Pseudaestuariivita rosea sp. nov., a novel species of genus Pseudaestuariivita, isolated from Acmaea, a kind of marine mollusea

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Pseudaestuariivita rosea sp. nov., a novel species of genus *Pseudaestuariivita*, isolated from Acmaea, a kind of marine mollusea.

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Abbreviations:

AAI, Average Amino Acid identity

ANI, Average Nucleotide Identity

cAAI, core-gene average amino acid identity

COG, Cluster of Orthologous Group

dDDH, digital DNA-DNA Hybridisation;

GGDC, Genome-to-Genome Distance Calculator

HPLC, High Performance Liquid Chromatography

KCTC, Korean Collection for Type Cultures

KEGG, Kyoto Encyclopedia of Genes and Genomes

LPS, lipopolysaccharide

MA, Marine agar 2216

MB, Marine broth 2216

MCCC, Marine Culture Collection of China

MEGA, Molecular Evolutionary Genetics Analysis

NCBI, National Center for Biotechnology Information

PC, phosphatidylcholine

PE, phosphatidylethanolamine

PG, phosphatidylglycerol

POCP, percent of conserved proteins

TLC, Two-dimensional thin-layer chromatogram

VFDB, Virulence Factors of Pathogenic Bacteria.

Abstract

A Gram-stain-negative, pink-pigmented, facultatively anaerobic, gliding and rod-shaped bacterium, showing optimum growth at 33 °C, designated as strain H15^T, was isolated from the gut microbial of the Acmaea from Weihai, Shandong Province, China and characterised phylogenetically, phenotypically and chemotaxonomically. Phylogenetic analysis based on 16S rRNA gene sequence comparisons revealed that the strain belonged to the family *Rhodobacteraceae* and was associated with members of the recognized genera, the closest relative was the type strain of *Pseudaestuariivita atlantica* (96.7%). Genome analysis showed that the genome size was 3893398 bp and the DNA G + C content obtained from the draft genome sequence was 56.7%. The secondary metabolites based on genome predicated that the strain H15^T contained one cluster of lassopeptide, one cluster of bacteriocin, two clusters of terpene production, two clusters of homoserine lactone (Hserlactone) production and one cluster of beta lactone. The average amino acid identity, average nucleotide identity and digital DNA–DNA hybridization values between genome sequences of strain

H15^T and all the type strains of the recognized taxa compared were lower than 63.1, 72.0 and 19.7%, respectively. Based on the analysis of chemical components, the predominant cellular fatty acids were summed featured 8 ($C_{18:1}\omega7c/\omega6c$, 46.1%), $C_{20:1}$ $\omega7c$ (17.1%), the major polar lipids contained phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine and an unidentified lipid and the predominant menaquinone was Q10. Therefore, the combined chemotaxonomic, phenotypic and phylogenetic data indicated that the strain was considered to represent a novel species of the genus *Pseudaestuariivita* and the name *Pseudaestuariivita rosea* sp. nov. was proposed for strain H15^T (MCCC 1K04420^T = KCTC 82505^T).

Keywords: Genome · Marine creature · Polyphasic analysis · *Pseudaestuariivita* · 16S rRNA

Declarations

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Authors' contribution

YJY contributed to the sample collection and biochemical characterization. STY analysed the data and drafted the manuscript. ZC contributed to the construction of

phylogenetic tree. MJZ contributed to revision of this article. SKG contributed to genome submission and analyses. YXZ designed the experiments and revised the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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

Introduction

The genus *Aestuariivita*, was first established in 2014 by Sooyeon Park as a novel genus and embraced 2 species, including *Aestuariivita boseongensis* (Park et al. 2014), and *Aestuariivita atlantica* (Li et al. 2015). Until 2018, the boundaries of bacterial genera based upon the multi-faceted analysis were redefined by Wirth and Whitman and proposed that *Aestuariivita atlantica* be reassigned to the novel genus *Pseudaestuariivita* with the type species *Pseudaestuariivita atlantica* on the basis of the low core-gene average amino acid identity (cAAI) and percent of conserved proteins (POCP) (Wirth and Whitman 2018). Therefore, the novel genus *Pseudaestuariivita* was constructed and at the time of writing in 2021, the genus *Pseudaestuariivita* comprised only one species with validly published names. The species was in description of Gram-staining-negative, oxidase-positive and catalase-negative, oval-to rod-shaped, and non-motile with grey-white-colored

colonies (Li et al. 2015).

