Microvirga Terrestris sp. nov., and *Microvirga arvi* sp. nov., Two Novel Species Isolated from Soil in South Korea

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Abstract

Two novel Gram-stain-negative, aerobic, rod shaped bacterial strains BT290^T and BT689^T were isolated from soil collected in South Korea. Colony morphologies of both strains were circular and convex while the colors of BT290^T and BT689^T were light-pink and white, respectively. Phylogenetic analysis based on 16S rRNA gene sequences revealed that BT290^T and BT689^T belong to a distinct lineage within the genus *Microvirga* (family *Methylobacteriaceae*, order *Rhizobiales*, class *Alphaproteobacteria*, phylum *Proteobacteria*, kingdom *Bacteria*). The 16S rRNA gene sequence similarity between two strains was 97.9%. Both strains had the similar quinone system, with ubiquinone 10 (Q-10) as the major respiratory quinone. The major polar lipids of strains BT290^T and BT689^T were phosphatidylethanolamine (PE), diphosphatydilglycerol (DPG), phosphatidylcholine (PC), and phosphatydilglycerol (PG). The major cellular fatty acids of strain BT290^T were $C_{18:1}$ *ω7c* (58.2%) and $C_{16:0}$ (17.7%), while those of strain BT689^T were $C_{18:1}$ *ω7c* (61.8%) and $C_{16:0}$ (10.8%).

On the bases of polyphasic analysis (phylogenetic, chemotaxonomic, and biochemical), strains BT290^T and BT689^T can be suggested as novel bacterial species within the genus *Microvirga* and the proposed names are *Microvirga terrestris* and *Microvirga arvi*, respectively. The type strain of *Microvirga terrestris* is BT290^T (= KCTC 72367^T=NBRC 114844^T) and the type strain of *Microvirga arvi* is BT689^T (= KACC 22016^T = NBRC 114858^T), respectively.

Introduction

The genus *Microvirga* was first described by Kanso and Patel (2003) and was afterwards emended by Zhang et al. (2009) and Weon et al. (2010) allocated to the family *Methylobacteriaceae*, order *Rhizobiales* and class *Alphaproteobacteria*. At the time of writing (Feb 2022), the genus comprises 27 published species and not validly published 9 (https://lpsn.dsmz.de/genus/microvirga). *Microvirga* species have been retrieved from various polar environments in the last years, e.g. from Tibet hot spring sediments (Liu et al. 2020), roots of rapeseed plants (Jimenez-Gomez et al. 2019), root nodule (Wang et al. 2019; Msaddak et al. 2019), forest soil (Zhang et al. 2019) and rhizospheric soil (Li et al. 2020). Usually, cells of genus *Microvirga* are Gram-strain-negative and contain $C_{18:1}$ *ω7c* and cyclo-$C_{19:0}$ *ω8c* as major fatty acids. The average genome size and DNA G+C content are 3.53 - 9.63 Mb and 61.1 - 65.1%, respectively (Zhang et al. 2019).

In this study, two strains BT290^T and BT689^T were newly isolated from soil samples collected in South Korea. Phylogenetic analysis was conducted based on the 16S rRNA gene sequences and phenotypic, genotypic, and chemotaxonomic characteristics were performed to determine the taxonomic position of strains BT290^T and BT689^T. The results suggested that strains BT290^T and BT689^T represent two novel species of the genus *Microvirga*, for which the name *Microvirga terrestris* sp. nov. and *Microvirga arvi* sp. nov. are proposed, respectively.

Materials And Methods

Isolation and culture conditions of bacteria

Two soil samples were obtained in South Korea to isolate new strains. The strain BT290^T was isolated from Jeongseon province (37° 22' 45" N, 128° 39' 53" E) soil and strain BT689^T was isolated from Uijeongbu city (37° 44' 55" N, 127° 2' 20" E) soil, respectively. Colonies were isolated using Reasoner's 2A (R2A) agar medium (Difco) after incubation at 25°C for 10 days and single colony purification were performed under the same condition. The strains were routinely sub-cultured on R2A agar at 25°C, maintained at 4°C and stock solutions were stored in 10% (w/v) glycerol suspension at −80°C before use.

