

Methylobacterium frigidaeris sp. nov., isolated from an air conditioning system

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Abstract

A reddish pink-pigmented, Gram-stain-negative, aerobic and methylotrophic bacterial strain, designated strain IER25-16^T, was isolated from a laboratory air conditioning system in the Republic of Korea. Cells were motile rods showing catalase- and oxidase-positive reactions. Strain IER25-16^T grew at 10–40 °C (optimum, 30 °C), at pH 4.0–7.0 (optimum, pH 5.0–7.0) and in the presence of 0–1.0 % (w/v) NaCl (optimum, 0 %). The major respiratory quinone was ubiquinone-10 and ubiquinone-9 was also detected as the minor respiratory quinone. Summed feature 8 (comprising $C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$) was detected as the predominant fatty acids. The genomic DNA G+C content of strain IER25-16^T was 70.0 mol%. Phylogenetic analysis based on 16S rRNA gene sequence comparison revealed that strain IER25-16^T belonged to the genus *Methylobacterium* of the class *Alphaproteobacteria*. Strain IER25-16^T was most closely related to *Methylobacterium platani* PMB02^T (97.9 %), *Methylobacterium aquaticum* GR16^T (97.9 %) and *Methylobacterium tarhaniae* N4211^T (97.5 %). The average nucleotide identity and *in silico* DNA–DNA hybridization values between strain IER25-16^T and *M. platani*, *M. aquaticum* and *M. tarhaniae* were 88.3, 88.8 and 89.6 % and 36.2, 37.3 and 39.3 %, respectively. The phenotypic and chemotaxonomic features and the phylogenetic inference clearly suggested that strain IER25-16^T represents a novel species of the genus *Methylobacterium*, for which the name *Methylobacterium frigidaeris* sp. nov. is proposed. The type strain is strain IER25-16^T (=KACC 19280^T=JCM 32048^T).

The genus Methylobacterium belonging to the class Alphaproteobacteria was first proposed by Patt et al. [1] with Methylobacterium organophilum as the type species [1] and thereafter its emended genus descriptions were provided by Green and Bousfield [2]. Members of the genus Methylobacterium are typically strictly aerobic, Gram-stain-negative, rod-shaped, pink pigmented, facultatively methylotrophic bacteria containing ubiquinone-10 as the major respiratory quinone. They are able to grow on single carbon compounds such as formate, formaldehyde, methanol and methylamine as sole sources of carbon and energy, as well as on a wide range of multi-carbon growth substrates [3, 4], and thus they are especially present on plant surfaces, where they assimilate methanol emitted from plants as a product of pectin degradation [5]. Members of the genus Methylobacterium have been found in various natural or man-made environments, including plants [6-12], freshwater [13], seawater [14], drinking water [15-17], air samples [18], soil [19], a traditional Korean fermented seafood [20] and a polluted environment [21]. At the time of writing, the genus Methylobacterium comprises 52 validated species (www.

bacterio.net/methylobacterium.html). In this study, we isolated a putative novel species belonging to the genus *Methylobacterium*, designated strain IER25-16^T, from an air conditioning system, and characterized it further taxonomically using a polyphasic approach.

Strain KER25-12^T was isolated from an air conditioning system that had been operating in a laboratory of Chung-Ang University in Seoul, Republic of Korea (37° 30' 14.93" N $126^{\circ} 57' 25.03''$ E). In brief, a piece of filter obtained from the air conditioning system was resuspended in phosphatebuffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.2). The aliquots of each serial dilution were spread on R2A agar (BD, USA) and incubated aerobically at 25 °C for 3 days. The 16S rRNA genes of colonies grown on R2A agar were PCR-amplified using the universal primers, F1 (5'-AGA GTT TGA TCM TGG CTC AG-3') and R13 (5'-TAC GGY TAC CTT GTT ACG ACT T-3'), and double-digested with HaeIII and HhaI, as described previously [22]. The PCR amplicons showing distinct fragment patterns were partially sequenced using the universal primer 340F (5'-CCT ACG GGA GGC

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Abbreviations: ANI, average nucleotide identity; DDH, DNA-DNA hybridization; ML, maximum-likelihood; MP, maximum-parsimony; NJ, neighbour-joining; Q, ubiquinone.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and genome sequences of strain IER25- 16^{T} are KY864396 and PELK00000000, respectively.

