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ORIGINAL PAPER

A new *Rhizobium* species isolated from the water of a crater lake, description of *Rhizobium aquaticum* sp. nov.

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Abstract A novel isolate, strain SA-276^T, was isolated from the water of Lake St. Ana, a crater lake which is located in Romania. Phylogenetic analysis based on the 16S rRNA gene revealed that the new strain is a member of the family *Rhizobiaceae*, showing a high pairwise similarity value (97.65%) to *Rhizobium tubonense* CCBAU 85046^T (= DSM 25379^T), *Rhizobium leguminosarum* USDA 2370^T (= LMG 14904^T), *Rhizobium anhuiense* CCBAU 23252^T and *Rhizobium laguerreae* FB206^T. Cells of strain SA-276^T were rod-shaped, motile, oxidase negative and weakly catalase positive. The predominant fatty acids were $C_{18:1}\omega7c$ and cyclo $C_{19:0}\omega8c$, the major respiratory quinones were Q-10 and Q-9, and the main polar lipids were phosphatidylmonomethylethanolamine, phosphatidylglycerol and phosphatidylcholine. The G + C content of the genomic DNA of strain SA-276^T was 60.8 mol%. The novel isolate can be distinguished from the closest related type strain *R. tubonense* DSM 25379^T based on its broader substrate specificity and positive trypsin enzyme activity. On the basis of the phenotypic, chemotaxonomic and molecular data, strain SA-276^T is considered to represent a new species, for which the name *Rhizobium aquaticum* sp. nov. is proposed. The type strain is SA-276^T (= DSM 29780^T = JCM 31760^T).

The GenBank/EMBL/DDBJ Accession Number for the 16S rRNA, *atpD*, *glnII* and *recA* gene sequences of strain SA-276^T are KM083136, KY947543, KY947544 and KY947545, respectively.

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Keywords *Rhizobium* · Crater lake · *Alphaproteobacteria* · *Rhizobiacaeae*

Abbreviations

PC	Phosphatidylcholine
PG	Phosphatidylglycerol
PMME	Phosphatidylmonomethylethanolamine

Introduction

The family *Rhizobiaceae* (class *Alphaproteobacteria*) currently consists more than 100 species with validly published names (Parte 2014). Parallel to the accelerated description of new species in recent years, taxonomic revisions have also been performed: e.g. some *Rhizobium* species were reclassified in the genera *Agrobacterium* and *Allorhizobium*, and species formerly belonging to *Rhizobium* were proposed to be transferred into newly established genera, such as *Pararhizobium* and *Neorhizobium* (Mousavi et al. 2014, 2015). Considering the species with validly published names, the genus *Rhizobium* is clearly polyphyletic based on 16S rRNA gene sequences, and although genomic data are available for many species within the family *Rhizobiaceae*, the status of some *Rhizobium* species is still under debate (Carrareto Alves et al. 2014; Mousavi et al. 2015; Ormeño-Orrillo et al. 2015). The taxonomy of *Rhizobium* and related genera is complicated by the combination of several factors: in the case of many species, a large proportion of the genome is harbored in extrachromosomal replicons (chromids and plasmids), the loss or gain of which may significantly influence phenotypic results (Slater et al. 2009; Ormeño-Orrillo and Martínez-Romero 2013; Althabegoiti et al. 2014); 16S rRNA gene-based interspecies similarity values are high in many cases (Hunter et al. 2007), which hinders the species-level identification based on the most-widely used taxonomic marker gene; and the agricultural significance of many strains (Carrareto Alves et al. 2014) asserts the retention of classical systematics rather than modern taxonomy. As a result, strains of rhizobia (i.e. bacteria capable of nodulating leguminous plants and form nitrogen fixing symbioses) belonging or closely related to the genus *Rhizobium* are referred to as members of the '*Rhizobium/Agrobacterium* group' (or cluster) in recent systematic works (Carrareto Alves et al. 2014; Ormeño-Orrillo et al. 2015).

Although almost all *Rhizobium* species were isolated from root nodules, new species belonging to family *Rhizobiaceae* have been described recently based on type strains originating from aquatic habitats, e.g. *Rhizobium alvei* was isolated from river water (Sheu et al. 2015), *Shinella granuli*, *Rhizobium daejeonense* and *Rhizobium selenitireducens* were isolated from bioreactors (Quan et al. 2005; An et al. 2006; Hunter et al. 2007), *Rhizobium marinum* was isolated from seawater (Liu et al. 2015) and *Gellertiella hungarica* from thermal bath (Tóth et al. 2017). This study presents the polyphasic characterization of a new aquatic strain, SA-276^T, which was isolated from a freshwater lake and is closely related to members of the genus *Rhizobium*.

