

Oceaniglobus Trochenteri sp. nov., Isolated from the Gut Microflora of top Shell (*Trochus Maculatus Linnaeus*).

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Abstract

A Gram-stain negative, non-flagellated, beige-pigmented, circular, catalase-positive, oxidase-positive bacterium, designated G4^T, was isolated from gut microflora of top shell (*Trochus maculatus Linnaeus*) collected from Diwanggong market, Weihai, PR China. The novel isolate was able to grow at 4–42°C (optimum 25–33°C), pH 7.0–9.0 (optimum 6.5–7.0) and with 0.0–11.0% NaCl (optimum 2.0–3.0%, w/v). Analysis of 16S rRNA gene sequence revealed that strain G4^T shared the highest 16S rRNA gene sequence similarities with *Oceaniglobus* YLY08^T (96.6%), followed by *Oceaniglobus indicus* 1-19b^T (95.3%). The genome of strain G4^T, with 32 assembled contigs, was 4.5 Mb long with a G + C content of 65.3 mol%. DNA–DNA hybridization values of the isolate against the closely related type strains were far below the 70% limit for species delineation. The average amino acid identity, average nucleotide identity and digital DNA–DNA genome hybridization relatedness between strain G4^T and the closely related members of the genus *Oceaniglobus*, *Oceaniglobus indicus*1-19b^T and *Oceaniglobus ichthyenteri* YLY08^T were 71.3, 76.4 and 20.0%, and 75.0, 76.3 and 19.4%. The major cellular fatty acid was summed feature 8 (C_{18:1} ω7c and/or C_{18:1} ω6c). The sole respiratory quinone detected was Q-10. The polar lipid profile consisted of diphosphatidylglycerol, phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol and phosphatidylidimethylethanolamine. The results of phenotypical, phylogenetic and biochemical analyses indicated that strain G4^T represents a novel species in genus *Oceaniglobus* within the family *Rhodobacteraceae*, for which the name *Oceaniglobus trochenteri* sp. nov. is proposed. The type strain is G4^T (= MCCC 1K04356^T = KCTC 82506^T).

Introduction

Marine bacteria play an important role in marine ecology, many bacterial strains have been isolated and characterized taxonomically from coastal marine environments and marine organisms. The genus *Oceaniglobus* belongs to the family *Rhodobacteraceae*, was initially established by Li et al. (2017). At the time of writing, the genus *Oceaniglobus* consists of two species both from the marine environment, including *Oceaniglobus indicus* (type species) from sea water (Li et al. 2017) and *Oceaniglobus ichthyenteri* from the gut of sea bass (Wang et al. 2019). For researching bacterial diversity in the gut of top shell and isolating potential probiotics, we carried out a culture-dependent bacterial isolation from the gastrointestinal tract of *Trochus maculatus Linnaeus*. During this research, a novel bacterial strain was isolated and designated strain G4^T. The aim of this study was to validate the existence of novel species of the genus *Oceaniglobus* on the basis of phylogenetic, genotypic and phenotypic data.

Materials And Methods

Isolation and culture conditions

For the bacterial isolation, the intestinal tissue sample was processed by an enriched culture technique (Du et al. 2014). The enriched culture (30 days) was diluted serially in sterile seawater and samples of

each serial dilution were spread on marine agar 2216 (MA; Becton Dickinson) plates for cultivation at 28°C. After incubation for a week, a beige-pigmented colony was picked up and subcultured several times to get a pure culture, later designated as strain G4^T. The isolate was purified by repeated subculture and stored at - 80 °C in sterile 15% (v/v) glycerol supplemented with 1% (v/v) NaCl. The type strains, *Oceaniglobus indicus* MCCC 1A11863^T and *Oceaniglobus ichthyenteri* MCCC 1H00318^T were purchased from their respective collection institutions. All strains were cultured under comparable conditions for physiological and chemotaxonomic characterizations, unless otherwise specified. They were preserved at - 80°C in sterile distilled water supplemented with 1.0 % NaCl (w/v) and 15.0 % (v/v) glycerol.