As described above, the novel strain H15^T was isolated from Acmaea, a kind of mollusk creature, with a description of Gram-stain-negative, pink-pigmented, motile and rod-shaped. The 16S rRNA gene sequence indicated that strain H15^T belonged to the family *Rhodobacteraceae* and showed the highest sequence similarity value to *Pseudaestuariivita atlantica* KCTC 42276^T (96.7%). And other analyses were presented in this study for the specific description of the novel strain and determinization of the exact taxonomic position.

Materials and methods

Isolation and culture conditions

Strain H15^T was isolated from Acmaea, a mollusk collected from Weihai, Shandong Province, China (121°58'39.9″ E, 37°28'29.7″ N). Sampling was processed in the laboratory and purified by ten-fold dilution series method. The dilution was then inoculated on marine agar 2216 (MA, Becton Dickinson). Pink colonies were obtained after incubation at 30 °C for 5 days and designated as strain H15^T. The strain was then deposited at Korean Collection for Type Cultures (KCTC) and Marine Culture Collection of China (MCCC) and the experimental reference strain *Pseudaestuariivita atlantica* KCTC 42276^T was purchased from KCTC. After purification, the isolated pure cultures were cultured under comparable conditions for physiological and chemotaxonomic features. And they were preserved frozen at –80 °C in sterile distilled water supplemented with 1.0% NaCl (w/v) and 15.0% (v/v)

105 glycerol. When necessary, they were grown for 3–5 days at 30 °C under aerobic
106 condition on MA medium.

107 Phylogenetic and genome characterization

108 The 16S rRNA sequences of strain H15^T obtained was determined by PCR using
109 bacterial universal primers (Kim et al. 2014), and the PCR product was purified using
110 a PCR purification kit (Sangon Biotech, China). Obtained sequences were assembled
111 using Bioedit (Tippmann 2004) and compared with those of validly published species
112 in the EzBioCloud web service (Yoon et al. 2017a) and National Center for
113 Biotechnology Information (NCBI) database by using BLAST search. Based on all
114 the strains with 16S rRNA sequence similarity of 95.0% above, multiple alignments
115 of their sequences were performed using Clustal_X version 1.83 with default settings
116 (Thompson et al. 1997). Maximum-likelihood (Felsenstein 1981) and
117 Neighbor-Joining (Saitou and Nei 1987) phylogenetic tree was constructed for the
118 analyses of phylogenetic and molecular evolutionary in the software package MEGA
119 version 7.0 (Kumar et al. 2016) with the Kimura two-parameter model (Kimura 1980),
120 and the topology of each tree was evaluated by the bootstrap analysis based on 1000
121 random re-samplings of the sequences.
122 The draft genome sequence of strain H15^T was determined using paired-end
123 sequencing method with the Illumina HiSeq-PE150 platform. Assembly of raw reads
124 was performed using the SOAP and Abyss software and CISA software for
125 integration. The annotation of genome sequence was processed using the GeneMarkS,

126 rRNAmmer, and Rfam software. These operations were all implemented by Beijing
127 Novogene Bioinformatics Technology Co, Ltd. (Beijing, China). Functional
128 annotation of sequences was performed by using Blast against the non-redundant
129 GenBank database. The genes involved in metabolic pathways were analysed using
130 the Kyoto Encyclopedia of Genes and Genomes (KEGG) databases (Kanehisa et al.
131 2016). Protein-encoding regions were identified and annotated with the Rapid
132 Annotations using Subsystems Technology (RAST) server
133 (http://rast.nmpdr.org/rast.cgi) (Aziz et al. 2008) and the UniProtKB / Swiss-Prot
134 (Consortium 2018). Secondary metabolite was presented by antiSMASH 5.0 (Blin et
135 al. 2019). In order to assess the pathogenic potential of the strain H15^T, Virulence
136 Factors of Pathogenic Bacteria (VFDB) database(Chen et al. 2005) was used to offer
137 the species information and basic description of virulence genes, detailed descriptions
138 of virulence gene functions and pathogenic mechanisms provided as well. The DNA
139 G + C content of strain H15^T was determined from the genome sequence.
140 The average amino acid identity (AAI) (Konstantinidis and Tiedje 2005; Rodriguez-R
141 and Konstantinidis 2014) calculator was carried out between strain H15^T and its
142 closely related species (http://enve-omics.ce.gatech.edu/aai/). The average nucleotide
143 identity (ANI) values and Digital DNA–DNA hybridization (dDDH) values were
144 determined for the genome-based similarities, using the EzBioCloud integrated
145 database (Yoon et al. 2017a) OrthoANIu algorithm of the EzGenome web service
146 (Yoon et al. 2017b) and the Genome-to-Genome Distance Calculator (GGDC) version
147 (Meier-Kolthoff et al. 2013).

