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Nocardioides sambongensis sp. nov., isolated from Dokdo Islands soil

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Abstract

Strain KUDC5002^T was isolated from soil sampled on the Dokdo Islands, Republic of Korea. This bacterial strain was Gram stain-positive, non-motile, rod-shaped, capable of growing at 25–37°C and pH 5.0–12.0, and showed optimal growth at 30 °C and pH 7.0–8.0. Strain KUDC5002^T could be grown in tryptic soy broth containing less than 7.0 % NaCl (w/v). The cell width ranged from 0.5 to 0.6 µm and length ranged from 0.8 to 1.0 µm. Strain KUDC5002^T was catalase- and oxidase-positive. Its genomic G+C content was 72.2 mol%. Its major fatty acids were $C_{18:1}\omega9c$ (17.3 %), iso-$C_{16:0}$ (16.0 %) and iso-$C_{17:0}$ (11.4 %). Phylogenetic analysis, based on 16S rRNA gene sequences, showed that strain KUDC5002^T belongs to the genus *Nocardioides* and is most closely related to strain *Nocardioides humi* DCY24^T (97.0 %). Based on its phenotypic, phylogenetic, genetic and chemotaxonomic features, strain KUDC5002^T should be considered a novel species in the genus *Nocardioides*, for which we have proposed the name *Nocardioides sambongensis* sp. nov. The type strain is KUDC5002^T (=KCTC 39855^T=DSM 106604^T).

The genus *Nocardioides* belongs to the family *Nocardiaceae* [1], with *Nocardioides albus* [2] as the type strain. At the time of writing, according to LPSN (www.bacterio.net/index.html; [3]), this genus contains more than 104 recognised species. Members of this genus are Gram-stain-positive and mesophilic, and have LL-diaminopimelic acid cell walls and a high DNA G+C content (68–74 mol%) [4]. The major respiratory quinone is menaquinone MK-8(H_4) and the fatty acid profile contains both branched and straight-chain fatty acids [5]. *Nocardioides* species have been isolated from industrial wastewater [6], groundwater [7], alkaline soil [8–10], marine sediment [11–16] and desert soil [17].

The Dokdo Islands are a group of volcanic islands located in the middle of the East Sea, east of the Republic of Korea (37° 14′ 24.2″ N, 131° 52′ 12.2″ E; Uljin-gun, Gyeongsangbuk-do). Dokdo is characterized by barren environmental conditions, including shallow soil depth, steep inclinations, drought, high soil salinity, and high uric acid and low organic matter content in soil [18]. From 2005 to the present day, 57 novel bacterial species have been isolated from the Dokdo Islands. Furthermore, these include several *Nocardioides* type strains (*Nocardioides insulae* [19], *Nocardioides terrigena* [20], *Nocardioides hankookensis* [21], *Nocardioides dokdonensis* [16] and *Nocardioides paucivorans* [22]).

Here, we report the taxonomic analysis of a novel bacterial strain (KUDC5002^T) isolated from dry soil sampled on these islands and propose the name *Nocardioides sambongensis* sp. nov.

In November 2011, soil samples were collected from the rhizosphere of plants distributed across Dong-do, one of the two Dokdo Islands (Ulleung-gun, Gyeongsangbuk-Do). The samples were collected and stored as described previously [23]. They were suspended in a saline solution (0.85 %, w/v, NaCl) and serial dilutions (10^{-4}–10^{-6}) were prepared. A 100 µl aliquot of each dilution was plated onto tryptic soy agar (TSA; Difco) and incubated at 25 °C (under ambient conditions) for 7 days [24]. Morphologically different colonies were picked up and individual colonies were further purified by repeated streaking on TSA. Strain KUDC5002^T was cultivated on TSA and maintained at −70 °C in a 0.85 % NaCl solution supplemented with 15 % glycerol (v/

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Keywords: *Nocardioides*; Dokdo Islands; Soil bacteria.
Abbreviations: ANI, average nucleotide identity; dDDH, digital DNA–DNA hybridization; R2A, Reasoner's 2A; TSA, tryptic soy agar; TSB, tryptic soy broth.
The GenBank/EMBL/DDBJ accession numbers for 16S rRNA gene sequence and the whole genome sequence of strain KUDC5002^T are KX858534 and CP041091, respectively.
Five supplementary figures are available with the online version of this article.

v). The strains used in this study were subcultured on Reasoner's 2A (R2A) agar at 30 °C.

