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Marimonas arenosa gen. nov., sp. nov., isolated from sea sand

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Abstract

A Gram-stain-negative, non-motile, non-spore-forming, aerobic and short rod-shaped bacterial strain, CAU 1311^T, was isolated from sea sand in the Republic of Korea. Strain CAU 1311^T grew at temperatures from 20–37 °C, in the range of pH 6.5–10.0, and under various NaCl concentrations from 0–6 % (w/v). Phylogenetic analysis based on the 16S rRNA gene sequence of CAU 1311^T showed that it formed a distinct lineage within the family *Rhodobacteraceae* as a separate deep branch, with 96.2 % or lower sequence identity to representatives of the genera *Marivita*, *Aestuariivita*, *Mameliella*, *Sulfitobacter* and *Maritimibacter*. The major fatty acid was $C_{18:1}\omega7c$ and the predominant respiratory quinone was Q-10. The major polar lipids were phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, an unidentified aminolipid and an unidentified glycolipid. The DNA G+C content was 60.7 mol%. On the basis of genotypic, phenotypic and chemotaxonomic findings, strain CAU 1311^T could be classified as representing a novel species of a new genus of the family *Rhodobacteraceae*, for which the name *Marimonas arenosa* gen. nov., sp. nov. is proposed. The type strain of the type species is CAU 1311^T (=KCTC 52189^T=NBRC 111988^T).

Alphaproteobacteria are a diverse class of organisms, including the most abundant groups of marine environment micro-organisms, and are present in diverse marine habitats, from coastal to open oceans or sea ice to sea floor, representing up to 25 % of the total bacterial community [1, 2]. The family *Rhodobacteraceae* was established by Garrity *et al.* [3] in the order *Rhodobacterales* within the class *Alphaproteobacteria* [4]. At the time of writing, the family *Rhodobacteraceae* comprises more than 100 genera (http://www.bacterio.net/). Many novel genera belonging to this family have been described recently, for example *Alkalimicrobium* [5], *Frigidibacter* [6], *Halovulum* [7], *Lacimonas* [8], *Pontivivens* [9], *Pseudohalocynthiibacter* [10], *Pseudoseohaeicola* [11], *Pseudooctadecabacter* [12], *Litorisediminivivens* [13], *Maliponia* [14], *Pseudoroseicyclus* [15], *Silicimonas* [16] and *Xuhuaishuia* [17]. Members of the family *Rhodobacteraceae* are Gram-stain-negative and rod-shaped with Q-10 as the predominant quinone and $C_{18:1}\omega7c$ as the major fatty acid. The G+C content of genomic DNA ranges from 50–70 mol%.

In this study, a novel bacterial strain, CAU 1311^T was isolated from a sea sand sample from Eurwangri beach in Incheon, Republic of Korea. Comparative 16S rRNA gene sequence analysis indicated that strain CAU 1311^T belonged to the family *Rhodobacteraceae*. Accordingly, the objective of the present study was to determine the exact taxonomic position of strain CAU 1311^T using a polyphasic approach involving the determination of phenotypic, genotypic and chemotaxonomic properties, and 16S rRNA gene sequence analysis.

In the part of screening, strain CAU 1311^T was isolated by the standard dilution plating technique according to Gordon and Mihm [18] on marine agar (MA; Difco). The sample was diluted with sterilized saline solution and incubated under aerobic conditions at 30 °C for 7 days on MA. The pure colony of strain CAU 1311^T was picked and preserved at −80 °C in 30 % (v/v) glycerol. The most closely related strains, *Marivita geojedonensis* DPG-138^T and *Aestuariivita boseongensis* BS-B2^T, were purchased from the Korean Collection for Type Cultures (KCTC; Jeongeup, Korea) and used as reference strains for biochemical characterization and fatty acid analysis.