Materials and methods

Strain isolation and growth conditions

Strain SA-276^T was isolated from the water of Lake St. Ana (a crater lake in the Ciomad Mountains, Harghita County, Romania; in Romanian: Lacul Sfânta Ana) in August 2012. A detailed site description including the physical and chemical characteristics of lake water is given by Felföldi et al. (2016). For isolation, plates containing lake water solidified with 20 g l^{-1} agar were used. The standard dilution plating technique was applied to obtain isolates by incubation at room temperature (20–22 °C). Subsequently, strain SA-276^T was maintained on a modified R2A agar medium (pH 7.0), which contained only half amount of carbon sources as given in the original description (DSMZ medium 830, www.dsmz.de). Later, strain SA-276^T showed effective growth on R2A agar, YMA agar (DSMZ medium 1031) and Rhizobium agar (DSMZ medium 98) media. For side-by-side analyses, the new strain and strains *Rhizobium tubonense* DSM 25379^T (= CCBAU 85046^T) and *Rhizobium leguminosarum* LMG 14904^T (= USDA 2370^T) were maintained on YMA agar at 28 °C.

Morphological and physiological analyses

Optimal temperature, pH and salt concentration values were determined based on the growth intensity

observed at 4, 10, 20, 25, 30, 37, 45 and 55 °C, at pH from 4 to 11 (with intervals of 1) and with NaCl concentration from 0 to 5% (w/v, with intervals of 1%), respectively, as described previously (Felföldi et al. 2014). For testing the nitrate reduction of strains under anaerobic conditions, R2A liquid medium supplemented with 1 g l^{-1} KNO_3 and Nitrate Broth (with Durham tubes; Barrow and Feltham 2003) was used. An anoxic atmosphere was created by using an Anaerocult A Mini (Merck) gas generator system.

Colony morphology of strain SA-276^T was tested by direct observation of single colonies. Cell morphology was observed after Gram staining according to Claus (1992), while the presence of flagella was assessed as described by Heimbrook et al. (1989). Oxidase activity and catalase reaction were examined as given by Tarrand and Gröschel (1982) and Cowan and Steel (1974), respectively. Caseinase, urease and starch hydrolysis activities were determined as described by Smibert and Krieg (1994), while acid production from D-glucose was checked by the oxidative and fermentative tests according to Hugh and Leifson (1953). Additional metabolic tests were performed with API 50 CH, API 20 NE and API ZYM (bioMérieux) systems following the instructions given by the manufacturer. Susceptibility of the strains to antibiotics was studied on YMA plates using antibiotic-containing discs (Bio-Rad) after incubation for 3 days at 28 °C.

Chemotaxonomic analyses

Analyses of cell wall diamino acids, isoprenoid quinones, cellular fatty acids, polar lipids and the determination of DNA base composition were performed as described by Felföldi et al. (2011).

DNA sequence analyses

The 16S rRNA gene sequence of strain SA-276^T was amplified and sequenced as described by Máthé et al. (2014) using the primers given previously (Felföldi et al. 2017). Amplification of protein-coding genes was performed with primers atpD-255F (5′-GCT SGG CCG CAT CMT SAA CGT C-3′) and atpD-782R (5′-GCC GAC ACT TCM GAA CCN GCC TG-3′) in the case of the beta subunit of ATP synthase (*atpD* gene), glnII-12F (5′-YAA GCT CGA GTA CAT YTG GCT-3′) and glnII-689R (5′-TGC ATG CCS GAG CCG TTC CA-3′) in the case of glutamine synthetase II (*glnII* gene), recA-41F (5′-TTC GGC AAG GGM TCG RTS ATG-3′) and recA-640R (5′-ACA TSA CRC CGA TCT TCA TGC-3′) in the case of recombinase (*recA* gene), following the protocols given by Vinuesa et al. (2005). Amplicons were purified with the PCR-M^{TM} Clean Up System (Viogene, Sijhih, Taiwan), and sequencing of the PCR products was carried out through a service provided by the Biomi Ltd. (Gödöllő, Hungary).

Sequence alignment of the 16S rRNA gene with the closest related type strains was performed with the SINA Alignment Service (Pruesse et al. 2012). Sequence alignment of protein-coding genes was performed with the MEGA 7.0 software (Kumar et al. 2016), multiple fasta files were created with MergeAlign (Collingridge and Kelly 2012), and concatenation was conducted with SequenceMatrix 1.8 (Vaidya et al. 2011). Phylogenetic analyses (which included the search for the best-fit model parameters) were performed with the MEGA 7.0 software.

