Fluviispira sanaruensis sp., nov., Isolated from a Brackish Lake in Hamamatsu, Japan

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Running title: *Fluviispira sanaruensis* sp., nov.,

Yoshiaki Maejima[1†], Takao Iino[2†], Ryota Moriuchi[3†], Koya Kushimoto[1], Yusuke Muraguchi[1], Kohei Fukuda[4], Hideaki Nojiri[5], Moriya Ohkuma[2], Hideo Dohra[3], Kazuhide Kimbara[1], Masaki Shintani[1,2,3,4*]

Author affiliations: [1]Department of Engineering, Graduate School of Integrated Science and Technology, Shizuoka University, 3-5-1 Johoku, Naka-ku, Hamamatsu, 432-8561, Shizuoka, Japan, [2]Japan Collection of Microorganisms, RIKEN BioResource Research Center, 3-1-1 Koyadai, Tsukuba, Ibaraki, 305-0074, Japan, [3]Research Institute of Green Science and Technology, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka, Shizuoka, 422-8529, Japan. [4]Department of Bioscience, Graduate School of Science and Technology, Shizuoka University, 3-5-1 Johoku, Naka-ku, Hamamatsu, 432-8561, Shizuoka, Japan, [5]Biotechnology Research Center, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo, 113-8561, Tokyo, Japan,

[*]**Corresponding author:** Masaki Shintani, shintani.masaki@shizuoka.ac.jp

ORCID Masaki Shintani (https://orcid.org/0000-0002-6505-9850)

[†]Yoshiaki Maejima, Takao Iino, and Ryota Moriuchi contributed equally to this work.

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Code availability: Not Applicable.

Author contributions: MS conceived, designed, and supervised the study. YM, TI, MY, FK, and MS performed the experiments and analyzed the data. RM and HD designed and performed the experiments and analyses of next generation sequencing. MY, TI, MO, KK, and MS wrote, reviewed, and edited the manuscript. All authors read and approved the final manuscript.

Abbreviations: JCM, Japan Collection of Microorganisms; LMG, BCCM/LMG Bacteria Collection, Ghent, Belgium; COD, chemical oxygen demand; PBS, phosphate-buffered saline; TEM, transmission electron microscopy; ANI, average nucleotide identity; AAI, average amino acid identity, POCP, percentage of conserved proteins; dDDH, digital DNA–DNA hybridization; DDH, DNA–DNA hybridization; ME, minimum-evolution; ML, maximum-likelihood; MP, maximum-parsimony; NJ, neighbor-joining; CDS, coding sequence

Abstract

Strain RF1110005^T, which was isolated from brackish lake water sampled at Lake Sanaru in Japan as a 'filterable' bacterial strain, was characterized as a novel species in the genus *Fluviispira*, family *Silvanigrellaceae*, order *Silvanigrellales*, the class *Oligoflexia* and the phylum *Bdellovibrionota*. Cells of RF1110005^T were aerobic, Gram stain negative, and show a pleomorphic morphology of spiral, filamentous and rod shapes. Catalase reaction was positive. Strain RF1110005^T grew optimally at 30ºC, pH 7.0-8.0 and 0.5% NaCl (w/v). The major polar lipids in RF1110005^T were phosphatidylethanolamine and phosphatidylglycerol. The predominant cellular fatty acids were iso-$C_{15:0}$ and anteiso-$C_{15:0}$. Phylogenetic analysis based on 16S rRNA gene sequences and concatenates of core gene sequence showed that the nearest neighbor of strain RF1110005^T was *Fluviispira multicolorata* strain 33A1-SZDP^T with 98.4% 16S rRNA gene sequence similarity. The genome size of strain RF1110005^T was 3.5 Mbp with two plasmids (80 kb and 69 kb), and the G + C content was 33.7 mol%. Comparisons with genome wide analyses and chemotaxonomic characters clearly showed that strain RF1110005^T differed from *F. multicolorata*. Therefore, a novel species in *Fluviispira sanaruensis*, sp. nov., is proposed for strain RF1110005^T (=JCM 31447^T =LMG 30360^T).

Introduction

The genus *Fluviispira* was established by Pitt et al. as a novel genus belonging to the novel family *Silvanigrellaceae* [1], assigned to the order *Silvanigrellales*, the class *Oligoflexia* and the phylum *Bdellovibrionota* [2–4]. The class *Oligoflexia* includes four orders, *Bacteriovoracales, Bdellovibrionales*, *Oligoflexales* and *Silvanigrellales* [3]. The *Silvanigrellales* includes the sole family *Silvanigrellaceae* composed of the two

genera *Silvanigrella* and *Fluviispira* [1]. The former genus includes two validly described species, *Silvanigrella aquatica* and *Silvanigrella paludirubra* with type strains MWH-Nonnen-W8red^T and SP-Ram-0.45-NSY-1^T, respectively [1], while only *Fluviispira multicolorata* 33A1-SZDP^T is within the latter [1]. The three strains were isolated from freshwater samples in Germany and Austria [1, 3]. Recently, *Pigmentibacter ruber* was proposed as a novel genus and species of the family *Silvanigrellaceae*, which was isolated from human blood [5]. Here we describe strain RF1110005, which was isolated from lake water sampled at Lake Sanaru, a brackish lake in Hamamatsu, Shizuoka, Japan [6]. This strain was one of filterable bacteria through a 0.22 μm pore size filter [7]. We propose to establish for this strain the species name *Fluviispira sanaruensis* sp. nov.

Materials and Methods

Isolation, Cultivation Conditions and Maintenance of Strains

Strain RF1110005 was isolated from lake water sampled at Lake Sanaru on Nov. 10^{th,} 2015, a brackish lake in Hamamatsu, Shizuoka, Japan (area: 1.1 km^2, maximum depth: 2.5 m, altitude: 0.1 m, salinity: around 1%, E137°41′15″, N34°42′30″), as one of 141 filterable bacteria in 2014-2015 [6]. The water sample was collected from the surface of the lake (0-20 cm depth), the temperature was 13°C and pH was around 8.0. The lake Sanaru is surrounded by upstream river with freshwater, and downstream tidal river connected to downstream another larger brackish lake Hamana, which is connected to the sea (area: 65 km^2, maximum depth: 16.6 m, altitude: 0 m, salinity 1-3%). The geographic features probably make the chemical oxygen demand (COD) of the lake water high, being one of the highest among

92 Japanese lakes (> 8 mg/L; Japanese report for the quality of environmental water in
93 Shizuoka prefecture in Japan (http://www.hamamatsu-
94 kankyo.jp/suishitsu/district/naka_situation.htm). Strain RF1110005^T was routinely
95 cultivated on R2A agar (Difco, BD Biosciences, Franklin Lakes, NJ, USA) for 6 days
96 at 30ºC. As for the liquid culture, DAIGO R2A liquid medium (Nihon Pharmaceutical
97 Co., Ltd, Tokyo, Japan) was used because the former R2A agar contains agarose.