The phylogenetic position of the isolate was determined based on comparative analysis of the 16S rRNA gene. The 16S rRNA gene was amplified and PCR products were purified as described previously [25]. Universal primer sets 27F/1492R [26] and 518F/800R [27] were used. Direct sequencing of the 16S rRNA gene was carried out by Macrogen with sequencing primers (27F, 1492R, 518F and 800R) and an ABI 3730xl automated sequencer (Applied Biosystems). The EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/) [28] was used to identify the closest phylogenetic neighbours and calculate pairwise 16S rRNA gene sequence similarity values with respect to the novel strain.

The complete genome of strain KUDC5002^T and *Nocardioides humi* DCY24^T were sequenced using the PacBio RSII sequencing system (Pacific Biosciences) by Macrogen. The reads were assembled *de novo* using Hierarchical Genome Assembly Process version 3.0 (HGAP3.0) in SMRT analysis version 2.3.0 [29]. The complete genome sequence was annotated using the combined results from the automatic NCBI Prokaryotic Genomes Annotation Pipeline (PGAP).

The 16S rRNA gene sequence of strain KUDC5002^T (1462 bp) was determined, as described previously [30]. This strain showed the highest 16S rRNA gene sequence similarity (96.96 %) to *N. humi* DCY24^T, followed by *Nocardioides daecheongensis* KIS2-16^T (96.95 %), *Nocardioides panacisoli* GSoil 346^T (96.74 %), *Nocardioides kongjuensis* A2-4^T (96.60 %) and *Nocardioides nitrophenolicus* NSP 41^T (96.60 %). A comparison of the preliminary 16S rRNA gene sequences showed that strain KUDC5002^T was related to members of the genus *Nocardioides*.

The complete genomes determined in this study have been deposited in the NCBI GenBank database under the accession numbers CP041091 and CP041146. The complete genome of strain KUDC5002^T consists of a circular 4 434 294 bp chromosome. Strain KUDC5002^T contained 71.7 mol% G+C in its DNA. This value was similar to the value of 72.6 mol% for *N. humi* DCY24^T.

The average nucleotide identity (ANI) values based on the BLAST+ algorithm (ANIb) and the MUMmer ultra-rapid aligning tool (ANIm) were calculated through the website of JSpeciesWS (http://jspecies.ribohost.com/jspeciesws) [30]. The average amino acid identity (AAI) values were obtained through the Kostas lab website (http://enve-omics.ce.gatech.edu/). The digital DNA–DNA hybridization (dDDH) values between strain KUDC5002^T and *N. humi* DCY24^T were calculated using the server-based Genome-to-Genome Distance Calculator version 2.1 (http://ggdc.dsmz.de/distcalc2.php) [31]. The dDDH results were based on recommended formula 2 (identities/HSP length).

The ANIb and ANIm values of strain KUDC5002^T between the most closely related strain, *N. humi* DCY24^T, were 76.5 and 85.1 %. Also, the in dDDH value of strain KUDC5002^T compared with *N. humi* DCY24^T was 22.00 %. The values were clearly lower than their recommended thresholds (ANI,~95 %; dDDH, 70 %) [32, 33]. The AAI value between strain KUDC5002^T and *N. humi* DCY24^T was 69.1 %. Based on these genomic data, it is clearly indicated that strain KUDC5002^T represents a novel species of the genus *Nocardioides*.

