Sphingopyxis rigui sp. nov. and Sphingopyxis wooponensis sp. nov., isolated from wetland freshwater, and emended description of the genus Sphingopyxis

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Two yellow-pigmented, Gram-reaction-negative strains, designated 01SU5-PT and 03SU3-PT, were isolated from the freshwater of Woopo wetland, Republic of Korea. Both strains were aerobic, non-motile and catalase-negative. Phylogenetic analysis based on 16S rRNA gene sequences indicated that the two isolates belong to the genus Sphingopyxis, showing the highest level of sequence similarity with respect to Sphingopyxis witflariensis V-50T (95.4–95.7 %). The two novel isolates shared 99.4 % sequence similarity. DNA–DNA hybridization between the isolates and the type strain of S. witflariensis clearly suggested that strains 01SU5-PT and 03SU3-PT represent two separate novel species in the genus Sphingopyxis. The two strains displayed different fingerprints after PCR analysis using the repetitive primers BOX, ERIC and REP. Several phenotypic characteristics served to differentiate these two isolates from recognized members of the genus Sphingopyxis. The data from the polyphasic study presented here indicated that strains 01SU5-PT and 03SU3-PT should be classified as representing novel species in the genus Sphingopyxis, for which the names Sphingopyxis rigui sp. nov. and Sphingopyxis wooponensis sp. nov., respectively, are proposed. The type strain of Sphingopyxis rigui sp. nov. is 01SU5-PT (=KCTC 23326T=JCM 17509T) and the type strain of Sphingopyxis wooponensis sp. nov. is 03SU3-PT (=KCTC 23340T=JCM 17547T).

Sphingopyxis was introduced after the subdivision of the genus Sphingomonas by Takeuchi et al. (2001). Members of the genus Sphingopyxis are Gram-negative, non-fermentative, aerobic, non-spore-forming, yellow-pigmented or whitish-brown and contain sphingoglycolipids as cell envelope components (Yabuuchi et al., 1990; Takeuchi et al., 2001). Sphingopyxis species have been isolated from wastewater treatment systems (Takeuchi et al., 1993; Kämpfer et al., 2002; Kim et al., 2005), various types of soil (Takeuchi et al., 1993; Godoy et al., 2003; Lee et al., 2008a, b; Srinivasan et al., 2010; Choi et al., 2010; Zhang et al., 2010; Sharma et al., 2010), seawater (Vancanneyt et al., 2001; Yoon & Oh, 2005; Yoon et al., 2005; Kim et al., 2008) and natural mineral water (Lee et al., 2001). Most of the soils where the members of the genus Sphingopyxis have been isolated were contaminated with hydrocarbon or phenolic compounds or used as a landfill or ginseng field. At the time of writing, the genus comprises 17 species with validly published names. Strains 01SU5-PT and 03SU3-PT were isolated from a sample of surface freshwater collected from Woopo wetland (35° 33’ N 128° 25’ E) located in the Republic of Korea. At the time of water sampling, this wetland was full of vegetation that was in flower. Isolation was achieved with the standard dilution plating technique using PYGV (Staley, 1968) and R2A agar (Becton Dickinson) at 25 °C for 7 days. The isolate was routinely cultured on R2A agar and preserved at −80 °C as a suspension in distilled water containing 20 % glycerol (w/v). Sphingopyxis baekryungensis KACC 12342T, Sphingopyxis flavimaris KACC 12343T, Sphingopyxis litoris KACC 13026T, Sphingopyxis marina

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains 01SU5-PT and 03SU3-PT are HQ436492 and HQ436493, respectively.

One supplementary table and three supplementary figures are available with the online version of this paper.
KCTC 1276T and *Sphingopyxis* witflariensis KACC 12339T were used as reference strains.