98 **Microscopy Observation**

99 A single colony of the strain RF1110005^T on R2A agar plate after 6 days of
100 cultivation at 30°C was used for microscopy observation. The observation of the cells
101 with fluorescent and/or phase contrast imaging was performed with BZ-X700
102 (KEYENCE Corp.) for the cells resuspended in phosphate-buffered saline (PBS, pH
103 7.4) after staining the cells with SYBR Green (Lonza Rockland, ME, USA). The cells
104 stained with 2% (w/v) phosphotungstic acid were observed on electron microscopy
105 grids and observed with transmission electron microscopy (TEM: JEM-2000RXII).
106 The TEM images were taken on JEM-2000FX-II (JEOL) electron microscope at 160
107 kV after fixation of strain RF1110005^T.

108 **Flow cytometry and Cell Sorter**

109 The cells with different sizes of strain RF1110005^T were separately collected at the
110 single cell level using flow cytometry and cell sorter MoFlo XDP® IntelliSort II
111 instrument (Beckman Coulter Inc., Denver, MA, USA) equipped with a CyClone
112 robotic arm for plate sorting, using a 488-nm argon laser and a 70-µm nozzle orifice.
113 The sorting was performed under previously described conditions [8] with a
114 modification for the gate setting, which was based on forward scatter and side
115 scatter after taking the logarithm. Each single cell was separately sorted onto R2A

agar plates and then incubated at 30ºC. After the cell formed a colony, the colony was suspended in PBS, and a part of the resultant suspension was observed by phase contrast microscopy (BZ-X700; KEYENCE Corp.).

Phylogenetic Analysis

The total DNA of strain RF1110005^{T} was extracted using Nucleospin® Tissue Kit (Takara Bio). The 16S rRNA gene of one of the colonies was amplified with primers (27F, 5´-AGAGTTTGATCMTGGCTCAG-3´ and 1492R, 5´-TACGGYTACCTTGTTACGACTT-3´) using ExTaq DNA polymerase, and the amplicon was again cloned into pMD20 (Takara Bio). Partial nucleotide sequences of the resultant plasmids were determined with 805R primer (5´-GACTACCAGGGTATCTAATC-3′), while the full length of the one among inserts was determined with primers (357F, 5´-CTCCTACGGGAGGCAGCAG-3´; 518R, 5´-GTATTACCGCGGCTGCTGG-3´; 1100R, 5´-AGGGTTGCGCTCGTTG-3´) in addition to 27F and 1492R by the Sanger sequencing method. Comparisons of the 16S rRNA gene sequences with related strains were performed with EzBioCloud server (https://www.ezbiocloud.net/identify) [9]. Phylogenetic analyses were performed by the maximum likelihood method (Kimura 2-parameter method [10]), neighbor-joining method [11] (Tamura-Nei method [11]), and the minimum evolution method [12] (Tamura-Nei method [11]) by MEGA 7.0 [13] after alignment of the sequences by ClustalW [14].

Genome Sequencing and Their Characterization

The genome sequences of the strain RF1110005^{T} were determined using MiSeq technology with a paired-end library (2 x 301 bp) prepared using the TruSeq DNA PCR-free library preparation kit (Illumina Inc., San Diego, CA, USA). The high-quality

reads were assembled in the SPAdes software [15] with a default set of k-mer sizes. The finishing was aided by GenoFinisher and AceFileViewer [16]. Genome-wide comparisons were performed between strain RF1110005^T (GenBank accession no. AP019368) and other related strains (Table S1). Phylogenetic analysis based on whole genome sequences was performed using GTDB-Tk v. 1.4.1. [17] to identify core genes and generate concatenated alignment of their translated amino acid sequences, which was used for construction of a phylogenetic tree using a randomized axelerated maximum likelihood (RAxML) tool [18]. The average nucleotide identity (ANI) [19], average amino acid identity (AAI) [20] and percentage of conserved proteins (POCP) [21] was calculated as described previously [22]. Digital DNA-DNA hybridization (dDDH) analyses were performed by using Genome-to-Genome Distance Calculator 2.1 (http://ggdc.dsmz.de/ggdc.php) [23].

Reference Strain

Based on the phylogenetic analyses and 16S rRNA gene sequence similarity, *Fluviispira multicolorata* 33A1-SZDP^T was selected as a reference strain for comparative study. The reference strain 33A1-SZDP^T was obtained from JCM (designated as JCM 32978^T).

Morphological and Physiological Characterization, Biochemical Analysis, and Chemotaxonomic Characteristics

Gram staining was performed with the Gram-staining kit (FUJIFILM Wako Pure Chemical Corp.) according to the manufacturer's instruction. Temperature ranges (4ºC and 34 ºC, 10-50ºC at 5ºC intervals) and pH ranges (3.5-10.5, in increments of 0.5 pH units by the addition of HCl or NaOH) for growth of strain RF1110005^T were tested using DAIGO R2A liquid medium (Nihon Pharmaceutical Co., Ltd, Tokyo,

Japan). The pH of each medium was adjusted after autoclaving. The pH was also measured when the strain RF1110005^T began growing in the media to confirm that pH was maintained at that time. The longest incubation time was 4 months. Catalase reaction was tested by placing drops of 3% (v/v) H_2O_2 solution directly on cells cultivated on R2A agar and observing gas evolution. Oxidase reaction was tested using a Cytochrome Oxidase Test Strip (Nissui) according to the manufacturer's instructions. Other characters were compared by using API 50 CH, API20NE, and API ZYM (bioMérieux, Marcy-l'Étoile, France) according to the manufacturer's instructions except for the cell biomass (twice number of cells (McFarland Standard point 4 instead of point 2) were used for assays in API 50 CH).

Chemotaxonomic Characterization

The cells of strain RF1110005^T and *F. multicolorata* JCM 32978^T grown on R2A agar at 30 and 25 °C for seven days were harvested for the determination of the chemotaxonomic characteristics. The polar lipids pattern of the strain was determined by using two-dimensional TLC and spraying with 5% ethanolic molybdophosphoric acid, ninhydrin, Dittmer & Lester reagent, anisaldehyde reagent and Dragendoff's reagent, as described previously [24, 25]. The major isoprenoid quinone was determined by the HPLC method described by Komagata & Suzuki [26]. The Sherlock Microbial Identification System (MIDI) version 6.0 (MIDI Inc., Agilent Technologies, Newark, NJ, USA) was used for identifying and quantifying the cellular fatty acids based on the method described by Sasser [27].

Accession Numbers: The DDBJ/EMBL/GenBank accession numbers for the 16S rRNA gene and complete genome sequence of *Fluviispira sanaruensis* RF1110005^T are LC349851 (16S rRNA gene), AP019368 (chromosome), AP019369 (79K

188 plasmid), and AP019370 (68K plasmid).