148 Morphology, physiology and biochemical analysis

149 The cell morphology, cell size, and presence of flagella were observed via scanning
150 electron microscopy (Jem-1200; JEOL) after incubating on MA at 30 °C for 5 days.
151 Basic phenotypic characterization such as gram staining, optimal pH and motility
152 were presented as follows: growing status of strain H15^T was measured on MA at
153 different temperatures (4, 10, 15, 20, 25, 30, 33, 37, 40, 42, and 45 °C), and the
154 optimum growth temperature was obtained from above. The pH tolerance range (pH
155 5.5–9.5) was determined in MB with a concentration of 20 mM using the buffer
156 systems as described by Yin (2021). Test on the effect of NaCl was implemented in
157 NaCl-free artificial seawater medium supplemented with various concentrations of
158 NaCl (final concentration 0.0–10.0%, in increments of 0.5%) (Yang and Cho 2008).
159 Gram staining was performed as described by Smibert and Krieg (Smibert and Krieg
160 1994). Investigation on anaerobic growth was carried out after the strain H15^T was
161 cultured on MA with or without 0.1% (w/v) $NaNO_3$ under anaerobic conditions at
162 30 °C for 2 weeks. Gliding motility was observed by oil-immersion phase-contrast
163 microscopy (AX70; Olympus) according to the method by Bowman (Bowman 2000).
164 In addition to the fundamental activities of catalase and oxidase, hydrolysis of
165 cellulose, agar, casein, and Tween 80 tested by the conventional procedures as
166 described by Tindall (2007), additional physiological tests were also carried out using
167 API 20E, API 20NE and API ZYM strips (bioMérieux) and the oxidation and
168 fermentation of carbohydrates were detected after growth on MA at 30 °C for 3 days
169 using the Biolog GEN III Micro Plates and API 50CHB Fermentation Kit

(bioMérieux) according to the manufacturer's instructions. Antibiotic sensitivity was assessed on MA plates with discs (Tianhe) containing various antibiotics for 3 days at 30 °C.

Chemotaxonomy

Chemotaxonomic characteristics of strain H15^T and the reference strain were determined under similar conditions for the sake of avoiding large differences caused by the cultured environments. And the freeze-drilled strains were also needed. For the analysis of fatty acid, both of strains were grown on MA medium at 30 °C. The cellular fatty acids were extracted, methylated and analysed by Sherlock Microbial Identification System (MIDI) (Sawant et al. 2015) with the TSBA6.0 database. Detection of respiratory quinone and polar lipids were completed with High Performance Liquid Chromatography (HPLC) (Hiraishi et al. 1996) and the two dimensional thin-layer chromatography using the method of Minnikin (1984), respectively.

Results and discussion

Phylogenetic analysis

Based on 16S rRNA gene sequences extracted from the draft genome, phylogenetic analysis showed that strain H15^T shared the highest similarity with *P. atlantica* (96.7%). In the neighbor-joining phylogenetic tree based on 16S rRNA gene sequences, strain H15^T formed a separate branch from *P. atlantica* (Fig. 1), and the

Maximum-Likelihood phylogenetic tree showing the similar taxonomic position given in Fig. S1.

AAI, ANI and dDDH values between strain H15^T and *P. atlantica* were 63.1%, 72.0% and 19.7%, respectively. Commonly, it was suggested that AAI value of 90% and 60% were probably the threshold value for species and genus, respectively (Rodriguez-R and Konstantinidis 2014). AAI value of 63.1% supported that strain H15^T represented a new species. Besides, the ANI and dDDH values of the strains were far below the recommended cut off values (95–96% cut off for ANI and 70% for dDDH) for prokaryotic species delineation (Chun et al. 2018), and the detailed information between strain H15^T and neighbor type strains were shown in supplementary materials (Table S1).

Genome features

General features of strain H15^T genome were summarised in Table S2. The genome of strain H15^T had 4066 predicted genes of 3 rRNA operons and 40 tRNA coding with a total length of 3494520 bp. The total coverage depth was 394 ×. The N50 and N90 value was 114996 bp and 24994 bp, respectively.

