are Gram-negative, non-spore-forming rods. After 24 h growth on yeast extract-mannitol agar at 28 °C, the mean cell size is about 0.5–0.8 × 0.8–2.2 μm. Growth is observed at 28, 30 and 37 °C. Catalase- and oxidase-positive. Assimilation of glucose, arabinose, mannose, mannitol, *N*-acetylglucosamine, gluconate, caprate, adipate, citrate, malate and phenylacetate is observed. No indole production, gelatin hydrolysis, aesculin hydrolysis, glucose fermentation or assimilation of maltose is observed. Additional characteristics are listed above. Known strains were isolated from root nodules of *Mimosa bimucronata* and *Mimosa scabrella.*

The type strain is strain Br3437^T (=BCRC 17575^T=LMG 23741^T). Phenotypic characteristics of the type strain are the same as described for the species. Its DNA G+C content is 62.8 mol% and the genome size is approximately 9.0 Mb. Strains Br3461 (=R-22632) and Br3470 (=R-25486) are also assigned to this species.

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