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# Molecular Characterization of *Burkholderia* Strains Isolated from Rice Cultivars (*Oryza sativa* L.) for Species Identification and Phylogenetic Grouping

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**The genus *Burkholderia* consists of extremely versatile bacteria that occupy diverse niches and are commonly encountered in the rhizosphere of crop plants. In this study, we characterized three plant growth promoting strains assigned as *Burkholderia* sp. using biochemical and molecular characterization. The *Burkholderia* spp. strains CBMB40, CBPB-HIM, and CBPB-HOD were characterized using biochemical tests, BIOLOG carbon substrate utilization, fatty acid methyl ester analysis, analysis of *recA* gene sequences, and DNA-DNA hybridization. The results from these studies indicated that the strains CBMB40, CBPB-HIM, and CBPB-HOD can be assigned under *Burkholderia vietnamiensis*, *Burkholderia ubonensis*, and *Burkholderia pyrrocinia*, respectively.**

**Keywords:** *Burkholderia*, DNA-DNA hybridization, 16S rDNA sequence, *recA* gene

The genus *Burkholderia*, a heterogeneous group of genotypically different strains with an exceptional metabolic versatility, presently consists of over 30 species. Commonly encountered as rhizosphere bacteria, they promote plant-growth through one or more mechanisms like nitrogen fixation, or production of plant hormones [1, 3, 24]. For a long time, $N_2$-fixing ability in the genus *Burkholderia* was recognized only in the species *Burkholderia vietnamiensis* [5]. Analysis of maize, sorghum, and coffee plants grown under field conditions revealed the richness of the genus *Burkholderia* in unknown diazotrophs and the $N_2$-fixing ability of *Burkholderia kururiensis* [3]. Strains of *Burkholderia* that are endophytic and are nitrogen fixing, representing some novel species, have been reported from maize and teosinte [4, 20]. They are also known for their role in biocontrol through their antifungal activity and suppression of plant diseases [13, 14, 27]. Furthermore they act as potential degraders of organic pollutants finding applications in bioremediation [2, 12] and a recent investigation has documented the role of *Burkholderia* sp. in the biological removal of sulfur compounds, finding applications in large-scale industrial processes [8].

In this study, we report three strains, CBMB40 (KACC91210P), CBPB-HIM (KACC91311P), and CBPB-HOD (KACC91312P), isolated from roots and rhizosphere soil of rice, as *Burkholderia* sp. Strain CBMB40 is a methylotroph utilizing methanol as a carbon source. It produced indole-3-acetic acid (IAA) and 1-aminocyclopropane-1-carboxylate (ACC) deaminase, possessed antagonism against the phytopathogen *Erwinia caratovora* subsp. *caratovora*, and has phosphate solubilizing ability. Strains CBPB-HOD and CBPB-HIM, originally isolated as phosphate solubilizing bacteria, also produced IAA, ACC deaminase, and siderophores. Furthermore, these strains produce cell-to-cell communicating quorum-sensing signal molecules under *in vitro* and *in planta* conditions [19]. Cell-to-cell communication and multicellular coordination derived through quorum sensing provide bacteria with advantages that are not attainable as autonomous agents, including collective defense against antagonists, access to the resources and niches that would not be available to isolated cells, and improved population survival through differentiation into distinct cell types [6]. Identification and description of the taxonomic status gain importance before a bacterial strain could be commercially exploited as bioinoculants. Hence, in this study, attempts were made to identify the three strains by both phenotypic and molecular characterization.