189

190 **Results and Discussion**

191 A pure colony was obtained after several rounds of purification plated each time with
192 a single colony of strain RF1110005^T (Fig. S1a). The cells showed spiral,
193 filamentous, and rod-shaped forms (Fig. S1b, c). To confirm culture purity, cells with
194 different sizes were separately sorted onto R2A agar plates, using flow cytometry
195 and cell sorter. The cell shapes of them were observed by phase contrast
196 microscopy after they formed colonies. The main cell shapes were spirals (Fig. 1a)
197 but some filamentous or rod forms were observed (Fig. 1b), even though they were
198 all collected from a single colony on an R2A agar plate, on which the single cell of
199 strain RF1110005^T had been sorted. These different cell forms were usually
200 observed regardless of initial sizes of the sorted cells (data not shown). The total 28
201 clones of the 16S rRNA gene showed almost identical sequences for 531 bp (the five
202 clones had one or two nucleotide differences, which might be due to PCR error,
203 because the mismatch sites were different in each clone). The purity of strain
204 RF1110005^T was also confirmed by its complete genomic sequencing, and it
205 contained five 16S rRNA genes, four of them showed identical sequences whereas
206 the other one had one different nucleotide (see below). Considering these results,
207 we concluded that strain RF1110005^T showed pleomorphic cell morphology.

208 The 16S rRNA gene sequence of strain RF1110005^T has been deposited in
209 GenBank (accession number LC349851). Phylogenetic analysis of the 16S rRNA
210 gene sequences of strain RF1110005^T and other related strains showed that the
211 strain RF1110005^T located nearest to *Fluviispira multicolorata* 33A1-SZDP^T (Fig. 2).

Its 16S rRNA gene sequence showed 98.4% similarity to *F. multicolorata* 33A1-SZDP^T. As for other related strains, genera *Silvanigrella* [1, 3] and *Pigmentibacter* [5] were found, which were closely related to "*Candidatus* Spirobacillus cienkowskii", which is an uncultured pathogen of water fleas (*Daphnia* spp.) morphologically described in the 19th century [28].

Complete genome sequence of strain RF1110005^T was successfully obtained. It had a single circular chromosome (3.52 Mb) and two plasmids [pRF1110005S (68.8 kb) and pRF1110005L (79.5 kb)], which have been deposited in GenBank [accession no. AP019368 (chromosome), AP019369 (79K plasmid), and AP019370 (68K plasmid)]. Three replicons were obtained with 234X (chromosome), 321X (79K plasmid), 313X (68K plasmid) coverage. The chromosome had 2,933 coding sequences (CDSs), while 65 and 76 CDSs were found in the respective plasmids. Strain RF1110005^T had five ribosomal operons and 43 tRNA genes. The DNA G+C content of the chromosome was 33.7 mol% based on its nucleotide sequences. Of the 120 bacterial single-copy marker genes previously proposed for genome phylogeny [29], 108–115 marker genes (108 for strain RF1110005, Table S2) were identified from the 13 genome sequences using GTDB-Tk [17]. Their translated amino acid sequences were concatenated into a single multiple sequence alignment, trimmed to 5,040 amino acids, and used for the construction of a RAxML phylogenetic tree (Fig. 3). The results of dDDH showed 20.7% to *F. multicolorata* 33A1-SZDP^T (Table S1). The ANI value of genome sequence of strain RF1110005^T was 79.6-79.8% to *F. multicolorata* 33A1-SZDP^T (Fig. S2a). This value was lower than 95-96%, which is a general ANI cut-off value for different species [30]. The AAI value of strain RF1110005^T was 68.9-71.9% to strain 33A1-SZDP^T (Fig. S2b). The POCP value of strain RF1110005^T was 80.7% to *F. multicolorata* 33A1-SZDP^T (Fig.

S2c). In addition to the fact that the similarity of 16S rRNA genes between strain RF1110005^T and 33A1-SZDP^T was lower than the cut-off value recommended for species differentiation (98.7-99.0%) [31], the genome wide features indicated that strain RF1110005^T could represent a new species of the genus *Fluviispira*.

Cells of strain RF1110005^T were non-spore forming for all types of cells, and only spiral cells were motile. Gram staining was negative for all cells using the Gram-staining kit (FUJIFILM Wako Pure Chemical Corp., Osaka, Japan). To confirm that strain RF1110005^T was smaller than the pore size of the filter, the cells of the strain were observed with TEM after being stained with 2% (w/v) phosphotungstic acid. The major spiral form of the RF1110005^T cells was 0.1-0.2 μm wide, 1.8-3.2 μm long, and 0.4-0.6 μm in diameter of spirals (Fig. 1c, d). A single polar flagellum was observed from spiral cells under transmission electron microscopy (Fig. 1d). No flagella were observed for filamentous or rod forms (Fig. 1c). The filamentous formed cells were 0.3-0.5 μm wide and 8.1-36.0 μm long, and the rod formed cells were 0.3-0.8 μm wide and 1.1-3.1 μm long (Fig. 1c). These facts indicated that the spiral cells could go through the pore size of the filter.

The temperature range for growth of strain RF1110005^T was from 10-30ºC, with an optimum temperature of 30ºC; the pH range for growth was 6.5-9.0, with an optimum pH range of 7.0-8.0. Growth in R2A liquid medium with 0, 0.5, 1.0, 2.0 and 3.0% (w/v) NaCl was investigated, and growth occurred at 1.0% (w/v) or less, with an optimum salinity of 0.5% (w/v) NaCl.

Physiological and biochemical characterizations were compared between strain RF1110005^T and *F. multicolorata* JCM 32978^T and the phenotypic traits characterizing the two strains are shown in Table 1. As described above, the

morphology of strain RF1110005^T was variable, which and these features were similar to those of *F. multicolorata* [1]. Both strains were aerobic. Colonies of strain RF1110005^T are circular, flat, entire, or undulate and salmon pink with 1.0-3.0 mm diameter on R2A agar plate after 5 days of cultivation.

Catalase reaction was positive for strain RF1110005^T but negative for JCM 32978^T. Oxidase reaction was negative for RF1110005^T and JCM 32978^T. Acid productions were detected from D-galactose, D-glucose, D-mannose, D-lactose by the strain RF1110005^T, but not by *F. multicolorata* JCM 32978^T (Table 1).

The polar lipids of strain RF1110005^T mainly comprised phosphatidylethanolamine and phosphatidylglycerol with unidentified amino-phospholipid and unidentified glycolipids (Fig. S3). The isoprenoid quinone could not be detected in strain RF1110005^T, while menaquinone 8 was detected in *F. multicolorata* 33A1-SZDP^T [1]. The fatty acids are shown in Table 2. The predominant cellular fatty acids composition of strain RF1110005^T were iso-$C_{15:0}$ (23.1%), anteiso-$C_{15:0}$ (10.0%).