Phylogenetic analysis and genome sequencing

For 16S rRNA gene sequencing and phylogenetic analysis, the genomic DNA of strain G4^T was extracted using a commercial genomic DNA extraction kit (Takara) according to the manufacturer's instructions. The 16S rRNA gene sequence was amplified by PCR with universal bacterial primers 27F and 1492R (Liu and Shao 2005). The PCR product was purified and cloned into pMD18-T vector (Takara) according to the manufacturer's instructions. Plasmids were sequenced using universal M13 primers. The identification of phylogenetic neighbours and the calculation of 16S rRNA gene sequence similarity were performed using the EzBioCloud tool (<http://www.ezbiocloud.net/>) (Kim et al. 2012) and the blast tool in the NCBI database (www.ncbi.nlm.nih.gov/blast/). Sequences of related taxa were obtained from the GenBank database. Based on 16S rRNA gene sequences, phylogenetic analysis was performed using mega version 7 (Kumar et al. 2016) with distance option according to the default parameter models and clustering with the neighbour-joining (Saitou and Nei 1986), maximum-parsimony (Fitch 1971) and maximum-likelihood (Felsenstein 1981) methods, with bootstrap values based on 1000 replications (Felsenstein 1985).

The draft genome of strain G4^T was sequenced at Beijing Novogene Bioinformatics Technology Co, Ltd. (Beijing, PR China), using a HiSeq-PE150 platform (Illumina) with massively parallel sequencing technology (Illumina). The genes involved in metabolic pathways were analyzed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) databases (Kanehisa et al. 2016). Protein-encoding regions were identified and annotated with the Rapid Annotations using Subsystems Technology (RAST) server (<http://rast.nmpdr.org/rast.cgi>) (Aziz et al. 2008) and the UniProtKB/Swiss-Prot (Consortium 2019). Secondary metabolite was presented by antiSMASH 5.0 (Blin et al. 2019). The DNA G + C content of strain G4^T was determined from the genome sequence. Genomic data of other type strains within the family *Rhodobacteraceae* were obtained from the GenBank/ENA/DDBJ databanks. The average amino acid identity (AAI) values were calculated using EzBioCloud integrated database (Yoon et al. 2017a). Average nucleotide identity (ANI) values between two genomes were calculated using OrthoANlu algorithm (Yoon et al. 2017b). The digital DNA-DNA hybridization (dDDH) values were calculated using the Genome-to-Genome Distance Calculator (GGDC 2.0) (Meier-Kolthoff et al. 2013).

Morphology, physiology, and biochemical analysis

The morphological and physiological characteristics of strain G4^T were observed on MA at 30 °C for 2 days. Cell morphology, size and the presence of flagella were examined by transmission electron microscopy (JEM-1200, JEOL). Gram reaction was determined as described by according to Park et al. (2014). The activities of catalase and oxidase, hydrolysis of cellulose, agar, casein, and Tween 80, were tested according to the method of Tindall (2007). Hydrolysis of alginate was tested on MA with 0.2% (w/v) sodium alginate as described by Takeshita et al. (1991). Motility was determined using the hanging-drop method and gliding motility was determined as described by Bowman (2000). Temperature-dependent growth was tested at 4, 10, 15, 20, 25, 30, 33, 37, 40, 42, and 45°C on MA. The pH range for growth was determined in MB adjusted to pH 5.5–9.5 with a concentration of 20 mM using the following buffer systems: MES (pH 5.5 and 6.0), PIPES (pH 6.5 and 7.0), HEPES (pH 7.5 and 8.0), Tricine (pH 8.5), and CAPSO (pH 9.0 and 9.5). The effect of NaCl on growth was tested in NaCl-free artificial seawater medium supplemented with 5.0 g peptone, 1.0 g yeast extract, and various concentrations of NaCl (final concentration 0.0–10.0%, in increments of 0.5%) (Yang and Cho 2008). To examine O₂ metabolism, growth under strictly anaerobic conditions was tested on MA with or without 0.1% NaNO₃ for 7 days at 30 °C. Oxidase activity was determined with an oxidase reagent (bioMérieux). Catalase activity was tested by the observation of gas bubble safter the addition of a few drops of 3.0% (v/v) H₂O₂ to fresh biomass grown on an agar plate. Antibiotic susceptibility tests were performed by the disc-diffusion method on MA according to the protocol of Du et al. (2014). The presence of PHB granules were determined according to the protocols of Ostle *et al.* (1982). According to the manufacturers' instructions, the oxidation and fermentation of carbohydrates were determined after growth on MA at 30°C for 2 days using the Biolog GEN III Micro Plates and API 50CHB Fermentation Kit (bioMérieux). Other physiological tests were carried out using API 20E, API 20NE, and API ZYM strips (bioMérieux).

