

Burkholderia humptydooensis sp. nov., a *Burkholderia thailandensis*-like species and the fifth member of the *pseudomallei* complex.

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Running title: *Burkholderia humptydooensis* sp. nov.

Abbreviations: BCC, MLST, FAME, MALDI-TOF, GGDC, ASA

Footnote: GenBank accession numbers for the 16S rRNA gene sequences of *B. humptydooensis* sp. nov. strains MSMB121, MSMB122, are KF378608 – KF378609, respectively. The complete whole genome sequence of the strain MSMB121 was published under GenBank accession nos. CP004095 and CP004096.

MSMB43: GCA_001513745

MSMB122: SRA (SRR1956040); LOYF000000000

Summary

During routine screening for endemic *Burkholderia pseudomallei* from water wells in northern Australia, Gram-negative bacteria (strains MSMB43^T, MSMB121, and MSMB122) with a similar morphology and biochemical pattern to *B. pseudomallei* and *B. thailandensis* were co-isolated with *B. pseudomallei* on Ashdown's selective agar. To determine the exact taxonomic position of these strains and to distinguish them from *B. pseudomallei* and *B. thailandensis*, they were subjected to a series of phenotypic and molecular analyses. Biochemical and fatty acid methyl esters analysis was unable to distinguish *B. humptydooensis* sp. nov. from closely related species. In MALDI-TOF analysis, all isolates grouped together in a cluster separate from other *Burkholderia* spp. 16S rRNA and *recA* sequence analysis demonstrated phylogenetic placement for *B. humptydooensis* sp. nov. in a novel clade within the *B. pseudomallei* group. MLST analysis of the three isolates in comparison with MLST data from 3,340 *B. pseudomallei* strains and related taxa revealed a new sequence type (ST318). Genome to genome distance calculations and average nucleotide identity of all isolates to both *B. thailandensis* and *B. pseudomallei*, based on whole genome sequences, also confirmed *B. humptydooensis* sp. nov. as a novel *Burkholderia* species within the *pseudomallei* complex. Molecular analyses clearly demonstrate that strains MSMB43^T, MSMB121, and MSMB122 belong to a novel *Burkholderia* species for which the name *Burkholderia humptydooensis* sp. nov. is proposed with the type strain MSMB43^T (=ATCCXXXXX^T; LMGXXXX, CP013380-CP013382).

Burkholderia species are abundant and occupy diverse ecological niches, including soil, plants, animals, and humans. Probably the most diverse and environmentally adaptable plant-associated bacteria also belong to the genus *Burkholderia* (Compant et al., 2008). Many species of *Burkholderia* have been described since the discovery of *B. cepacia* by W. H. Burkholder in

1949 as the cause of onion rot (Burkholder, 1950), and later recognized as a human pathogen. Currently, there are more than 90 identified species in this genus (Vial et al., 2011, Gu et al., 2015). There has been a proposal to divide the species into two genera, one of which would retain the *Burkholderia* name and the other *Paraburkholderia* gen. nov. (Sawana et al., 2014). At least 17 closely-related species belong to the *Burkholderia cepacia* complex (BCC), including nine well-known species comprising the genomovars I-IX, respectively: *B. cepacia*, *B. multivorans*, *B. cenocepacia*, *B. stabilis*, *B. vietnamiensis*, *B. dolosa*, *B. ambifaria*, *B. anthina*, *B. pyrrocinia* (Coenye and Vandamme, 2003); seven recently identified species: *B. latens*, *B. diffusa*, *B. arboris*, *B. seminalis*, *B. metallica*, *B. contaminans*, and *B. lata* (Vanlaere et al., 2009, Vanlaere et al., 2008); and *B. ubonensis* (Yabuuchi et al., 2000). Most of these species reside in soils and are associated with plants.

Notably, there are two *Burkholderia* species that can cause severe human and animal diseases: *B. pseudomallei* and *B. mallei*, the causative agents of melioidosis and glanders, respectively. *B. pseudomallei* is a major cause of community acquired sepsis in Northeast Thailand and northern Australia (Cheng and Currie, 2005). Due to the concerns of their potential use as weapons of mass destruction, federal health agencies in the United States have recently classified these species as Tier 1 (Top Tier) disease agents (Butler, 2012). It has been well established that *B. mallei* is a clone of *B. pseudomallei* that became a host-adapted pathogen in equines resulting in massive genome reduction (Godoy et al., 2003). Genetically, both species are members of the *pseudomallei* phylogenetic group or complex (<http://www.ncbi.nlm.nih.gov/Taxonomy/>). Three additional closely related species have been identified so far in this group, including *B. thailandensis* (Brett et al., 1997), *B. oklahomensis* (Glass et al., 2006b), and a newly identified *B. thailandensis*-like species (Currie, 2015, Gee et

al., 2008). These closely-related species are soil saprophytes and are considered non-pathogenic, although a few strains of *B. thailandensis* and *B. oklahomensis* have been described as causing clinical infection in humans (Glass et al., 2006a, Glass et al., 2006b).