*Burkholderia* sp. strains including the reference strains were grown on trypticase soy agar (TSA, tryptic soy broth with 15 g/l agar; Difco Laboratories, Detroit, MI, U.S.A.) at 30°C unless stated otherwise specifically. Lipolytic and proteolytic activities were investigated on tributyrin agar with 1% glycerol tributyrate and 10% TSA with 1.5% skim milk, respectively. The plates were incubated for 3 to

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5 days and examined for the presence of clearance zones around the growing colonies. The polygalacturonases and the cellulose activity were checked using standard procedures [11]. Siderophore activity was determined using Chromazurol S (CAS) assay on culture supernatants [22]. Swarming motility assay was performed on nutrient broth with 0.5% glucose and 0.5% agar. The intrinsic antibiotic resistance pattern was determined using the antibiotic disc assay on TSA containing the bacterial strains. The plates were incubated for 3 days and observed for growth inhibition. The strains were further characterized by their metabolic fingerprints, using BIOLOG GN2 plates (BIOLOG Inc., Hayward, CA, U.S.A.) and fatty acid methyl ester (FAME) analysis according to the procedures previously described [18]. FAME analysis was performed by the Microbial identification system TSBA50 method version 5 (Microbial ID Inc., Newark, DE, U.S.A.).

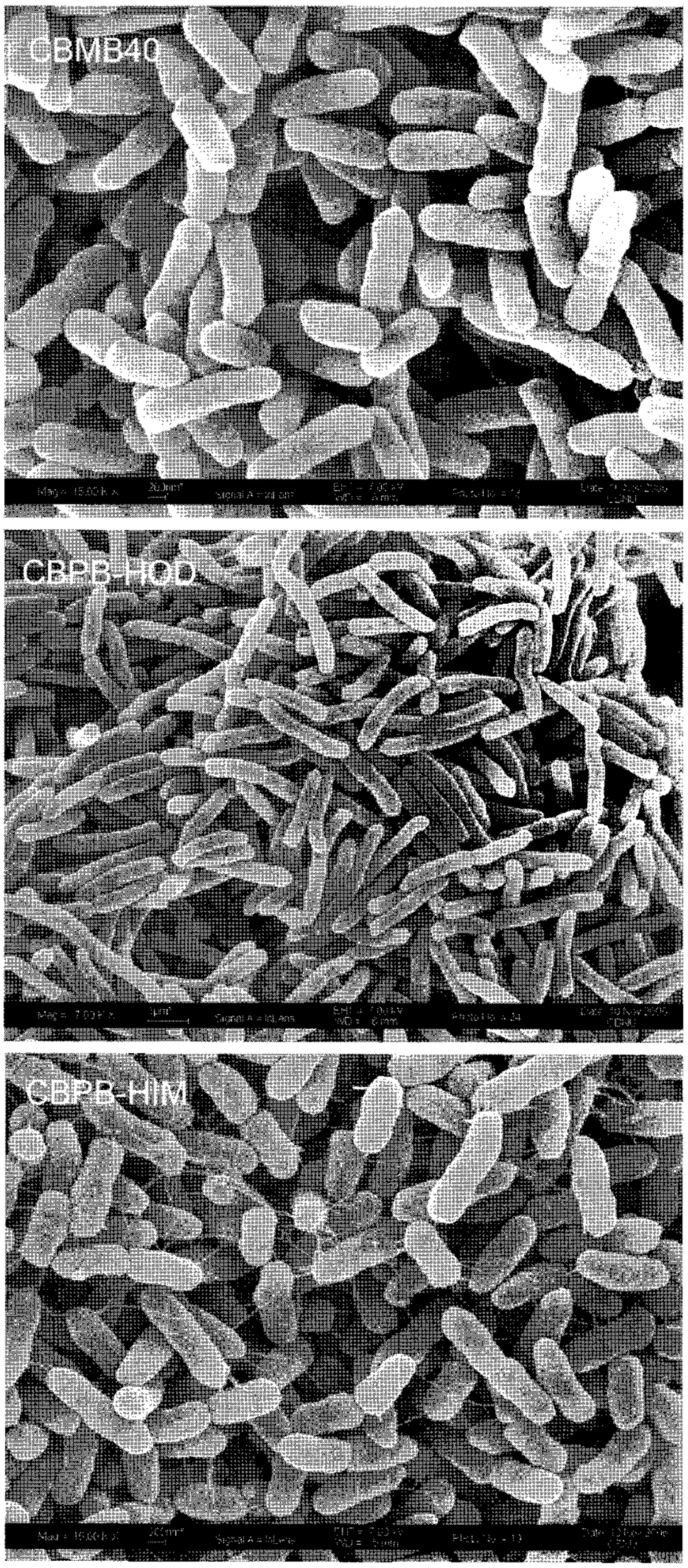


**Fig. 1.** Scanning electron microscope (SEM) photomicrographs of *B. vietnamiensis* strain CBMB40 and *B. ubonensis* strains CBPB-HOD and CBPB-HIM on TSA medium (glutaraldehyde/ osmium tetroxide fixation, gold/palladium coating: Hitachi S-2500C).
Bar, 200 nm, 1 μm, and 200 nm, respectively.