Chemotaxonomic characterisation

Chemotaxonomic characteristics of strain G4^T and the reference strain were determined under similar conditions. Fatty acid extraction and analyses were performed according to procedures described by Sasser (1990) with a Microbial Identification system (MIDI; Microbial ID). The respiratory isoprenoid quinones were purified according to the protocol described by Hiraishi et al. (1996) and analysed using HPLC. Polar lipid analysis was performed by the Marine Culture Collection of China (MCCC), Xiamen, Fujian Province, P. R. China.

Results And Discussion

Phylogenetic analysis and genome sequencing

Analysis of the 16S rRNA gene sequences revealed that strain G4^T belonged to the genus *Oceaniglobus*, within the family *Rhodobacteraceae*. Strain G4^T shared the highest sequence similarity with *O. ichthyenteri* (96.6%), followed by *O.indicus* (95.3%). The neighbour-joining phylogenetic tree (Fig. 1) revealed that strain G4^T, *O. ichthyenteri* and *O. indicus* formed an monophyletic cluster, with high

bootstrap support (99%). It indicated that strain G4^T might represent a novel species of the genus *Oceaniglobus*.

The genome of strain G4^T was comprised of 4472 genes and 33 contigs with a total length of 4588953 bp. The main coverage was 360 ×. The N50 value was 226458 bp. There were 5S rRNAs of 3, 16S rRNA of 2, 23S rRNAs of 2, tRNAs of 47 and sRNAs of 3. Complete genome analysis revealed that the 4,099 protein-coding genes constituted 91.7% of the total genes in the genome. Furthermore, there were 3,965 genes (88.7%) connected to KEGG pathways, 3,320 genes (74.2%) assigned to 24 different clusters of orthologous groups (COGs) and 1,458 genes (32.6%) connected to SwissProt pathways. Based on the genome sequence annotation, the genome contained several genes coding for Glycoside Hydrolases (GHs), Glycosyl transferases (GTs) and Carbohydrate esterases (CEs). There were 3965 genes annotated by KEGG pathway annotation, involving cellular Processes, environmental information processing, genetic information processing, human diseases, metabolism and organismal systems and a large number of genes were found to be focus on metabolism and membrane transport. The genes that encoded the enzymes required for PHB synthesis were present in the genome, the presence of genes encoding *phaZ* (EC3.1.1.75) and *phaC* (EC 2.3.1.-) showed the metabolic capacity of strain G4^T to accumulate PHB intracellularly.

According to the genome sequence, the DNA G + C content was 65.3 mol%, which was higher than the related strains *O. indicus* (59.0 mol%) and *O. ichthyenteri* (64.2 mol%). The AAI values were 71.3% with *O. indicus* and 75.0% with *O. ichthyenteri*, which were far below the 90% cut-off value for prokaryotic species delineation (Rodriguez-R and Konstantinidis 2014). AAI values of 71.3% and 75.0% indicated that strain G4^T represents a new species. The ANI values were 76.4% with *O. indicus* and 76.3% with *O. ichthyenteri*, which were lower than the 95–96% cut-off value for species demarcation (Richter and Rossello-Mora 2009). The dDDH values were 20.0% with *O. indicus*, 19.4% with *O. ichthyenteri*, which were below the standard cut-off value (70%) (Meier-Kolthoff et al. 2013). These results confirmed that strain G4^T represented a novel species of the genus *Oceaniglobus*.

Morphology, physiology, and biochemical analysis

Cells were Gram-stain negative, aerobic, motile, gliding, lacking flagella, and oval (0.5–0.8 μm in width, 0.6–1.0 μm in length) (Fig. S1). Colonies were 0.8–1.0 mm in diameter, creamy, circular and convex with smooth surfaces after incubation on MA at 30°C for 2 days. NaCl was not essential for growth, which distinguished strain G4^T from the related strains, *O. ichthyenteri* and *O. indicus*. The accumulation of PHB granules was observed by fluorescence microscopy. Besides, it was sensitive to ampicillin, penicillin, erythromycin, chloramphenicol, ciprofloxacin, gentamicin and cefazolin. Other phenotypic characteristics of the strain G4^T and related strains are shown in Table 1.

