Kangiella Shandongensis Sp. Nov., A Novel Species Isolated From Saltern In Yantai, China

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Abstract

A Gram-stain-negative, wheat, rod-shaped, motile, non-spore forming, and aerobic bacterium, designated as strain PI^T, was isolated from saline silt samples collected in saltern in Yantai, Shandong, China, and subjected to a polyphasic taxonomic study. The 16S rRNA gene sequence analysis revealed a clear affiliation of strain PI^T with the genus *Kangiella*. Strain PI^T showed the closest phylogenetic relationship with *Kangiella taiwanensis* KT1^T with the highest 16S rRNA gene sequence similarity of 98.3%. The genome size of the strain PI^T was 2809662 bp consisting of 2653 genes which include 39 tRNAs genes, 2 sRNA genes and 4 rRNA genes. The major cellular fatty acids of strain PI^T were identified as iso-$C_{15:0}$, iso-$C_{11:0}$, iso-$C_{11:0}$ 3-OH and Summed Feature 9 (iso-$C_{17:1}\omega 9c$), and the major respiratory isoprenoid quinone was Q8. The major polar lipids were diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylmonomethylethanolamine (PME) and phosphatidylinositol (PI). The G+C content of the genomic DNA was 45.8 mol%. The Average Amino Acid identity (AAI) value between strain PI^T and *Kangiella sediminilitoris* was 83.9%. The Average Nucleotide Identity (ANI) values between strain PI^T and *Kangiella sediminilitoris*, *Kangiella aquimarina* and *Kangiella taiwanensis* were 76.9%, 71.0% and 74.6%, respectively. The digital DNA-DNA Hybridization (dDDH) values between strain PI^T and *Kangiella sediminilitoris*, *Kangiella aquimarina* and *Kangiella taiwanensis* were 19.8%, 19.1% and 18.7%, respectively. The results of 16S rRNA gene phylogeny, phenotypic characterizations, chemotaxonomic characterization and genome features indicate that strain PI^T represents a novel specie of the genus *Kangiella*, for which the name *Kangiella shandongensis* sp. nov. is proposed. The type strain is PI^T (KCTC 82509^T = MCCC 1K04352^T).

Introduction

The genus *Kangiella*, a member of the class *Gammaproteobacteria*, was first proposed by Yoon *et al*. (2004) to accommodate two species, *Kangiella koreensis* (type species) and *Kangiella aquimarina* (Yoon et al. 2004). Over the last about seventeen years, the genus description was emended to accommodate eight further *Kangiella* species, *Kangiella japonica* (Romanenko et al. 2010), *Kangiella spongicola* (Ahn et al. 2011), *Kangiella geojedonensis* (Yoon et al. 2012), *Kangiella taiwanensis* (Jean et al. 2012), *Kangiella marina* (Jean et al. 2012), *Kangiella sediminilitoris* (Lee et al. 2013), *Kangiella chungangensis* (Kim et al. 2015) and *Kangiella profundi* (Xu et al. 2015) isolated from the aquatic environment in different regions. Commonly, most species of this genus were Gram-stain-negative, non-motile, rod-shaped and aerobic bacteria, with Q-8 as the predominant isoprenoid quinone and iso-$C_{15:0}$ as the predominant cellular fatty acid (Yoon et al. 2004; Romanenko et al. 2010; Ahn et al. 2011; Yoon et al. 2012; Jean et al. 2012; Lee et al. 2013; Kim et al. 2015; Xu et al. 2015).

In this study, a novel isolate strain PI^T was isolated from saltern in Yantai, Shandong, China, which presented most of features as above. The purpose of this study was to determine the exact taxonomic position of strain PI^T by using a polyphasic approach, which included morphology, physiology,

biochemical and detailed phylogenetic analysis based on the 16S rRNA gene sequence. The results of this study showed that strain PI^T should be accommodated as the eleventh member of the genus *Kangiella*.

Materials And Methods

Isolation procedure, maintenance and cultural conditions

Saline silt samples was collected from Yantai, Shandong Province, China (121°40'20" E, 37°26'39" N) and used as source for isolating bacterial strains. Strain PI^T was isolated by the dilution plating technique on marine agar 2216 (MA, Becton Dickinson) at 30 ℃. After incubating at 30 ℃ for 2 days, wheat colonies were observed and then picked from the plate and purified on MA. The obtained single colony was designated as strain PI^T. Subcultivation was carried out on MA at 30 ℃. Three reference strains, *K. aquimarina* (KCTC 12183^T), *K. sediminilitoris* (= KCTC 23892^T) and *K. taiwanensis* (= BCRC 80330^T) were all investigated for morphological, physiological and chemotaxonomic characterizations at the same conditions with strain PI^T. The bacterial culture were preserved at − 80℃ in sterile distilled water consisted of 1.0% NaCl (w/v) and 15.0% (v/v) glycerol.