Morphology, physiology, and biochemical analysis

The cell morphologies of strains BT290^T and BT689^T were examined using transmission electron microscopy (JEOL, JEM1010) after grown on R2A agar for 3 days at 25°C with negative staining method. The Gram-staining was performed using a commercialized kit, following the manufacturer’s instruction (bioMérieux). Catalase activity was examined with 3% (w/v) hydrogen peroxide and oxidase activity was examined by addition of 1% (w/v) tetramethyl-p-phenylenediamine (Cappucino et al. 2002). Bacterial growths of both strains were tested on Reasoner’s 2A (R2A) agar, Luria-Bertani (LB) agar, Tryptic Soy Agar (TSA), Nutrient Agar (NA), and on MacConkey (MAC) agar, respectively. Cell viability was observed under various temperatures (10, 15, 25 and 30°C), different pH conditions (pH 5 to 9, by pH 1 intervals), and different NaCl concentrations (1–5% [w/v %], by 1% intervals). API 20NE and API ZYM tests were performed according to the manufacturer’s instruction (bioMérieux).

Phylogenetic Analysis

The 16S rRNA genes of strains BT290^T (1,417 bp) and BT689^T (1,451 bp) were amplified by PCR using two universal bacterial primers 27F and 1492R (Weisburg et al. 1991) with genomic DNA as templates. Then, sequencing was performed using four universal primers; 337F and 518R for BT290^T, 785F and 926R for BT689^T (Macrogen). To determine the taxonomic positions of strains BT290^T and BT689^T, similar 16S rRNA sequences were obtained from EzBioCloud (Yoon et al. 2017) and compared with those of strains BT290^T and BT689^T using EzEditor2 server. Phylogenetic trees were constructed using the MEGAX program (Kumar et al. 2018) with the neighbor-joining (Saitou et sl. 1987), maximum-likelihood (Felsenstein et al. 1981), and maximum-parsimony algorithms (Fitch et al. 1971). The stability of tree topologies was calculated based on 1,000 replications (Felsenstein et al. 1985) and evolutionary distances were calculated using Kimura’s two-parameter model (Kimura et al. 1983).

Whole-genome Sequence Analysis

Genomic DNA was extracted using a genomic DNA extraction kit according to the manufacturer's instruction (Solgent). Sequencing libraries were prepared using the Nextera DNA Flex Library Prep Kit (Illumina) and whole-genome sequencing was performed by iSeq 100 system (Illumina). The obtained genome sequences were assembled using a SPAdes 3.10.1 (Algorithmic Biology Lab, St. Petersburg Academic University of the Russian Academy of Sciences). Whole-genome sequences of strains BT290^T and BT689^T were deposited in GenBank (www.ncbi.nlm.nih.gov/) database, respectively. The genome sequences of strains BT290^T and BT689^T were annotated by the National Center for Biotechnology Information Prokaryotic Genome Annotation Pipeline (PGAP) (Kimura et al. 1983). The average nucleotide identity (ANI) was calculated using the EzBioCloud (https://www.ezbiocloud.net) and digital DNA-DNA hybridization (dDDH) values were obtained using the Genome-to Genome Distance Calculator (GGDC) with the recommended formula 2 (Table 1) (Meier-Kolthoff et al. 2013).

Chemotaxonomic characteristics

To analyze the cellular composition of polar lipid, fatty acid, and quinone of strains BT290^T and BT689^T, both strains were grown on R2A agar at 25°C for three days and then cells were freeze-dried. Polar lipids were extracted as described previously (Minnikin et al. 1984). Total lipids, glycolipids, phosphatidylcholine, and amino groups were separated using two-dimensional thin-layer chromatography (TLC). The polar lipid spots were detected by spraying the proper detection reagents (Komagata et al. 1987; Minnikin et al. 1984). The fatty acids were purified by saponification, methylation, and extraction procedures and analyzed by Sherlock Microbial Identification System V6.01 (MIS, database TSBA6, MIDI Inc., Newark, DE, USA) (Sasser et al. 1990). The quinones of strains BT290^T and BT689^T were extracted using the Sep-Pak Vac cartridges (Waters) and analyzed by high-performance lipid chromatography (HPLC) based on the previous methods (Hiraishi et al. 1996). The fatty acid methyl esters (FAME) were identified using the Sherlock Microbial Identification System V6.01 (MIS, database TSBA6, MIDI Inc).

Results And Discussion

Morphology, physiology, and biochemical analysis

Strains BT290^T and BT689^T were Gram-staining-negative bacteria and they showed rod-shaped morphology (Fig. 1). Colonies of strains BT290^T and BT689^T were circular, convex, smooth, light-pink and white colored after incubation for three days at 25°C. Cells of strains BT290^T and BT689^T could survive at 10 to 30°C (optimum 25°C) and pH 6.0 - 9.0 (optimum pH 8.0) on R2A medium. Distinct features of new strains and reference strains were presented in Table 1. The negative reaction of strains BT290^T and BT689^T by API analysis were provided as supplementary tables (Table S1 and S2, respectively).