The strains were Gram negative, strictly aerobic, nonspore-forming, motile rods, and the colonies were convex, glossy, and 3.0 to 6.0 mm in diameter after 48 h incubation. They grew in a pH range of 5.5–8.5 (optimum, 6.5 and 7.0), in the absence of NaCl and in the presence of 1.5% (w/v) NaCl but not in the presence of 5% NaCl. All the strains were more sensitive to kanamycin and tetracycline. The cells of CBPB-HIM and CBMB40 were medium in length whereas CBPB-HOD was elongated rods when observed under scanning electron microscope, and in general, the cells were about 0.2–0.4 μm wide and 0.6–2.5 μm long (Fig. 1). They were able to swarm in 0.3% agar and the strains CBPB-HOD and CBPB-HIM produced siderophores. All the strains were positive for catalase and oxidase. Other phenotypic and chemotaxonomic characteristics that differentiated the three strains are listed in Table 1. The fatty acids 14:0, 16:0, 17:0 cyclo, 16:1 2OH, 16:0 2OH, 16:0 3-OH, C18:0, summed feature 2 (comprising 14:0 3OH 16:1 iso I or 12:0 ALDE or any combination of these fatty acids), summed feature 3 (comprising 16:1w7c or $C_{15}$ iso2OH or both) that are reported to be characteristic to *Burkholderia* sp. [26] were present in all three strains. The amount of fatty acids varied between the strains, and strain CBMB40 had more 18:0 and less 16:0 than the other two strains (Table 2).

The 16S rDNA sequences of the strains CBMB40, CBPB-HIM, and CBPB-HOD were retrieved from the GenBank database and compared with the published 16S rDNA sequences of other *Burkholderia* species. The sequences were aligned using ClustalV software [7] and homologies of sequences were determined using the basic alignment search BLAST against the NCBI database. The distance matrices and phylogenetic tree were calculated with Kimura 2 parameter [9] and neighbor-joining [21] algorithms using MEGA version 3.10 software [10]. Comparative 16S rRNA gene sequence analysis of all the strains showed their similarity to Burkholderiaceae, showing higher similarity to *B. vietnamiensis* TVV75$^T$. The strain CBMB40 showed 99.0% similarity to *B. vietnamiensis* TVV75$^T$ followed by *B. glumae* (97.7%) and *B. plantarii* (97.4%). Strain CBPB-HOD showed close similarity to *B. vietnamiensis* TVV75$^T$ (97.7%) and to *B. gladioli* (97.6%)