One supplementary table and three supplementary figures are available with the online version of this article.

AGC AG-3'). The resulting 16S rRNA gene sequences were compared with those of all reported type strains using the Nucleotide Similarity Search program in the EzTaxon-e server (https://www.ezbiocloud.net/identify) [23]. From the comparative 16S rRNA gene sequence analysis, a putative novel strain belonging to the genus Methylobacterium, designated strain IER25-16^T, was selected for further phenotypic and phylogenetic analyses. Strain IER25- 16^{T} was routinely cultured aerobically on R2A agar at 30°C for 3 days, except where indicated, and stored at -80 °C in R2A broth containing 15 % (v/v) glycerol for a long-term preservation. The type strains of Methylobacterium platani (KACC 14558^T), Methylobacterium aquaticum (KACC 11543^T), Methylobacterium tarhaniae (DSM 25844^T) and Methylobacterium organophilum (KACC 15217^T, the type species of the genus Methylobacterium) were used as reference strains for the comparison of phenotypic properties and fatty acid compositions.

The 16S rRNA gene amplicon of strain IER25-16^T that was PCR-amplified using the F1 and R13 primers was further sequenced using the universal primers, 518R (5'-ATT ACC GCG GCT GCT GG-3') and 805F (5'-GAT TAG ATA CCC TGG TAG TC-3') at Macrogen (Repubic of Korea) to obtain an almost-complete 16S rRNA gene sequence (1406 nucleotides). The identification of phylogenetic neighbours for the phylogenetic tree reconstructions and the calculations of pairwise 16S rRNA gene sequence similarities were accomplished by using the EzTaxon-e database (https:// www.ezbiocloud.net/identify) [23]. The 16S rRNA gene sequence of strain IER25-16^T and closely related type strains were aligned using the fast secondary-structure aware Infernal aligner in the Ribosomal Database Project (https://pyro. cme.msu.edu/login.spr) [24]. Phylogenetic relationships of strain IER25-16^T with closely related type strains were inferred using the DNADIST and DNAPARS programs based on the neighbour-joining (NJ) algorithm with the Kimura twoparameter model and the maximum-parsimony (MP) algorithm through a heuristic search, respectively, in the PHYLIP software (version 3.695) [25] and their tree topologies were evaluated through bootstrap analyses based on a 1,000resampled dataset. The maximum-likelihood (ML) analysis with bootstrap values was performed using RAxML-HPC BlackBox (version 8.2.9) available in the Cyber-Infrastructure for Phylogenetic Research project (www.phylo.org) [26]. The whole genome of strain IER25-16^T was extracted using the Wizard Genomic DNA Purification Kit (Promega) and sequenced by an Illumina HiSeq 2500 platform at Macrogen GenBank accession no. PELK00000000). The whole genome sequence of strain IER25-16^T was compared with those of *M. platani* PMB02^T (GenBank accession no. NZ_LWHQ00000000), M. aquaticum DSM 16371^T (Gen-Bank accession no. NZ LABX00000000) and M. tarhaniae DSM25844^T (GenBank accession no. NZ_LABZ0000000) available in GenBank based on average nucleotide identity (ANI) and in silico DNA-DNA hybridization (DDH). The ANI values between strain IER25-16^T and the reference strains was calculated using standalone software available in the EZGenome web server (www.ezbiocloud.net/sw/oat) [27]. *In silico* DDH values between strain IER25-16^T and the reference strains were computed with the server-based genome-to-genome distance calculator version 2.1 (http://ggdc.dsmz.de/distcalc2.php) [28].