DNA preparation, PCR amplification and sequencing of the 16S rRNA gene was carried out as described previously (Chun & Goodfellow, 1995). Sequence similarities were determined from pairwise 16S rRNA gene sequence comparisons using the EzTaxon-e server (Kim et al., 2012). Sequences were aligned by using CLUSTAL X (Thompson et al., 1997), and the alignment was refined using PHYDIT (Chun, 1995). A total of 1329 nt in unambiguously aligned positions was used for tree reconstruction. Phylogenetic analyses was performed by using PAUP* 4.0 (Swoford, 1998). Phylogenetic trees were inferred using the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) algorithms. Distance matrices for the neighbour-joining method were generated according to the model of Jukes & Cantor (1969). The robustness of the topology in the neighbour-joining phylogenetic tree was evaluated by bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings.

The 16S rRNA gene sequences of strains 01SU5-P and 03SU3-P were continuous stretches of 1435 nt. Sequence comparisons with 16S rRNA gene sequences held in GenBank indicated that the two isolates were closely related to the genus *Sphingopyxis*. Strains 01SU5-P and 03SU3-P shared 99.4 % sequence similarity. *S. witflariensis* W-50T showed the highest level of 16S rRNA gene sequence similarity with respect to strains 01SU5-P (95.4 %) and 03SU3-P (95.7 %). The sequence contained four of five signature nucleotides defined for the genus *Sphingopyxis* (Cluster IV) reported by Takeuchi et al. (2001), i.e. C–G at position 52 : 359, G at position 134, U at position 593, G–C at position 987 : 1218 and U–A at position 990 : 1215 (Escherichia coli numbering system).

The levels of 16S rRNA gene sequence similarity between strain 01SU5-P and the type strains of other *Sphingopyxis* species were in the range 93.7–95.4 % and the corresponding values for strain 03SU3-P were 93.9–95.8 %. The neighbour-joining tree showed that the two isolates formed a monophyletic clade but joined weakly with the marine isolate clade comprising the type strains of *S. baikryungensis*, *S. flavimarist*, *S. litoris* and *S. marina*, with 68 % bootstrap support (Fig. 1). This relationship was confirmed in the maximum-likelihood tree. Members of other clades that shared higher sequence similarity than those of the marine isolate clade were mainly isolated from soil and wastewater. This result suggested that the new isolates could form a new clade when more novel isolates are available from freshwater.

DNA–DNA hybridization was performed by the membrane filter technique using the DIG High Prime DNA Labelling and Detection Starter kit II (Roche Molecular Biochemicals) according to the method described in detail by Lee et al. (2003), with the modification that the hybridization temperature was 60 °C. The two new isolates shared a low DNA–DNA relatedness value (35 %), below the threshold (70 %) for determining bacterial species (Wayne et al., 1987); the finding strongly suggested that the two isolates should belong to separate genomic species in the genus *Sphingopyxis*.

We performed PCRs using the repetitive primers BOX, ERIC and REP to fingerprint the genomes of strains

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**Fig. 1.** Neighbour-joining tree based on 1329 nt in unambiguously aligned 16S rRNA gene sequences, showing the relationships between strains 01SU5-P and 03SU3-P, members of the genus *Sphingopyxis* and some other related taxa. Bootstrap values (>50 %) based on 1000 resamplings are shown as percentages at branch nodes. Circles indicate that corresponding nodes were recovered in trees generated with the maximum-likelihood method (filled circles) and the maximum-parsimony method (open circles). *Rhodospirillum rubrum* ATCC 11170T (CP000230) was used as an out-group (not shown). Bar, 0.01 nt substitutions per position.
01SU5-P^T and 03SU3-P^T using TaKaRa LA Taq polymerase (TAKARA BIO) and primers REP1R-I, REP2-I, ERIC1R, ERIC2 and BOXA1R (Louws et al., 1994). The banding patterns from these PCR experiments clearly showed differences between the two strains (Fig. S1 in IJSEM Online).