**Table 1.** Differential phenotypic characteristics of *Burkholderia* strains isolated from rice.

| Characteristics | CBMB40 | CBPB-HIM | CBPB-HOD |
|---|---|---|---|
| Colony morphology | Smooth, round, glossy | Smooth, round, glossy | Smooth, round, glossy |
| Cell shape | Rods | Rods | Slightly curved rods |
| Cell size | 0.3–0.4×0.9–1.2 μm | 0.2–0.3×0.6–1.0 μm | 0.3–0.4×1.5–2.5 μm |
| Gram reaction | –ve | –ve | –ve |
| Protease | – | + | + |
| Lipase | + | + | – |
| Carbon source (BIOLOG) | | | |
| Dextrin | + | – | – |
| Glycogen | + | b | – |
| *N*-Acetyl-D-galactosamine | – | + | – |
| Adonitol | – | – | + |
| L-Fucose | + | – | + |
| α-D-Lactose | b | – | – |
| D-Psicose | + | – | – |
| D-Raffinose | b | + | – |
| Xylitol | – | – | b |
| Acetate | + | + | b |
| D-Galactonic acid lactone | + | – | + |
| α-Hydroxybutyrate | + | – | + |
| γ-Hydroxybutyrate | – | – | + |
| Itaconic acid | + | – | – |
| α-Ketoglutarate | b | + | b |
| α-Ketovaleric acid | + | – | b |
| Succinamic acid | + | – | + |
| Glucuronamide | + | + | b |
| L-Alaninamide | + | – | + |
| Glycyl-L-glutamate | b | b | – |
| L-Ornithine | + | – | + |
| D-Serine | b | + | + |
| D,L-Carnitine | + | b | + |
| Inosine | + | – | – |
| Thymidine | – | – | + |
| Phenylethyl-amine | + | b | + |
| Putrescine | + | – | + |
| 2,3-Butanediol | + | – | – |
| α-D-Glucose-1-phosphate | b | + | + |
| D-Glucose-6-phosphate | + | + | – |

*All the three strains utilized 55 carbon sources when tested using BIOLOG GN2 plate and they were not able to utilized α-cyclodextrin, D-cellobiose, i-erythritol, gentiobiose, lactolose, maltose, D-meilbiose, C2-β-methyl-D-glucoside, L-rharonose, turanose, glycyl-L-aspartate, and uridine and were variable for other carbon sources mentioned here.

+, Positive; –, negative; b, variation/borderline.

and *B. thailandensis* (97.5%). Strain CBPB-HIM showed a higher percentage of similarity to *B. vietnamiensis* accounting to 98.4% followed by *B. thailandensis* (96.5%) and *B. pseudomallei* (96.3%) (Fig. 2).

Sequence polymorphism within the *recA* gene has proven very useful in defining the taxonomy of the *B. cepacia* complex, and in this study, PCRs for the *recA* gene were carried out with BUR1/BUR2 and BUR3/BUR4 primers as previously described [16, 17]. DNA was prepared for PCR amplification from overnight cultures as described previously [15]. Thermal cycling was carried out in a PCR machine, ABI 9700 (Applied Biosystems, U.S.A.), for 30 cycles of 30 s at 94°C, annealing for 30 s at 60°C, and extension at 72°C for 45 s, with a final 5-min extension at 72°C. None of the three strains showed positive amplifications for BUR1/BUR2 primers, although they would produce