Multiple sequence alignment and gap removal were carried out using CLUSTAL_W [34] and the sequence data were analysed with the software package BioEdit [35]. Gaps at the 5′ and 3′ ends of the alignment were omitted from further analyses. The Jukes and Cantor model [36] was used to generate an evolutionary distance matrix for the neighbour-joining algorithm. Phylogenetic trees were reconstructed using the neighbour-joining [37] algorithm in PHYLIP version 3.696 [38]. The SEQBOOT and CONSENSE programs in the PHYLIP package was used to evaluate the resulting tree topologies by bootstrap analysis with 1000 replications [39]. Phylogenetic trees were inferred using the maximum-parsimony [40] and maximum-likelihood [41] tree-making algorithms in the MEGA7 software [42] with 1000 bootstrap replicates. Maximum-likelihood phylogeny was generated by using Kimura's two-parameter model [43]. *Kribbella flavida* was used as an outgroup. Trees were rooted and drawn using DRAWGRAM in PHYLIP, MEGA7 in the Newick format.

In the neighbour-joining phylogenetic (Fig. 1) and maximum-likelihood trees (Fig. S1, available in the online version of this article), strain KUDC5002^T was grouped with *N. humi* DCY24^T, whereas *N. daecheongensis* KIS2-16^T and other related strains were located in another cluster. In the maximum-parsimony tree (Fig. S2), strain KUDC5002^T was located within a group containing *N. humi* DCY24^T, *N. daecheongensis* KIS2-16^T, *N. kongjuensis* A2-4^T, *N. nitrophenolicus* NSP 41^T and ten other *Nocardioides* species. Furthermore, strain KUDC5002^T exhibited high 16S rRNA gene similarity values and a distinct phylogenetic lineage, corresponding to a novel species in the genus *Nocardioides* (Figs 1 S1, S2).

Cells morphology was observed under a stereomicroscope equipped with a Zentech digital camera (Sw 804255, Samwon Optics and Seige), a scanning electron microscope (SU 8220, Hitachi) and a transmission electron microscope (H-7100, Hitachi) after incubation on R2A agar at 30 °C for 2 days. Gram-staining was performed using a Gram-stain kit (bioMérieux), according to the manufacturer's instructions. Growth of this strain at different temperatures (4, 10, 18, 25, 30, 37, 40 and 45 °C) was assessed on TSA, and the pH tolerance was tested in tryptic soy broth (TSB; Difco) by varying the pH (pH 4–11, in 0.5 pH unit increments). The pH values were adjusted as described by Kämpfer *et al.* [44]. The NaCl tolerance and growth in the absence of NaCl were tested at 0–8 % NaCl concentration (w/v; in 0.5 % increments) in TSB. For phenotypic characterization and comparative purposes, three reference strains, *N. humi* DCY24^T,