Genomic DNA was extracted according to the method of Marmur [19] and the 16S rRNA gene was amplified by PCR following established procedures [20]. The amplified 16S rRNA gene was sequenced directly using a BigDye Terminator Cycle Sequencing kit and an automatic DNA sequencer (model 3730; Applied Biosystems). The 16S rRNA gene sequences of strain CAU 1311^T and closely related taxa were obtained and compared with available 16S rRNA gene sequences by using the EzTaxon-e database (www.ezbiocloud.net) and were aligned with CLUSTAL X 2.1 [21].

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Two supplementary figures are available with the online Supplementary Material.

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Evolutionary distance matrices were created by the neighbour-joining method described by Jukes and Cantor [22]. Phylogenetic trees were reconstructed using the neighbour-joining [23], least-squares [24], maximum-likelihood [25] and maximum-parsimony [26] algorithms in the PHYLIP package [27]. Tree topology was calculated by the bootstrap resampling method [28] with 1000 replicates of the neighbour-joining dataset with the SEQBOOT and CONSENSE programs from the PHYLIP package. The G+C content of the genomic DNA was determined using HPLC by the method of Tamaoka and Komagata [29].

For the investigation of morphological, physiological and biochemical characteristics, strain CAU 1311^T and other reference strains were cultivated on MA at 30 °C for 3 days as an exponential growth phase. Cell morphology was examined by light microscopy (DM 1000; Leica Microsystems) and transmission electron microscopy (JEM 1010; JEOL), using cells from an exponentially growing culture on MA at 30 °C for 3 days. For transmission electron microscopy, cells were negatively stained with 1 % (w/v) phosphotungstic acid and the grids were examined after being air-dried. Gram staining was performed using the Gram staining kit (bioMérieux) according to the manufacturer's instructions. Gliding motility was examined using the hanging-drop method on a 72-h marine broth culture (MB; Difco) as followed by Bowman [30]. To observe the growth conditions, strain CAU 1311^T was cultured on MA and incubated at 4, 10, 20, 30, 37, 40 and 45 °C for 3 days in an aerobic incubator (MIR-253; Sanyo) and an anaerobic chamber (Bactron; Sheldon). The pH range for growth was determined at 30 °C in MB adjusted to pH 4.0–11.5 at increments of 0.5 pH unit by using sodium acetate/acetic acid for pH 4.0–8.0 and Na_2CO_3 buffers for pH 8.0–11.5 [31]. The ability to grow under various NaCl concentrations was investigated in MB supplemented with 0–15.0 % (w/v) NaCl, with the MB prepared according to the formula of the Difco medium except that NaCl was excluded and 0.45 % (w/v) $MgCl_2.6H_2O$ and 0.06 % (w/v) KCl were added. Oxidase activity was tested with 0.1 % (w/v) tetramethyl-*p*-phenylenediamine [32]. Catalase activity was determined by observing bubble production in 3 % (v/v) H_2O_2 solution. Hydrolysis of gelatin, casein, starch and citrate were determined according to Lányi [33], and; Smibert and Krieg [34]. Biochemical characterization tests were carried out using the API 20E and API ZYM systems (bioMérieux) according to the manufacturer's instructions. The strips were read after 24 and 48 h, respectively. Antibiotic susceptibility was determined by disc diffusion method, using Sensi-Disc susceptibility test discs (BD BBL) on MA and incubated at 30 °C. The antibiotics were as follows (µg per disc unless otherwise stated): ampicillin (10), cephalothin (30), cefoxitin (30), gentamicin (10), nalidixic acid (30), penicillin (10 U), polymyxin B (300 U), tetracycline (30), tobramycin (10), trimethoprim/sulfamethoxazole (1.25/23.75) and streptomycin (10). Inhibition zone diameter >10 mm indicated susceptibility and absence or <10 mm indicated resistance [35].