Cluster of Orthologous Groups (COG), a protein database created and maintained by NCBI, was based on the complete genomes of bacteria, algae and eukaryotes, and the results of the COG annotation of strain H15^T were listed in Table S3. The genome contained several genes coding for amino acid transport and metabolism, translation, ribosomal structure and biogenesis, inorganic ion transport and metabolism and

several functions unknown, which participated in several aspects of cell life activities. And the similar results were shown in the reference strain as well. Particularly, genes coding for extracellular structure, nuclear structure and cytoskeleton were not found in reference strain, which presented differences with the strain H15^T. And the detailed function classification annotated by COG was shown in Fig. S2.

Specially, strain H15^T contained genes relating to the synthesis and accumulation of carotenoid pigments, which may explain why the strain H15^T was pink-pigmented. Among them, several genes such as *crt*I, *crt*F and *crt*B played a vital role in synthesis of lycopene, which was a red carotene and carotenoid pigment, one of the most potent antioxidants according to Narges Hedayati et al(2019). Several genes encoding enzymes involved in bioremediation were also detected in the novel strain, such as 2-halo acid dehalogenase and haloalkane dehalogenase, two key enzymes participated in chloroalkane and chloroalkene degradation, which may show activity to degrade diesel fuel.

Among Virulence Factors of Pathogenic Bacteria (VFDB) database, several genes were linked to adherence, polysaccharide capsule, pyrimidine biosynthesis, immune system evasion, flagella, protease, enzyme and others. The categories with more genes predicted were protease, such as catalase-peroxidase and urease, which was an important colonization factor, contributing to acid resistance, epithelial cell damage, chemotactic behavior and nitrogen metabolism, and also by the way of adherence associated with flagella and lipopolysaccharide (LPS) (Table S4). In addition, the secondary metabolite of strain H15^T were predicted to terpene, hserlactone,

bacteriocin, lasso peptide and beta lactone, which were considered as important raw materials for drug synthesis.

Morphology and Phenotypic characterization

Cells were observed to be Gram-stain-negative, facultatively anaerobic, motile, gliding, lacking flagella, and rod-shaped. A pink colony with a diameter of 1.5 mm was obtained after incubating on MA for 3 days at 30 °C (Fig. S3). Growth was observed at 4–37 °C (optimum 33 °C), pH 5.5–9.0 (optimum 7.0). The NaCl concentrations for growth were 0.0–7.0% (optimum 4.0%). Strain H15^T possessed the enzymes catalase and oxidase, different from *P. atlantica*, but it was cellulase-negative and alginate-negative. Enzymatic activities determined for strain H15^T indicated positive results for alkaline phosphatase, leucine aryl amidase and acid phosphatase, with negative results for trypsin, α-chymotrypsin, α-galactosidase, β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase, and other results such as assimilation of substrates also shown highly consistency with *P. atlantica* (Li et al. 2015). They all shown negative results for assimilation of phenylacetic acid, citric acid, malic acid, adipic acid, capric acid, gluconate, maltose, N-acetyl-glucosamine, mannose, mannitol, gelatin and arabinose. Moreover, utilization of urease, esterase (C4), esterase lipase (C8), lipase (C14) and β-galactosidase were the notable characteristics to distinguish the strain H15^T from type strain of *P. atlantica.* And other phenotypic characteristics of the strain H15^T and the related strain were shown in Table 1. Furthermore, the result for negative for the

utilization as sole carbon and energy sources a are described in Table S5. Besides, it was sensitive to penicillin, ampicillin, cephalosporin, gentamicin, erythromycin and amphenicol, resistant to amikacin.

Chemotaxonomy

The major fatty acids of strain H15^T were identified as summed featured 8 ($C_{18:1}\omega7c/\omega6c$, 46.1%), $C_{20:1}$ $\omega7c$ (17.6%), along with small amounts of $C_{18:0}$ (8.5%), $C_{16:0}$ (5.3%), $C_{18:1}$ 2-OH (5.9%) and 11-methyl 18:1 $\omega7c$ (4.9%). And the results were basically identical with the description of the reference strain (Li et al. 2015). But the presence of $C_{20:1}$ $\omega7c$ and $C_{18:1}$ 2-OH can be used to differentiate the novel strain from the reference strain (Table 2). The polar lipids of strain H15^T were found to include phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidylcholine (PC) and an unidentified lipid (L) (Fig. S4). Q10 was the predominant quinone of strain H15^T.