Strain MSMB43^T (proposed type strain) was isolated from a water sample from an automated water bore (well) collected in 1995 and examined for *B. pseudomallei* in the Northern Territory (NT) of Australia (Gee et al., 2008). It was initially thought to be *B. thailandensis* because this strain was able to assimilate arabinose as a sole carbon source, which is a trait used to discriminate *B. thailandensis* from *B. pseudomallei* (arabinose negative) (Gee et al., 2008). The bore from which MSMB43^T was discovered is located in Humpty Doo, a region of rural properties outside the capital of the NT, Darwin. The Top End of the NT has a high incidence rate of melioidosis (Parameswaran et al., 2012). Indeed the water sample from which MSMB43^T was recovered also yielded *B. pseudomallei*. An additional two strains (MSMB121 and MSMB122) were both isolated in 2007, from a single separate bore water sample within the NT collected approximately 950 km south of the territory capital, Darwin, resulting in a 910 km separation between the two sample sites of MSMB121/MSMB122 and MSMB43^T. To date, *B. humptydooensis* sp. nov. has not been identified outside the NT and it has not been isolated from any clinical specimens from patients within the NT.

The specific epithet “*humptydooensis*” given to this new species was adopted from the location name “Humpty Doo,” the location where this new species was first discovered. The name “*humptydooensis*” has been used previously for a new species of beetle (*Australoxenella humptydooensis*) from this same geographic region (Howden and Storey, 1992).

As described previously (Gee et al., 2008), MSMB43^T did not grow while incubated at a temperature greater than 42°C and also produced little or no gas from nitrate. All three strains were grown at 8, 25, 37, 42, and 45°C for 24, 48, 72, and 144 h on Columbia blood agar, MacConkey agar, Ashdown's selective agar, and Standard I with and without supplementary CO₂. On Columbia blood agar smooth and creamy-white colonies were observed after 24 h, whereas red, convex and small (1-2mm) colonies were observed on MacConkey after 48h. Dry and wrinkled colonies were observed on Ashdown's agar after 72 h of growth (Fig. 1), similar to the appearance of *B. pseudomallei*, while slimy-confluent honey-like growth appeared on Standard I after 48h (Fig. 1). Bacterial growth was visible on all media after incubation at 25-42°C for at least 24 h with best growth observed on Columbia blood agar. No growth was observed at 8°C and 45°C. The optimal temperatures for growth were between 28-37°C aerobically.

Cell morphology was examined using a Zeiss light microscope at 1000x magnification with cells grown for 2 days at 37 °C. All strains showed a Gram-negative bipolar staining appearing as rods of 2-3 µm in length and 0.4-0.8 µm in diameter. All strains were motile in semi-solid media.

Biochemical data were obtained for all three strains of *B. humptydooensis* sp. nov. (MSMB43^T, MSMB121, and MSMB122) and compared to the type strains of *B. pseudomallei* (K96243) (Wuthiekanun et al., 1996) and *B. thailandensis* (E264) using the API NE and API Zym (bioMérieux) systems according to the manufacturer's instructions. Differentiation of *B. humptydooensis* sp. nov from *B. thailandensis* was not possible based on the biochemical reactions (Table 1).

Matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was performed on all three *B. humptydooensis* sp. nov strains and they clustered with other members of the *B. pseudomallei* complex (see SI doc and Fig. S1). Fatty acid methyl esters analysis was unable to distinguish among the fatty acid profiles from the three *B. humptydooensis* sp. nov. strains and its closely related species (five *B. thailandensis*, two *B. oklahomensis*, and three *B. ubonensis* strains) (see SI doc and Fig. S2).

Minimum inhibitory concentrations (MICs) were determined by broth microdilution method using commercially available CE-certified MICRONAUT-S 96-well microtitre plates (Merlin, Bornheim-Hersel, Germany) containing a 2-fold serial dilution of the following antibiotics: amoxicillin/clavulanic acid (0.5–64/0.25–32 mg/L), ceftazidime (0.5–64 mg/L), imipenem (0.25–32 mg/L), rifampicin (0.0625–8 mg/L), chloramphenicol (0.5–64 mg/L), trimethoprim/sulfamethoxazole (0.25–32/4.75–608 mg/L), streptomycin (0.25–32 mg/L), gentamicin (0.25–32 mg/L), doxycycline (0.25–32 mg/L), tigecycline (0.03125–4 mg/L), ciprofloxacin (0.03125–4 mg/L), and levofloxacin (0.0625–4 mg/L). One well without antibiotic was used as a growth control. All plates containing the lyophilized antimicrobial substances were stored at room temperature until use.

Testing conditions were in accordance with the current Clinical & Laboratory Standards Institute (CLSI) recommendations for *B. pseudomallei* (CLSI M45-A2 Volume 30 No. 18). Single colonies of MSMB43^T, MSMB121, and MSMB122 were picked from agar plates and inoculated in physiological saline until the turbidity matched that of a 0.5 McFarland standard. The suspension was diluted 221-fold in cation-adjusted Mueller Hinton II broth (Becton Dickinson, catalog #297701). After incubation for 24 h at 37°C in a 5% CO₂ atmosphere bacterial growth was verified photometrically at a wavelength of 620 nm using a commercial

photometer (MERLIN, Bornheim-Hersel, Germany) and each strain was tested in triplicate. Additionally, a gradient strip method (Etest, bioMérieux) was applied to investigate a broader range of antibiotic concentrations.