**Table 2.** Cellular fatty acid content (%) of *Burkholderia* strains and its close relatives.

| Fatty acid | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| Saturated | | | | | | | | | |
| $C_{12:0}$ | – | 0.10 | 3.06 | – | – | – | – | 0.09 | 0.08 |
| $C_{14:0}$ | 3.97 | 4.28 | 0.91 | 4.7 | 4.5 | 3.8 | 4.6 | 4.78 | 4.65 |
| $C_{15:0}$ | 0.21 | 0.17 | 0.31 | – | – | – | – | 0.28 | 0.39 |
| $C_{16:0}$ | 18.43 | 24.65 | 23.86 | 27.0 | 33.6 | 19.5 | 14.7 | 25.72 | 23.58 |
| $C_{17:0}$ | 0.62 | 0.27 | 0.35 | – | – | – | – | 0.27 | 0.53 |
| $C_{18:0}$ | 10.29 | 0.92 | 1.08 | Tr | 1.2 | 5.2 | – | 0.69 | 0.92 |
| $C_{18:1}$ | – | – | – | 22.2 | 5.3 | 19.7 | – | – | – |
| Hydroxy | | | | | | | | | |
| $C_{14:0}$ 3OH | – | – | – | 6.1 | 5.2 | 6.3 | – | – | – |
| $C_{16:1}$ 2OH | 0.525 | 1.03 | 0.59 | 1.1 | Tr | 1.3 | 3.5 | 1.24 | 0.95 |
| $C_{16:0}$ 2OH | 1.23 | 1.04 | 1.68 | 3.0 | 1.4 | 2.8 | 3.6 | 1.31 | 1.42 |
| $C_{16:0}$ 3OH | 6.195 | 6.52 | 5.39 | 5.6 | 6.3 | 6.4 | 5.6 | 6.65 | 6.71 |
| $C_{18:1}$ 2OH | 0.11 | – | – | 3.5 | 4.4 | 3.4 | 1.7 | – | – |
| Cyclo | | | | | | | | | |
| $C_{17:0}$ cyclo | 5.3 | 3.98 | 9.57 | 11.8 | 25.2 | 14.0 | 5.1 | 14.72 | 9.48 |
| $C_{19:0}$ cyclo w8c | 2.91 | 1.43 | 4.4 | 8.5 | 10.3 | 5.8 | 2.5 | 12.42 | 3.71 |
| Monosaturated | | | | | | | | | |
| $C_{16:1}$ w5c | – | – | 0.43 | – | – | – | – | 0.24 | 0.18 |
| $C_{16:1}$ w7c | – | – | – | 4.5 | 1.2 | 9.8 | – | – | – |
| $C_{18:1}$ w7c | 27.61 | 27.58 | 27.27 | – | – | – | 35.6 | 17.47 | 26.71 |
| Summed features* | | | | | | | | | |
| 2:$C_{14:0}$ 3OH/$C_{16:1}$ ISOI | 4.91 | 5.48 | 5.57 | – | – | – | 8.1 | 6.09 | 5.6 |
| 2:$C_{12:0}$ ALDE? | 4.91 | 5.48 | 5.57 | – | – | – | – | 6.09 | 5.6 |
| 3:$C_{16:1}$ w7c/$C_{15}$ iso2OH | 16.32 | 20.91 | 14.32 | – | – | – | 13.6 | 6.74 | 14.34 |
| 3:$C_{16:1}$ w7c/$C_{15:0}$ iso2OH | 16.32 | 20.91 | 14.32 | – | – | – | – | 6.74 | 14.34 |

*Summed features are groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. 1, CBMB40; 2, CBPB-HIM; 3, CBPB-HOD; 4, *B. glumae*; 5, *B. plantari*; 6, *B. vietnamiensis*; 7, *B. fungorum*; 8, *B. thailandensis*, 9, *B. ubonensis*.

an 869 bp *recA* fragment not absolutely specific to *Burkholderia* species [16]. However, all three strains produced the expected 385 bp *recA* fragment, when BUR3/BUR4 primers were used. The products were directly sequenced for analyzing the taxonomic position of these strains. The BUR3 and BUR4 primers were found to be specific for the genus, and analysis of just a 300-bp region of the *recA* sequence would produce phylogenetic trees with the same topology and discrimination as nearly the full-length sequences [16]. Sequencing reactions were prepared using the Applied Biosystems Big Dye Terminator ready reaction mix version 3.1 and analyzed using Applied Biosystems ABI-Prism 3730xl genetic analyzer. The consensus sequence of the products was aligned using the Seqman program (DNASTAR) and CLUSTAL W [25] and analyzed through BLAST. Phylogenetic and molecular evolutionary analyses were carried out as already mentioned. A phylogenetic tree constructed with deduced amino acid sequences of the *recA* gene product from CBMB40 showed 100% similarity to *B. vietnamiensis* and 97.5% to *B. ubonensis*. The similarity of *recA* products was higher between CBPB-HIM and *B. cepacia* (99.2%), and CBPB-HOD and *B. pyrrocinia* (Fig. 3).

