Burkholderia dilworthii sp. nov., isolated from *Lebeckia ambigua* root nodules

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Three strains of Gram-stain-negative, rod-shaped bacteria were isolated from *Lebeckia ambigua* root nodules and authenticated on this host. Based on the 16S rRNA gene sequence phylogeny, they were shown to belong to the genus *Burkholderia*, with the representative strain WSM3556^T being most closely related to *Burkholderia caledonica* LMG 23644^T (98.70 % 16S rRNA gene sequence similarity) and *Burkholderia rhynchosiae* WSM3937^T (98.50 %). Additionally, these strains formed a distinct group in phylogenetic trees of the housekeeping genes *gyrB* and *recA*. Chemotaxonomic data, including fatty acid profiles and analysis of respiratory quinones, supported the assignment of our strains to the genus *Burkholderia*. Results of DNA–DNA hybridizations, MALDI-TOF MS analysis and physiological and biochemical tests allowed genotypic and phenotypic differentiation of our strains from their nearest neighbour species. Therefore, these strains represent a novel species, for which the name *Burkholderia dilworthii* sp. nov. is proposed, with the type strain WSM3556^T (=LMG 27173^T=HAMBI 3353^T).

Over the past decade, several novel betaproteobacterial species have been isolated from legume root nodules and shown to nodulate their host (Gyaneshwar *et al.*, 2011). Most of these species, including *Burkholderia diazotrophica* (Sheu *et al.*, 2013), *B. mimosarum* (Chen *et al.*, 2006), *B. nodosa* (Chen *et al.*, 2007), *B. phymatum* (Elliott *et al.*, 2007b; Vandamme *et al.*, 2002), *B. sabiae* (Chen *et al.*, 2008) and *B. symbiotica* (Sheu *et al.*, 2012), form associations with *Mimosa* species (subfamily Mimosoideae). However, the description of *Burkholderia tuberum*, which was originally isolated from the South African legume *Aspalathus carnosa* (Vandamme *et al.*, 2002) and found to nodulate several *Cyclopia* species (subfamily Papilionoideae) (Elliott *et al.*, 2007a), confirmed that species of the genus *Burkholderia* are not exclusively *Mimosa* symbionts. Garau *et al.* (2009) also reported nitrogen fixation from strains of *Burkholderia* in nodules of herbaceous legumes from the fynbos biome in South Africa. These strains, isolated from *Rhynchosia ferulifolia* nodules, have recently been described as *Burkholderia rhynchosiae* (De Meyer *et al.*, 2013a).

As part of a continuing study on fynbos legumes that can potentially be used as perennial pasture legumes, and their associated rhizobia, 23 strains were isolated from surface-sterilized root nodules of *Lebeckia ambigua* originating from the South African Western Cape (Howieson *et al.*, 2013; le Roux & Van Wyk, 2007). Sequence analysis showed that all isolates belonged to the genus *Burkholderia* and that they grouped in five different clusters, of which the strains belonging to group four have recently been described as *Burkholderia sprentiae* (De Meyer *et al.*, 2013b). In the present study, three strains originating from *Lebeckia ambigua* root nodules collected near the Modder river (Howieson *et al.*, 2013) were selected for further investigation using a polyphasic approach. Strain WSM3556^T has been deposited in the BCCM/LMG Bacteria Collection (http://www.belspo.be/bccm) and the HAMBI Culture Collection, University of Helsinki, Finland (http://www.helsinki.fi/hambi/). All strains were subcultured on 1/2 LA medium (Yates *et al.*, 2007) at 28 °C unless otherwise indicated.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was performed as described previously (Wieme *et al.*, 2012). All conditions

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *gyrB* and *recA* gene sequences of strains WSM3556^T, WSM4204 and WSM4206 are respectively HQ698908, HE994041 and HE994060 (WSM3556^T), HQ698906, HE994057 and HE994075 (WSM4204) and HQ698907, HG422552 and HG422553 (WSM4206).