Adopting the CLSI breakpoints of *B. pseudomallei*, all strains were determined to be *in vitro* susceptible to ceftazidime, imipenem, trimethoprim/sulfamethoxazole, and doxycycline, whereas a resistance to amoxicillin/clavulanic acid was observed. (Table 2). The antimicrobial susceptibility pattern of *B. humptydooensis* sp. nov. generally resembles that of *B. pseudomallei* (Ahmad et al., 2013, Thibault et al., 2004, Trunck et al., 2009, Crowe et al., 2014), except for the elevated MICs of amoxicillin/clavulanic acid (Table 2). No significant differences were observed either among the three strains or between the two different susceptibility testing methods. As the maximum concentration of aminoglycosides in the microtiter plates was 32 mg/L, high-level streptomycin resistance but low-level gentamicin resistance was confirmed using the Etest method (data not shown).

The pathogenic potential of *B. humptydooensis* sp. nov., MSMB43^T, was investigated in a BALB/c mouse model and compared to *B. thailandensis* (type strain E264). Live culture was grown to logarithmic phase ($OD_{600} \sim 1.0$) in Luria-Bertani (LB) broth as described in (Morici et al., 2010). Sterile 1xPBS was used to wash cells twice before making dilutions for injecting mice. Viability counts of the final inocula were made on LB agar plates. Six to eight week old female BALB/c mice in treatment groups of 5 mice per cage were used. Food and water were provided *ad libitum*. All mice in a single cage received the same infectious dose (*B. humptydooensis* sp. nov.: 1.05×10^4 , 10^5 or 10^6 CFU; *B. thailandensis*: 3.4×10^4 , 10^5 or 10^6 CFU) via a single subcutaneous (s.c.) injection in the scruff of the neck. Mice were monitored daily for health status. All mice were euthanized on day 21 post injection. This work was

conducted under approved protocols from the NAU IACUC (Protocol 14-011) and DOD ACURO (HDTRA1-12-C-0066_Wagner). Neither species caused mortality in any mice when delivered via the s.c. route, nor did any mice show outward signs of illness. In comparison, s.c. infections of fully virulent *B. pseudomallei* results in 50% mortality within 10 days at a dose of 10^3 CFU (Barnes and Ketheesan, 2005). It remains unknown if the inhalation route increases the pathogenicity of species tested in the same way as *B. thailandensis* E264, which can cause high mortality in mice at doses of 10^4 - 10^6 CFU when delivered as an aerosol (Morici et al., 2010, West et al., 2008, Wiersinga et al., 2008).

16S rRNA and *recA* gene sequencing analyses were performed on three *B. humptydooensis* sp. nov. strains: MSMB43^T, MSMB121, and MSMB122 as previously described (Brett et al., 1997, Payne et al., 2005). From whole genome analysis of strain MSMB43^T using SSU-ALIGN (Nawrocki et al., 2009) we determined that four rRNA operons are present on its chromosomes of which two unique versions were found (AQ610_12930, AQ610_01425; AQ610_21350, AQ610_02540). These two copies of the 16S rRNA genes are different, which leads to ambiguities in conventional sequencing (see Fig. S3). The 16S rRNA gene sequence similarities of *B. humptydooensis* sp. nov. to other members of the *pseudomallei* complex (*B. thailandensis*, *B. mallei*, *B. oklahomensis*) were 99%. Phylogenetic reconstruction of 16S rRNA and *recA* sequences using MEGA version 6 (Tamura et al., 2013) confirmed genetic proximity to the *pseudomallei* complex but also determined that all *B. humptydooensis* sp. nov. strains formed their own group within this complex (see Fig. S3-S4).

MLST was performed on all three *B. humptydooensis* sp. nov. strains as previously described (Godoy et al., 2003). As of October 6, 2015, a total of 1,429 sequence types (STs) had been identified in *B. pseudomallei* and closely related species by MLST (<http://www.MLST.net>).

All three *B. humptydooensis* sp. nov. strains, MSMB43^T, MSMB121, and MSMB122 are ST318 and there are no other representatives of this ST. Overall, phylogenetic analysis using MLST data supports the separation of *B. humptydooensis* sp. nov. from the other *pseudomallei* complex members, as described previously (Gee et al., 2008).

Two genomes were sequenced on the PacBio platform, resulting in one finished assembly (MSMB43^T) and one mostly finished assembly with 4 contigs (MSMB122) (Table 3). Two other genomes that group with *B. humptydooensis* sp. nov. are present in GenBank, see Figure 2 (*B. thailandensis* MSMB121 and *Burkholderia* spp. MSMB43 (2002721687) with GenBank assembly accession numbers of GCA_000385525 and GCA_000959325 respectively). The MSMB43^T genome had one circular contig ~305Kb long that appears to be a plasmid; this same sequence is also present in the completed genome of *Burkholderia* spp. MSMB43 (2002721687). A comparative genomics approach using LS-BSR (Sahl et al., 2014) demonstrated that a large stretch of the *B. pseudomallei* K96243 genome (BPSS1165 – BPSS1184) on chromosome 2 is highly conserved (>98% identity) in the plasmid sequence, suggesting a shared origin for these regions.