The presence of the *nifH* gene was assessed with the PCR-based method of Bürgmann et al. (2004).

Results and discussion

Morphological and physiological characteristics

Cells of strain SA-276^T are Gram-stain negative, motile, facultatively anaerobic and mesophilic with a characteristic heterotrophic metabolism (Table 1). Based on the enzyme activities and substrates tested for utilization, the new strain could be distinguished from the closely related type strain, *R. tubonense* DSM 25379^T, based on its broader substrate specificity (capable of utilizing D/L-arabinose, D-fructose, D-galactose, D-glucose, glycerol, D-lyxose, malic acid, D-maltose, D-mannitol, D-mannose, L-rhamnose, D-ribose, sucrose, trehalose, turanose, D/L-xylose), positive trypsin enzyme activity (Table 1) and penicillin sensitivity (Table S1, available in the online Supplementary Material).

Chemotaxonomic characteristics

The major respiratory quinones of SA-276^T were Q-10 and Q-9 in a ratio of 47:29. The fatty acid pattern of strain SA-276^T was dominated by $C_{18:1}\omega7c$ (41.0%)

Table 1 Differential phenotypic and biochemical characteristics of SA-276^T and related type strains

Characteristic	1	2	3
Temperature range (optimum) (°C)	10–45 (20–30)	4–55 (20–30)	10–37 (20–30)
pH range (optimum)	6–10 (7–9)	5–9 (5–7)	5–10 (6–7)
Salt tolerance (NaCl, %)	0–2	0–2	0–1
Oxidase activity	−	−	w
Catalase activity	w	+	+
Nitrate reduction to nitrite (under anaerobic conditions)	+	−	−
Assimilation of			
D-Arabinose	+	−	−
L-Arabinose	+	−	−
D-Fructose	+	−	−
D-Fucose	+	+	−
D-Galactose	+	−	−
D-Glucose	+	−	−
Glycerol	+	−	−
5-Ketogluconate	–	+	−
D-Lyxose	+	−	−
Malic acid	+	−	−
D-Maltose	+	–	–
D-Mannitol	+	−	−
D-Mannose	+	−	−
L-Rhamnose	+	−	−
D-Ribose	+	−	−
Sucrose	+	−	−
Trehalose	+	−	−
Turanose	+	−	−
D-Xylose	+	−	−
L-Xylose	+	−	−
Enzyme activity			
α-Fucosidase	−	−	+
α-Mannosidase	−	−	+
N-acetyl-β-glucosaminidase	+	+	−
Trypsin	+	−	+
Valine arylamidase	−	−	+
DNA G + C content (mol%)	60.8	59.5*	62.5*

Strains: 1, SA-276^T; 2, *Rhizobium tubonense* DSM 25379^T; 3, *Rhizobium leguminosarum* LMG 14904^T. All strains were motile, positive for acid phosphatase, aesculin hydrolysis, alkaline phosphatase, esterase (C4), esterase lipase (C8), β-galactosidase, α-glucosidase, β-glucosidase, leucine arylamidase, naphthol-AS-BI-phosphohydrolase and urease; negative for arginine dihydrolase, caseinase, α-galactosidase, α-chymotrypsin, cystine arylamidase, gelatine hydrolysis, glucose fermentation, β-glucuronidase, indole production and lipase (C14) activities; positive for the assimilation of aesculin; negative for the assimilation of adipic acid, D-adonitol, amygdalin, D-arabitol, L-arabitol, arbutin, capric acid, cellobiose, citrate, dulcitol, erythritol, L-fucose, gentiobiose, gluconate, glycogen, inositol, inulin, 2-ketogluconate, lactose, melezitose, melibiose, methyl α-D-glucopyranoside, methyl α-D-mannopyranoside, methyl β-D-xylopyranoside, phenylacetic acid, raffinose, salicin, L-sorbose, D-sorbitol, starch, D-tagatose and xylitol. Symbols: +, present; −, absent; w, weak reaction. Data are from the present study, unless otherwise indicated. Asterisks mark data from Zhang et al. (2011) and Ramírez-Bahena et al. (2008)