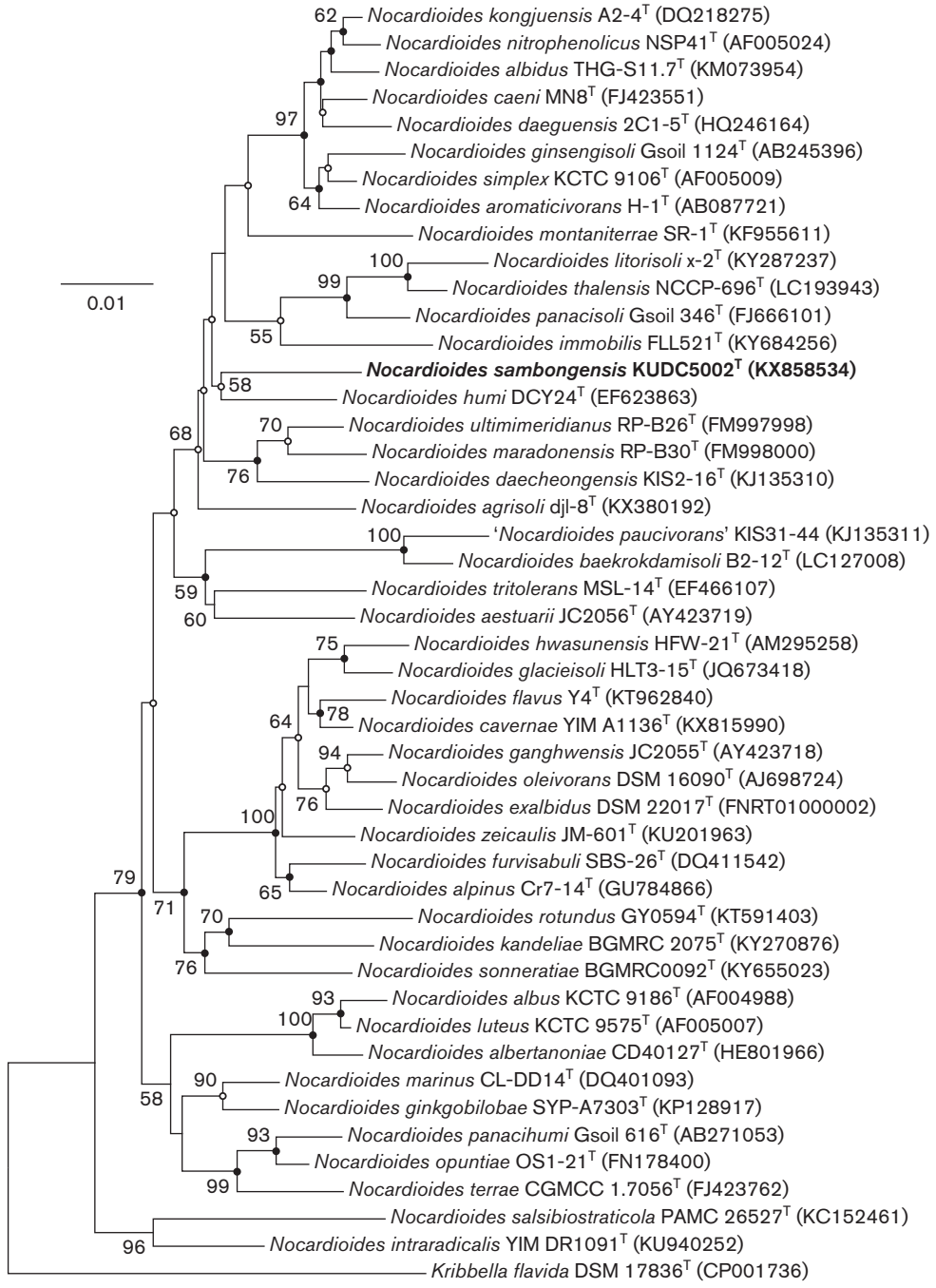

Fig. 1. Neighbour-joining phylogenetic tree reconstructed based on a comparative analysis of 16S rRNA gene sequences showing the relationships between strain KUDC5002^T and related species. Numbers at the nodes indicate the levels of bootstrap support (%) based on 1000 resampled datasets. The 16S rRNA gene sequence of *Kribbella flavida* DSM 17836^T was used as an outgroup. Solid circles indicate that the corresponding nodes were also obtained in both the maximum-likelihood and maximum-parsimony trees. Open circles indicate that the corresponding nodes were also obtained from the maximum-likelihood tree. Bar, 0.01 nucleotide substitutions per position.

N. daecheongensis KIS2-16^T and *N. panacisoli* GSoil 346^T were used.

Catalase activity was determined in a solution of 3 % hydrogen peroxide (v/v) and oxidase activity was tested using BBL Oxidase Reagent (Becton Dickinson). To assess anaerobic growth, strains were cultured on R2A agar for 7 days at 30 °C under anaerobic conditions in a GasPak EZ Standard Incubation Container (Becton Dickinson). Motility was

tested by observing cell growth on motility test medium agar with triphenyltetrazolium chloride (Carolina Biological Supply Company). Hydrolysis of casein, starch, Tween 20, Tween 40, Tween 60 and Tween 80 was determined as described by Cowan and Steel [45]. Hydrolysis of hypoxanthine, tyrosine and xanthine, at the substrate concentrations specified by Cowan and Steel [45], were assessed with an incubation in TSA at 30 °C. Other physiological and biochemical tests were carried out using API 50 CH and API 20 NE kits (bioMérieux) after incubation at 30 °C for 2 days. Enzyme activity was tested using an API ZYM kit (bioMérieux) after incubation at 25 °C for 10 h. To detect endospore formation, strain KUDC5002^T was grown on R2A for 2 days and spores were visualised using a scanning electron microscope (SU 8220, Hitachi) and a transmission electron microscope (H-7100, Hitachi).