Conclusion

Strain H15^T shared the highest similarity of 16S rRNA gene sequence with *P. atlantica* KCTC 42276^T. The results of the phylogenetic analysis, phenotypic analysis and chemotaxonomic studies described in this study supported the view that strain H15^T should be assigned to the genus *Pseudaestuariivita*. However, strain H15^T could be distinguished from the reference strain by some phenotypic characteristics given in Table 1. Considering its features when compared with the most closely related specie, the strain cannot be assigned to *P. atlantica*. Therefore, strain H15^T represents a novel

species of the genus *Pseudaestuariivita*, for which the name *Pseudaestuariivita rosea* sp. nov. is proposed.

Description of *Pseudaestuariivita rosea* sp. nov.

Pseudaestuariivita rosea (ro'se.a. L. fem. adj. rosea referring to the color characteristic of bacteria).

Cells are observed to be Gram-stain-negative, facultatively anaerobic, motile, gliding, lacking flagella, and rod-shaped. The pH range for growth is pH 5.5–9.0 (optimum 7.0). Growth temperature range is between 4–37 °C with optimum growth at 33 °C. The optimum concentration of NaCl for growth is 4.0% (w/v). Basic enzyme activities show positive for oxidase and catalase, negative for cellulase, alginate and amylase. Furthermore, the major fatty acids are summed featured 8 ($C_{18:1}$ ω7*c*/ω6*c*, 46.1%) and $C_{20:1}$ ω7*c* (17.1%). Phosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine and an unidentified lipid are depicted as polar lipids. Q10 is the respiratory quinone.

The type strain H15^T (= MCCC 1K04420^T = KCTC 82505^T) was isolated from Acmaea collected from Weihai, Shandong Province, China. The genomic DNA G + C content is 56.7%. The Genbank accession numbers for the 16S rRNA gene sequence is MW407011 and the number for the whole genome sequence is JACNMP000000000.

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Table 1. Differential characteristics of strain H15^T and other closely related member. Strains: 1, H15^T; 2, *Pseudaestuariivita atlantica* KCTC 42276^T. +, Positive; -, negative; w, weak.

Characteristics	1	2
Colony color	pink	Grey-white
Gram-staining	–	–
Cell size (μm)	2.5–3.0	0.8–1.0×1.3–1.8
Gliding motility	+	–
Growth range:		
Temperature range (optimum, °C)	4–37 (33)	10–45 (28)
NaCl range for growth (optimum, w/v)	0–7 (4)	1–9 (3–5)
pH range (optimum, %)	5.5–9.0 (7.0)	5–10 (7–8)
Enzyme activity:		
Catalase activity	+	–
Oxidase activity	+	–
Esterase (C4)	w	–
Lipase (C14)	–	w
β-galactosidase	+	–
Reduction of nitrate	–	–
Ureas	+	–
Valine arylamidase	–	w
Cystine arylamidase	–	w
Acids from:		
D-mannose	+	–
D-glucose	+	–
D-melibiose	+	–
Carbon source oxidation:		
Citric acid	–	+

413 **Table 2.** Cellular fatty acid composition of strain H15^{T}and the closest relatives.

414 Strains: 1, H15^T; 2, *Pseudaestuariivita atlantica* KCTC 42276^T. All data were taken

415 from this study. TR, Traces (<1.0%); -, not detected. Fatty acids amounting to <1.0 %

416 of the total fatty acids in both strains are not shown.

417

Fatty acid	**1**	**2**
Saturated fatty acids		
$C_{9:0}$	TR	TR
$C_{10:0}$	TR	1.4
$C_{14:0}$	TR	TR
$C_{16:0}$	5.3	**17.6**
$C_{18:0}$	8.5	5.1
$C_{20:0}$	TR	TR
Branched fatty acids		
iso-$C_{10:0}$	-	TR
iso-$C_{12:0}$	TR	-
iso-$C_{16:1}$ H	-	TR
Hydroxy fatty acids		
$C_{10:0}$ 3-OH	2.1	TR
$C_{12:0}$ 3-OH	TR	TR
$C_{12:1}$ 3-OH	-	6.4
$C_{16:0}$ 2-OH	TR	TR
$C_{18:1}$ 2-OH	5.9	-
$C_{18:0}$ 2-OH	3.5	-
Unsaturated fatty acids		
$C_{18:1}$ ω9*c*	TR	TR
$C_{20:1}$ ω7*c*	**17.1**	-
iso-$C_{17:1}$ ω5*c*	TR	TR
$C_{19:0}$ cyclo ω8*c*	-	**22.3**
Summed Feature 3[#]	TR	1.1
Summed Feature 8*	**46.1**	**26.2**