For the determination of fatty acids in whole cells, the cell mass of strain CAU 1311^T and the reference strains were harvested from MA after cultivation for 3 days at 30 °C. The physiological age of the biomass harvested for fatty acid analysis was standardized by observing growth development during incubation of the cultures and choosing the moment of harvesting according to the standard MIDI protocol (Sherlock Microbial Identification System version 6.1). Cellular fatty acid methyl esters were obtained according to Minnikin *et al.* [36], and separated by a 6890N automated gas chromatography system (Agilent Technologies). Peaks were identified by using the MIDI database TSBA6 (MOORE library version 5.0). Respiratory quinones were extracted according to Komagata and Suzuki [37] and analysed by HPLC (Waters). The quinones were eluted by an isocratic solvent system [methanol/isopropyl ether (3 : 1, v/v)] using a flow rate of 1 ml min^{-1}. The major polar lipids of strain CAU 1311^T were separated by using a two-dimensional TLC method according to Minnikin *et al.* [38]. Lipids were characterized by spraying plates with 10 % ethanolic molybdatophosphoric acid, molybdenum blue, ninhydrin, Dragendorff's reagent and α-naphthol/sulphuric acid reagent to detect total lipids, phospholipids, aminolipids, phosphatidylcholine, and glycolipids, respectively (Sigma-Aldrich).

The almost-complete 16S rRNA gene sequence of strain CAU 1311^T (1437 nt) was compared with corresponding sequences of related bacterial strains in the EzTaxon databases (accessed September 2016). The phylogenetic tree based on 16S rRNA gene sequences showed that strain CAU 1311^T was distinct from a clade represented by members of the genera *Marivita*, *Aestuariivita*, *Mameliella*, *Sulfitobacter* and *Maritimibacter* (Fig. 1). In pairwise analyses, *Marivita geojedonensis* DPG-138^T (96.2 %16S rRNA gene sequence similarity), *Aestuariivita boseongensis* BS-B2^T (96.1 %), *Mameliella atlantica* L6M1-5^T (95.7 %), *Sulfitobacter pseudonitzschiae* H3^T (95.7 %) and *Maritimibacter alkaliphilus* HTCC2654^T (95.6 %) were the recognized species that appeared most closely related to strain CAU 1311^T. The trees generated using the least-squares, maximum-likelihood and maximum-parsimony algorithms showed similar topologies with the neighbour-joining tree (data not shown). The G+C content of the DNA of strain CAU 1311^T was 60.7 mol%.

The phenotypic properties of strain CAU 1311^T are given in Table 1. Colonies on MA of CAU 1311^T were 0.1–0.2 mm in diameter, circular, slightly convex and beige after cultivation at 30 °C for 3 days. Cells were Gram-stain-negative, non-motile, non-spore forming, aerobic and short rod-shaped (0.3–0.6 µm wide and 1.4–2.0 µm long) (Fig. S1, available in the online Supplementary Material). Strain CAU 1311^T did not grow under anaerobic conditions. Strain CAU 1311^T grew at 20–37 °C (optimum 30 °C), at pH 6.5–10.0 (optimum pH 7.5), and with 0–6 % (w/v) NaCl (optimum 2 %). The optimum growth temperature distinguished strain CAU 1311^T (30 °C) from the genera *Mameliella* and *Sulfitobacter* (28 °C). Strain CAU 1311^T differed from the