Morphological, biochemical and physiological characteristics of strain RF1110005^T, along with those of *F. multicolorata* are summarized in Table 1. Notably, strain RF1110005^T was catalase-positive, but *F. multicolorata* was negative. The color of colonies was salmon pink for RF1110005^T, while that was purple for *F. multicolorata* (Table 1). Acid production was clearly detected from L-arabinose, D-xylose, D-galactose, D-glucose, or D-mannose, weakly from D-fucose for RF1110005^T, while no production was detected for JCM 32978^T but just weakly from D-glucose, or D-mannose (Tables 1, S3-1). As for assays with API20NE, both strains were positive for gelatin hydrolysis and weakly positive for esculin hydrolysis and D-

285 glucose fermentation (Table S3-2). In assays with API ZYM MicroPlates, both strains
286 were positive for alkaline phosphatase, acid phosphatase, naphthol-AS-BI-
287 phosphohydrolase and *N*-acetyl-β-glucosaminidase, weakly positive for esterase
288 (C4) and esterase lipase (C8) (Table S3-3). The fatty acid patterns of strains
289 RF1110005^T and JCM 32978^T were almost identical, but slightly differed in their ratio
290 as shown in Table 2. The levels of genome similarity were sufficient to differentiate
291 strain RF1110005^T from *F. multicolorata*, which represents a new species in the
292 genus *Fluviispira.*

293 The major cell morphology of RF1110005^T was a spiral (Figure 1), which
294 could be categorized as 'slender filamentous bacteria' by Nakai [7]. Similar shaped
295 cells were found in other filterable bacteria includes *F. multicolorata* 33A1-SZDP^T,
296 *Silvanigrella aquatica* MWH-Nonnen-W8red^T, *Silvanigrella paludirubra* SP-Ram-
297 0.45-NSY-1^T and *Oligoflexus tunisiensis* Shr3^T [1, 2] as well as *Hylemonella gracilis*
298 CB, which was isolated from filtrates of freshwater samples [32, 33]. The 'slender
299 filamentous bacteria' including RF1110005^T were thought to be able to go through
300 the filter pore by "squeezing" [7]. It should be noted that the former three strains and
301 strain RF1110005^T showed pleomorphism, including slender filamentous, spiral,
302 spherical or curled, or curved rod morphology [1, 2, 34]. The pleomorphism could be
303 one of characteristic features of the bacterium within class *Oligoflexia*. The reason
304 why these strains showed variable cell morphology was not clear, but it might be
305 beneficial to adapt to various environments (pH, salinity, and/or nutrients), by
306 changing their cell shapes.

307 Interestingly, including strain RF1110005^T, we successfully isolated various
308 filterable bacteria from one lake, Lake Sanaru [6], whereas the others were isolated

309 from various environmental sites in the other studies reviewed by Nakai [7]. Among
310 the isolates from Lake Sanaru, two other strains have been already proposed as
311 novel genus and/or species, although they did not show pleomorphism. One of them
312 is including a novel genus named *Chryseotalea* (*C. sanaruensis* Ys^T, rod-shaped
313 [35], within the family *Cytophagaceae*, and the other is a novel species of genus
314 *Algoriphagus* (*A. sanaruensis* M8-2^T, curved-rod-shaped [36]). This could be due to
315 geographical characteristics of the connecting point of Lake Sanaru and tidal river,
316 where the high salinity water and fresh water are mixed, in which the former is from
317 the downstream another brackish lake Hamana and the latter is from the upstream
318 lake and river. Further in-depth analyses of the lake and surroundings and ecology of
319 them will be necessary to understand the role(s) of these filterable bacteria in the
320 lake.

321 In conclusion, we proposed that strain RF1110005^T represents a novel
322 species of *Fluviispira*, for which, the name *F. sanaruensis* sp. nov., is proposed.

323 **Description of *Fluviispira sanaruensis* sp. nov.**

324 *Fluviispira sanaruensis* sp. nov. (sa.na.ru.en´sis. N.L. fem. adj. *sanaruensis* of or
325 belonging to Lake Sanaru, Hamamatsu, Japan, referring to the isolation source of
326 the type strain).

327 Gram-strain-negative, non-sporulating, motile, mesophilic, neutrophilic,
328 heterotrophic, aerobic bacterium. Cells form spirals, sometimes filamentous or rods.
329 Catalase-positive and oxidase-negative. Majority of cells are spirals (0.1-0.2 μm in
330 width, 1.8-3.2 μm in length and 0.4-0.6 μm in diameter of spirals), while some are
331 filamentous (0.3-0.5 μm in width, 8.1-36.0 μm in length) and rods (0.3-0.8 μm in
332 width, 1.1-3.1 μm in length). Colonies are circular, flat, entire or undulate and salmon

pink on R2A agar plate after 5 days of cultivation. The temperature for growth is 10-30ºC with an optimum growth at 30ºC. Grows at pH 6.5-9.0 with an optimum around pH 7.0-8.0. Growth occurs 1% (w/v) NaCl or less. In assays with API 50 CH, positive for L-arabinose, D-xylose, D-galactose, D-glucose, and D-mannose, weakly positive for D-fucose. As for assays with API20NE, positive for gelatin hydrolysis and weakly positive for esculin hydrolysis and D-glucose fermentation. In assays with API ZYM MicroPlates, positive for alkaline phosphatase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and *N*-acetyl-β-glucosaminidase, weakly positive for esterase (C4) and esterase lipase (C8). The predominant cellular fatty acids are iso-$C_{15:0}$ and anteiso-$C_{15:0}$. The major polar lipids are phosphatidylethanolamine and phosphatidylglycerol.

The type strain is RF1110005^T (=JCM 31447^T =LMG 30360^T), which was isolated from lake water sampled at a brackish lake (Lake Sanaru) in Hamamatsu, Japan. The DNA G+C content of the type strain is 33.7 mol% (Genome sequence). The DDBJ/EMBL/GenBank accession numbers for the 16S rRNA gene and complete genome sequence of *Fluviispira sanaruensis* RF1110005^T are LC349851 (16S rRNA gene), AP019368 (chromosome), AP019369 (79K plasmid), and AP019370 (68K plasmid).

Emend description of *Fluviispira multicolorata* Pitt *et al.* 2020

The following characteristics are emended in addition to the species *Fluviispira multicolorata*. Catalase-negative and oxidase-negative.

Declarations

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

Ethical Approval: Ethical Approval This study does not describe any experimental work related to humans.

Consent to participate: Not Applicable

Availability of data and material: The DDBJ/EMBL/GenBank accession numbers for the 16S rRNA gene and complete genome sequence of *Fluviispira sanaruensis* RF1110005^T are LC349851 (16S rRNA gene), AP019368 (chromosome), AP019369 (79K plasmid), and AP019370 (68K plasmid). Strain RF1110005^T have been deposited in bacterial culture collection centers, JCM and BCCM/LMG.

Code availability: Not Applicable.

Author contributions: MS conceived, designed, and supervised the study. YM, TI, MY, FK, and MS performed the experiments and analyzed the data. RM and HD designed and performed the experiments and analyses of next generation sequencing. MY, TI, MO, KK, and MS wrote, reviewed, and edited the manuscript. All authors read and approved the final manuscript.

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Table 1. Differential characteristics of strain RF1110005^T from *Fluviispira multicolorata*.

Strains: 1, RF1110005^T; 2, *Fluviispira multicolorata* JCM 32978^T (=33A1-SZDP^T). +, positive; w, weakly positive; -, negative.

Characteristics	**1**	**2**
Catalase	+	-
Cell size (μm)	0.1-0.2×1.8-3.2 μm, 0.4-0.6 μm in diameter of spirals (spiral) 0.3-0.5×8.1-36.0 μm (filamentous) 0.3-0.8×1.1-3.1 μm (rod)	Pleomorphic*
Pigmentation (agar plates)	Salmon pink	Purple
Temperature for growth (°C)		

Range	10-34	6-34 (w)*
NaCl requirement for growth (%, w/v)		
Range	0-1	1.0-1.2 (w)*
Acid production from		
L-Arabinose	+	-
D-Xylose	+	-
D-Galactose	+	-
D-Glucose	+	w
D-Mannose	+	w
D-Fucose	w	-

DNA G+C (mol%)	33.7	32.2

*Data from Pitt *et al.* [1]

Table 2. Cellular fatty acid composition (ratio %) of strain RF1110005^T and *Fluviispira multicolorata*. Strains: 1, RF1110005^T; 2, JCM 32978^T (=33A1-SZDP^T). 'tr' and '-' indicates trace (<0.2%) and not detected, respectively, and fatty acids with more than 10% are in bold.