Five supplementary figures and a supplementary table are available with the online version of this paper.

were exactly as described previously except that 1/2 LA growth medium was used to culture the strains prior to protein extraction (Wieme *et al.*, 2012). All strains formed one separate cluster that could be distinguished from the strains representing the closest neighbour, *B. rhynchosiae* (Fig. S1, available in the online Supplementary Material). For PCR, genomic DNA of all isolates was prepared using the GES method as described by Pitcher *et al.* (1989). $(GTG)_5$-PCR analysis was performed as described previously (Gevers *et al.*, 2001). The fingerprints were analysed using the BioNumerics 5.1 software package (Applied Maths). Similarity among the digitized profiles was calculated using Pearson's correlation coefficient (expressed for convenience as a percentage similarity) and a UPGMA dendrogram was derived from the similarity matrix. Fig. S2 shows the $(GTG)_5$-PCR fingerprints of the three novel isolates. The DNA fingerprints suggest that the isolates represent three genetically different strains and can be distinguished from their closest neighbour.

Nearly full-length amplicons for the 16S rRNA gene were obtained for all three strains using the primers and conditions described previously by Vancanneyt *et al.* (2004). The resulting 16S rRNA gene sequences were aligned using ARB (Ludwig *et al.*, 2004) and were added to the alignment of the SILVA SSURef version 106 ARB database (Pruesse *et al.*, 2007). Phylogenetic trees were reconstructed using the MEGA 5 software package with the maximum-likelihood (ML) method with the general time reversible (GTR) model and the neighbour-joining (NJ) method with Kimura's two-parameter model (Tamura *et al.*, 2011). Bootstrap analysis with 1000 replicate datasets was performed to assess the support of the clusters. The overall topologies of the phylogenetic trees obtained with the ML and NJ methods were similar (not shown). The three strains formed a novel branch within the genus *Burkholderia* (Fig. S3) and shared sequence similarities of 98.70–98.77 % with *Burkholderia caledonica* LMG 23644^T and 98.50–95.57 % with *B. rhynchosiae* WSM3937^T, as determined with the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim *et al.*, 2012). *gyrB* (482 bp) and *recA* (388 bp) gene sequence analysis was based on the method described by Spilker *et al.* (2009) with slight modifications: the 25 μl PCR consisted of 1 × PCR buffer (Qiagen), 1 U *Taq* polymerase (Qiagen), 250 μM each dNTP (Applied Biosystems), 1 × Q-solution (Qiagen) and 0.4 μM *gyrB* or 1 μM *recA* primer. The resulting gene sequences were aligned using the MEGA 5 software package (Tamura *et al.*, 2011) and phylogenetic trees were reconstructed using the ML method with the GTR model. Bootstrap analysis with 1000 replicates was performed to assess the support of the clusters. The phylogenetic trees based on the *gyrB* and *recA* gene sequences of the three strains (Figs S4 and S5) revealed a monophyletic cluster supported by a high bootstrap value (100 %). Levels of gene sequence similarity between the three strains and their closest neighbours *B. caledonica* LMG 19076^T and *B. rhynchosiae* WSM3937^T were 93.0–93.6 and 91.1–91.7 %, respectively, for *gyrB*, and 94.3–94.7 and 96.5–96.8 % respectively, for *recA*. The phylogenetic tree based on concatenated 16S rRNA, *gyrB* and *recA* gene sequences is shown in Fig. 1.