Table 2 Major fatty acids of SA-276^T and related type strains

Fatty acid	1	2	3
$C_{18:1}\omega7c$	41.0	25.2	32.9
cyclo $C_{19:0}\omega8c$	29.2	35.5	34.4
$C_{14:0}$ 3–OH	7.8	6.1	5.5
$C_{16:0}$	7.5	16.4	5.9
11-methyl $C_{18:1}\omega7c$	3.6	9.0	7.8
$C_{18:0}$	1.9	–	7.8
cyclo $C_{17:0}$	1.9	TR	TR
$C_{20:2}\omega6,9c$	TR	1.5	–
$C_{13:1}$ AT 12–13	TR	–	1.2
$C_{17:0}$	TR	–	1.0
$C_{16:0}$ 3–OH	–	1.6	–

Strains: 1, SA-276^T; 2, *Rhizobium tubonense* DSM 25379^T; 3, *Rhizobium leguminosarum* LMG 14904^T; –, not detected; TR, < 1.0%. Data are from the present study. All strains were grown on Rhizobium medium for 3 days at 28 °C

and cyclo $C_{19:0}\omega8c$ (29.2%), and in lower amounts $C_{14:0}$ 3-OH (7.8%), $C_{16:0}$ (7.5%) and other minor components (< 5%) (Table 2). Comparing these data with the related strains analyzed, their fatty acid compositions were similar and the dominance of fatty acids $C_{18:1}\omega7c$, cyclo $C_{19:0}\omega8c$ and $C_{16:0}$ has been reported in many *Rhizobium, Pararhizobium* and *Shinella* species by other authors (Lee et al. 2011; Zhang et al. 2011, 2015; Behrendt et al. 2016; Puławska et al. 2016), which confirmed that the new strain belongs to family *Rhizobiaceae*.

The polar lipid pattern of strain SA-276^T was dominated by phosphatidylmonomethylethanolamine (PMME), phosphatidylglycerol (PG) and phosphatidylcholine (PC), while phosphatidylethanolamine, an unidentified aminophospholipid and most likely diphosphatidylglycerol were detected as minor components (Fig. S1). Although *Rhizobium* species polar lipid data have not been previously reported for all species (Young et al. 2001; Kuykendall et al. 2005; Carrareto Alves et al. 2014), including recent descriptions (Saïdi et al. 2014; Zhang et al. 2015; Behrendt et al. 2016); PG, PC and/or PMME have been detected as characteristic polar lipids in various other *Rhizobium* and *Shinella* species (Liu et al. 2015; Sheu et al. 2015, 2016; Román-Ponce et al. 2016; Subhash and Lee 2016).

The cell wall of strain SA-276^T contained the diagnostic diamino acid, *meso*-2,6-diaminopimelic acid.

The genomic G + C content value of strain SA-276^T is 60.8 mol%, which falls within the range (57–66%) reported for the *Rhizobium/Agrobacterium* cluster (Carrareto Alves et al. 2014).

Results of DNA sequence analyses

Sequencing the 16S rRNA gene of strain SA-276^T resulted in a stretch of 1408 nucleotides. Based on this data, the currently most closely related species (represented by type strains) were identified with EzBioCloud's online service (Yoon et al. 2017). *R. tubonense* CCBAU 85046^T (= DSM 25379^T), *R. leguminosarum* USDA 2370^T (= LMG 14904^T), *Rhizobium anhuiense* CCBAU 23252^T and *Rhizobium laguerreae* FB206^T showed the highest, 97.65%, pairwise similarity value based on the 16S rRNA gene, while 22 other *Rhizobium*, 2 *Ensifer* and 3 *Pararhizobium* type strains shared lower similarities (but higher than 97.0%) to strain SA-276^T (see details in Table S2). These values are higher than the value (95%) suggested for general genus delineation by Tindall et al. (2010). However, according to the recommendation of Kim et al. (2014), based on pairwise comparison of bacterial genomes, the species level threshold should be increased to the level of 98.65% 16S rRNA gene sequence similarity. Furthermore, it has been previously noted by others (Hunter et al. 2007) that some strains showing < 1% sequence dissimilarity based on their 16S rRNA gene sequences may represent different *Rhizobium* species.

Phylogenetic analysis of the 16S rRNA gene (Fig. 1; Fig. S2) revealed that strain SA-276^T clustered with the two sensu stricto *Rhizobium* sub-clusters (the 'Rhizobium tropici' and the 'Rhizobium leguminosarum' groups; Mousavi et al. 2015). Housekeeping genes were also applied to aid resolving the phylogeny of rhizobia (Mousavi et al. 2014). Based on the sequence analysis of *atpD*, *recA* and *glnII* genes (Fig. 2), strain SA-276^T was positioned in a separate clade from nodule-forming *Rhizobium* strains.