DNA-DNA hybridization for the identified strains was carried out through a filter hybridization method [23]. Probe labeling was conducted using the nonradioactive DIG-High prime system (Roche), and hybridized DNA was visualized using the DIG luminescent detection kit (Roche). DNA-DNA relatedness was quantified by using a densitometer (Bio-Rad Laboratories). The results revealed binding values of 70.41–75.61% between the three strains. The DNA-DNA binding value between CBPB-HOD and *B. pyrrocinia* was higher (118.75%). CBPB-HIM and CBMB40 had higher DNA-DNA binding values with *B. ubonensis*. The relatedness values were not higher than 70% with the other *Burkholderia* strains studied (Table 3). These data clearly indicated that CBPB-HOD may be assigned under *B. pyrrocinia* and CBPB-HIM as *B. ubonensis*. However, for CBMB40, although the DNA-DNA association values were higher for *B. ubonensis*, the sequencing of other genes such as *recA*, *nifK*, *etc*., for other studies revealed its close association with *B. vietnamiensis*. To conclude, the

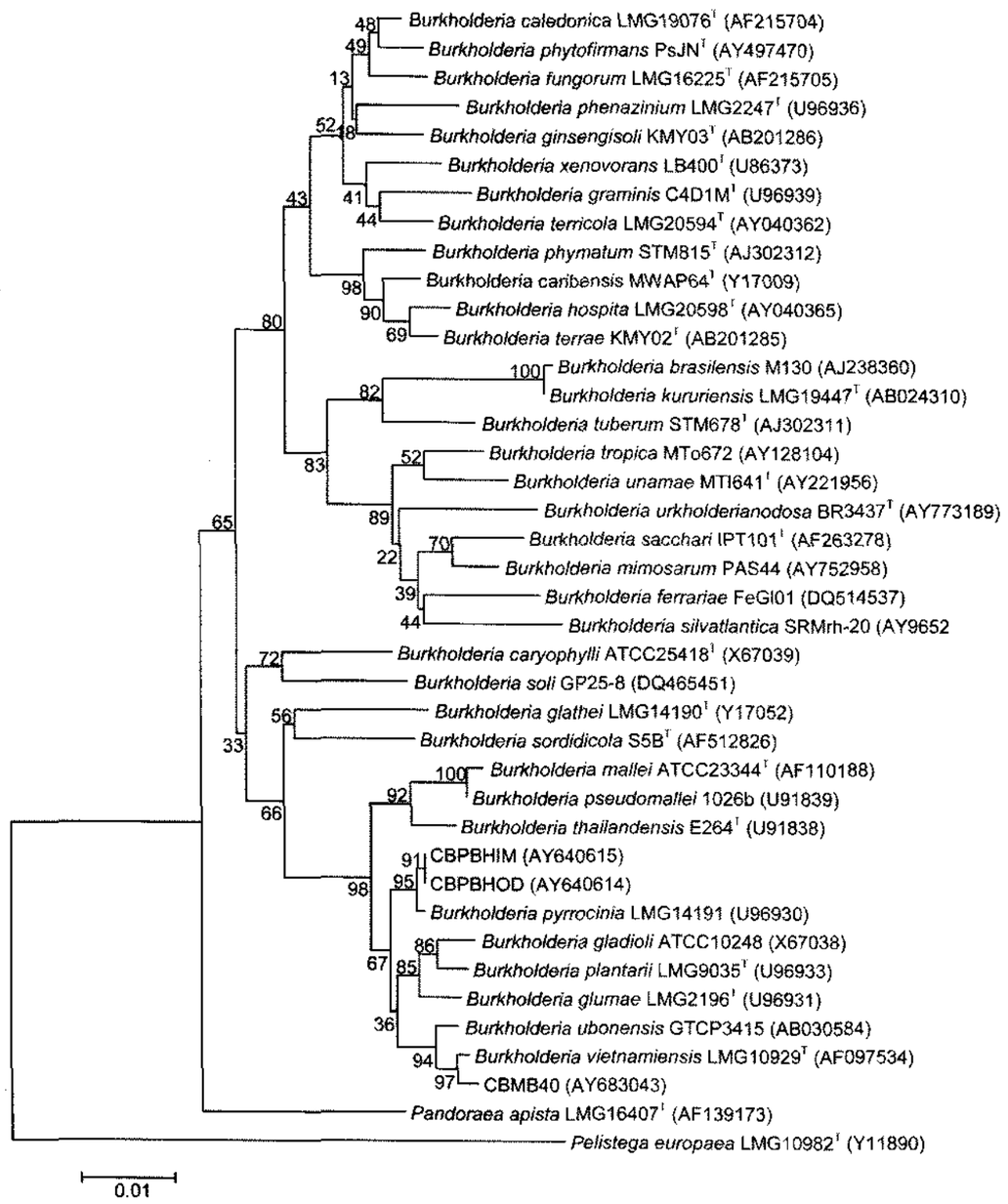


**Fig. 2.** Phylogenetic tree based on 16S rDNA gene sequence comparison showing the position of strains CBMB40, CBPB-HIM, and CBPB-HOD and other related species of the genus *Burkholderia*.

Numbers at nodes indicate percentages of bootstrap support based on a neighbor-joining analysis of 1,000 resampled datasets. Values below 50% are not indicated. Bar, 0.01 substitutions per site.