Phenotypic analysis was performed on 1/2 LA medium at 28 °C unless otherwise indicated. Cells were Gram stained according to Vincent (1970). Cell morphology and motility were observed by phase-contrast microscopy. Oxidase activity was detected by immersion of cells in 1 % *N*,*N*,*N*′,*N*′-tetramethyl *p*-phenylenediamine solution and catalase activity was determined by flooding a colony with 10 % H_2O_2 and checking for the presence of bubbles. Other biochemical tests were performed by inoculating API 20NE and API 20E strips (bioMérieux) according to the manufacturer's instructions and incubating for 48 h at 28 °C. Growth was tested at 28 °C in nutrient broth (NB; BD Difco) with 0–10 % NaCl and at pH 2–9, measured using an Orion 420A pH meter and adjusted with 35 % HCl or 5 M NaOH. Growth on 1/2 LA medium (Yates *et al.*, 2007) was tested at 4, 10, 15, 21, 28, 30, 37 and 40 °C. Colonies were visible after 24 h of growth at 21–30 °C on 1/2 LA medium; growth at 28 °C was also observed on LMG medium 14 (http://bccm.belspo.be/db/media_search_form.php) after 48 h. The results of the phenotypic and biochemical tests are given in the species description and in Table 1. Most notably, positive reactions were recorded for tryptophan deaminase and assimilation of adipic acid and trisodium citrate and negative reactions for the assimilation of capric acid. Antibiotic susceptibility tests were performed on nutrient agar (Oxoid) using the antibiotic Sensi-disc dispenser system (Oxoid) with bio-discs (Oxoid) containing ampicillin (10 μg), chloramphenicol (30 μg), gentamicin (10 μg), kanamycin (30 μg), penicillin G (10 μg), streptomycin (10 μg) and tetracycline (30 μg). The three strains were grown on 1/2 LA medium for 48 h prior to testing. The plates were incubated at 28 °C and read between 2 and 5 days. All strains investigated were resistant to ampicillin, chloramphenicol, gentamicin and penicillin and sensitive to tetracycline. Strain-dependent reactions were recorded for both kanamycin and streptomycin.

The whole-cell fatty acid composition was analysed and fatty acid methyl esters were extracted according to the MIDI protocol (http://www.microbialid.com/PDF/TechNote_101.pdf). All characteristics such as temperature, medium and physiological age (overlap area of the second and third quadrant from a quadrant streak) were as in the MIDI protocol. The profiles were generated using an Agilent Technologies 6890N gas chromatograph and identified and clustered using the Microbial Identification System software and MIDI TSBA database version 5.0. Fatty acid profiles are listed in Table 2. The most abundant fatty acids for our strains were $C_{18:1}\omega7c$ (20.6 %), $C_{16:0}$ (18.0 %), summed feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH; 13.1 %) and $C_{17:0}$ cyclo (12.3 %). The presence of $C_{16:0}$ 3-OH supports the placement of our strains in the genus *Burkholderia* (Garrity *et al.*, 2005; Yabuuchi *et al.*, 1992). Additionally, there were noticeable differences between the fatty acid profiles of the strains isolated from *L. ambigua* and other type strains of the genus *Burkholderia* (Table 2). Cell biomass for respiratory lipoquinone analysis was obtained from a late-exponential phase