The cells of strain KUDC5002^T were Gram-positive non-endospore forming rods (Figs S3 and S4) that proliferated well on TSA and R2A agar plates. Single cells were 0.5–0.6×0.8–1.0 µm. Flagella and endospores were not observed by transmission or scanning electron microscopy. The cells grew at 25–37 °C (optimally at 30 °C) in the presence of 0–7.0% NaCl (w/v; optimally in 0.5–2.0 %) and at pH 5–12 (optimally at pH 7–8) under aerobic conditions on TSA and R2A agar. Cells were positive for catalase and oxidase activity. The characteristics of strain KUDC5002^T are listed in the species description and phenotypic differences between this strain and the reference strain are presented in Table 1. Strain KUDC5002^T is non-motile and does not possess flagella in contrast to the motile flagella of *N. humi* DCY24^T. Strain KUDC5002^T and other closely related strains differed considerably with respect to utilization of different substrates as sole carbon sources via hydrolysis and other enzymatic activities. Strain KUDC5002^T can grow in culture media containing more than 3 % NaCl.

Strain KUDC5002^T was further characterized using chemotaxonomic analysis. Strain KUDC5002^T and the closely related *Nocardioides* type strains were collected from R2A plates after culturing at 30 °C for 2 days for analysing fatty acid methyl esters. Fatty acid methyl esters were saponified, methylated and extracted using the standard MIDI protocol (Sherlock Microbial Identification System, version 4.0). The fatty acids were analysed by gas chromatography (6890, Hewlett Packard) and identified using the Microbial Identification System TSBA 40 database [46]. Polar lipids were examined by two-dimensional TLC and identified using previously described methods [47]. For analysing cell-wall peptidoglycans, all strains were cultured in TSB at 37 °C for 48 h until OD_{600} was 0.8 (the mid-exponential phase). Peptidoglycans were hydrolysed according to a previously described method [48]. Amino acids and isomers in the cell-wall hydrolysates were analysed as described by Hamada *et al.* [49]. Isoprenoid quinones were extracted using a previously described method [50] and analysed by reversed-phase HPLC on a YMC ODS-A (250×4.6 mm) column [51].

MK-8(H_4) was the only predominant isoprenoid quinone in strain KUDC5002^T cells. This is consistent with the previous finding that MK-8(H_4) is the major quinone in the majority of the *Nocardioides* species [48]. The cellular fatty acid profiles of strain KUDC5002^T and the most closely related type strains are presented in Table 2. The major fatty acids in the novel strain were $C_{18:1}\omega9c$ (17.3 %), iso-$C_{16:0}$ (16.0 %) and iso-$C_{17:0}$ (11.4 %). Strain KUDC5002^T contained $C_{19:0}$, $C_{17:0}$ 2-OH and $C_{19:1}\omega9c$ and/or $C_{19:1}\omega11c$ fatty acid fractions and a high abundance of saturated $C_{17:0}$ fatty acid fractions, but did not contain $C_{16:0}$ 10-methyl, $C_{16:0}$ 2-OH, $C_{16:1}\omega6c$ or $C_{16:1}\omega7c$, which was different from the fatty acid compositions of *N. humi* DCY24^T and *N. panacisoli* GSoil 346^T. Strain KUDC5002^T contained the unsaturated fatty acid $C_{16:1}\omega11c$ (1.8 %). The polar lipids of strain KUDC5002^T were diphosphatidylglycerol, phosphatidylglycerol, two unidentified aminophospholipids, three unidentified aminolipids, one unidentified phospholipid and and one unidentified lipid. (Fig. S5). The cell-wall peptidoglycan contained LL-2,6-diaminopimelic acid as the diagnostic amino acid, which is in agreement with the other *Nocardioides* strains [52]. These features are common among members of *Nocardioides* [53].

Based on the phenotypic, chemotaxonomic, phylogenetic and genetic data presented here, strain KUDC5002^T should be considered a novel species affiliated with the genus *Nocardioides* for which we propose the name *Nocardioides sambongensis* sp. nov.