418 [#]Summed feature 3, $C_{16:1}$ ω7*c* and/or $C_{16:1}$ ω6*c*

419 *****Summed feature 8, $C_{18:1}$ ω7*c* and/or $C_{18:1}$ ω6*c*

Figures

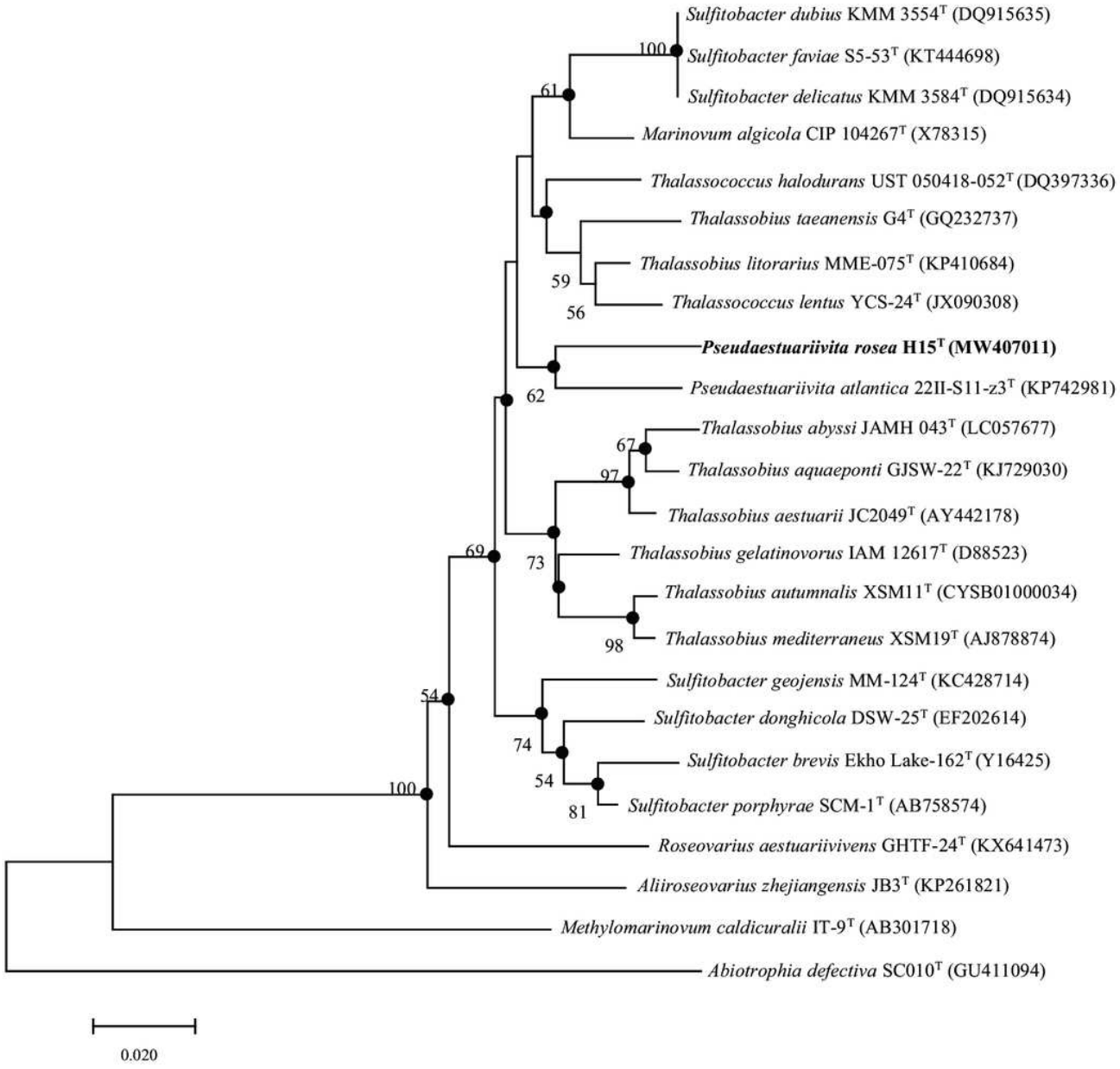

Figure 1

Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences of strain MST and high similarity of genus. Bootstrap support values (1000 replications) above 50% are shown at nodes. Abiotrophia defectiva SCO 10T was used as an outgroup. Bar, 0.02 substitutions per nucleotide position.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarymaterials.pdf