16S rRNA gene phylogeny

Two universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') were used to amplify the 16S rRNA gene of strain PI^T as described previously (Weisburg et al. 1991). The 16S rRNA gene sequence of strain PI^T was compiled using the BioEdit software (Hall 1999). NCBI BLAST and EzBioCloud were used to perform the comparison of 16S rRNA gene sequences (Yoon et al. 2017). Clustal_X version 1.83 with default settings was used to accomplish the multiple alignments of the sequences based on all genus-type strains with 16S rRNA sequence similarity of 95.0% above (Thompson et al. 1997). Phylogenetic trees were constructed using the neighbour-joining (Saitou and Nei 1987), maximum-likelihood (Takahashi and Nei 2000) and maximum-parsimony (FITCH 1971) algorithms with the Kimura two-parameter model (Kimura 1980) in the MEGA (version 7.0) software (Kumar et al. 2016).

Phenotypic characterizations

Cell morphology and flagella were observed through an electron microscope (Jem-1200; JEOL) after negative staining with 0.5% (w/v) uranyl acetate and air drying using cells incubated on MA at 30 ℃ for 3 days. Gliding motility was tested using an oil-immersion phase-contrast microscopy (AX70; Olympus) as described by Bowman (Bowman 2000). The growth temperature of strain PI^T was determined in MA at 4, 10, 15, 20, 25, 30, 33, 37, 40, 42, and 45℃. The test of pH range for growth was performed in marine broth 2216 (MB, Becton Dickinson) by changing pH from pH 5.5 to 9.5 using the following buffer systems with a concentration of 20 mM: 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). Salt tolerance was tested in artificial seawater broth composed of 1.0 g yeast extract, 5.0 g peptone and NaCl with different concentrations (concentration

range 0–10.0%, in increments of 0.5%) (Yang and Cho 2008). Gram-staining test was carried out according to the instructions by Smibert and Krieg *et al*. (1994). After incubating on MA in the presence and absence of 0.1% (w/v) $NaNO_3$ under anaerobic conditions at 30 ℃ for 3 days, the growth of strain PI^T was observed to determine whether it could grow under anaerobic conditions. Antibiotic sensitivity test was performed on MA plates with discs (Tianhe) composed of various antibiotics (Penicillin, Ampicillin, Cefazolin, Amikacin, Gentamicin, Erythromycin, Norfloxacin, Ciprofloxacin, Bactrim and Chloramphenicol) at 30 ℃ for 3 days. The oxidation and fermentation test of carbohydrates were performed using cells grown for 2 days on MA at 30℃ through the Biolog GEN III Micro Plates and API 50CHB Fermentation Kit (bioMé rieux) according to the manufacturer's instructions. Other physiological tests were performed using API 20NE, API 20E, and API ZYM strips (bioMé rieux).

Chemotaxonomic characterization

To determine the fatty acid composition of strain PI^T and reference strains, they were cultured in MB at 28 ℃ for 5 days approximately and the absorbance of biomass was measured by Elisa to ensure that the cells were at the mid-exponential phase. The composition of fatty acids was analysed by gas chromatography, according to the standard Microbial Identification System (Sherlock version 6.1MIDI; TSBA6.0 database) (Sasser 1990). Respiratory quinone of strain PI^T and reference strains were measured by using HPLC after grown in MB at 28 ℃ for 3 days (Kroppenstedt 1982). The polar lipids were separated by two-dimensional thin-layer chromatography (TLC) using chloroform/methanol/water (65:25:4, by vol) for the first dimension and chloroform/acetic acid/methanol/water (80:15:12:4, by vol) for the second dimension. Molybdatophosphoric acid (10%) was used to detect total lipid material and spray reagents specific for each part were used to identify specific functional groups as described by Tindall *et al.* (2014).