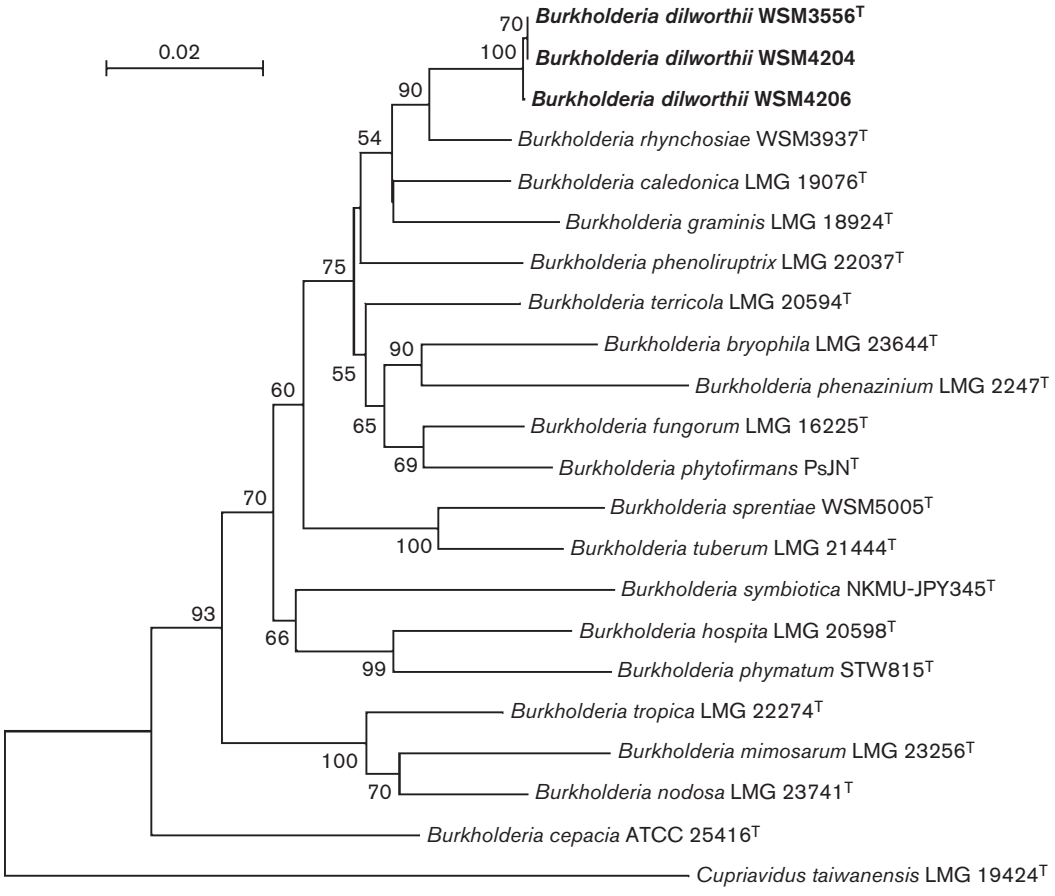

Fig. 1. ML tree based on concatenated 16S rRNA, *gyrB* and *recA* gene sequences of the novel strains and members of phylogenetically related species. Bootstrap values after 1000 replicates are expressed as percentages; values less than 50 % are not shown. *Cupriavidus taiwanensis* LMG 19424^T is included as an outgroup. Bar, 0.02 substitutions per site.

culture grown in 1/2 LA broth. Lipoquinones were extracted from lyophilized biomass by a modified one-phase Bligh/Dyer extraction method and analysed using an HPLC/electrospray/tandem mass spectrometry method as described by Ardley *et al.* (2012). For strain WSM3556^T, ubiquinone Q-8 was the major respiratory lipoquinone (approx. 61 %), with Q-10 (approx. 19 %) and Q-7 (approx. 1 %) also present. The identification of Q-8 as the major respiratory lipoquinone is in agreement with results found for other species of the genus *Burkholderia* (Aizawa *et al.*, 2010a, b, 2011; Sheu *et al.*, 2012, 2013; Valverde *et al.*, 2006).

For DNA–DNA hybridization and the determination of the DNA G+C content, high-molecular-mass DNA was prepared as described by Pitcher *et al.* (1989). DNA–DNA hybridizations were performed using a microplate method and biotinylated probe DNA (Ezaki *et al.*, 1989). The hybridization temperature was 45±1 °C. Reciprocal reactions (A × B and B × A) were performed for each DNA pair and their variation was within the limits of this method (Goris *et al.*, 1998). A summary of the hybridization values is given in Table S1. The values presented are the means of at least three replicates. The DNA–DNA relatedness between strain WSM3556^T and its closest neighbours *B. rhynchosiae* WSM3937^T and *B. caledonica* LMG 19076^T was respectively 44 and 45 %. The G+C content of the DNA was determined by HPLC according to the method of Mesbah *et al.* (1989) using a Waters Breeze HPLC system and XBridge Shield RP18 column thermostabilized at 37 °C. The solvent was 0.02 M $NH_4H_2PO_4$ (pH 4.0) with 1.5 % (v/v) acetonitrile. Non-methylated lambda phage (Sigma) and *Escherichia coli* DNA were used as calibration reference and control, respectively. The DNA G+C content of the novel strains was 61.4–61.9 mol% (Table 1), which is within the range reported for *Burkholderia* (59–69.9 mol%) (Garrity *et al.*, 2005; Gillis *et al.*, 1995; Yabuuchi *et al.*, 1992).

