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Simplicispira piscis sp. nov., isolated from the gut of a Korean rockfish, *Sebastes schlegelii*

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A novel Gram-stain-negative, aerobic, motile and rod-shaped bacterium, designated strain RSG39^T, was isolated from the gut of a Korean rockfish, *Sebastes schlegelii*. The 16S rRNA gene sequence analysis revealed that strain RSG39^T belonged to the genus *Simplicispira* in the class *Betaproteobacteria* and its highest sequence similarity was shared with *S. psychrophila* (98.4 %). The isolate grew optimally at 20 °C, at pH 7 and with 0 % (w/v) NaCl. The main respiratory quinone of the isolate was ubiquinone Q-8. The major cellular fatty acids were $C_{16:0}$, summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$) and summed feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$). The polar lipids of the isolate were phosphatidylglycerol, phosphatidylethanolamine, diphosphatidylglycerol and six unidentified lipids. The DNA–DNA hybridization values showed <7.4 % genomic relatedness with closely related strains. The genomic DNA G+C content was 65.2 mol %. Based on phylogenetic, phenotypic, chemotaxonomic and genotypic analyses, strain RSG39^T represents a novel species of the genus *Simplicispira*, for which the name *Simplocospira piscis* sp. nov. is proposed. The type strain is RSG39^T (=KACC 17539^T=JCM 19291^T).

The genus *Simplicispira* belongs to the family *Comamonadaceae* within the class *Betaproteobacteria*, and was proposed by Grabovich *et al.* (2006). The members of the genus *Simplicispira* are defined as Gram-staining-negative, aerobic, rod-shaped bacteria that contain ubiquinone-8 (Q-8) as a major quinone. At the time of writing, the genus *Simplicispira* comprises three species with validly published names: *Simplicispira metamorpha*, *Simplicispira psychrophila* (Grabovich *et al.*, 2006) and *Simplicispira limi* (Lu *et al.*, 2007).

The Korean rockfish *Sebastes schlegelii* is recognized as a commercially important ovoviviparous marine species in Korea and is distributed throughout shallow rocky shores along the coasts of Korea, Japan and China. Aquaculture of *Sebastes schlegelii* has been widely developed in Korea due to several beneficial characteristics for farming such as viviparous reproduction and environmental adaptation abilities (Lee *et al.*, 2000). Commensal or foreign microbes, which have probiotic or pathogenic effects on hosts, affect aquaculture efficiency and determine success or failure of aquaculture (Korkea-aho *et al.*, 2011; Santander *et al.*, 2013; Vine *et al.*, 2004). Due to the importance of gut commensal microbiota on the fishery industry, we have investigated microbiota of marine species and reported novel intestinal bacteria (Hyun *et al.*, 2013, 2014). During this ongoing study, we isolated a novel bacterial strain, designated strain RSG39^T, from *Sebastes schlegelii.* This paper describes the taxonomic characterization of this novel bacterial isolate based on a polyphasic analysis, which suggests that the isolate represents a novel species.

To isolate intestinal bacteria, the detached and homogenized intestinal tissue of farmed Korean rockfish *Sebastes schlegelii* was inoculated onto R2A agar (Difco) plates using the dilution-plating technique and incubated at 20 °C. Strain RSG39^T was isolated from a 10^{-2}-diluted sample after cultivation at 20 °C for 72 h. The isolate was purified by repeated subculture and stored at −80 °C as a suspension in R2A broth (MBcell) containing 40 % (v/v) glycerol. All physiological, biochemical, chemotaxonomic and genotypic analyses were repeated at least three times.

A phylogenetic analysis was performed based on 16S rRNA gene sequences. The 16S rRNA gene sequence of the isolate

Abbreviations: DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain RSG39^T is KC884002.

Four supplementary figures and a supplementary table are available with the online Supplementary Material.