DESCRIPTION OF *NOCARDIOIDES SAMBONGENSIS* SP. NOV.

Nocardioides sambongensis (sam.bong.en′sis. N.L. masc. adj. *sambongensis* of or pertaining to Sambong Islands. Sambong, ancient name of the Dokdo Islands, located on the coast of the East Sea in Korea, from where the organism was isolated).

Cells are aerobic, Gram-stain-positive, non-motile and non-endospore-forming rods. The cells grow well under aerobic conditions on R2A agar and TSA plates. The individual cells are 0.5–0.6×0.8–1.0 µm in size. Colonies are circular, smooth, pale yellow in colour, and are approximately 1.0–2.5 mm in diameter after 2 days of incubation on R2A agar at 30 °C. Growth occurs between 25 and 37 °C, but not at 40 °C and above. The pH range for growth is pH 5–12 (optimally at pH 7–8). Grows in the presence of 0–7% NaCl (w/v; optimally in 0.5–2 %), but not in 8 % NaCl. The cells are catalase- and oxidase-positive. The cells hydrolyse Tween 20, Tween 40, Tween 60 and Tween 80, but not casein, DNA, hypoxanthine, starch, L-tyrosine or xanthine.

Cells are positive for nitrate reduction, aesculin hydrolysis, gelatin hydrolysisand glucose fermentation, but negative for indole production, arginine dihydrolase and urease (API 20NE). D-Glucose is utilized, but adipate, L-arabinose, caproate, citrate, gluconate, *N*-acetyl-D-glucosamine, malate, maltose, D-mannitol, D-mannose and phenylacetate are not

Table 1. Differential physiological properties of strain KUDC5002^T and the type strains of closely related species in the genus *Nocardioides*

Strains: 1, KUDC5002^T; 2, *Nocardioides humi* DCY24^T; 3, *Nocardioides daecheongensis* KIS2-16^T; 4, *Nocardioides panacisoli* GSoil 346^T. −, Negative result; +, positive result.

	1	2	3	4
Habitat	Island soil	Ginseng field soil*	Forest soil†	Ginseng field soil‡
Colony colour	Pale yellow	Pale yellow	Whitish	Pale yellow
Cell size (μm)	0.5–0.6×0.8–1.0	0.3–0.5×0.8–1.0*	0.5–0.7×1.2–2.2†	0.2–0.4×0.8–1.2‡
Growth range:				
Temperature (°C)	25–37	25–42*	10–35†	10–42‡
pH	5–12	5–11*	4–10†	5.5–8.5‡
NaCl (%)	0–7	0–3	0–3†	0–2‡
Motility	−	+*	−†	−‡
Catalase activity	+	−	+	+
Oxidase activity	+	+	−	+
Reduction of nitrates to nitrites	+	−	+	+
Assimilation of:				
Adipic acid	−	−	−	+
D-Mannitol	−	+	−	−
L-Arabinose	−	−	+	−
Malate	−	+	+	+
Potassium gluconate	−	+	−	+
Trisodium citrate	−	−	+	+
Enzyme activity:				
Acid phosphatase	−	+	+	+
Alkaline phosphatase	−	+	−	+
Cystine arylamidase	+	+	−	−
Esterase	+	+	+	−
Esterase lipase	−	+	+	−
Leucine arylamidase	−	+	+	−
Lipase	−	−	+	−
Trypsin	+	+	−	+
Valine arylamidase	+	+	−	−
α-Chymotrypsin	−	+	−	+
α-Glucosidase	+	+	−	+
β-Galactosidase	+	+	−	−
β-Glucosidase	−	+	−	−
β-Glucuronidase	+	−	−	−
Hydrolysis of:				
Casein	−	−	+	−
Aesculin	+	+	+	−
Gelatine	+	−	−	−
L-Tyrosin	−	+	+	+
Tween 20	+	−	−	−
Tween 40	+	−	+	−
Tween 60	+	+	−	−

*Data obtained from Kim *et al.* [54].