The comparison of 16S rRNA gene sequences revealed that strain IER25-16^T was most closely related to *M. platani* PMB02^T (97.93 %), M. aquaticum GR16^T (97.86 %), M. tarhaniae N4211^T (97.50%) and Methylobacterium variabile GR3^T (96.92%). The phylogenetic analysis using the NJ algorithm indicated that strain IER25-16^T formed a phylogenic lineage within the genus Methylobacterium of the class Alphaproteobacteria (Fig. 1), which was also supported by the MP and ML algorithms (Fig. S1, available in the online version of this article). The ANI values between strain IER25-16^T and *M. platani* PMB02^T, *M. aquaticum* DSM 16371^T and *M. tarhaniae* DSM 25844^T were 88.3, 88.8 and 89.6%, respectively, which were clearly lower than the threshold of 94-96% for prokaryotic species delineation [29, 30]. In addition, in silico DDH values between strain IER25-16^T and *M. platani* PMB02^T, *M. aquaticum* DSM 16371^T and *M. tarhaniae* DSM 25844^T were 36.2, 37.3 and 39.3 %, respectively, which were also clearly below the 70 % threshold of DDH generally accepted for delineation of different species [31, 32]. These results clearly suggest that strain IER25-16^T can represent a novel species of the genus Methylobacterium.

Growth of strain IER25-16^T was assessed at 30 °C for 3 days on several bacteriological agar media: R2A agar, LB agar (MP Biomedicals), nutrient agar (BD), and tryptic soy agar (BD). Growth of strain IER25-16^T was tested on R2A agar at different temperatures (5-50 °C at 5 °C intervals). Both pH and NaCl tolerances were determined in R2A broth with different pH values (3.0-9.0 at 1.0 pH unit intervals) and NaCl concentrations (0-5% at 1% intervals) for 3 days, respectively. The pH values below 7.0 and pH 8.0-9.0 were prepared using the Na₂HPO₄-NaH₂PO₄ and Tris-HCl buffers, respectively, according to the method of Lányí [33] and their pH values were adjusted again if necessary after sterilization (121 °C for 15 min). The methylotrophic growth of strain IER25-16^T was assessed at 30°C for 5 days in M9 minimal broth supplemented with 0.5 and 1.0 % methanol as the sole carbon and energy source, as described previously [6]. NaCl concentrations were prepared in the laboratory according to the BD formula. Gram-staining was conducted using the Gram-stain kit (bioMérieux) according to the manufacturer's instructions. Anaerobic growth was assessed on R2A agar at 30°C for 3 weeks under the anaerobic condition (with 4-10 % CO₂) using the GasPak Plus system (BBL). Cell morphology and motility were observed using transmission electron microscopy (JEM-1010, JEOL) and phase contrast microscopy using cells grown in R2A broth at 30 °C for 2 days. Catalase and oxidase activities were tested by the production of oxygen bubbles in 3 % (v/v) aqueous hydrogen peroxide solution and the oxidation of 1 % (w/v) tetramethyl-p-phenylenediamine (Merck), respectively [34]. Pigments were extracted from lyophilized



Fig. 1. A neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships of strain IER25-16^T and the type strains of closely related *Methylobacterium* species. Bootstrap values with more than 70 % are shown on the nodes as percentages of 1000 replicates. Filled circles (•) indicate that the corresponding nodes were also recovered in the trees reconstructed by the ML and MP algorithms. *Methylorhabdus multivorans* DM13^T (AF004845) was used as an outgroup. The scale bar equals 0.01 changes per nucleotide position.

cells by addition of methanol for 60 min and analysed by using a UV-visible spectrometer (BioTek). The following properties of strain IER25-16^T and reference strains were investigated in parallel under the same conditions in this study. Hydrolysis of Tween 20, Tween 80, casein, starch, tyrosine and aesculin was checked on R2A agar following the methods described by Lányí [33] and Smibert and Krieg [34]. Additional enzymatic activities, biochemical features and oxidations of carbon compounds were tested using the API ZYM and API 20NE kits (bioMérieux) and the GN2 MicroPlate system (Biolog), respectively, according to the manufacturers' instructions; cells of strain IER25-16^T and reference strains resuspended in 0.85 % (w/v) saline were used as inocula for the tests.