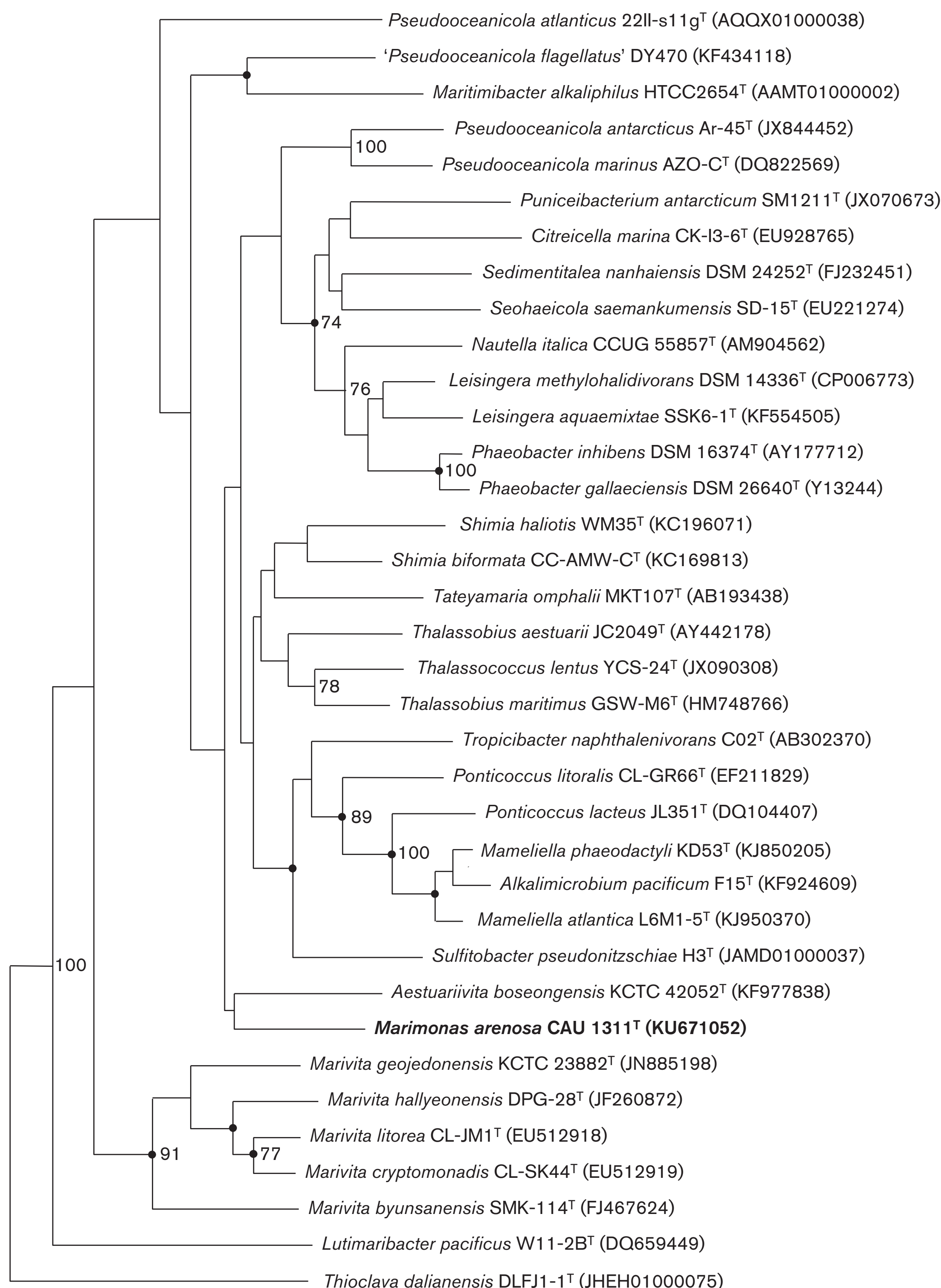

Fig. 1. Neighbour-joining tree showing the phylogenetic positions of strain CAU 1311^T and representatives of some other related taxa based on 16S rRNA gene sequences. Filled circles indicate nodes that were also recovered in maximum-likelihood, maximum-parsimony and least squares algorithms. Bootstrap values >70 % are shown based on a neighbour-joining analysis of 1000 resampled datasets. *Thioclava dalianensis* DLFJ1-1^T (GenBank accession number JHEH01000075) was used as an outgroup organism. Bar, 0.1 substitutions per nucleotide position.