	1	2
Saturated straight-chain:		
$C_{13:0}$	0.0	1.0
$C_{14:0}$	7.2	5.7
$C_{15:0}$	5.6	4.5
$C_{16:0}$	9.5	7.2
$C_{17:0}$	3.3	3.3
Unsaturated straight-chain:		
$C_{13:1}$ at 12-13	1.3	-

$C_{15:1}\omega 6c$	tr	1.7
$C_{17:1}\omega 8c$	6.5	5.3
Saturated branched-chain:		
iso-$C_{14:0}$	3.2	9.4
iso-$C_{15:0}$	**20.2**	**19.3**
anteiso-$C_{15:0}$	**12.0**	**14.1**
iso-$C_{16:0}$	-	2.2
iso-$C_{17:0}$	-	-
anteiso-$C_{17:0}$	-	-
Hydroxy acids:		
$C_{8:0}$ 3-OH	-	1.3

$C_{9:0}$ 3-OH	tr	2.2
$C_{10:0}$ 3-OH	3.4	2.4
$C_{11:0}$ 3-OH	3.1	-
iso-$C_{11:0}$ 3-OH	0.9	2.5
$C_{12:0}$ 3-OH	1.8	2.1
iso-$C_{13:0}$ 3-OH	-	-
iso-$C_{14:0}$ 3-OH	3.1	tr
$C_{16:0}$ 3-OH	2.4	tr
$C_{17:0}$ 3-OH	3.0	-
*Summed feature:		
1 ($C_{13:0}$ 3OH/$C_{15:1}$ I H)	1.9	-

2 ($C_{16:1}$ iso I/$C_{14:0}$ 3OH)	tr	-
3 ($C_{16:1}\omega7c$/$C_{16:1}\omega6c$)	**11.5**	**15.8**