Strain IER25-16^T grew well on R2A agar, tryptic soy agar and nutrient agar (optimum, R2A agar), but did not grow on LB agar. Colonies were reddish pink-pigmented, circular, convex and smooth with a diameter of approximately 0.5 mm on R2A agar after 3 days of incubation. The pink pigments were water-insoluble and had absorption maxima at 359, 495 and 525 nm in chloroform/methanol (1:1, v/v) extracts (Fig. S2) [11]. Cells of strain IER25-16^T were Gram-stain-negative, oxidase- and catalase-positive, and motile rods with approximately 1.7-1.8 µm wide and 2.1-3.4 μ m long (Fig. S3). Strain IER25-16^T grew well in M9 minimal broth with methanol as a sole carbon and energy source, indicating that the strain is methylotrophic. Anaerobic growth of strain IER25-16^T was not observed after 3 weeks incubation on R2A agar at 30 °C. In Biolog GN2 MicroPlate, strain IER25-16^T oxidized acetic acid, maltose, D-mannose, β -methyl-D-glucoside, D-sorbitol, trehalose, xylitol, D-glucuronic acid, itaconic acid, quinic acid, L-alanine, L-aspartic acid, glycyl-L-glutamic acid, hydroxy-L-proline, L-threonine, L-ornitine, L-phenylalanine, D-psicose, Tween 40, Tween 80, α -cyclodextrin, β -hydroxy butyric acid, L-fucose, L-arabinose, cis-aconitic acid, D-fructose and bromo succinic acid, but did not oxidize dextrin, glycogen, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, adonitol, D-arabitol, cellobiose, i-erythritol, m-inositol, lactose,

lactulose, D-mannitol, melibiose, raffinose, raffinose, sucrose, turanose, citric acid, D-galacturonic acid, D-gluconic acid, *p*-hydroxy phenylacetic acid, α -keto valeric acid, sebacic acid, L-alanyl-glycine, L-glutamic acid, glycyl-L-aspartic acid, L-histidine, L-proline, D-serine, L-serine, D,Lcarnitine, γ -hydroxy butyric acid, urocanic acid, inosine, uridine, thymidine, putrescine, 2-aminoethanol, 2,3-butanediol, D,L- α -glycerol phosphate, glucose-1-phosphate, glucose-6-phosphate, gentiobiose, L-leucine, α -keto glutaric acid, succinamic acid, D-galactonic acid lactone, D-glucosaminic acid, D-saccharic acid, glucuronamide, D-alanine, L-asparagine, L-pyroglutamic acid, formic acid, α -hydroxy butyric acid, D,L-lactic acid, malonic acid, propionic acid, succinic acid, D-galactose, α -D-glucose, methyl pyruvate, mono-methyl-succinate, α -keto butyric acid, L-alaninamide and γ -hydroxy butyric acid (Table S1). Many characteristics of IER25-16^T such as pink pigmentation, Gram reaction, motility, indole production, glucose fermentation and catalase activity were in good agreement with those of reference strains of the genus *Methylobacterium*, whereas other properties such as oxidase activity, reduction of nitrate and other many phenotypic properties allowed the differentiation of strain IER25-16^T from other closely related *Methylobacterium* species (Table 1). Phenotypic characteristics of strain IER25-16^T are presented in the species description and compared with those of the closely related type strains in Tables 1 and S1.

Isoprenoid quinones of strain IER25-16^T were analysed with a high-performance liquid chromatography system (model LC-20A, Shimadzu) equipped with a reversedphase column (250 Í 4.6 mm, Kromasil, Akzo Nobel) and a diode array detector (SPD-M20A, Shimadzu) using methanol-isopropanol (2:1, v/v) as an eluent (1 ml min⁻¹), as described by Komagata and Suzuki [35]. Isoprenoid quinones of *Shingobium limneticum* DSM 25076^T that was known as a ubiquinone (Q)-10 and Q-9 producing

Table 1. Comparisons of phenotypic properties between strain IER25-16^T and the type strains of closely related *Methylobacterium* species