Table 1. Differential characteristics between strain CAU 1311^T and the most closely related genera of the family *Rhodobacteraceae*

Strains: 1, CAU 1311^T (data from this study); 2, *Marivita geojedonensis* DPG-138^T (this study); 3, *Aestuariivita boseongensis* BS-B2^T (this study); 4, *Mameliella atlantica* L6M1-5^T [40]; 5, *Sulfitobacter pseudonitzschiae* H3^T [41]; 6, *Maritimibacter alkaliphilus* HTCC2654^T [42]. All strains are positive for oxidase, catalase, esterase and leucine arylamidase. All strains are negative for arginine dihydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-mannosidase and α-fucosidase. +, Positive; –, negative; W, weakly positive; NA, data not available; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; AL, unidentified aminolipid; PL, phospholipid; GL, unidentified glycolipid.

Characteristic	1	2	3	4	5	6
Optimum growth conditions						
Temperature (°C)	30	30	30	28	28	30
NaCl (%, w/v)	2	2–3	2	3–5	1–3	2.5–3.0
pH	7.5	7.0–7.5	7.0–8.0	7.0	6.0–12.0	10.0
Nitrate reduction	–	–	+	+	–	–
Hydrolysis of:						
Citrate	–	+	+	+	+	–
Gelatin	+	–	–	+	–	–
Aesculin	–	+	–	–	–	+
Urease activity	–	–	+	+	+	+
PNPG activity	–	+	–	+	–	–
Production of:						
Indole	–	–	+	+	–	–
Acetoin	–	+	+	+	–	–
Utilization of sole carbon source						
Glucose	–	+	+	+	+	+
Mannose	–	–	+	+	–	–
N-Acetyl-glucosamine	–	–	+	+	–	–
Maltose	–	–	+	+	–	+
Potassium 5-ketogluconate	+	W	–	–	–	–
Enzyme activity (API ZYM)						
Alkaline phosphatase	–	+	+	+	+	+
Lipase	–	–	–	+	W	–
Valine arylamidase	W	+	+	+	+	–
Acid phosphatase	–	+	–	+	+	–
β-Glucosidase	–	+	–	W	–	–
Major polar lipids	PC, PE, PG, AL, GL	PC, PE, PG, AL, L	PC, PE, PG, DPG, AL, L, PL	PG, PE, AL, PL	PE, PG, AL, PL	PC, PE, PG*
DNA G+C content (mol%)	60.74	59.9	62.2	66	61.7	64.1*

*Data from Lee *et al.* [43].

close relatives *Marivita geojedonensis* DPG-138^T, *Aestuariivita boseongensis* BS-B2^T, *Mameliella atlantica* L6M1-5^T, *Sulfitobacter pseudonitzschiae* H3^T, *Maritimibacter alkaliphilus* HTCC2654^T by activity of alkaline phosphatase and valine arylamidase, and utilization of potassium 5-ketogluconate.

The major polar lipids of strain CAU 1311^T were found to comprise phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, one unidentified aminolipid and one unidentified glycolipid (Fig. S2). The presence of an unidentified glycolipid in the polar lipid profile of strain CAU 1311^T was different from the polar lipid profiles of other related genera. The only respiratory quinone of strain CAU 1311^T was identified as a Q-10, which was consistent with the other genera of the family *Rhodobacteraceae*. The fatty acids of strain CAU 1311^T and a detailed comparison of the fatty acid profiles among the two reference strains are listed in Table 2. Strain CAU 1311^T contained saturated, unsaturated and hydroxyl fatty acids The fatty acid $C_{18:1}\omega 7c$ was a major component in strain CAU 1311^T and closely related genera, which is characteristic of members of the class *Alphaproteobacteria* [39], although there were differences in the proportions of some fatty acids. However, strain CAU 1311^{T}could be distinguished from the most closely related genera *Marivita* and *Aestuariivita* by the presence of $C_{12:0}$ 3-OH. Strain CAU 1311^T is susceptible to cephalothin, streptomycin, cefoxitin, tetracycline, tobramycin and gentamicin, but resistance to trimethoprim/sulfamethoxazole, nalidixic acid, penicillin, ampicillin and polymyxin B.

