MALDI-TOF:

Preparations of bacterial isolates (MSMB43\textsuperscript{T}, 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 (1). 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 × 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 × 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-cinnaminic acid in 50% aqueous acetonitrile containing 2.5% trifluoroacetic acid) per spot were 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 \textit{B. humptydooensis} sp. nov. strains grouped together with \textit{B. ubonensis} from the Bcc and other members of the \textit{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.

References:

Fig. S1. Dendrogram demonstrating strain relatedness revealed by MALDI – TOF analysis.
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.