In the case of the new strain, PCR failed to detect the *nifH* gene. Genes required for nitrogen fixation in legumes are encoded in unstable plasmids (Ormeño-Orrillo et al. 2015; Remigi et al. 2016), offering a possible explanation for the absence of *nifH*. Consequently, planktonic species may lose these traits since they probably do not establish nitrogen-fixing symbiosis with plants.

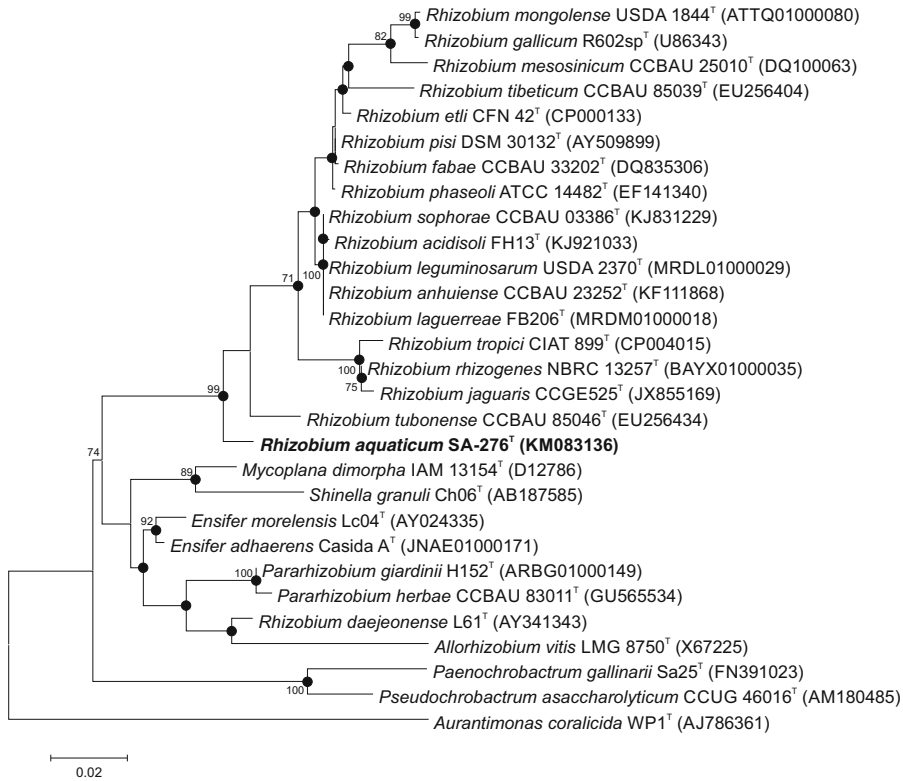

Fig. 1 Phylogenetic tree of SA-276^T and related type strains based on the 16S rRNA gene. Phylogenetic tree has been reconstructed based on 1319 nucleotide positions using the maximum likelihood method. Only bootstrap values > 70% are shown. GenBank accession numbers are given in parentheses. Filled circles indicate that the corresponding nodes were also recovered by the neighbor-joining method

Taxonomic conclusion

Strain SA-276^T shared the main physiological characteristics of family *Rhizobiaceae* (Carrareto Alves et al. 2014): aerobic, Gram-stain negative and rod-shaped. However, unlike many other members of this group, the new strain was not associated with soil and plants, and lacked the ability of nitrogen fixation. Chemotaxonomic data (polar lipid pattern, fatty acid composition) support the conclusion that strain SA-276^T belongs to family *Rhizobiaceae*, and phylogenetic analyses have confirmed that it is closely related to members of genus *Rhizobium*.

In conclusion, based on the data discussed above, strain SA-276^T is considered to represent a new species, for which the name *Rhizobium aquaticum* sp. nov. is proposed.

Description of *Rhizobium aquaticum* sp. nov.

Rhizobium aquaticum (a.qua'ti.cum. L. neut. adj. *aquaticum* living in water, aquatic; referring to the isolation source of the type strain).