Chemotaxonomic characterisation

The major fatty acid was summed feature 8 (C_{18:1} ω 7c and/or C_{18:1} ω 6c). The cellular fatty acid composition was similar to that of the related taxa of the genus *Oceaniglobus*. The detailed results of the fatty acids are shown in Table 2. The sole isoprenoid quinone found in strain G4^T, ubiquinone-10 (Q-10), was in accordance with the properties of the genus *Oceaniglobus*. The polar lipid components of strain G4^T were iphosphatidylglycerol, phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol and phosphatidylidimethylethanolamine. Further detailed polar lipid images of the different strains are given in Fig. S1 (available in the online version of this article).

Conclusion

The combined results of the phylogenetic and chemotaxonomic analyses supported that it is reasonable to assign strain G4^T as a member of the genus *Oceaniglobus*. Strain G4^T was distinguished from the type strain of *O. indicus* by differences in several phenotypic characteristics, including growth without NaCl, activity of some enzymes and susceptibility to some antibiotics (Table 1). On the basis of the phenotypic chemotaxonomic phylogenetic and genetic data, therefore, strain G4^T is considered to represent a novel species of the genus *Oceaniglobus*, for which the name *Oceaniglobus trochenteri* sp. nov. is proposed.

Description of *Oceaniglobus trochenteri* sp. nov.

Oceaniglobus trochenteri (troch.en'te.ri. N.L. masc. n. *Trochus* a conch genus; Gr. neut. n. *enteron* gut; N.L. gen. n. *trochenteri* of a conch gut).

Cells are Gram-stain negative, catalase-positive, oxidase-positive, motile, non-flagellated and ovoid-shaped, approximately 0.5–0.8 μ m wide and 0.6–1.0 μ m long. Colonies are elevated with a smooth surface, beige-pigmented and uniformly circular with a diameter of approximately 0.8–1.0 mm after incubation on MA at 30°C for 2 days. The pH and temperature ranges for growth are pH 6.0–9.0 and 4–42°C (optimum at pH 6.5–7.0 and 25–30°C). Growth occurs in the presence of 0.0–11.0% NaCl (optimum 2.0–3.0%). Cells hydrolyze Tween 40, cellulose, agar and starch, but not alginate. Acids are produced from glycerol, aesculin, L-rhamnose, L-xylose, L-fucose, D-arabinose, D-lyxose, D-ribose, D-xylose, D-fructose and D-fucose. It is positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α and β -galactosidase, α and β -glucosidase. but negative for lipase (C14), trypsin, β -glucuronidase, N-acetyl- β -glucosaminidase, α -mannosidase, and α -fucosidase. Carbon source oxidation tests show positive results for trehalose, maltose, cellobiose, sucrose, lactose, raffinose, N-acetyl-D-glucoside, D-glucose, D-mannose, D-fructose, D-galactose, D-fucose, myo-inositol and D-gluconic acid. All negative traits of commercial kits are given in Table S1. The sole isoprenoid quinone is Q-10. The major cellular fatty acid is summed feature 8 (C_{18:1} ω 7c and/or C_{18:1} ω 6c). The polar lipid profile consists of diphosphatidylglycerol, phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol and phosphatidylidimethylethanolamine. The DNA G + C content is 65.3 mol%.

The type strain, G4^T (= MCCC 1K04356^T = KCTC 82506^T) was isolated from gut microflora of top shell collected from Diwanggong market, at Weihai, Shandong Province, People's Republic of China.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain G4^T is MW555789 and the number for the whole genome sequence is JACNMM000000000.