Taxa: 1, strain IER25-16^T(this study); 2, *M. platani* KACC 14558^T [12]; 3, *M. aquaticum* KACC 11543^T [17]; 4, *M. tarhaniae* DSM 25844^T [19]; 5, *M. organophilum* KACC 15217^T [1]. All strains are positive for the following characteristics: motility and activity* of urease, alkaline phosphatase, esterase (C4), esterase lipase (C8), acid phospatase and naphtol-AS-BI-phosphohydrolase. All strains are negative for the following characteristics: Gram reaction, indole production, glucose fermentation, anaerobic growth*, activity* of arginine dihydrolase, 4-nitrophenyl- β -D-galactopyranoside, lipase (C14), cystine arylamidase, α -chymotrypsin, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase, assimilation* of D-mannitol, *N*-acetyl-glucosamine, maltose and capric acid, and hydrolysis* of casein, tyrosine, starch, aesculin and Tween 80. +, Positive; –, negative; w, weakly positive; ND, not determined.

Characteristic	1	2	3	4	5
Isolation source	Air conditioning system	Leaf	Drinking water	Soil	Lake
Catalase	+	+	+	+	ND
Oxidase	+	+	-	ND	+
Reduction of nitrate to nitrite*	W	W	W	W	-
Pigmentation*	Pink to red	Pink	Pink	Pink	Light pink
Colony diameter (mm)	0.5-2.0	0.2-1.5	1.0-2.0	0.3-1.2	1.0-1.6
Cell length (µm)	2.1-3.4	2.0-6.5	4.5-8.0	2.6-5.6	1.5-2.0
Cell width (µm)	1.7-1.8	1.4-1.5	1.5-1.7	1.0-1.6	0.8-1.0
Hydrolysis* of					
Tween 20	+	+	+	+	-
Gelatin	+	-	-	-	-
Assimilation (API 20NE)* of:					
D-Mannose	-	-	W	-	-
Phenylacetic acid	-	-	W	W	-
Malic acid	W	-	-	+	+
Trisodium citrate	W	-	W	+	-
Adipic acid	-	+	+	+	-
D-Glucose	-	-	W	+	+
L-Arabinose and potassium gluconate	+	+	+	+	-
Enzyme activity (API ZYM)* of:					
Valine arylamidase	-	-	+	+	+
Leucine arylamidase	-	+	+	+	+
Trypsin	-	W	W	+	+
DNA G+C content (mol%)	70.0†	71.1†	70.9†	70.4†	69.6

*These analyses were conducted under the same conditions in this study.

+The DNA G+C contents were calculated based on their genomes in this study.

bacterium were used as standards [36, 37]. For the analysis of cellular fatty acids, strain IER25-16^T and reference strains were cultivated in R2A broth at 30 °C and the cells were harvested at the same growth phase (exponential phase, optical density was 0.8 at 600 nm). The fatty acids were saponified, methylated, extracted and washed using the standard MIDI protocol and the fatty acid methyl esters were analysed using a gas chromatography system (model 6890; Hewlett Packard) based on the TSBA6 database in the Microbial Identification System (Sherlock version 6.2B) [38]. The DNA G+C contents of strain IER25-16^T and the type strains of *M. platani*, *M. aquaticum* and *M. tarhaniae* were calculated using the EditSeq module of the Lasergene package based on their genome sequences.

The predominant respiratory quinone of strain IER25-16^T was ubiquinone (Q)-10 (>90%), like in all other members of the genus Methylobacterium. Q-9 was also detected as the minor respiratory quinone. Summed feature 8 (comprising $C_{18:1}\omega 7c$ and/or $C_{18:1}\omega 6c$) was the predominant cellular fatty acid in strain IER25-16^T (73.8%), which was in line with those of reference strains of the genus Methylobacterium (Table 2). However, there were some differences in the respective compositions of some fatty acid components. For example, C_{12:0} was detected from strain IER25-16^T as the second dominant fatty acid (7.0 %), but it was detected as a trace component in the other reference strains. The DNA G+C content of strain IER25-16^T was 70.0 mol%, which was similar with those of reference strains (Table 1). In conclusion, the phenotypic and chemotaxonomic features and the phylogenetic inference support the proposition that strain IER25-16^T represents a novel species of the genus *Methylo*bacterium, for which the name Methylobacterium frigidaeris sp. nov. is proposed.