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was amplified using Ex *Taq* PCR premix (Takara) with the universal bacterial primer pair, forward primer 8F and reverse primer 1492R (Lane, 1991). The 16S rRNA gene amplicon was sequenced using a BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems), according to the manufacturer's instructions. The reaction mixtures were analysed using an automated DNA analyser (3730xl DNA Analyser; Applied Biosystems). The sequenced 16S rRNA gene sequence fragments were assembled using SeqMan 5.0 (DNASTAR) and compared with the sequences of type strains in the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim *et al.*, 2012). The 16S rRNA gene sequences comparison revealed that strain RSG39^T shared 98.4 % similarity with *S. psychrophila* LMG 5408^T, 97.9 % similarity with *S. limi* KCTC 12608^T and 97.3 % similarity with *S. metamorpha* LMG 23381^T. A phylogenetic consensus tree was reconstructed to clarify the phylogenetic relationships between the isolate and closely related species. The sequences were aligned using CLUSTAL W (Thompson *et al.*, 1994) and used to reconstruct phylogenetic trees with MEGA 6 (Tamura *et al.*, 2013). The phylogenetic distances were calculated using the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1972) and maximum-likelihood (Felsenstein, 1981) algorithms based on 1000 bootstrap replicates. The phylogenetic trees showed that strain RSG39^T formed a cluster with members of the genus *Simplicispira* (Fig. 1 and Figs S1-2, available in the online Supplementary Material). To facilitate a more comprehensive and comparative characterization of strain RSG39^T, type strains of all species of the genus *Simplicispira* were used as reference strains. *S. psychrophila* LMG 5408^T and *S. metamorpha* LMG 23381^T were obtained from the Belgian Co-ordinated Collections of Microorganisms/Laboratorium voor Microbiologie, Universiteit Gent (BCCM/LMG), and *S. limi* KCTC 12608^T was obtained from the Korean Collection for Type Cultures (KCTC).