The nodulation and nitrogen fixation capacity of all three strains was studied previously (Howieson *et al.*, 2013) on their original host (*Lebeckia ambigua*), as well as on *Lebeckia sepiaria*, using the axenic sand-culture system described by Yates *et al.* (2007). These results confirmed that they can form effective N_2-fixing symbioses with *L. ambigua* and *L. sepiaria*.

Table 1. Phenotypic characteristics that distinguish the novel strains from the type strains of other species of the genus *Burkholderia*

Strains: 1, *B. dilworthii* sp. nov. (n=3); 2, *B. caledonica* LMG 19076^T (data from this study); 3, *B. diazotrophica* LMG 26031^T (Sheu *et al.*, 2013); 4, *B. mimosarum* LMG 23256^T (Chen *et al.*, 2006, 2007); 5, *B. nodosa* LMG 23741^T (Chen *et al.*, 2007); 6, *B. phymatum* LMG 21445^T (Vandamme *et al.*, 2002; Chen *et al.*, 2008); 7, *B. rhynchosiae* WSM3937^T (this study); 8, *B. sabiae* LMG 24235^T (Chen *et al.*, 2008); 9, *B. sprentiae* WSM5005^T (this study); 10, *B. symbiotica* JPY345^T (Sheu *et al.*, 2012); 11, *B. tuberum* LMG 21444^T (this study). +, Positive; +^{w}, weak; −, negative; V, variable; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Isolation source*	RN	RH	RN	RN	RN	RN	RN	RN	RH	RN	RN
Nitrate reduction	+	+	+	V	+	+	−	+	−	+	−
Activity of:											
Arginine dihydrolase	+^{w}	−	−	−	ND	−	+^{w}	−	+	−	−
Tryptophan deaminase	+	−	ND	ND	ND	−	−	ND	+^{w}	ND	−
Urease	−	−	+	V	+	−	−	+	−	+	−
β-Galactosidase	+^{w}	+	+	−	+	+	+	+	+	+	+
Assimilation of:											
Adipic acid	+	−	−	−	+	−	−	−	−	−	−
Capric acid	−	+	−	−	+	+	+^{w}	+	−	−	+
D-Glucose	+	+	+	+	+	+	+	+	+	+	+
D-Mannitol	+	+	+	+	+	+	+	+	+	+	+
D-Mannose	+	+	+	V	+	+	+	+	+	+	+
L-Arabinose	+	+	+	ND	+	+	+	+	+	+	+
N-Acetylglucosamine	+	+	+	+	+	+	+	+	+	+	+
Trisodium citrate	+	−	+	−	+	−	+	+	+^{w}	+	ND
Fermentation/oxidation of:											
D-Glucose	+^{w}	−	−	−	−	−	−	−	−	−	−
D-Mannitol	−	−	+	+	+	+	−	+	−	+	ND
D-Sorbitol	−	−	+	+	+	+	−	+	−	+	ND
Inositol	−	−	ND	+	+	ND	−	+^{w}	−	+	ND
L-Arabinose	+^{w}	−	+	+	+	ND	−	+	−	+	ND
DNA G+C content (mol%)	61.4–61.9	62.0	63–65	64.8	62.8	62.1	61.2	64.5	61.6	64.2–65.7	62.8

*RH, Rhizosphere; RN, root nodule.

Table 2. Fatty acid compositions of members of the genus *Burkholderia*

Strains: 1, *B. dilworthii* sp. nov. (means from three strains); 2, *B. caledonica* LMG 19076^T; 3, *B. diazotrophica* LMG 26031^T; 4, *B. phymatum* LMG 21445^T; 5, *B. mimosarum* LMG 23256^T; 6, *B. nodosa* LMG 23741^T; 7, *B. rhynchosiae* WSM3937^T; 8, *B. sabiae* LMG 24235^T; 9, *B. sprentiae* WSM5005^T; 10, *B. terricola* LMG 20594^T; 11, *B. tuberum* LMG 21444^T. All values are given as percentages of the total composition. ND, Not detected; TR, trace amounts (<1 %). All strains were grown on BCCM/LMG medium 14. All data were obtained in this study.