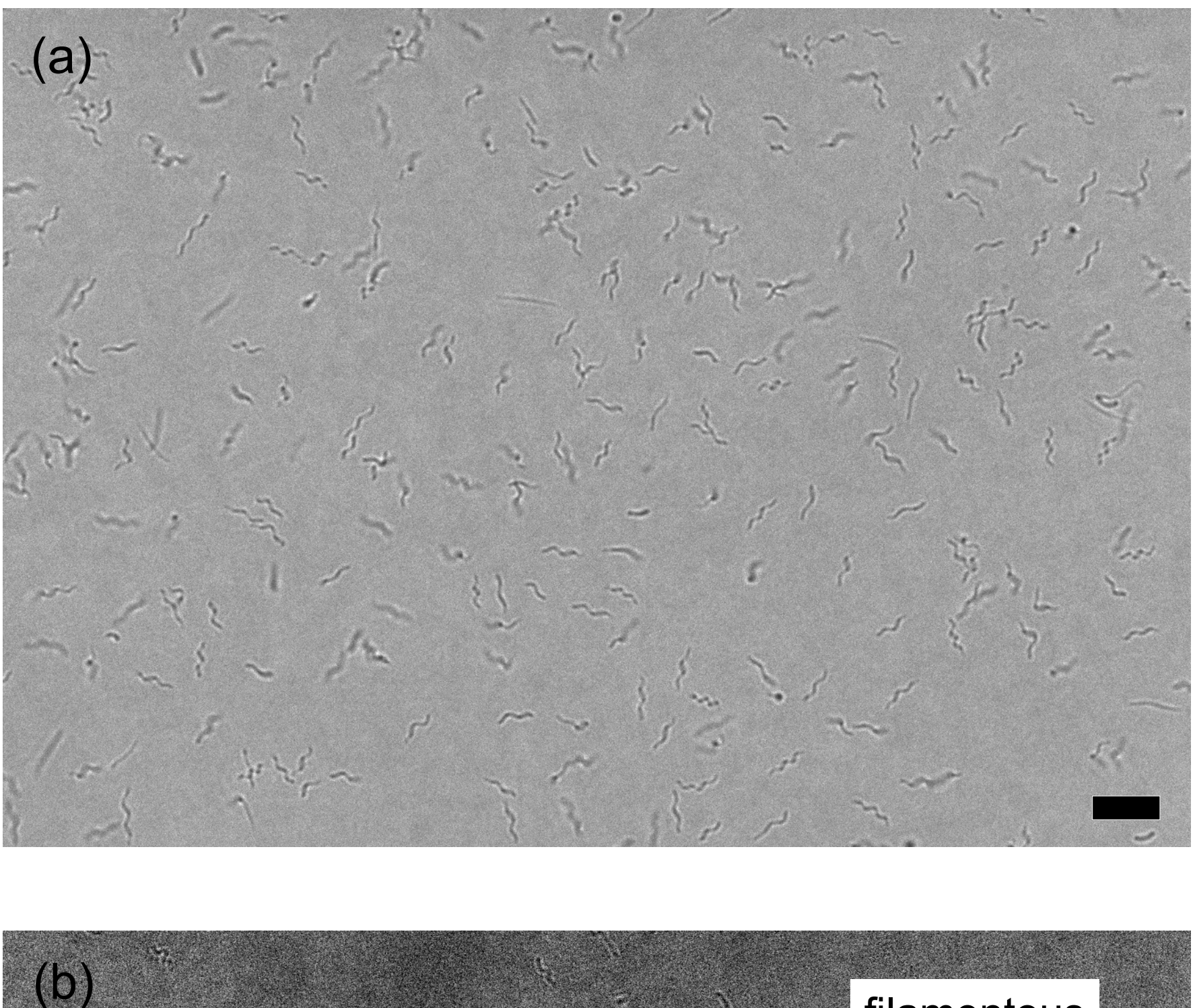
492

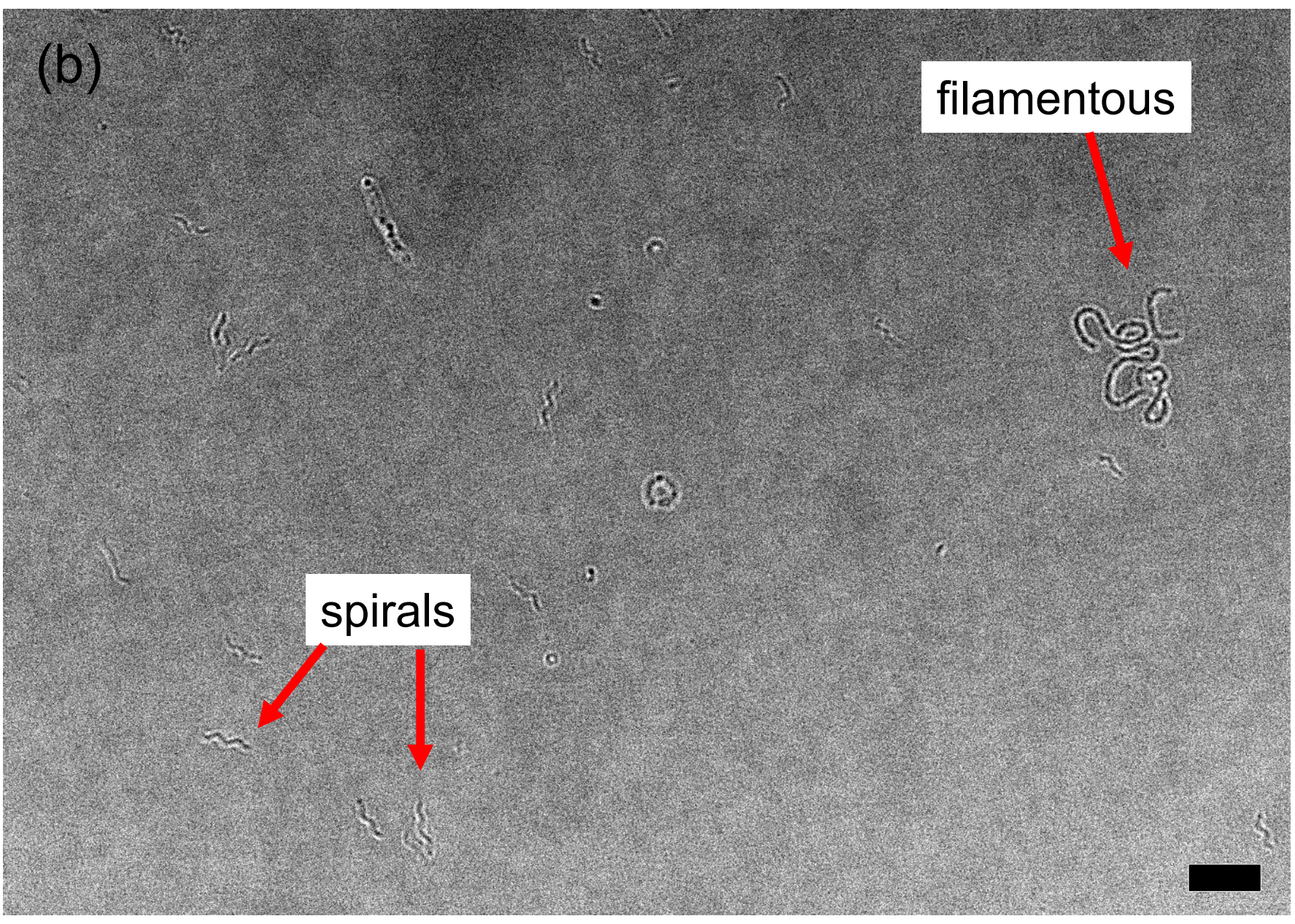
Figure legends

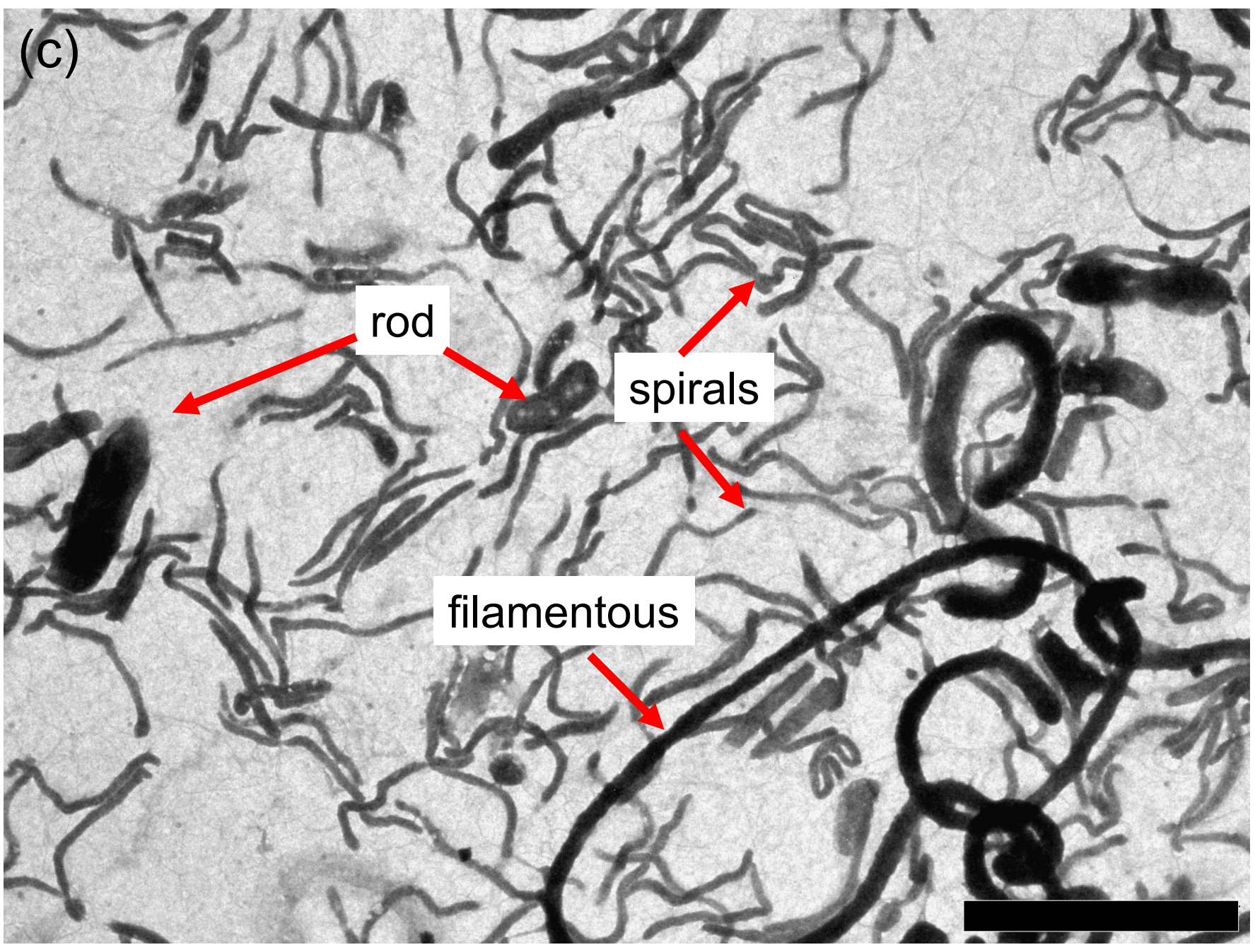
Fig. 1. (a) Morphological characterization of strain RF1110005^T observed by phase contrast microscopy after 5 days of incubation in R2A agar plate at 30ºC. (b) Strain RF1110005^T cells collected from a single colony on R2A agar plate, on which a single cell was sorted, were observed under phase contrast microscopy. (c) and (d) Morphological characterization of strain RF1110005^T observed under transmission electron microscopy after 5 days of incubation in R2A agar plate at 30ºC. Bars indicate 5 µm in (a) and (b), 4 µm in (c) and 2 µm in (d).