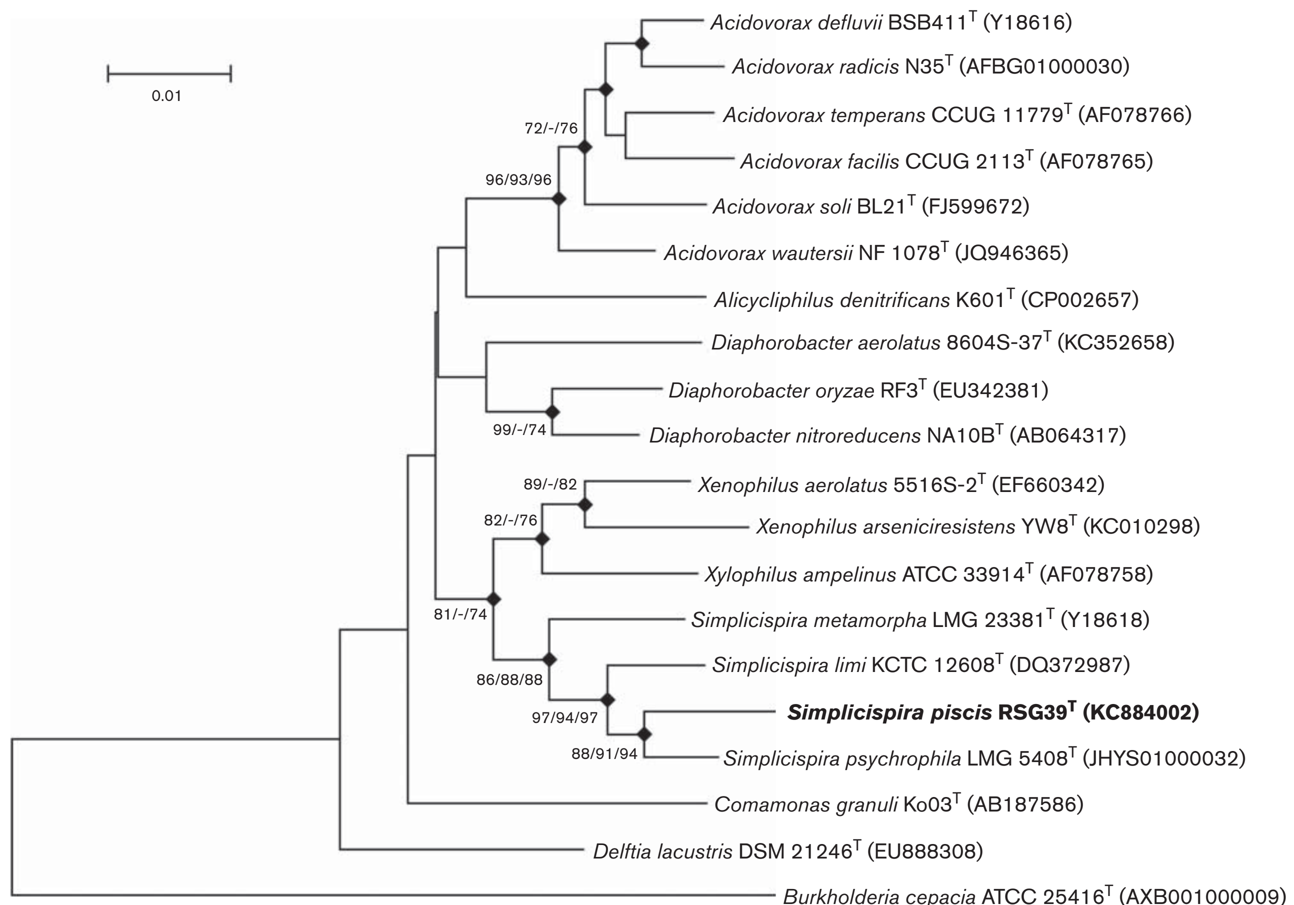

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences, showing the phylogenetic position of strain RSG39^T and closely related species. The tree was reconstructed using the neighbour-joining (NJ) algorithm. Filled diamonds represent identical branches generated using three algorithms: NJ, maximum-parsimony (MP) and maximum-likelihood (ML). MP-and ML-based phylogenetic trees are presented in Figs S1 and S2, respectively. Numbers at nodes indicate bootstrap values (NJ/MP/ML) as percentages of 1000 replicates; values <70 % are not shown. *Burkholderia cepacia* ATCC 25416^T (GenBank accession no. AXBO01000009) was used as an outgroup. Bar, 0.01 accumulated changes per nucleotide.

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The following characteristics were determined using cells of strain RSG39^T grown on R2A at 20 °C for 48 h. Gram-staining was performed using a Gram staining kit (bioMérieux) according to the manufacturer's instructions. Gram-staining and cell morphology were observed using a light microscope (Eclipse 50*i*; Nikon). Cell motility was tested in semi-solid R2A broth containing 0.4 % agar (Tittsler & Sandholzer, 1936). A transmission electron microscope (SUPRA VP55; Zeiss) was used to detect the presence of flagella. Cells of strain RSG39^T were Gram-stain-negative, rod-shaped (0.7–0.9 µm wide and 1.0–2.8 µm long) and motile with a polar flagellum (Fig S3). Strain RSG39^T forms translucent, circular, convex colonies with an entire margin that were slightly viscous and white–beige with a diameter of 0.5–1.0 mm on R2A. Growth of strain RSG39^T was tested at different temperatures, pH, and salinities. The isolate was cultured in R2A broth (MBcell) at 4, 10, 15, 20, 25, 30, 37, 40, 45, 55 and 65 °C, and at pH 4–11 (at intervals of 1.0 pH unit). The adjustment of the pH of R2A broth was achieved using 10 mM MES (for pH 4–6), 10 mM TAPS (for pH 7–8), or 10 mM Na_2HPO_4 (for pH 9–11). For the salinity test, the isolate was incubated in an R2A broth supplemented with 0, 1, 2, 3, 4, 5, 6, 8, 10, 12 or 15 % (w/v) NaCl. The turbidity of each individual culture was assessed by measuring the optical density at 600 nm in a spectrophotometer (Synergy MX; BioTek), according to the method of Hyun *et al.* (2015). Anaerobic growth of the isolate was tested after cultivation for 7 days at 20 °C on R2A agar plates in an anaerobic chamber filled with a $N_2/CO_2/H_2$ atmosphere (90 : 5 : 5, by vol.). Strain RSG39^T grew at 4–37 °C, at pH 5–9 and in the presence of 0–1 % (w/v) NaCl. The optimum growth conditions were 20 °C, pH 7 and 0 % (w/v) NaCl. Anaerobic growth was not observed. Unless stated otherwise, all of the tests used to characterize the isolate were conducted under optimal growth conditions.

To characterize the biochemical properties of strain RSG39^T, enzyme activities, utilization of sole carbon sources and acid production from different carbohydrates were compared with those of the type strains of *S. psychrophila*, *S. metamorpha* and *S. limi*. The biochemical tests were performed using cultures grown on R2A under optimal conditions for 48 h. Catalase activity was conducted according to bubble production in the presence of 3 % (v/v) hydrogen peroxide solution, and the presence of oxidase was determined by indophenol blue production using 1 % (w/v) tetramethyl-*p*-phenylenediamine (bioMérieux). Acid production from carbohydrates was tested using API 50 CH test strips (bioMérieux) with 50 CHB/E medium, according to the manufacturer's instructions. Utilization of various sole carbon sources was assessed using GN2 MicroPlates (Biolog) and GN/GP inoculating fluid (Biolog), according to the manufacturer's instructions. The enzyme activities were tested using API ZYM and API 20NE test strips (bioMérieux), according to the manufacturer's instructions. Strain RSG39^T was positive for catalase and oxidase activities, and could be distinguished from the reference strains by the utilization of various carbon sources, enzyme activities and acid production from various carbohydrates (Table 1). The results of the biochemical tests for the isolate are detailed in the species description.