Genome features

The software (SOAP, Spades and Abyss) were used to assemble sequence of genome and CISA was used for integration. The GeneMarkS (http://topaz.gatech.edu/) and IslandPath-DIOMB software was used to predict the coding genes and genomic island of strain PI^T, respectively. The CPISPR gene cluster was forecasted using CRISPR digger. In order to analyse the antibiotic gene cluster of the strain PI^T, the amino acid sequences of strain PI^T were compared with Comprehensive Antibiotic Research Database (CARD) to obtain the names of tolerated antibiotics and related drug-resistance genes. By using Diamond software, the amino acid sequences of strain PI^T were compared with Kyoto Encyclopedia of Genes and Genomes (KEGG) and the genes of strain PI^T were combined with their corresponding functional annotation information to analyse the genes involved in metabolic pathways (Kanehisa et al. 2016). In addition, Cluster of Orthologous Groups of proteins (COG) and Virulence Factors of Pathogenic Bacteria (VFDB) were also used to analyse the function classifications of COG and virulence factors, respectively. The antiSMASH-4.0.2 program (Blin et al. 2017) was used to analyse secondary metabolic gene cluster. The DNA G + C content of strain PI^T was calculated from the genome sequence. The average nucleotide identity (ANI) values (https://www.ezbiocloud.net/tools/ani), digital DNA–DNA hybridization (dDDH)

values (http://ggdc.dsmz.de/ggdc.php#) and average amino acid identity (AAI) (Konstantinidis and Tiedje 2005; Rodríguez-R and Konstantinidis 2014b) calculator (http://enve-omics.ce.gatech.edu/aai/) were used to calculate the genome-based similarities between strain PI^T and the closest phylogenetic neighbours..

Results And Discussion

Phylogenetic analysis

The almost-complete 16S rRNA coding sequence of strain PI^T (1564 bp) was measured in this study. The phylogenetic tree based on 16S rRNA gene sequence using neighbour-joining tree (Fig. 1), revealed the novel strain formed a new phyletic line closely associated with the type strain of *K. sediminilitoris* BB-Mw22^T.The result of phylogenetic analysis according to neighbour-joining tree showed that strain PI^T belongs to the genus *Kangiella* (Fig. 1). The closest phylogenetic neighbours of strain PI^T were also depicted in the maximum-likelihood tree, which displayed the same tree topologies (Supplementary Fig. S1). The 16S rRNA gene sequences analysis indicated that strain PI^T had the highest similarity with *K. taiwanensis* KT1^T (98.3%) and *K. sediminilitoris* BB-Mw22^T (98.3%), followed by *K. marina* KM1^T (97.1%), *K. japonica* KMM3899^T (99.0%), *K. geojedonensis* YCS-5^T (97.0%), *K. koreensis* SW-125^T (96.0%), *K. spongicola* A79^T (95.9%), *K. aquimarina* SW-154^T (95.9%), *K. chungangensis* CAU 1040^T (93.7%) and other related species in the family *Kangiellaceae*.

Phenotypic characteristics

The phenotypic characteristics of strain PI^T were almost similar to three reference strains, *K. aquimarina* KCTC 12183^T (Yoon et al. 2004), *K. taiwanensis* JCM 17727^T (Jean et al. 2012) and *K. sediminilitoris* KCTC 23892^T (Lee et al. 2013), as well as other *Kangiella* species (Yoon et al. 2004; Romanenko et al. 2010; Ahn et al. 2011; Yoon et al. 2012; Jean et al. 2012; Kim et al. 2015; Xu et al. 2015). Cells of strain PI^T were determined to be Gram-stain-negative, wheat, rod-shaped, aerobic, non-spore-forming, oxidase and catalase-positive, which are consistent with these *Kangiella* species. Under the electron microscope, strain PI^T was 0.4–0.5 μm in width, 1.0–2.0 μm in length (Supplementary Fig. S2). Growth of strain PI^T was observed over a pH range of 6.0–9.0 and NaCl concentrations range of 1.0–11.0% (w/v), which was consistent with the species mentioned above. Different from other *Kangiella* species, strain PI^T was a motile life form which could grow at lower and higher temperatures, ranging from 4 ℃ to 45 ℃. Other differential phenotypic characteristics were provided in Table 1. The differences above determined that strain PI^T was a distinct *Kangiella* species.

Table 1 Differential phenotypic characteristics of strain PI^T and other closely related members of the genus *Kangiella*.

Strains: 1, PI^T; 2, *Kangiella aquimarina* KCTC 12183^T; 3, *Kangiella sediminilitoris* KCTC 23892^T; 4, *Kangiella taiwanensis* JCM 17727^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: 2 (Yoon et al. 2004), 3 (Lee et al. 2013), 4 (Jean et al. 2012).