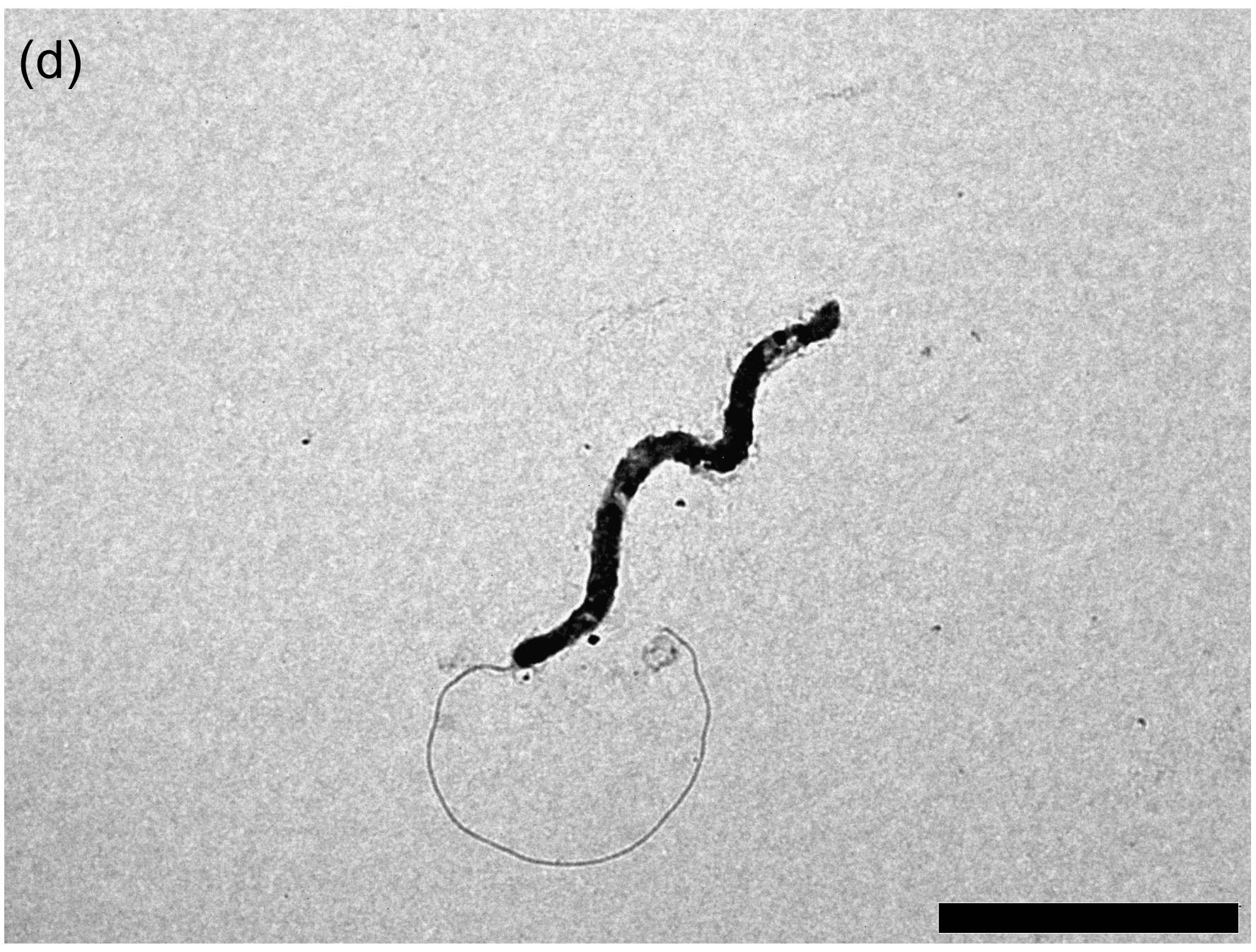
Fig. 2. Maximum-likelihood tree of partial 16S rRNA gene sequences of strain RF1110005^T with representative members of selected genera belonging to the class *Oligoflexia*. Bootstrap values (1000 replications) are shown as percentages at nodes (neighbour-joining, maximum-likelihood, and minimum evolution methods). The tree was reconstructed using MEGA software. *Geobacter metallireducens* DSM 7210^T was used as the outgroup. Bar, 0.020 substitutions per nucleotide position.

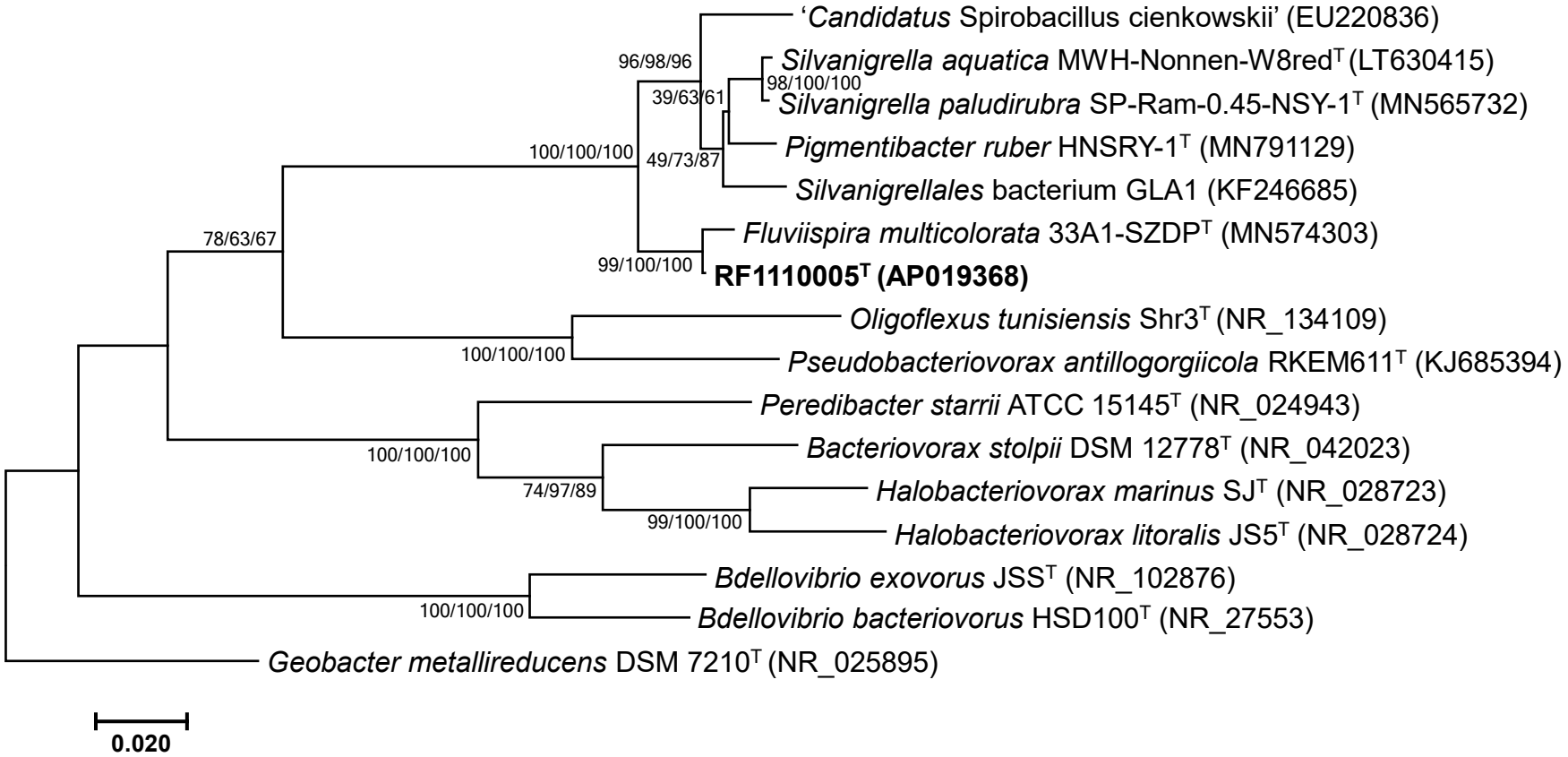
Fig. 3. RAxML tree of the concatenated amino acid sequences of the core genes of strain RF1110005^T and representative members of selected genera belonging to the class *Oligoflexia*. Bootstrap values (100 replications) are shown as percentages at nodes. *Geobacter metallireducens* DSM 7210^T was used as the outgroup. Bar, 0.2 substitutions per amino acid position.