Cells are rod-shaped (0.5–0.7 × 1.6–1.9 μm) and motile. Colonies on YMA agar medium are beige-coloured, circular and raised. Growth occurs after 1–2 days of incubation at 10–45 °C (optimum, 20–30 °C), at pH 6–10 (optimum, pH 7–9) and 0–2% (w/v) NaCl concentration. Capable of growth under anaerobic conditions with nitrate. Positive for acid phosphatase, aesculin hydrolysis, alkaline phosphatase, catalase (weak), esterase (C4), esterase lipase (C8), β-galactosidase, α-glucosidase, β-glucosidase, leucine arylamidase, *N*-acetyl-β-glucosaminidase, naphthol-AS-BI-phosphohydrolase, nitrate reduction to nitrite, trypsin and urease. Negative for the following enzyme activities: arginine dihydrolase, caseinase, α-chymotrypsin, cystine arylamidase, α-fucosidase, α-

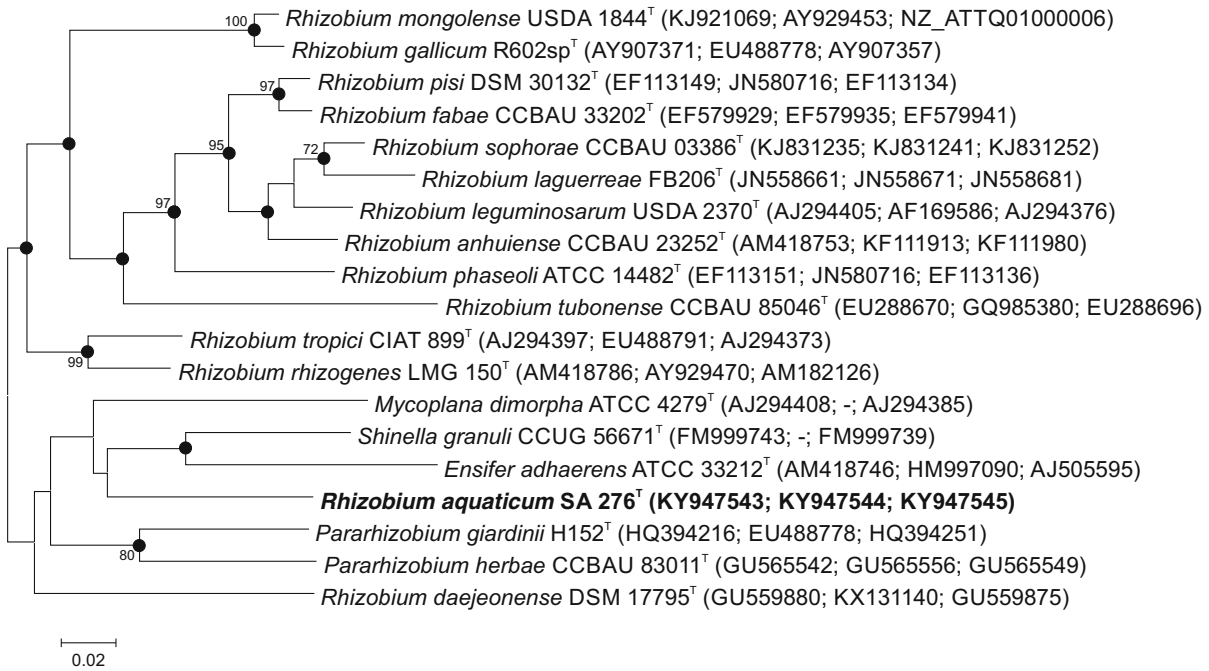

Fig. 2 Phylogenetic tree of SA-276^T and related type strains based on concatenated sequences of genes *atpD*, *glnII* and *recA*. Phylogenetic tree has been reconstructed based on 1236 nucleotide positions using the maximum likelihood method. Only bootstrap values > 70% are shown. GenBank accession numbers are given in parentheses (order: *atpD*, *glnII* and *recA*). Filled circles indicate that the corresponding nodes were also recovered by the neighbor-joining method

galactosidase, gelatine hydrolysis, glucose fermentation, β-glucuronidase, indole production, lipase (C14), α-mannosidase, oxidase and valine arylamidase. The major respiratory quinones are Q-10 and Q-9. The major fatty acids are $C_{18:1}\omega7c$ (41.0%) and cyclo $C_{19:0}\omega8c$ (29.2%). The major polar lipids are PMME, PG and PC. The cell wall contains *meso*-2,6-diaminopimelic acid. The G + C content of the genomic DNA of the type strain is 60.8 mol%.

The type strain is SA-276^T (= DSM 29780^T = JCM 31760^T) which was isolated from the water of a crater lake. The TaxonNumber of strain SA-276^T is TA00355.

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Compliance with ethical standards

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

Ethical approval The article does not contain any studies with humans or animals.

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