Characteristic	1	2	3	4
Facultatively anaerobic	-	+	+	+
Temperature range for growth (℃)	4-45	10-48	10-40	15-40
Gliding motility	+	-	-	-
NaCl range for growth (%, w/v)	1-11	0.5-12	0.5-13	0.5-12
pH range (optimum, %)	6.0-9.0	5.5-8.0	5.5-7.0	7.0-9.0
Nitrate reduction	-	+	-	+
Hydrolysis of:				
β-galactosidase	-	-	+	-
Gelatin	+	+	-	+
cystine arylamidase	-	-	w	+
trypsin	+	+	w	+
Esterase (C4)	w	+	+	+
Esterase lipase (C8)	w	+	+	+
α-Chymotrypsin	-	-	w	+
Acid phosphatase	+	+	-	-
naphthol-AS-BI-phosphohydrolase	+	+	w	-
alkaline phosphatase	+	+	+	-
Trypsin	+	+	w	+
Acids from:				
arabinose	+	-	-	-
sucrose	-	-	+	-
DNA G+C content (mol%)	45.8	44.0	48.9	43.9

Chemotaxonomic characteristics

As shown in Table 2, the main fatty acids of strain PI^T composed of iso-$C_{15:0}$ (52.7%) and Summed Feature 9 (iso-$C_{17:1}\omega 9c$) (11.8%), which was similar to those of the genus *Kangiella*. However, the amount

of iso-$C_{15:0}$, iso-$C_{11:0}$ 3-OH and Summed Feature 9 (iso-$C_{17:1}$ $\omega 9c$) could be used to different it from the reference strains. The predominant ubiquinone of strain PI^T was Q-8, which was consistent with other members of the genus *Kangiella* (Yoon et al. 2004; Romanenko et al. 2010; Ahn et al. 2011; Yoon et al. 2012; Jean et al. 2012; Lee et al. 2013; Kim et al. 2015; Xu et al. 2015). The polar lipids included diphosphatidylglycerol (DPG); phosphatidylethanolamine (PE); phosphatidylmonomethylethanolamine (PME) and phosphatidylinositol (PI) (Supplementary Fig. S3). The major lipid (PME) was similar to the reference strains *K. sediminilitoris* (Lee et al. 2013) and *K. taiwanensis* (Jean et al. 2012). PI detected in strain PI^T was different from the reference strains.

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

Strains: 1, PI^T; 2, *K. sediminilitoris* KCTC 23892^T; 3, *K.aquimarina* KCTC 12183^T; 4, *K. taiwanensis* JCM 17727^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	4
Saturated fatty acids				
$C_{14:0}$	1.4	1.7	TR	1.5
$C_{16:0}$	4.9	1.2	4.4	2.7
Branched fatty acids				
iso-$C_{11:0}$	5.2	8.2	2.9	5.7
iso-$C_{13:0}$	TR	3.8	TR	1.0
iso-$C_{14:0}$	TR	TR	TR	2.3
iso-$C_{15:0}$	**52.7**	**53.0**	**49.4**	**47.7**
iso-$C_{15:1}$ F	2.5	2.0	3.6	2.0
anteiso-$C_{15:0}$	TR	3.3	TR	TR
iso-$C_{16:0}$	TR	TR	1.4	3.2
iso-$C_{17:0}$	4.9	1.8	6.7	4.2
iso-$C_{16:1}$ G	-	-	-	2.9
Hydroxy fatty acids				
iso-$C_{11:0}$ 3-OH	8.6	**14.0**	6.2	**11.2**
Summed Feature 1*	TR	2.5	1.3	1.8
Summed Feature 9&	**11.8**	3.9	**16.4**	**10.1**

*Summed Feature 1, $C_{13:0}$ 3-OH and/or iso- $C_{15:1}$ H/i;

&Summed Feature 9, iso-$C_{17:1}\omega 9c$

Genome features

Genome features of strain PI^T were depicted in Supplementary Table S1. The genome size of the strain PI^T was 2809662 bp consisting of 2653 genes which include 39 tRNAs genes, 2 sRNA genes and 4 rRNA genes (one 5S, one 16S and two 23S rRNA genes). The N50 value was 308341 bp and the N90 value was 96858 bp. The depth of sequencing coverage was 549 ×. The gene length distribution of strain PI^T was mainly concentrated in the range of 300–800 bp and more than 2000 bp (more than 200 bp), the detailed information was depicted in supplementary materials (Fig. S4). The secondary function of strain PI^T