†Data obtained from Lim *et al.* [55].

‡Data obtained from Cho *et al.* [56].

utilized as sole carbon and energy sources (API 20NE). API 50 CH tests are positive for acid production from aesculin, cellobiose, glucose, 5-ketogluconate, rhamnose, sucrose, trehalose, turanose and D-xylose, but negative for that from 2-ketogluconate, adonitol, amygdalin, D-arabinose, D-arabitol, L-arabinose, L-arabitol, arbutin, dulcitol, erythritol, fructose, D-fucose, L-fucose, galactose, gluconate, glycerol, glycogen, inositol, inulin, lactose, maltose, mannitol, mannose,

Table 2. Cellular fatty acid profiles of strain KUDC5002^T and the type strains of closely related species in the genus *Nocardioides*

Strains: 1, KUDC5002^T; 2, *Nocardioides humi* DCY24^T; 3, *Nocardioides panacisoli* GSoil 346^T. Values are percentages of total fatty acids. –, Not detected or less than 1 %.

Fatty acid	1	2	3
Saturated:			
$C_{16:0}$	2.5	5.1	5.7
$C_{17:0}$	8.1	1.3	1.5
$C_{18:0}$	6.0	6.0	6.5
$C_{19:0}$	1.0	–	–
Branched:			
iso-$C_{15:0}$	2.3	3.2	3.1
iso-$C_{16:0}$	16.0	23.1	22.1
iso-$C_{17:0}$	11.4	16.2	12.2
iso-$C_{18:0}$	3.8	2.9	2.0
anteiso-$C_{17:0}$	3.0	4.4	6.1
Unsaturated:			
$C_{17:1}\omega 8c$	5.7	1.6	1.5
$C_{18:1}\omega 9c$	17.3	17.8	16.6
10-Methyl:			
$C_{16:0}$ 10-Methyl	–	1.4	–
$C_{17:0}$ 10-Methyl	2.2	1.9	1.3
$C_{18:0}$ 10-Methyl (TBSA)	8.7	9.5	10.0
Hydroxy:			
$C_{16:0}$ 2-OH	–	–	1.0
$C_{17:0}$ 2-OH	1.0	–	–
Summed features:*			
3	–	1.6	1.6
6	4.2	–	–
8	2.5	2.3	4.2

*Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 3 comprises $C_{16:1}\omega 6c$ and/or $C_{16:1}\omega 7c$. Summed feature 6 comprises $C_{19:1}\omega 9c$ and/or $C_{19:1}\omega 11c$. Summed feature 8 comprises $C_{18:1}\omega 6c$ and/or $C_{18:1}\omega 7c$.

melezitose, melibiose, methyl α-D-glucoside, methyl α-D-mannoside, methyl β-D-xyloside, *N*-acetylglucosamine, gentiobiose, D-lyxose, D-tagatose, L-xylose raffinose, ribose, salicin, sorbitol, sorbose, starch and xylitol (API 50CH). Test results are positive for cystine arylamidase, esterase, β-galactosidase, α-glucosidase, β-glucuronidase, naphthol-AS-BI-phosphohydrolase, trypsin and valine arylamidase, but negative for acid phosphatase, alkaline phosphatase, α-chymotrypsin, esterase lipase, α-fructosidase, α-galactosidase, , β-glucosidase, leucine arylamidase, lipase, α-mannosidase and *N*-acetyl-β-glucosaminidase activities (API ZYM). The predominant menaquinone is MK-8(H_4). The major fatty acids are $C_{18:1}\omega 9c$, iso-$C_{16:0}$ and iso-$C_{17:0}$. The DNA G+C content is 71.7 mol%.

The type strain is KUDC5002^T (=KCTC 39855^T=DSM 106604^T), which was isolated from soil collected from the Dokdo Islands, Republic of Korea. The 16S rRNA gene sequence and the whole genome sequence of strain KUDC5002^T has been deposited in GenBank/EMBL/DDBJ under the accession numbers KX858534 and CP041091. General features of the genome assembly are as follows: genome size, 4 434 294 bp; number of contigs, 1; coverage, 111.0×.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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