For the core genome phylogeny, genomes were aligned against *B. pseudomallei* K96243 with NUCmer (Delcher et al., 2003). The reference genome was also aligned against itself to identify duplicated regions, which were masked from subsequent analyses; the NASP pipeline was used to wrap these methods (<http://tgennorth.github.io/NASP/>). A phylogeny was inferred with RAxML v8 (Stamatakis, 2014) on a large set (n=331,000) of concatenated SNPs using a time reversible model incorporating the Lewis ascertainment bias correction. The phylogeny demonstrates the position of *B. humptydooensis* sp. nov. in relation to other clades in the *B. pseudomallei* complex and confirms the results from other methods (Fig. 2).

DNA-DNA hybridization (DDH) is the current gold-standard for bacterial species delineation. DDH is necessary for the description of a new species within a taxon when strains share more than 97% of 16S rRNA gene sequence similarity (Goebel and Stackebrandt, 1994, Tindall et al., 2010). If DNA-DNA relatedness between two strains is less than 70%, both strains are considered as different species. However, DDH is laborious and difficult to standardize and inter-laboratory reproducibility is relatively low. DDH was previously performed on MSMB43^T and has a relative binding ratio of 91% with *B. thailandensis* (ATCC 700388T) with a divergence of 4% (Gee et al., 2008). Because of the drawbacks of conventional DDH and the rapid progress in genome sequencing techniques, various *in silico* algorithms for calculating genome-to-genome similarities or distances have been developed. In addition to the commonly used average nucleotide identity (ANI) (Konstantinidis and Tiedje, 2005), recently a highly reliable estimator for the relatedness of genomes was developed by Jan P. Meier-Kolthoff and colleagues (Meier-Kolthoff et al., 2013). Genome to genome distance calculation (GGDC) produces digital DDH values that correlate well with values obtained by conventional DDH, which is of utmost importance for compatibility with the current species concept, and also provides confidence-interval estimation. Values reported in Table 4 were generated using formula 2, which summed the identities found in high-scoring segment pairs (HSP) and then were divided by the overall HSP length. The GGDC service is available from the German Collection of Microorganisms and Cell Cultures homepage (<http://ggdc.dsmz.de/distcalc2.php>). PacBio assemblies were used to determine the distances among *B. humptydooensis* sp. nov. and other closely related species, *B. humptydooensis* sp. nov. strains MSMB43^T, MSMB121, and MSMB122. The genomes were subjected to GGDC analysis and compared to the available

genome sequences of *B. pseudomallei*, *B. oklahomensis* and *B. thailandensis* reference strains (K96243, C6786^T, and E264, respectively). For comparison, the average nucleotide identity (ANI) values were also calculated for all reference sequences with JSpecies (Richter and Rossello-Mora, 2009); the authors of JSpecies determined that ANI values <95% indicate separate species.

Among the three tested *B. humptydooensis* sp. nov. genomes, the calculated GGDC and ANI values were in the range of 93-99% and 98-99%, respectively (Table 4), indicating that all of these tested strains belong to a single species, including the proposed *B. humptydooensis* sp. nov. type strain MSMB43^T. As expected from WGS analyses, strain MSMB43^T had a slightly lower GGDC similarity (93%) to the other two *B. humptydooensis* strains, which were approximately 97% similar. Further GGDC analysis (Table 4) has determined that the similarities of all *B. humptydooensis* strains compared to all other tested *Burkholderia* species in the *B. pseudomallei* complex was less than 70%, with the highest similarity detected to *B. thailandensis* (51.1% ($\pm$ 3.2%)). This confirms that the three tested strains are not *B. thailandensis*, but rather a distinct species. The GGDC similarity between *B. mallei* and *B. pseudomallei* was 92.5% which confirms previous conventional DDH results and demonstrates that, from a strict taxonomic point of view, they belong to a single species.

Description of *Burkholderia humptydooensis* sp. nov.

Burkholderia humptydooensis sp. nov. (hump.ty.doo.en'sis. L. gen. adj. *humptydooensis*, pertaining to Humpty Doo, a small town in Northern Territory of Australia, where the first member of this species was isolated).

Bacilli, 0.4-0.8 μ m in diameter and 2-3 μ m in length, arranged individually or in irregular clusters. The organism is Gram-negative with bipolar staining, motile, and non-spore forming.

Growth is observed in a temperature range of 25-42°C within 24-48 h on various standard solid media. Within 24 h, small colonies (0.5-1 mm) are formed on non-selective media (Columbia blood and Standard-I) and after 48 h also on selective media (Ashdown's, MaConkey). Best growth occurs at 28-37°C after ≥ 24 h. Colonies become confluent and honey-like in appearance on glycerol- containing medium (ST-I) after 48 h. On Ashdown's selective agar, highly wrinkled purple colonies are observed within ≥ 48 h, resembling the growth of *B. pseudomallei*.

Positive assimilation (API 20NE) was found for D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetyl-glucosamine, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate, and phenylacetic acid while negative for D-maltose. Aesculin and gelatin are hydrolyzed. Variable reactions are L-arginine and 4-Nitrophenyl- β D-galactopyranoside (PNPG).

Positive (API ZYM) for alkaline phosphatase, esterase, esterase lipase, lipase, leucine arylamidase, acidic phosphatase, and naphthol-AS-BI-phosphohydrolase. Enzymes absent on API ZYM are valine arylamidase, cystin arylamidase, trypsin, α -chymotrypsin, α - and β -galactosidase, β -glucuronidase, α - and β -glucosidase, N-acetyl- β -glucosaminidase, α -mannosidase, and α -fructosidase. This species is aerobic, catalase and oxidase-positive, urease and indole negative. Nitrate and nitrite are reduced (with no gas formation from nitrite) and no production of H₂S.