Abbreviations

AAI Average Amino Acid identity

ANI Average Nucleotide Identity

Dddh digital DNA-DNA Hybridization

HPLC High Performance Liquid Chromatography

HEPES N-(2-Hydroxyethyl)Piperazine-N'-2-Ethanesulfonic Acid

CAPSO 3-Cyclohexylamino-2-Hydroxypropanesulfonic Acid Sodium Salt

MES 2-(N-Morpholino) ethane-sulfonic acid

PIPES Piperazine-1,4-Bisethanesulfonic Acid

Tricine N-[Tris(Hydroxymethyl)Methyl] Glycine

KCTC Korean Collection for Type Cultures

KEGG Kyoto Encyclopedia of Genes and Genomes

MA Marine agar 2216; MB, Marine broth 2216

MEGA Molecular Evolutionary Genetics Analysis

MCCC Marine Culture Collection of China

PHB poly-β-hydroxybutyrate.

Declarations

Funding information

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Conflicts of interest

Authors declare that there is no conflict of interest.

Data availability statement

All data generated or analysed during this study are included in this published article, its supplementary information files and GenBank/EMBL/DDBJ. The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain G4^T is MW555789 and the number for the whole genome sequence is JACNMM000000000. Supplementary figures and Supplementary tables are available with the online version of this paper.

Authors' contributions

YXZ designed research and project outline. RY and ZC performed isolation, deposition, and identification. STY and MJZ performed genome analysis. ZC drafted the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1. Differential characteristics of strain G4^T and other closely related members of the genus *Oceaniglobus*.

Strains: 1, G4^T; 2, *O. indicus* MCCC 1A11863^T; 3, *O. ichthyenteri* MCCC 1H00318^T. +, Positive; -, negative; w, weak. All data from this study except DNA G+C contents of the related strains, which were from the original species description:

Characteristic	1	2	3
Isolation source	Gut of snail	Seawater	Gut of sea bass
Growth range(optimum)			
NaCl (w/v, %)	0.0–11.0 (3.0–4.0)	0.5–10.0 (3.0–4.0)	1.0–9.0 (2.0–3.0)
Temperature (°C)	4–42 (25–30)	5–37 (28–35)	4–40 (28–30)
pH	6.0–9.0 (6.5–7.0)	5.0–10.0 (7.0–8.0)	6.0–9.5 (7.0–7.5)
Nitrate reduction	-	+	-
Hydrolysis of starch	+	-	+
Acids production from (API 50 CHB):			
Glycerol	+	-	-
D-glucose	+	-	-
L-Arabinose	+	-	+
D-mannose	+	-	+
Maltose	+	-	+
Sucrose	+	-	+
Enzymic activities (API ZYM):			
Alkaline phosphatase	+	-	+
α -chymotrypsin	-	-	+
Acid phosphatase	+	+	-
Oxidation of (Biolog GEN III):			
Cellobiose	+	-	+
α -ketoglutaric acid	+	-	+
D-fructose	+	-	+
myo-inositol	+	-	+
D-maltose	+	-	+
D-trehalose	+	-	+
L-rhamnose	+	+	-

D-galacturonic acid	+	+	-
D-glucuronic acid	+	+	-
DNA G+C content (mol%)	65.3	64.2 ^a	59.0 ^b

^a Data from Li et al. (2017)

^b Data from Wang et al. (2019)

Table 2. Cellular fatty acid composition of strain G4^T and the closest relatives.

Strains: 1, G4^T; 2, *O. indicus* MCCC 1A11863^T; 3, *O. ichthyenteri* MCCC 1H00318^T. All data were taken from this study. TR, Traces (<1.0%); -, not detected. Fatty acids amounting to <1.0% of the total fatty acids in both strains are not shown.

Fatty acid	1	2	3
Straight-chain:			
C _{16:0}	10.97	6.15	8.27
C _{17:0}	TR	1.36	TR
C _{18:0}	3.05	1.35	1.94
Unsaturated fatty acids			
C _{20:2} ω6,9 <i>c</i>	TR	TR	-
C _{19:0} cycloω8 <i>c</i>	18.70	20.56	-
C _{18:1} ω7 <i>c</i> 11-methyl	3.76	3.24	6.6
Hydroxy fatty acids			
C _{10:0} 3-OH	2.81	2.74	3.62
C _{12:0} 3-OH	1.05	TR	3.02
Summed feature 3*	TR	1.12	1.52
Summed feature 7[#]	TR	1.87	-
Summed feature 8^{&}	55.12	54.87	67.47

*Summed feature 3, C_{16:1}ω7*c* and/or C_{16:1}ω6*c*.