To determine the chemotaxonomic characteristics of strain RSG39^T, the cellular fatty acid profile, isoprenoid quinone composition and polar lipid composition were compared with those of the reference strains. Chemotaxonomic analyses were performed using cell masses of the isolate and the reference strains after cultivation on R2A in the optimum growth conditions for 48 h. Cellular fatty acids were saponified, methylated and extracted, according to the protocol for the Sherlock Microbial Identification System (MIDI, 1999). Cellular fatty acid profiles of the isolate and the reference strains were determined using gas chromatography (6890 gas chromatograph; Agilent Technologies) and the Microbial Identification Software package (Sherlock version 6.2) with the TSBA6 database (Sasser, 1990). The major cellular fatty acids (>10 % of the total) of strain RSG39^T were summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$; 51.2 %), $C_{16:0}$ (19.4 %) and summed feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$; 16.9 %) (Table 2). Isoprenoid quinones were extracted from strain RSG39^T according to the method of Collins & Jones (1981a). The isoprenoid quinone extract was purified by one-dimensional thin-layer chromatography (TLC) on a silica gel 60 F_{254} plate (Merck) and identified by high-performance liquid chromatography (Collins & Jones, 1981b) using a reverse-phase Hydrosphere C18 (150×2.0 mm) column. A more comprehensive analysis of the isoprenoid quinone was performed by liquid chromatography (Ultimate 3000; Dionex) using an ion trap mass spectrometer, equipped with an electrospray ionization probe (HCT; Bruker), according to the method of Kaiser *et al.* (2012). The respiratory quinone of strain RSG39^T was ubiquinone-8 (Q-8), which is the major quinone in species of the genus *Simplicispira* (Grabovich *et al.*, 2006; Lu *et al.*, 2007). Polar lipids were extracted from the isolate and the reference strains according to the method of Xin *et al.* (2000), and separated by two-dimensional TLC on a silica gel 60 F_{254} plate (Merck). Two solvents were used for separation: chloroform/methanol/water (65 : 25 : 4, by vol.) for the first dimension and chloroform/methanol/acetic acid/water (80 : 12 : 15 : 4, by vol.) for the second dimension. Four spray reagents were used to detect the polar lipids (Tindall, 1990): 5 % ethanolic molybdatophosphoric acid for total lipids, ninhydrin for amino-group-containing lipids, Zinzadze reagent for phospholipids and α-naphthol for glycolipids. The phospholipids were identified by one-dimensional TLC on a silica gel 60 F_{254} plate (Merck) with chloroform/methanol/acetic acid/water (50 : 6 : 6 : 1, by vol.) using four standard compounds (Sigma): phosphatidylcholine, phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and diphosphatidylglycerol (DPG). Strain RSG39^T contained PG, PE, DPG and six unidentified lipids (Fig. S4). The isolate could be distinguished from *S. limi* based on presence of the major polar lipid component, PG.

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Table 1. Differential characteristics of strain RSG39^T and its closest phylogenetic relatives in the genus *Simplicispira*