Fatty acid	1	2	3	4	5	6	7	8	9	10	11
$C_{13:1}$ at 12–13	TR	TR	ND	ND	ND	ND	TR	ND	ND	ND	ND
$C_{14:0}$	4.7	4.7	4.72	4.2	5.6	4.2	3.7	4.6	4.7	4.8	3.9
$C_{16:0}$	18.0	13.6	19.0	20.9	15.2	20.1	14.4	18.3	19.9	14.3	17.6
$C_{16:0}$ 2-OH	2.9	2.4	TR	1.4	3.1	2.2	1.5	1.6	2.1	3.5	2.2
$C_{16:0}$ 3-OH	6.8	6.0	5.5	5.8	8.4	6.1	5.4	6.5	6.7	6.6	6.1
$C_{16:1}$ 2-OH	5.5	2.7	1.1	2.0	5.1	5.3	2.7	TR	3.3	3.1	1.8
$C_{17:0}$ cyclo	12.3	8.4	3.6	8.1	1.9	1.4	3.5	5.7	10.6	14.0	12.6
$C_{18:0}$	1.1	ND	TR	1.3	ND	ND	TR	1.1	TR	TR	ND
$C_{18:1}$ 2-OH	1.3	1.1	ND	TR	1.8	1.4	TR	TR	1.3	1.5	TR
$C_{18:1}\omega7c$	20.6	34.2	37.9	26.7	26.7	29.9	38.2	33.5	30.5	27.8	28.9
$C_{19:0}$ cyclo $\omega8c$	4.9	3.7	2.2	3.0	ND	ND	1.7	3.8	4.1	7.1	7.1
Summed feature 2*	9.2	7.4	7.0	8.3	11.9	7.9	6.4	8.2	8.5	8.2	8.2
Summed feature 3*	13.1	14.5	17.9	17.4	20.3	21.6	20.9	14.9	13.1	9.1	10.2

*Summed features are groups of two or three fatty acids that cannot be separated by GLC with the MIDI System. Summed feature 2 contains $C_{14:0}$ 3-OH and/or iso-$C_{16:1}$ I; summed feature 3 contains $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH.

The genotypic and phenotypic data presented in this study demonstrate that the three strains isolated from South African *L. ambigua* root nodules form a novel species in the genus *Burkholderia*. Therefore, we propose to assign the strains to *Burkholderia dilworthii* sp. nov.

Description of *Burkholderia dilworthii* sp. nov.

Burkholderia dilworthii (dil.wor′thi.i. N.L. masc. gen. n. *dilworthii* of Dilworth, named in honour of Mike Dilworth, who first described the acetylene reduction assay for assessment of nitrogen fixation in nodules).

Cells are rod-shaped and motile (approx. 0.9×2.0 μm). Gram-negative, catalase- and oxidase-positive. Colonies are white, smooth, round, 0.4–2.0 mm in diameter and convex with entire margins on 1/2 LA medium. Growth occurs on 1/2 LA medium at 15–37 °C but not at 4, 10 or 40 °C. Growth is visible in NB with 0–10 % NaCl and at pH 5.5–8 at 28 °C. Positive reactions are recorded for activity of β-galactosidase and tryptophan deaminase, citrate utilization, nitrate reduction and assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetylglucosamine, potassium gluconate, adipic acid, phenylacetic acid and malate. Weakly positive reactions are recorded for arginine dihydrolase and oxidation of D-glucose and L-arabinose. Negative reactions are recorded for activities of lysine and ornithine decarboxylases, urease, gelatinase and β-glucosidase, H_2S production, production of indole and acetoin, oxidation of D-mannitol, inositol, D-sorbitol, L-rhamnose, sucrose, melibiose and amygdalin and assimilation of maltose and capric acid. Resistant to ampicillin, chloramphenicol, gentamicin, penicillin and streptomycin, sensitive to tetracycline and partially resistant to kanamycin. The whole-cell fatty acid profile is given in Table 2.

The type strain, WSM3556^T (=LMG 27173^T=HAMBI 3353^T), was isolated from root nodules of *Lebeckia ambigua* from the Western Cape of South Africa. The DNA G+C content of the type strain is 61.4 mol%.

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