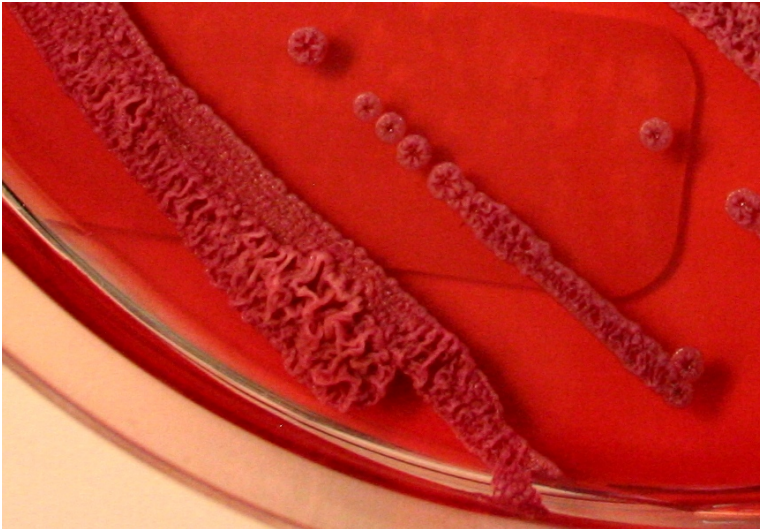
B. humptydooensis sp. nov. strains are resistant to aminoglycosides and amoxicillin/clavulanic acid, but susceptible to trimethoprim/sulfamethoxazole, doxycycline, imipenem, and ceftazidime. All *B. humptydooensis* sp. nov. strains are seroreactive with sera from melioidosis patients who were infected with *B. pseudomallei* serotype B strains. All strains produced O-antigen ladder type B2, except that strain MSMB43^T produced a novel O-antigen ladder type (Stone et al., 2012). The type strain, MSMB43^T, has been previously referred to as *B.*

thailandensis-like species in multiple studies (Currie, 2015, Gee et al., 2008). MSMB43^T was isolated in 1995 from an automated water well (bore) in Humpty Doo, Australia. Of interest, *B. pseudomallei* was also cultured from that same water sample. *B. humptydooensis* sp. nov., like *B. thailandensis*, is non-virulent in mice. In addition, MSMB43^T is known to produce Thailanstatins, which possess antiproliferative activities in representative human cancer cell lines (Liu et al., 2013). The type strain MSMB43^T has been deposited in the American Type Culture Collection as ATCC XXXXX and the Belgian Co-ordinated Collections of Micro-organisms as LMGXXXX.

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Figure 1. Colony morphology of *B. humptydooensis* sp. nov. MSMB43^T.

(a) Culture grown on Ashdown's agar and on (b) Standard I.



(b)



Table 1. Phenotypic characteristics of *B. humptydooensis* sp. nov. and closely related species within the *B. pseudomallei* group.

Strains: Bp, *Burkholderia pseudomallei* (K96243)*; Bt, *B. thailandensis* (E264); Bh-43, *B. humptydooensis* sp. nov. (MSMB43^T); Bh-121, *B. humptydooensis* sp. nov. (MSMB121); Bh-122, *B. humptydooensis* sp. nov. (MSMB122). +, Positive reaction; -, negative reaction. All strains were positive for nitrate, gelatin, glucose, mannose, mannitol, N-acetylglucosamine, gluconate, caprate, adipate, malate, citrate, and phenylacetate. All strains were negative for glucose (acidification) and urea.

Characteristic	Bp [*]	Bt	Bh-43	Bh-122	Bh-121
Tryptophan	+	-	-	-	-
Arginine	+	-	+	-	-
Aesculin	-	+	+	+	+
PNPG	-	-	-	-	+
Assimilation of:					
Arabinose	-	+	+	+	+
Maltose	-	+	-	-	-

* Data obtained from Wuthiekanun et al., (1996).

Table 2. Summary of MIC's determined in triplicate by broth microdilution method.

Concentrations are given in mg/L.

Antimicrobial substance	<i>B. pseudomallei</i> MIC ₉₀	MSMB43 ^T	MSMB121	MSMB122
Amoxicillin/clavulanic acid	8 ^a	32/16 [*]	32/16 [*]	32/16 [*]
Ceftazidime	4 ^a	4	2	4
Imipenem	1 ^a	0.5	0.5	0.5
Rifampicin	32 ^a	>8	>8	8
Chloramphenicol	16 ^a	8	4	4
Trimethoprim/sulfamethoxazole	1.5 ^c	0.5/9.5	≤0.25/4.75	≤0.25/4.75
Streptomycin	n/a	>32	>32	>32
Gentamicin	64 ^a	32	32	>32
Doxycycline	1 ^a	1	1	1
Tigecycline	4 ^b	2	4	4
Ciprofloxacin	4 ^a	0.5	1	1
Levofloxacin	4 ^a	0.5	1	1

^{*}Resistance was observed based upon the CLSI breakpoints of *B. pseudomallei*.

Data obtained from ^a Thibault *et al.*, (2004) ^b Ahmad *et al.*, (2013) ^c Crowe *et al.*, (2014).