In conclusion, it is evident from the phenotypic, chemotaxonomic and genotypic data that strain CAU 1311^T positioned within the family *Rhodobacteraceae* in the vicinity of

Table 2. Fatty acid contents (%) of strain CAU 1311^T, *Marivita geojedonensis* DPG138^T and *Aestuariivita boseongensis* BS-B2^T

Strains: 1, CAU 1311^T; 2, *Marivita geojedonensis* DPG-138^T; 3, *Aestuariivita boseongensis* BS-B2^T. All data are from this study. –, Not detected; TR, trace (<1.0 %).

Fatty acid	1	2	3
Saturated			
$C_{16:0}$	2.45	3.9	1.3
$C_{17:0}$	–	TR	TR
$C_{18:0}$	2.79	6.69	TR
Unsaturated			
$C_{18:1}\omega7c$ 11-methyl	6.57	13.81	2.4
$C_{18:1}\omega7c$	84.16	69.41	80.3
Hydroxy			
$C_{12:0}$ 3-OH	3.47	–	–
$C_{12:1}$ 3-OH	–	2.56	3.1

the genera *Marivita* and *Aestuariivita* (Fig. 1). The phylogenetic data and differential chemotaxonomic and other phenotypic data suggest that strain CAU 1311^T could be classified as a representative of a novel species of a new genus in the class *Alphaproteobacteria*, for which the name *Marimonas arenosa* gen. nov., sp. nov. is proposed.

DESCRIPTION OF *MARIMONAS* GEN. NOV.

Marimonas (Ma.ri.mo′nas. L. n. *mare* the sea; L. fem. n. *monas* a unit, monad; N.L. fem. n. *Marimonas* sea monad).

Gram-stain-negative and strictly aerobic bacteria. Cells are catalase- and oxidase-positive, short rod-shaped, non-spore forming and non-motile. The temperature range for growth is 20–37 °C. Growth occurs in the presence of 0–6 % (w/v) NaCl. The pH range for growth is 6.5–10.0. The respiratory quinone is Q-10. The polar lipids consist of phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, one unidentified aminolipid and one unidentified glycolipid. The major fatty acid is $C_{18:1}\omega7c$. The type species is *Marimonas arenosa*.

DESCRIPTION OF *MARIMONAS ARENOSA* SP. NOV.

Marimonas arenosa sp. nov. (a.re.no′sa. L. fem. adj. *arenosa* sandy).

Cells are approximately 0.3–0.6 µm wide and 1.4–2.0 µm long. Colonies on MA are beige, circular and slightly convex, and cells are small, straight, regular rods after incubation for 3 days at 30 °C. Optimum growth occurs at 30 °C, at pH 7.5 and with 2 % (w/v) NaCl. Gelatin is hydrolysed, but casein, starch and citrate are not. H_2S is not produced. Potassium 5-keto-gluconate is utilized as a sole carbon source. In the API 20E test, assimilation of L-tryptophan is positive, but L-arginine, L-lysine, L-ornithine, indole production, 2-nitrophenyl-β D-galactopyranoside (ONPG) and acetoin production (VP) are negative. In the API ZYM test, esterase (C4), esterase lipase (C8), naphtol-AS-BI-phosphohydrolase, leucine arylamidase and valine arylamidase (weakly) activities are positive, but alkaline phosphatase, lipase (C14), crystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, α-galatosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase activities are negative. The only respiratory quinone is Q-10. The major fatty acid (>10 % of the total fatty acid) is $C_{18:1}$ *ω7c*.

The type strain, CAU 1311^T (=KCTC 52189^T=NBRC 111988^T), was isolated from sea sand in Republic of Korea. The DNA G+C content of the type strain is 60.7 mol%.

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

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