according to KEGG consisted of metabolism, genetic information processing, environmental information processing, cellular processes, human diseases and organismal systems. There were 2730 genes described by KEGG result annotation, in which 444 genes were related to metabolic pathways, 203 genes were related to biosynthesis of secondary metabolites, 149 genes were related to biosynthesis of antibiotics and 113 genes were related to microbial metabolism in diverse environments. Virulence Factors of Pathogenic Bacteria (VFDB) analysis indicated that strain PI^T possessed virulence related genes, which involved in induction of apoptosis, proteolysis, immune system evasion, efflux pump, glycosylation of the flagellin, biofilm formation, cellular invasion, cell adhesion, resistance to serum killing and antibiotic susceptibility. The secondary metabolite of strain PI^T was predicted to be arylpolyene and siderophore, which was consisted 12 and 49 genes, respectively. The resistance genes of strain PI^T determined its resistance to fluoroquinolone, beta-lactam, isoniazid, aminoglycoside and glycopeptide (Details in Table S3). The Cluster of Orthologous Groups of proteins (COG) of strain PI^T included 24 function classifications, for instance, those involved in posttranslational modification, protein turnover, chaperones, transcription, lipid transport and metabolism, coenzyme transport and metabolism, secondary metabolites biosynthesis, transport and catabolism (detailed in Fig. S5).

The DNA G + C content of strain PI^T was 48.5 mol%, which was within the range of DNA G + C contents for species of the genus *Kangiella* (43–49 mol%) previously reported. The AAI value between strain PI^T and the most related type strain *K. sediminilitoris* was 83.9% (between 90% and 60%, which were probably the threshold value for species and genus, respectively) (Rodríguez-R and Konstantinidis 2014a). The ANI values between strain PI^T and the reference strains of *K. sediminilitoris*, *K. aquimarina* and *K. taiwanensis* were 76.9%, 71.0% and 74.6%, respectively, which were far below the described ANI values (95.0–96.0%) for species classification (Kim et al. 2014). The dDDH values between strain PI^T and *K. sediminilitoris*, *K. aquimarina* and *K. taiwanensis* were 19.8%, 19.1% and 18.7%, respectively, which were lower than the prescribed species delineation standard of 70% (Meier-Kolthoff et al. 2013). The specific result of ANI, dDDH and AAI values between strain PI^T and neighbour type strains were depicted in supplementary materials (Table S2). Based on the analysis of genome sequence, strain PI^T was proposed to accommodate the novel strains of genus *Kangiella*. A complete 16S rRNA gene sequence (1564 bp) obtained from the genome sequence was 99.8% similar with the clone sequence (1525 bp) deposited in GenBank under accession number JACNML000000000.

Description of Kangiella shandongensis **sp. nov**

Kangiella shandongensis (shan.dong.en'sis. N.L. fem. adj. *shandongensis* pertaining to Shandong, where the type strain was isolated).

Cells (0.4–0.5 μm×1.0–2.0 μm) are Gram-staining negative, aerobic, motile, rod-shaped and non-spore-forming. The colonies are wheat, circular, flat, smooth, opaque and 1–2 mm in diameter after cultured on MA at 30 ℃ for 3 days. Growth occurs at 4–45℃ (optimum 33℃), pH 6.0–9.0 (optimum 7.0) and 1.0–11.0% (w/v) NaCl (optimum 3.0%). The predominant ubiquinone is Q-8 and the major fatty acids are iso-

$C_{15:0}$, iso-$C_{11:0}$ 3-OH, and Summed Feature 9 (iso-$C_{17:1}\omega 9c$). The major polar lipids are diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylmonomethylethanolamine and phosphatidylinositol. The DNA G + C content is 45.8 mol%.

The type strain is PI^T (= KCTC 82509^T = MCCC $1K04352^T$), isolated from saline silt samples collected in saltern in Yantai, Shandong, China. The 16S rRNA gene and genome sequences of strain PI^T were submitted to GenBank with accession numbers MW407073 and JACNML000000000 respectively.