361 **Table 3.** Whole genome data for *B. pseudomallei* group.

Strain/species	GC content	Genome size	# CDS
MSMB43 ^T <i>B. humptydooensis</i>	67.1%	7.3* Mb	6324
MSMB121 <i>B. humptydooensis</i>	67.5%	6.7 Mb	5795
MSMB122 <i>B. humptydooensis</i>	67.5%	6.8 Mb	5845
E264 <i>B. thailandensis</i>	67.6%	6.7 Mb	5652
C6786 <i>B. oklahomensis</i>	66.9%	7.1 Mb	6097
K96243 <i>B. pseudomallei</i>	68.1%	7.2 Mb	5948
ATCC23344 <i>B. mallei</i>	68.5%	5.8 Mb	5006

Two chromosomes present for all genomes.

* One plasmid present.

CDS = coding DNA sequences

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Table 4. Genome to genome distance calculation (GGDC) in the bottom half matrix (with confidence intervals) and average nucleotide identity (ANI) in the top half matrix that demonstrate whole genome sequence similarities of *B. humptydooensis* sp. nov. strains and other species in the *pseudomallei* complex. Values highlighted yellow represent values above the similarity threshold that defines members of the same species.

	MSMB43 <i>B. humptydooensis</i>	MSMB121 <i>B. humptydooensis</i>	MSMB122 <i>B. humptydooensis</i>	E264 <i>B. thailandensis</i>	C6786 <i>B. oklahomensis</i>	K96243 <i>B. pseudomallei</i>	ATCC23344 <i>B. mallei</i>
MSMB43 <i>B. humptydooensis</i>	100	98.81	98.98	93.56	91.80	91.17	93.22
MSMB121 <i>B. humptydooensis</i>	93.1 ± 2.3	100	99.84	93.59	91.80	93.39	93.26
MSMB122 <i>B. humptydooensis</i>	93.1 ± 2.3	97.3 ± 0.02	100	93.60	91.86	93.23	93.25
E264 <i>B. thailandensis</i>	51.1 ± 3.2	51.4 ± 3.2	51.3 ± 3.2	100	91.48	93.01	93.05
C6786 <i>B. oklahomensis</i>	43 ± 3.1	43.1 ± 3.1	43.1 ± 3.1	40.7 ± 3.1	100	91.17	91.33
K96243 <i>B. pseudomallei</i>	48.6 ± 3.1	48.6 ± 3.2	48.6 ± 3.2	45.6 ± 3.3	39.6 ± 3.1	100	99.04
ATCC23344 <i>B. mallei</i>	48.7 ± 3.1	49.1 ± 3.1	49.1 ± 3.1	45.7 ± 3.2	40.3 ± 3.1	92.5 ± 2.8	100

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MALDI-TOF:

Preparations of bacterial isolates (MSMB43, MSMB121, MSMB122) for matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) were done according to the ethanol/formic acid extraction protocol recommended by the manufacturer (Bruker Daltonics) and as previously described (Mellmann et al., 2008) Briefly, a loopful of bacterial material was evenly dissolved in 300 µl analytical grade water, and 900 µl pure ethanol was added. The cell suspension was centrifuged at $13,000 \times g$ for 2 minutes, and the supernatant was discarded. The centrifugation was repeated, and the residual ethanol was discarded. The pellet was air dried and thoroughly resuspended in 5-50 µl 70% formic acid depending on the size, and, finally, an equal volume of acetonitrile was added. After centrifugation at $13,000 \times g$ for 2 minutes, 1 µl of the supernatant was transferred to the MALDI target plate. After air-drying at room temperature 1 µl of matrix solution (saturated solution of α -cyano-hydroxy-cinnamic acid in 50% aqueous acetonitrile containing 2.5% trifluoroacetic acid) per spot were was applied. MALDI-TOF MS was conducted using a Microflex LT mass spectrometer (Bruker Daltonics) equipped with a nitrogen laser. All spectra were recorded in linear, positive ion mode across a mass/charge ratio (m/z) of 2,000 to 20,000. The acceleration voltage was 20 kV. Spectra were collected as a sum of 240 shots across a spot. Main spectra were calculated from 8 spectra per strain and used for construction of a score oriented dendrogram by using the BioTyper software (version 2.3, Bruker Daltonics). In these analyses *B. humptydooensis* sp. nov. strains grouped together with *B. ubonensis* from the Bcc and other members of the *B. pseudomallei* complex (Fig. S1).

Fatty acid methyl ester (FAME) profile analysis:

Fatty acid methyl esters analysis was unable to distinguish among the fatty acid profiles from the three *B. humptydooensis* sp. nov. strains and its closely related species (five *B. thailandensis*, two *B. oklahomensis*, and three *B. ubonensis* strains). The fatty acid methyl esters were performed using the MIDI Sherlock[®] Microbial Identification System by Microbial ID, Inc. (Delaware, USA). Similarity index analysis was used to display the relatedness of FAME profiles produced by these species. The analysis revealed that FAME profiles of the four *B. humptydooensis* sp. nov. strains grouped into two sub-groups, along with strains from other species (Fig. S2). This finding suggests that *B. humptydooensis* sp. nov. had similar FAME profiles with other tested genetically related *Burkholderia* species and this technique cannot be used to distinguish *B. humptydooensis* sp. nov. or distinguish the other closely related species.