Growth on various standard bacteriological media was tested by using nutrient agar (NA; Becton Dickinson), R2A agar, plate-count agar (PCA; Becton Dickinson), glucose-yeast extract agar (GYEA; Gordon & Mihm, 1962), marine agar (MA; Becton Dickinson) and trypticase soy agar (TSA; Becton Dickinson) according to the manufacturers’ instructions. Cells grown on R2A agar at 25 °C for 2–3 days were used for physiological and biochemical tests. The Gram reaction test of cells grown on R2A agar at 25 °C for 2–14 days was performed by using the bioMérieux Gram stain kit according to the manufacturer’s instructions and the Ryu non-staining KOH method (Powers, 1995). Motility was examined by observing cells grown in wet mounts using phase-contrast microscopy (TMS-F; Nikon). Flagellation was determined by transmission electron microscopy (CM-20; Philips) using cells cultured for 48 h in R2A broth. Growth at various NaCl concentrations (0–10 %, w/v, using increments of 1.0 %) was investigated in R2A broth prepared according to the formula of the R2A agar medium except that no NaCl was used. Growth at pH 4–11 (increments of 1 pH unit) was determined using R2A broth medium containing 100 mM acetate buffer, 100 mM phosphate buffer and 100 mM NaHCO3/Na2CO3 buffer, at pH 4–5, 6–8 and 9–11, respectively. The optimal temperature and temperature range for growth was tested on R2A agar at 4–50 °C. Anaerobic growth was tested on R2A agar in a jar containing an AnaeroPack-Anaero (Mitsubishi Gas Chemical), which works as oxygen absorber and CO2 generator, for up to 10 days. Catalase and oxidase activities were tested in 3 % (v/v) hydrogen peroxide solution (Hanker & Rabin, 1975) and 1 % (w/v) p-tetramethyl phenylenediamine (bioMérieux), respectively. Acid production from sugars was tested as described by Yamaguchi & Yokoe (2000). Nitrate reduction was tested in 3 % (v/v) hydrogen peroxide solution according to the manufacturers’ instructions. Cells grown on R2A agar at 25 °C for 2–3 days were used for physiological and biochemical tests. The Gram reaction test of cells grown on R2A agar at 25 °C for 2–14 days was performed by using the bioMérieux Gram stain kit according to the manufacturer’s instructions and the Ryu non-staining KOH method (Powers, 1995). Motility was examined by observing cells grown in wet mounts using phase-contrast microscopy (TMS-F; Nikon). Flagellation was determined by transmission electron microscopy (CM-20; Philips) using cells cultured for 48 h in R2A broth. Growth at various NaCl concentrations (0–10 %, w/v, using increments of 1.0 %) was investigated in R2A broth prepared according to the formula of the R2A agar medium except that no NaCl was used. 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Degradation of the following macromolecules was tested using R2A agar as the basal medium and incubation at 25 °C for 10 days (all w/v; all Sigma): casein (2 % skimmed milk), starch (0.2 %), Tween 20 (1 %) and Tween 80 (1 %). Degradation was revealed by formation of clear zones around the colonies either directly or after flooding with adequate staining solutions (Smibert & Krieg, 1994). Hydrolysis of aesculin (0.01 %, w/v, aesculin; 0.05 %, w/v, ferric citrate), gelatin (12 %, w/v) and arginine dihydrolase was tested as described by Tindall et al. (2007) using R2A agar as the basal medium. Decomposition of tyrosine (0.5 %, w/v) was tested using R2A agar as the basal medium (Barrow & Feltham, 1993). DNase activity was determined with DNase test agar (Becton Dickinson). Some physiological characteristics and enzyme activities were determined using API 20NE and API ZYM kits (bioMérieux) according to the instructions of the manufacturer. Antibiotic resistance was determined with the disc diffusion method (Bauer et al., 1966) using commercial antibiotic-impregnated discs (Becton Dickinson). After 5 days of incubation at 25 °C on R2A agar, the results were interpreted according to the guidelines set out by the CLSI (2009).