Strains: 1, RSG39^T; 2, *S. psychrophila* LMG 5408^T; 3, *S. limi* KCTC 12608^T; 4, *S. metamorpha* LMG 23381^T. All data were obtained from this study except where indicated otherwsie. All strains were positive for the following: enzyme activities of leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase (API ZYM); acid production from aesculin and 5-ketogluconate (API 50 CHB); utilization of Tween 40, pyruvic acid methyl ester, succinic acid monomethyl ester, α-hydroxybutyric acid, β-hydroxybutyric acid, α-ketobutyric acid, α-ketovaleric acid, DL-lactic acid, propionic acid, L-aspartic acid, L-glutamic acid, L-leucine and L-threonine (Biolog GN2). All strains were negative for the following: enzyme activities of alkaline phosphatase, lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase (API ZYM); reduction of nitrates to nitrites, D-glucose fermentation, L-arginine dihydrolase, hydrolysis of aesculin and 4-nitrophenyl-β-D-galactopyranoside, assimilation of L-arabinose, D-mannose, D-mannitol, *N*-acetylglucosamine, maltose, potassium gluconate, capric acid, adipic acid and trisodium citrate (API 20 NE); acid production from erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, methyl β-D-xyloside, D-galactose, D-fructose, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl α-D-mannoside, methyl α-D-glucoside, *N*-acetylglucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose, glycogen, xylitol, gentiobiose, turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate and 2-ketogluconate (API 50 CHB); utilization of α-cyclodextrin, dextrin, glycogen, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, adonitol, L-arabinose, D-arabitol, cellobiose, *i*-erythritol, D-fructose, L-fucose, D-galactose, gentiobiose, *myo*-inositol, α-lactose, lactulose, maltose, D-mannitol, D-mannose, melibiose, methyl β-D-glucoside, D-psicose, raffinose, L-rhamnose, D-sorbitol, sucrose, trehalose, turanose, xylitol, *cis*-aconitic acid, citric acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, γ-hydroxybutyric acid, *p*-hydroxyphenylacetic acid, itaconic acid, malonic acid, quinic acid, D-saccharic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, L-histidine, L-phenylalanine, L-pyroglutamic acid, D-serine, DL-carnitine, inosine, uridine, thymidine, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, DL-α-glycerol phosphate, α-D-glucose 1-phosphate and D-glucose 6-phosphate (Biolog GN2). +, Positive or weakly positive; −, negative.

Characteristic	1	2	3	4
Cell morphology	Rods	Spirilla*[a]	Rods[b]	Spirilla[c]
Temperature for growth (°C)				
Range	4–37	2–25[a]	10–40[b]	3–38[c]
Optimum	20	20[a]	30[b]	30–32[c]
Enzyme activities (API ZYM / API 20 NE)				
Esterase lipase (C8)	+	+	+	−
Esterase (C4)	−	+	+	−
Urease	−	+	−	+
α-Chymotrypsin	−	−	+	−
Assimilation of (API 20 NE):				
Malic acid	+	+	+	−
Phenylacetic acid	−	−	+	+
D-Glucose	−	+	−	−
Acid production from (API 50 CHB):				
Glycerol	+	−	+	−
Starch	−	+	−	−
D-Glucose	−	−	+	−
Utilization of (Biolog GN2):				
Tween 80, L-proline	+	+	+	−
Acetic acid, succinamic acid	+	+	−	+
L-Asparagine	+	−	+	+
D-Alanine, L-alanine, hydroxy L-proline, L-serine, glycerol	+	−	+	−
Succinic acid	−	+	+	−
Formic acid	−	+	−	−
α-Keto glutaric acid	−	−	+	−
γ-Aminobutyric acid, urocanic acid	+	−	−	−
α-D-Glucose, bromosuccinic acid, glucuronamide, L-alaninamide, L-alanyl glycine, L-ornithine	−	−	+	−
Sebacic acid	−	−	−	+
Indole production	+	+	−	−
Gelatin hydrolysis	−	−	−	+
Reduction of nitrates to nitrogen	+	+	−	−
DNA G+C content (mol %)	65.2	65[a]	63.3[b]	63[c]
Isolation source	Korean rockfish	Municipal sewage plant[a]	Activated sludge[b]	Putrid infusion of freshwater shellfish[c]

*Data from: *a*, Mechichi *et al.* (2003) and Grabovich *et al.* (2006); *b*, Lu *et al.* (2007); *c*, Grabovich *et al.* (2006).

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Table 2. Cellular fatty acid contents (%) of strain RSG39T and the type strains of closely related species in the genus *Simplicispira*

Strains: 1, RSG39T; 2, *S. psychrophila* LMG 5408T; 3, *S. limi* KCTC 12608T; 4, *S. metamorpha* LMG 23381T. All data were obtained from this study. TR, Traces (<0.5 %); –, not detected.

Fatty acid	1	2	3	4
Saturated				
$C_{12:0}$	3.2	2.9	3.7	2.7
$C_{14:0}$	2.8	3.2	2.0	2.8
$C_{16:0}$	19.4	21.8	18.1	19.6
$C_{18:0}$	TR	0.7	TR	TR
Unsaturated				
$C_{18:1}\omega 9c$	–	0.5	–	–
Hydroxy				
$C_{8:0}$ 3-OH	TR	TR	0.7	–
$C_{10:0}$ 3-OH	3.4	2.8	4.3	4.1
Branched				
Cyclo $C_{17:0}$	1.2	0.7	–	TR
11-methyl $C_{18:1}\omega 7c$	TR	TR	0.7	–
Summed features*				
3	51.2	58.7	53.5	57.0
7	0.5		2.7	TR
8	16.9	8.1	13.9	12.9

*Summed features are groups of two or three fatty acids that could not be separated by the Microbial Identification System. Summed feature 3 comprises C16 : 1ω7c and/or C16 : 1ω6c; summed feature 7 comprises C19 : 1ω6c and/or unknown ECL (equivalent chain-length) 18.446; summed feature 8 comprises C18 : 1ω7c and/or C18 : 1ω6c.