In conclusion, we have utilized comprehensive genotyping techniques including 16S rRNA, *recA*, MLST, and whole genome based GGDC to further support the existence of a new species that is distinct but genetically related to the four members of the pseudomallei complex (*B. pseudomallei*, *B. mallei*, *B. thailandensis*, and *B. oklahomensis*). These analyses confirm the speciation of *B. humptydooensis* sp. nov., a soil bacterial saprophyte found in the Northern Territory of Australia where melioidosis is highly endemic. The addition of *B. humptydooensis* sp. nov. as a new member of the pseudomallei complex will benefit evolutionary studies of *B. pseudomallei*, the serious bacterial pathogen that shares a similar ecological niche with this new species.

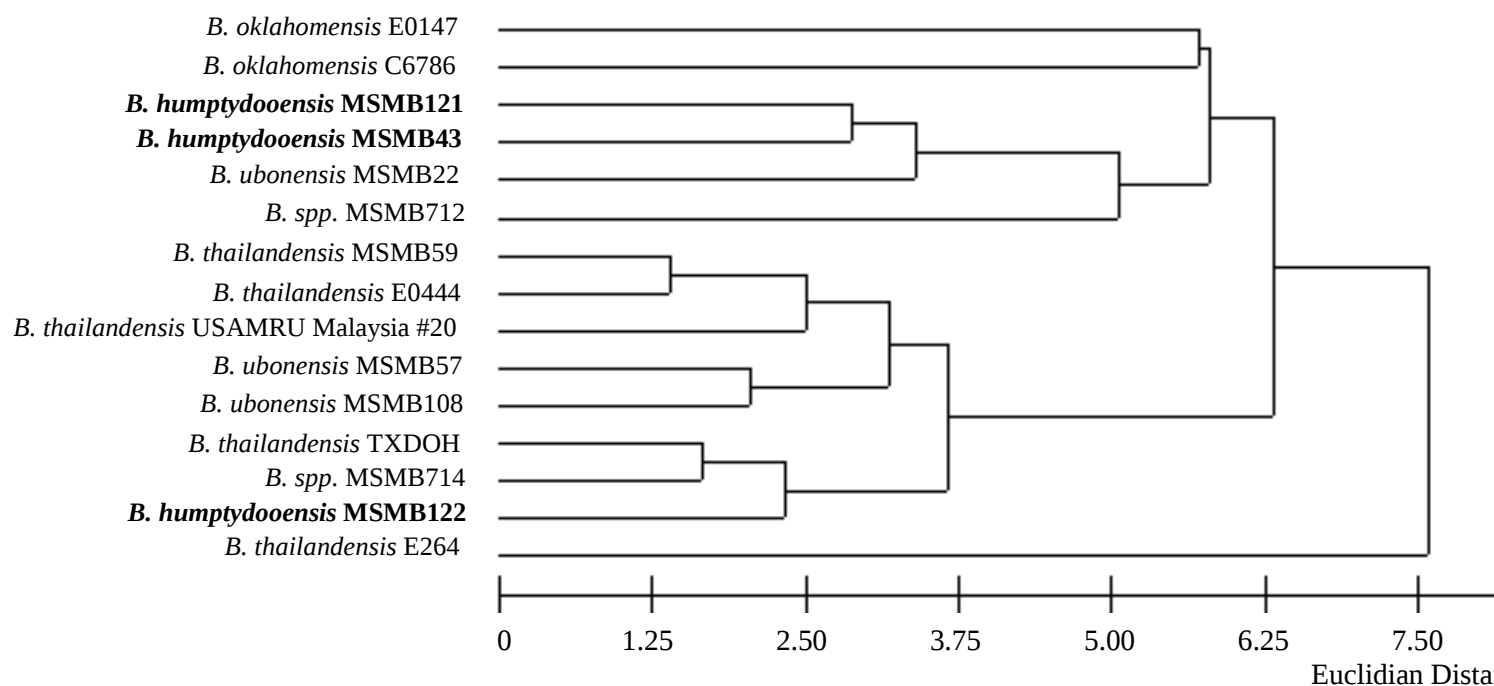


Fig. S2. A dendrogram demonstrating the relatedness of fatty acid compositions in *B. humptydooensis* sp. nov. and other closely related species. Similarity Index in the Microbial Identification System (MIS) was used to display how closely the fatty acid compositions found in *B. humptydooensis* sp. nov., a new species, compared with those from other genetically related species. FAME (Fatty Acid Methyl Esters) profiles were generated using GC-MS. The dendrogram was generated based upon the clustering analysis technique to produce unweighted pair matching based on FAME profiles. Our analysis has shown that FAME profiles from three tested *B. humptydooensis* sp. nov. strains were grouped into two sub-groups. The first group also contained one *B. ubonensis* and two *B. oklahomensis* strains, while the second group contained most of *B. thailandensis* strains and two *B. ubonensis* strains. We noted that *B. thailandensis* E264 had a distinct FAME profile to both subgroups.