The two new isolates were distinguished from closely related species with regard to motility and catalase activity: strains 01SU5-P^T and 03SU10-P^T were non-motile and catalase-negative. The two isolates showed differences between each other in terms of hydrolysis of casein, aesculin, tyrosine and Tween 20 and activities of leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase. The detailed results of physiological and biochemical analyses are given in Table 1 and the species descriptions.

For analyses of polar lipids, polyamine patterns and isoprenoid quinone, cells were grown in R2A broth for 5 days at 25 °C. Polar lipids were analysed by using standard procedures (Minnikin et al., 1984). Extracted lipids were separated by two-dimensional TLC (Minnikin et al., 1977) and identified by spraying with appropriate detection reagents (Embley & Wait, 1994). Polyamines were extracted according to Scherer & Kneifel (1983). The dansylated product was extracted with benzene and separated by TLC in chloroform/triethylamine (25:2: v/v; Wi et al., 2006). Isoprenoid quinone analysis was performed by reversed-phase TLC according to Collins (1994). For cellular fatty acid analysis, cells were grown in adequate media described below and harvested at late exponential phase. The two isolates and five reference type strains were grown on R2A agar (01SU5-P^T, 03SU10-P^T, S. baekryungensis, S. flavimaris and S. witflariensis) and MA (S. litoris and S. marina) for 5 days at 25 °C. The methyl esters were analysed by GLC (HP 6890; Hewlett Packard) and the MIDI aerobe method (Sherlock version 4.0) according to the instructions of the Microbial Identification System (Sasser, 1990; TSBA40 database). For G+C content calculations, the DNA sample was prepared in triplicate and determined by the thermal denaturation method of Marmur & Doty (1962).

Ubiquinone 10 (Q-10) was a major respiratory quinone with Q-8 and Q-9 as minor components in both of the new isolates. Diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol and sphingoglycolipid were found as major polar lipids in both isolates (Fig. S2). In addition to the common major polar lipids, strain 01SU5-P^T contained an unidentified glycolipid (GL1), while strain
**Table 1.** Phenotypic characteristics that differentiate strains 01SU5-PT and 03SU3-PT from their phylogenetic neighbours in the genus *Sphingopyxis*

<table>
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<th>Characteristic</th>
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<td>Reduction of nitrate to nitrite</td>
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<td>Catalase</td>
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<td>Gelatin</td>
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<td>Tween 80</td>
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<td>Decomposition of tyrosine</td>
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<td>Utilization of:</td>
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<td>D-Glucose</td>
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<td>Enzyme activity (API ZYM)</td>
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<td>Naphthol-AS-BI-phosphohydrolase</td>
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<td>Leucine arylamidase</td>
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<td>Trypsin</td>
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<td>Major fatty acids (&gt;10 %)</td>
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<td>C18:1ω7c, C16:1ω7c, C18:1ω7c, C16:1ω7c, C18:1ω7c, C16:1ω7c, C16:ω7c, C17:1ω6c, C15:0 2-OH, C17:ω8c, C15:0 2-OH, C17:1ω6c, C15:0 2-OH</td>
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<td>Signature nucleotides of 16S rRNA gene</td>
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<td>A</td>
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<td>G</td>
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<tr>
<td>DNA G+C content (mol%)†</td>
<td>57.3</td>
<td>53.3</td>
<td>63‡</td>
<td>58§</td>
<td>NDll†</td>
<td>NDll†</td>
<td>ND§</td>
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*Data different from Yoon et al. (2005).
†E. coli 16S rRNA numbering system was used.
‡Data from Yoon et al. (2005).
§Data from Yoon & Oh (2005).
¶Data from Kim et al. (2008).
§§Data from Kämpfer et al. (2002).
03SU3-P^T contained two unknown glycolipids (GL3 and 4). Spermidine was found as a major polyamine in both isolates.