Abbreviations

AAI Average Amino Acid identity

ANI Average Nucleotide Identity

COG Cluster of Orthologous Groups

dDDH digital DNA-DNA Hybridization

DPG diphosphatidylglycerol

GGDC Genome-to-Genome Distance Calculator

HPLC High Performance Liquid Chromatography

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

PE phosphatidylethanolamine

PI phosphatidylinositol

PME phosphatidylmonomethylethanolamine

VFDB Virulence Factors of Pathogenic Bacteria

Declarations

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Author's contributions LYP wrote the manuscript and performed the research. HNJ, XXL and YM performed isolation, deposition, and identification. LYP and SKG performed genome analysis. YXZ contributed to study design. YXZ and RY revised the paper. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest All the authors have declared no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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Figures

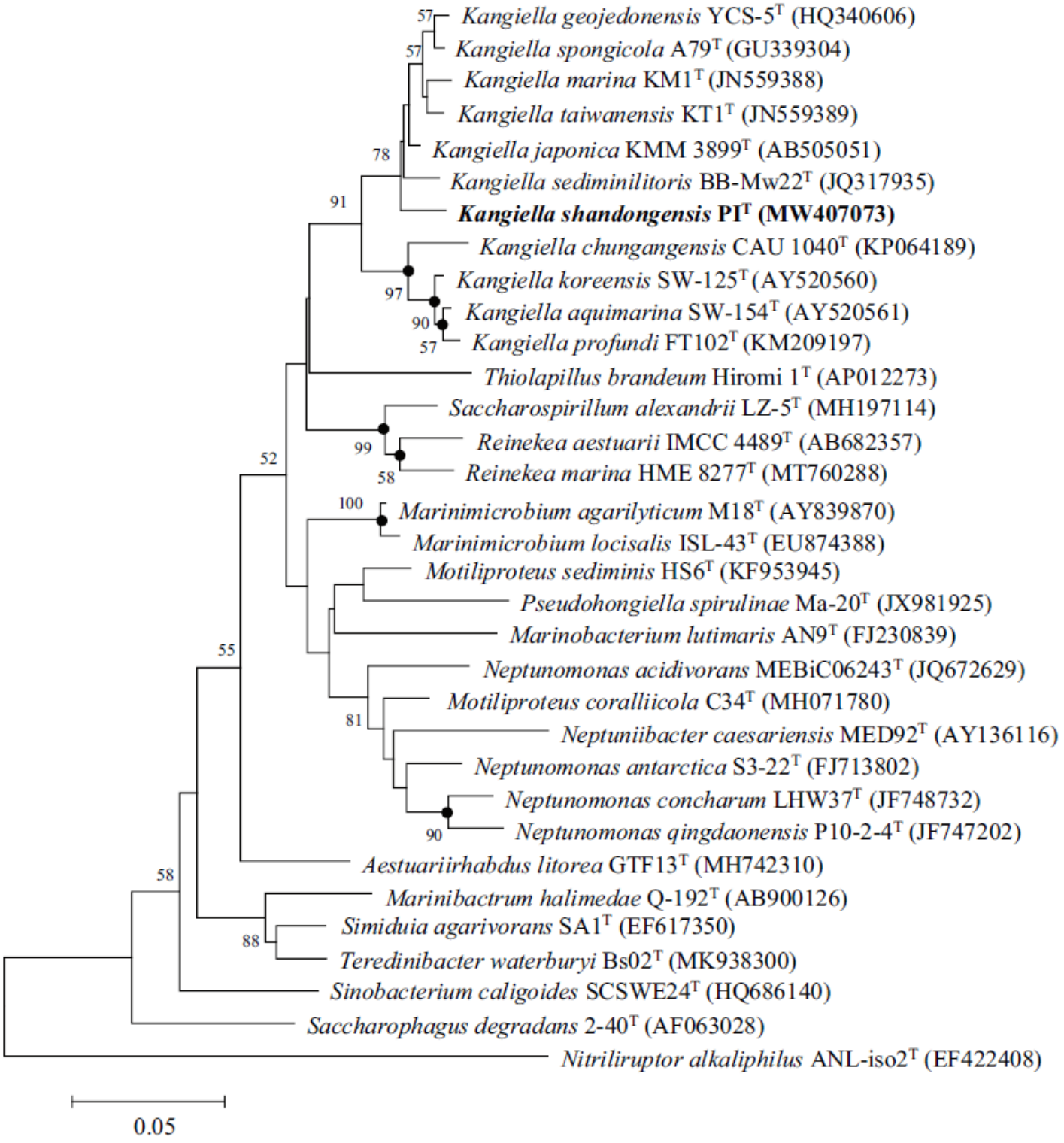

Figure 1

Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, depicting the relationships between strain PIT and closest phylogenetic neighbours. Bootstrap support values (1000 replications) above 50% are shown at nodes. The filled circles indicate that the corresponding node is also restored in the maximum-likelihood tree. Nitriliruptor alkaliphilus ANL-iso2T was used as an outgroup organism. Bar, 0.05 substitutions per nucleotide position.

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