To determine the genotypic characteristics of strain RSG39T, the genomic DNA G+C content was measured and DNA–DNA hybridizations were performed. Genomic DNA was extracted from strain RSG39T, *S. psychrophila* LMG 5408T, *S. limi* KCTC 12608T and *S. metamorpha* LMG 23381T according to the method of Rochelle *et al.* (1992). The DNA G+C content of the isolate was estimated by a fluorimetric method with SYBR Gold I (Invitrogen) using the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories) (Gonzalez & Saiz-Jimenez, 2002). Genomic DNA extracts from *Bacteroides thetaiotaomicron* VPI 5482T, *Streptococcus parasanguinis* ATCC 15912T, *Escherichia coli* K-12, *Ruegeria pomeroyi* DSS-3T and *Bacteroides fragilis* NCTC 9343T were used as calibration references. The DNA G+C content of the isolate was 65.2 mol%. To clarify the genetic relatedness between the isolate and the reference strains, DNA–DNA hybridization was performed using a genome-probing microarray (Bae *et al.*, 2005; Chang *et al.*, 2008). The DNA–DNA relatedness values were calculated based on the signal-to-noise ratios of the genomic probes (Loy *et al.*, 2005). The DNA–DNA relatedness values between strain RSG39T and *S. psychrophila* LMG 5408T, *S. limi* KCTC 12608T and *S. metamorpha* LMG 23381T were 7.4±1.2 % (reciprocal 6.8±0.4 %), 5.6±0.4 % (reciprocal 5.9±0.6 %) and 4.6±0.2 % (reciprocal 3.4±0.9 %), respectively (Table S1). These values were below the threshold of 70 %, indicating that the isolate is a distinct species (Wayne *et al.*, 1987).

The physiological, biochemical, chemotaxonomic, and genotypic analyses performed in this study suggest that strain RSG39T represents a novel species of the genus *Simplicispira*, for which the name *Simplicispira piscis* sp. nov. is proposed.

Description of *Simplicispira piscis* sp. nov.

Simplicispira piscis (pis′cis. L. gen. masc. n. *piscis* of a fish).

Cells are Gram-stain-negative, aerobic, motile with a polar flagellum and rod-shaped (0.7–0.9 µm×1.0–2.8 µm). Colonies are translucent, circular, convex with an entire margin, slightly viscous and white–beige with a dimeter of 0.5–1.0 mm after incubation on R2A for 48 h at 20 °C. Growth occurs at 4–37 °C (optimum 20 °C), at pH 5–9 (optimum pH 7) and with 0–1 % (w/v) NaCl (optimum 0 %). Positive for esterase lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase (API ZYM), catalase and oxidase activities. Acid is produced from glycerol, aesculin and 5-ketogluconate (API 50 CHB). Utilizes Tween 40, Tween 80, L-proline, acetic acid, succinamic acid, L-asparagine, D-alanine, L-alanine, hydroxy L-proline, L-serine, glycerol, γ-aminobutyric acid, urocanic acid, pyruvic acid methyl ester, succinic acid monomethyl ester, α-hydroxybutyric acid, β-hydroxybutyric acid, α-ketobutyric acid, α-ketovaleric acid, DL-lactic acid, propionic acid, L-aspartic acid, L-glutamic acid, L-leucine and L-threonine (Biolog GN2). Positive for indole production, reduction of nitrates to nitrogen, and malic acid assimilation (API 20 NE). The major fatty acids are summed feature 3 ($C_{16:1}\omega 7c$ and/or $C_{16:1}\omega 6c$), $C_{16:0}$ and summed feature 8 ($C_{18:1}\omega 7c$ and/or $C_{18:1}\omega 6c$). The sole ubiquinone is Q-8. The polar lipids comprise PG, PE, DPG and six unidentified lipids.

The type strain RSG39T (=KACC 17539T=JCM 19291T) was isolated from the gut of a Korean rockfish, *Sebastes schlegelii*. The DNA G+C content of the type strain is 65.2 mol%.

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