The cellular fatty acid profile of strains 01SU5-P^T and 03SU10-P^T was mainly dominated by monounsaturated components. In both strains, C_{18:1}ω7c, summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-0H), C_{17:0}ω6c, C_{17:1}ω8c and C_{16:0} were the major fatty acids. A comparison of the cellular fatty acid compositions of strains 01SU5-P^T and 03SU10-P^T and some related species in the genus *Sphingopyxis* is given in Table S1. The DNA G+C content of strains 01SU5-P^T and 03SU10-P^T was 57.3±0.5 and 53.3±0.3 mol% (mean ± SD of three determinations), respectively.

On the basis of the data from the present polyphasic study, it is evident that strains 01SU5-P^T and 03SU10-P^T represent separate novel species in the genus *Sphingopyxis*, for which the names *Sphingopyxis rigui* sp. nov. and *Sphingopyxis wooponensis* sp. nov., respectively, are proposed. A revised description of the genus *Sphingopyxis* is presented.

**Description of Sphingopyxis rigui** sp. nov.

*Sphingopyxis rigui* (ri’gu.i. L. gen. n. *rigui*, of a well-watered place).

Cells are Gram-reaction-negative, aerobic, oxidase-positive, non-motile, short rods (0.4×0.6–0.8 μm in size; Fig. S3). Cells grow best on media such as R2A agar, NA and GYEa; slowly on PCA; not on MA or TSA. Colonies on R2A agar are convex, circular, smooth, opaque with entire margins, light yellow and approximately 0.6 mm in diameter after 5 days at 25 °C (pH 7). Growth occurs in 0–1% (w/v) NaCl (optimum, 0%), at pH 5–11 (optimum, pH 7) and at 10–35 °C (optimum, 25 °C). Catalase activity is absent. Reduces nitrate. Negative for arginine dihydrolase. Does not produce H_2S or indole. Aesculin, casein and Tween 80 are hydrolysed, but chitin, DNA, gelatin, starch, Tween 20 and urea are not. Decomposes tyrosine. Acid production from arabinose, amygdalin, glucose, inositol, mannitol, melibiose, rhamnose, sorbitol and sucrose is negative. Negative reactions are obtained for arginine dihydrolase, protease, urease and assimilation of adipate, arabinose, caprate, gluconate, glucose, malate, maltose, mannitol, mannosone, N-acetylgalactosamine and phenylacetate. Positive for β-galactosidase (with API 20NE). In the API ZYM gallery, positive reactions are found for acid phosphatase, alkaline phosphatase, leucine arylamidase, naphthol-AS-Bl-phosphohydrolase and trypsin; weakly positive reactions are obtained for esterase lipase (C8); negative reactions are found for N-acetyl-β-glucosaminidase, α-chymotrypsin, cysteine arylamidase, esterase (C4), x-fucosidase, x-galactosidase, β-galactosidase, x-glucosidase, β-glucuronidase, lipase (C14), x-mannosidase and valine arylamidase. Cells are sensitive to (µg per disc, unless otherwise indicated) amikacin (30), ampicillin (10), chloramphenicol (30), erythromycin (15), kanamycin (30) and vancomycin (30), but resistant to gentamicin (10), nalidixic acid (30), penicillin (10 IU), polymyxin B (300 IU), streptomycin (10) and tetracycline (30). Major fatty acids are C_{18:1}ω7c, summed feature 3 (iso-C_{15:0} 2-0H and/or C_{16:1}ω7c), C_{17:0}ω6c and C_{17:1}ω8c; the complete fatty acid composition is given in Table S1. Ubiquinone Q-10 is the predominant quinone type; small amounts of ubiquinone Q-8 and Q-9 are also present. Major polar lipids are diphostidylglycerol, phosophatidylethanolamine, phosphatidylglycerol, sphingoglycolipid and unidentified glycolipid GL1; small amounts of phosphatidylcholine and unidentified glycolipid GL2 are also present. Spermidine is present as the major polyamine compound, whereas spermine is detected only in small amount.