**Table 3.** DNA-DNA hybridization values between *Burkholderia* strains CBMB40, CBPB-HIM, and CBPB-HOD and its closest relatives. The hybridization procedure was carried out according to the method of Seldin and Dubnau [23].

| Species | DNA-DNA hybridization (%) | | |
|---|---|---|---|
| | CBMB40 | HIM | HOD |
| CBMB40 | 100 | 43.29 | 75.18 |
| CBPB HIM | 71.16 | 100 | 75.61 |
| CBPB HOD | 73.00 | 70.41 | 100 |
| *Burkholderia tropica* Ppe8$^T$ | 40.21 | 45.10 | 76.47 |
| *Burkholderia unamae* MTI-641$^T$ | 38.34 | 26.66 | 51.89 |
| *Burkholderia vietnamiensis*TVV75$^T$ | 40.56 | 49.07 | 93.34 |
| *Burkholderia gladioli* KCTC 2967$^T$ | 29.93 | 51.58 | 92.00 |
| *Burkholderia pyrrocinia* KCTC 2973$^T$ | 40.72 | 55.94 | 118.78 |
| *Burkholderia glathei* KCTC 2968$^T$ | 16.42 | 31.93 | 37.37 |
| *Burkholderia sacchari* KCTC 12954$^T$ | 34.50 | 43.10 | 52.69 |
| *Burkholderia fungorum* KCTC 12917$^T$ | 41.00 | 42.49 | 45.04 |
| *Burkholderia glumae* KCTC 2969$^T$ | 62.67 | 48.04 | 67.72 |
| *Burkholderia plantarii* KCTC 2972$^T$ | 59.78 | 35.17 | 60.31 |
| *Burkholderia thailandensis* DSM 13276$^T$ | 44.22 | 43.23 | 38.04 |
| *Burkholderia ubonensis* DSM 17311$^T$ | 74.37 | 88.77 | 82.02 |