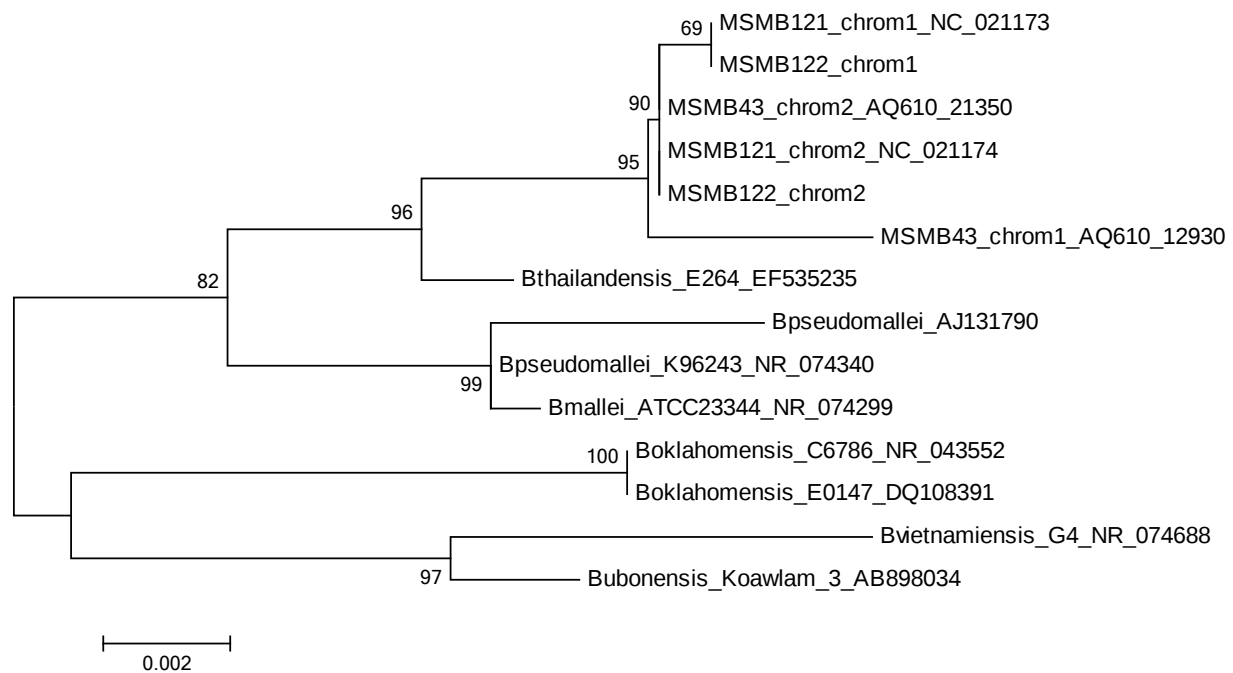


Fig. S3. 16S maximum likelihood (500 bootstrap) phylogeny using both copies of 16S from each *B. humptydooensis* sp. nov. strain (1420 bp and 14 sequences).

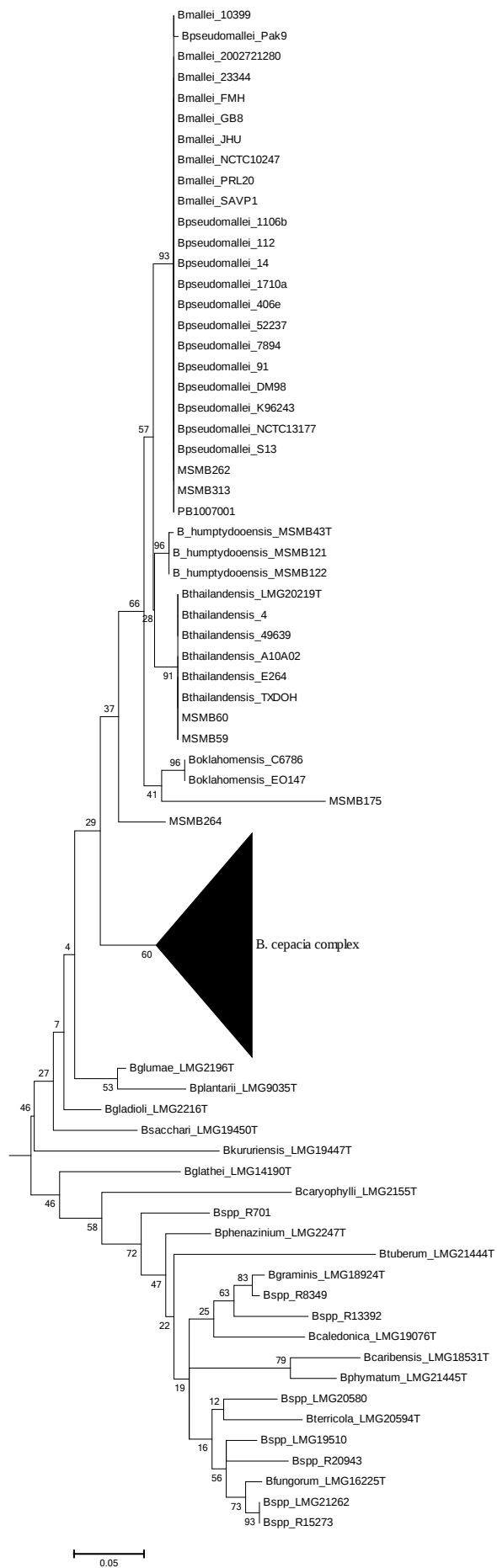


Fig. S4. Maximum likelihood phylogeny (1,500 bootstrap) of recA sequence (335 bp) using 193 sequences.