[#]Summed feature 7, un 18.846 and/or C_{19:1}ω6*c*.

Figures

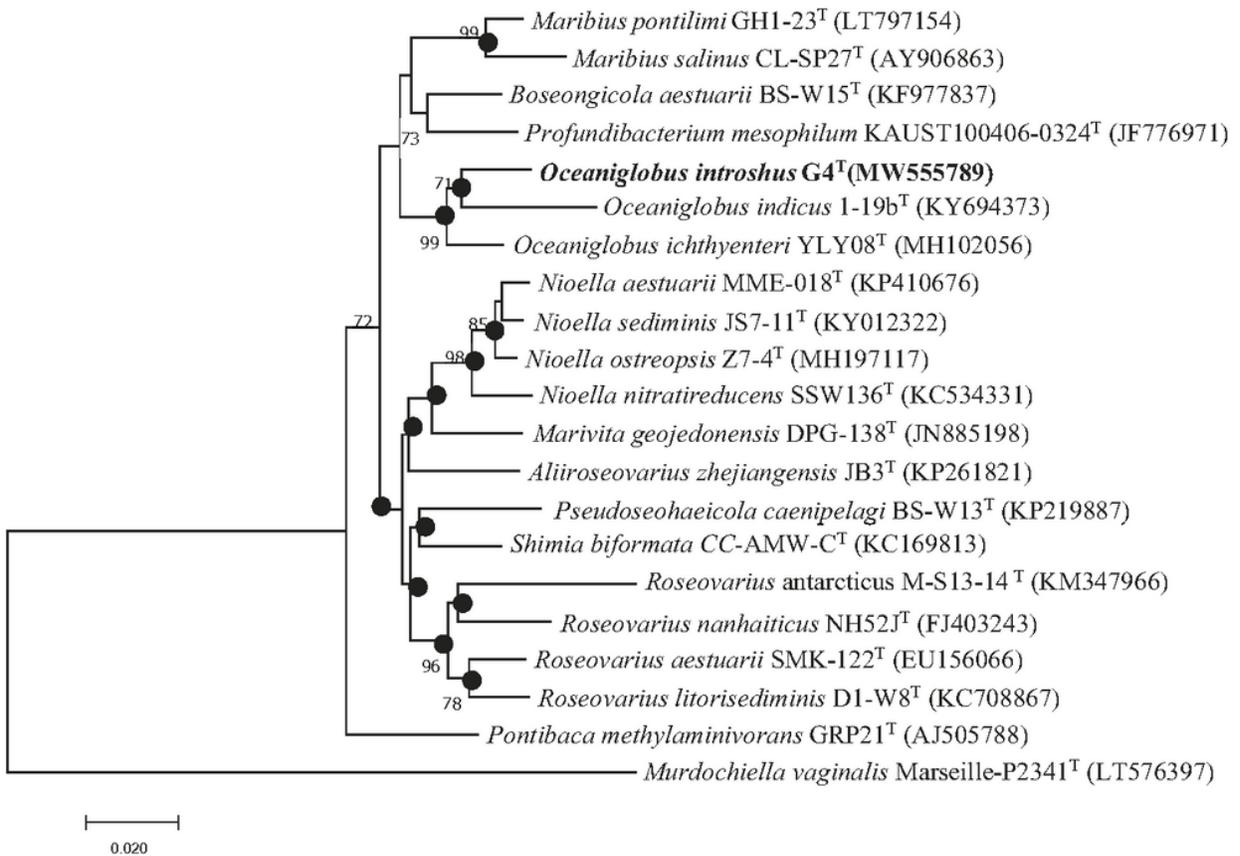


Figure 1

Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences of strain G4T and high similarity of genus. Bootstrap support values (1000 replications) above 50% are shown at nodes. Filled circles

indicate nodes that were also recovered in maximum-likelihood and neighbor-joining phylogenetic trees based on the same sequences. *Murdochiella vaginalis* Marseille-P2341T was used as an outgroup. Bar, 0.02 substitutions per nucleotide position.

Supplementary Files

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