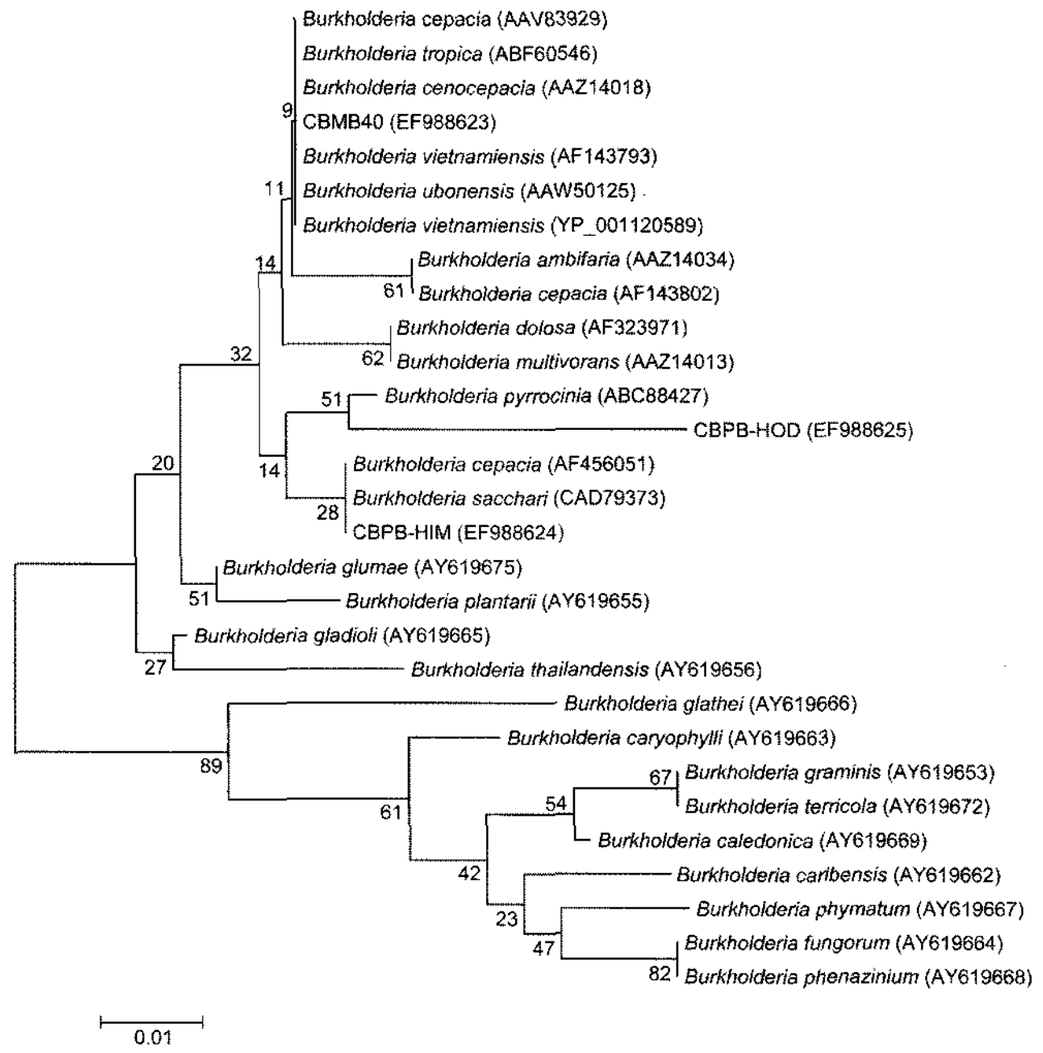


**Fig. 3.** Phylogenetic tree of *recA* gene sequences of CBMB40, CBPB-HIM, and CBPB-HOD obtained from field-grown rice.

Deduced amino acid sequence alignments of *recA* were constructed and analyzed phylogenetically using genetic-distance-based neighbour-joining algorithms (Jukes-Cantor matrix model; bootstrapping with 1,000 replications). The accession numbers of other *Burkholderia* species *recA* gene are shown in parentheses. Bootstrap values and genetic distance scales (number of substitutions per site) are indicated.

strains analyzed, *viz*, CBPB-HOD and CBPB-HIM, were assigned under *B. pyrrocinia* and *B. ubonensis*, respectively, and based on the 16S rDNA sequence analysis and *recA* gene analysis mentioned here, strain CBMB40 is classified as *B. vietnamiensis*.

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