(a)


(b)
filamentous
spirals


(c)
rod
spirals
filamentous


(d)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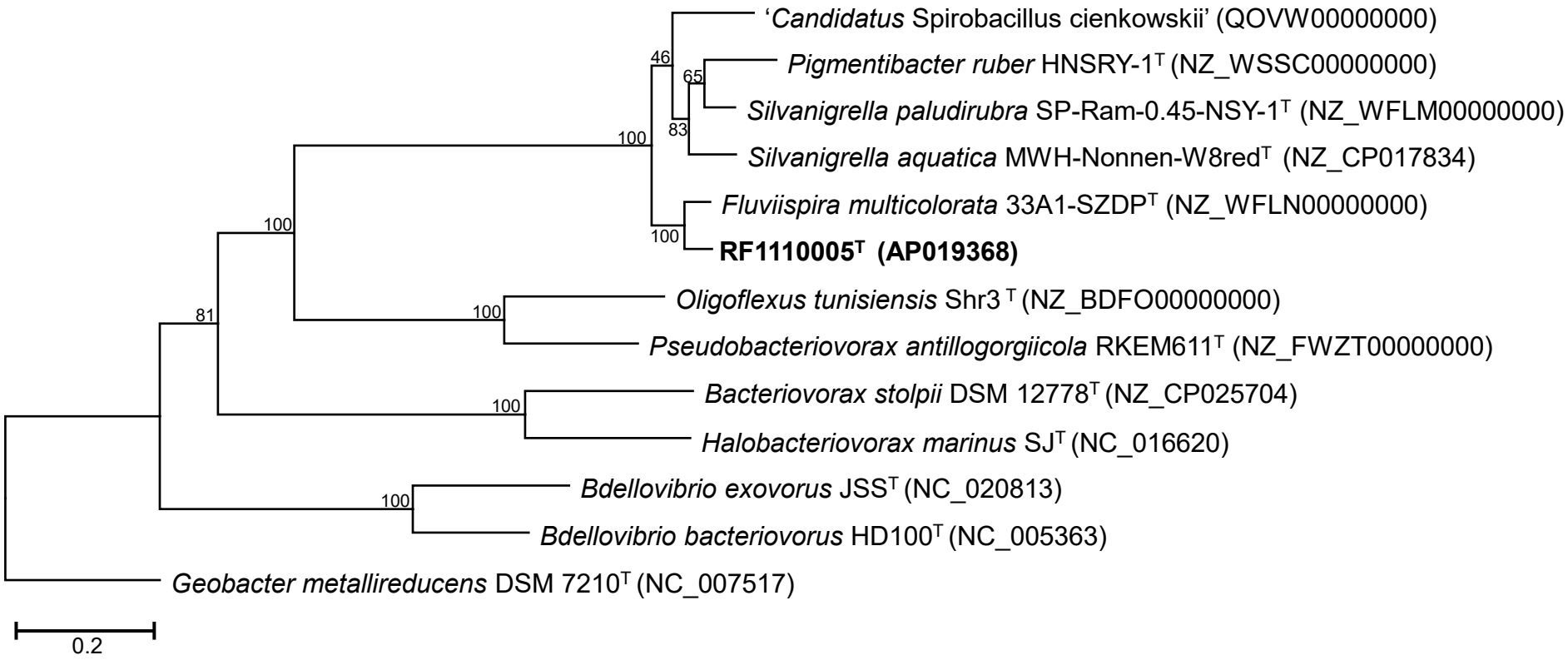
96/98/96
Silvanigrella aquatica MWH-Nonnen-W8red^{T} (LT630415)
98/100/100
39/63/61
Silvanigrella paludirubra SP-Ram-0.45-NSY-1^{T} (MN565732)
Pigmentibacter ruber HNSRY-1^{T} (MN791129)
100/100/100
49/73/87
Silvanigrellales bacterium GLA1 (KF246685)
78/63/67
Fluviispira multicolorata 33A1-SZDP^{T} (MN574303)
99/100/100
RF1110005^{T} (AP019368)
Oligoflexus tunisiensis Shr3^{T} (NR_134109)
100/100/100
Pseudobacteriovorax antillogorgiicola RKEM611^{T} (KJ685394)
Peredibacter starrii ATCC 15145^{T} (NR_024943)
100/100/100
Bacteriovorax stolpii DSM 12778^{T} (NR_042023)
74/97/89
Halobacteriovorax marinus SJ^{T} (NR_028723)
99/100/100
Halobacteriovorax litoralis JS5^{T} (NR_028724)
Bdellovibrio exovorus JSS^{T} (NR_102876)
100/100/100
Bdellovibrio bacteriovorus HSD100^{T} (NR_27553)
Geobacter metallireducens DSM 7210^{T} (NR_025895)
0.020


‘Candidatus Spirobacillus cienkowskii’ (QOVW00000000)
Pigmentibacter ruber HNSRY-1T (NZ_WSSC00000000)
Silvanigrella paludirubra SP-Ram-0.45-NSY-1T (NZ_WFLM00000000)
Silvanigrella aquatica MWH-Nonnen-W8redT (NZ_CP017834)
Fluviispira multicolorata 33A1-SZDPT (NZ_WFLN00000000)
RF1110005T (AP019368)
Oligoflexus tunisiensis Shr3 T (NZ_BDFO00000000)
Pseudobacteriovorax antillogorgiicola RKEM611T (NZ_FWZT00000000)
Bacteriovorax stolpii DSM 12778T (NZ_CP025704)
Halobacteriovorax marinus SJT (NC_016620)
Bdellovibrio exovorus JSST (NC_020813)
Bdellovibrio bacteriovorus HD100T (NC_005363)
Geobacter metallireducens DSM 7210T (NC_007517)
46
65
83
100
100
100
81
100
100
100
0.2