The type strain, 01SU5-P^T (=KCTC 23326^T=JCM 17509^T), was isolated from the freshwater of Woopo wetland in Gyeongnam Province, Republic of Korea. The DNA G+C content of the type strain is 57.3 mol%.

**Description of Sphingopyxis wooponensis** sp. nov.

*Sphingopyxis wooponensis* (woo.po.nen’sis. N.L. fem. adj. *wooponensis*, of or belonging to Woopo wetland, Republic of Korea, the geographical origin of the type strain of the species).

Cells are Gram-reaction-negative, aerobic, oxidase-positive, non-motile rods (0.3–0.4×0.7–1.1 μm in size; Fig. S1). Cells grow best on media such as R2A agar and GYEa; slowly on NA; not on MA, PCA or TSA. Colonies on R2A agar are convex, circular, smooth, opaque with entire margins, yellow and approximately 1.0 mm in diameter after 5 days at 25 °C (pH 7). Does not grow in 1% NaCl. Growth occurs at pH 6–11 (optimum, pH 7) and at 10–35 °C (optimum, 25 °C). Catalase activity is absent. Reduces nitrate. Negative for arginine dihydrolase. Does not produce H_2S or indole. Tween 20 and Tween 80 are hydrolysed, but aesculin, casein, chitin, DNA, gelatin, starch and urea are not. Does not decompose tyrosine. Acid production from arabinose, amygdalin, glucose, inositol, mannitol, melibiose, rhamnose, sorbitol and sucrose is negative. Negative reactions are obtained for arginine dihydrolase, protease, urease and assimilation of adipate, arabinose, caprate, glucuronate, glucose, malate, maltose, mannitol, mannosone, N-acetylglucosamine and phenyl-acetate. Positive for β-galactosidase (with API 20NE). In the API ZYM gallery, a positive reaction is found for alkaline phosphatase; weakly positive reactions are obtained for esterase lipase (C8) and trypsin; negative reactions are found for N-acetyl-β-glucosaminidase, acid phosphatase, α-chymotrypsin, cysteine arylamidase, esterase (C4), x-fucosidase, x-galactosidase, β-galactosidase, α-glucosidase, β-glucuronidase, lipase (C14), α-mannosidase and valine arylamidase. Cells are sensitive to (µg per disc, unless otherwise indicated) amikacin (30), ampicillin (10), chloramphenicol (30), erythromycin (15), gentamicin (10), kanamycin (30),...
penicillin (10 IU) and vancomycin (30), but resistant to nalidixic acid (30), polymyxin B (300 IU), streptomycin (10) and tetracycline (30). Major fatty acids are C_{18:1}ω7c, summed feature 3 (iso-C_{15:0}2-OH and/or C_{16:1}ω7c) and C_{17:1}ω6c; the complete fatty acid composition is given in Table S1. Ubiquinone Q-10 is the predominant quinone type; small amounts of ubiquinone Q-8 and Q-9 are also present. Major polar lipids are diphasphatidylglycerol, phospatidylethanolamine, phosphatidylglycerol, sphingolipid and unidentified glycolipids GL2 and GL3; a small amount of unidentified glycolipid GL4 is also present. Spermidine is present as the major polyamine compound, whereas spermine is detected only in small amount.

The type strain, 03SU3-p^{T} (=KCTC 23340^{T}=JCM 17547^{T}), was isolated from the freshwater of Woopo wetland in Gyeongnam Province, Republic of Korea. The DNA G+C content of the type strain is 53.3 mol%.

Emended description of the genus Sphingopyxis Takeuchi et al. 2001

The description of the genus is as given by Takeuchi et al. (2001) with the following modification. Cells are Gram-negative and catalase-variable.

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Two novel Sphingopyxis species