DESCRIPTION OF METHYLOBACTERIUM FRIGIDAERIS SP. NOV.

Methylobacterium frigidaeris (fri.gid.a'e.ris. L. adj. *frigidus* cold; L. n. *aer* air; N.L. gen. n. *frigidaeris* of cold air, as the strain was isolated from an air-conditioning system).

Cells are Gram-stain-negative, strictly aerobic and motile rods showing oxidase- and catalase-positive reactions. Cells are 1.7-1.8 µm wide and 2.1-3.4 µm long. Colonies on R2A agar are reddish pink-pigmented, circular, convex and smooth with a diameter of approximately 0.5 mm after 3 days of incubation on R2A agar. The pink pigments show maximum absorptions at 359, 495 and 525 nm. Grows well methylotrophically using methanol as a sole carbon and energy source. Growth occurs at 10–40 °C (optimum, 30 °C), at pH 4.0-7.0 (optimum, pH 5.0-6.0) and in the presence of 0-1.0% (w/v) NaCl (optimum, 0%). Tween 20 and gelatin are hydrolysed, but casein, tyrosine, starch, aesculin and Tween 80 are not. Weakly reduces nitrate to nitrite, but does not produce nitrogen gas. Alkaline phosphatase, esterase (C4), esterase lipase (C8), acid phospatase, naphtol-AS-BI-phosphohydrolase and urease activities are positive, but indole production, glucose fermentation, arginine dihydrolase, **Table 2.** Cellular fatty acid compositions (%) of strain IER25-16^T and the type strains of closely related *Methylobacterium* species

Taxa: 1, strain IER25-16^T; 2, *M. platani* KACC 14558^T; 3, *M. aquaticum* KACC 11543^T; 4, *M. tarhaniae* DSM 25844^T; 5, *M. organophilum* KACC 15217^T. All data were obtained from this study. The data are expressed as percentages of the total fatty acids. Major fatty acid components (>5.0%) are highlighted in bold. –, Not detected.

Fatty acid	1	2	3	4	5
Saturated:					
C _{9:0}	-	0.6	-	0.7	-
C _{12:0}	7.0	1.7	1.8	-	1.4
C _{14:0}	-	-	0.4	-	-
C _{16:0}	4.2	2.2	6.4	4.4	2.1
C _{18:0}	2.8	1.4	2.1	4.1	7.6
Unsaturated:					
$C_{14:1}\omega 5c$	-	-	-	0.5	0.4
C _{19:0} cyclo <i>w</i> 8c	1.5	-	-	-	-
C _{20:2} <i>ω</i> 6,9 <i>c</i>	1.5	-	-	-	0.6
Hydroxy:					
C _{8:0} 3-OH	-	0.5	-	0.9	0.9
iso-C _{15:0} 3-OH	1.6	0.6	-	0.7	0.7
C _{18:0} 3-OH	4.9	4.3	2.0	2.5	3.4
Summed feature*:					
2	1.9	2.3	2.3	2.7	1.5
3	0.9	0.7	1.5	1.2	-
8	73.8	85.9	83.4	82.4	81.6

*Summed features represent groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 2, C_{14:0} 3-OH and/or iso-C_{16:1} I; summed feature 3, C_{16:1} ω 7c; summed feature 8, C_{18:1} ω 6c and/or C_{18:1} ω 7c.

4-nitrophenyl- β -D-galactopyranoside, lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, N-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase activities are negative. Assimilation of L-arabinose, potassium gluconate, malic acid and trisodium citrate is positive, but assimilation of D-mannitol, N-acetyl-glucosamine, maltose, capric acid, D-mannose, phenylacetic acid, D-glucose and adipic acid is negative. Major fatty acids (>5 %) are $C_{12:0}$ and summed feature 8 (comprising $C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$). Q-10 is the major respiratory quinone and Q-9 is also detected as the minor respiratory quinone.

The type strain is $IER25-16^{T}$ (=KACC 19280^{T} =JCM 32048^{T}), isolated from an air conditioning system that operated in a laboratory in the Republic of Korea. The DNA G+C content is 70.0 mol%.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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