Phylogenetic analysis

Based on the 16S rRNA gene sequence similarities, strains BT290^T and BT689^T were affiliated with the family *Methylobacteriaceae* and they showed high sequence similarities with the genus *Microvirga*. The 16S rRNA gene sequence similarities of strains BT290^T and BT689^T with the most closely related species *M. aerilata* 5420S-16^T were 98.2% and 98.5%, respectively, and with other *Microvirga* species were 98.1%. These values were below the threshold for differentiating among bacterial species (98.7%), supporting that two strains are novel species (Chun et al. 2018). The results of neighbor-joining tree (Fig. 2), maximum-likelihood tree (Fig. S1), and maximum-parsimony tree (Fig. S2) showed that strains BT290^T and BT689^T were clustered with *M. soli* R491^T and *M. flocculans* ATCC BAA-817^T, respectively, at >70% bootstrap support (Fig. 2). The phylogenetic analysis results clearly showed that strains BT290^T and BT689^T are two new species within the genus *Microvirga*.

Genome sequence analysis

The draft genome of strain BT290^T was 4.20 Mb (38.6×) long and consisted of 3,867 protein-coding genes, 50 RNA genes (5 rRNA genes, 45 tRNA genes), and 41 pseudogenes. The draft genome of strain BT689^T was 6.10 Mb (29.9×) long and consisted of 4,014 protein-coding genes, 51 RNA genes (4 rRNA genes, 44 tRNA genes), and 66 pseudogenes. The genome sequences of strains BT290^T and BT689^T were deposited in GenBank database (accession numbers: NZ_JADQDN000000000 for BT290^T and NZ_JAFEMD000000000 for BT689^T, respectively). The DNA G+C contents of strains BT290^T and BT689^T were 62.3 mol% and 62.4 mol%, respectively. These values were within the range of the G+C contents for the genus *Microvirga* those previously reported (61.1 - 65.1 mol %) (Zhang e al. 2019), again supported that two strains belong to genus *Microvirga*. The digital DNA-DNA hybridization values between strains BT290^T and BT689^T and other related type strains of genus *Microvirga* were less than 26.7% and 28.4%, respectively (Table S3), which are below the cutoff (70%) point (Meier-Kolthoff et al. 2013). Average nucleotide identity (ANI) values between strains BT290^T and BT689^T and other related type strains of genus *Microvirga* were less than 82.9% and 83.9%, respectively (Table S3). These values are below the ANI species threshold (95 - 96% ANI value) as described by Ritcher and Rossello-Mora (Richter et al. 2009).

Chemotaxonomic characterization

Fatty acid profiles of strains BT290^T and BT689^T and three reference strains of genus *Microvirga* were presented in Table 2. The major fatty acids of strain BT290^T were $C_{18:1}$ *ω7c* (58.2%) and $C_{16:0}$ (17.7%). Interestingly, strain BT290^T has large amounts of $C_{18:0}$ (5.7%), cyclo-$C_{19:0}$ *ω8c* (2.2%), $C_{14:0}$ (1.6%) and $C_{18:1}$ *ω7c* 11-methyl (1.2%), whereas the most closely related species *M. aerilata* 5420S-16^T has smaller amounts of corresponding fatty acids. In addition, strain BT290^T did not contain $C_{15:1}$ *ω8c* while most closely related species *M. aerilata* 5420S-16^T contained that fatty acid. The polar lipids of strain BT290^T consisted of phosphatidylethanolamine (PE), diphosphatydilglycerol (DPG), phosphatidylcholine (PC), phosphatydilglycerol (PG), aminolipid (AL), and four unknown lipid (L) (Fig. S3). The major fatty acid profiles of strain BT689^T were $C_{18:1}$ *ω7c* (61.8%) and $C_{16:0}$ (10.8%). Strain BT689^T has large amounts of

cyclo-$C_{19:0}$ $\omega 8c$ (8.2%), $C_{18:0}$ (6.6%), $C_{18:1}$ $\omega 7c$ 11-methyl (2.3%), $C_{14:0}$ (1.3%) and $C_{18:0}$ 3OH (1.0%), whereas the most closely related species *M. aerilata* 5420S-16^T has smaller amounts of corresponding fatty acids. Strain BT689^T contained $C_{17:0}$, cyclo-$C_{17:0}$ and $C_{18:1}$ $\omega 9c$, but most closely related species *M. aerilata* 5420S-16^T did not contain those fatty acids. The polar lipids of strain BT689^T consisted of phosphatidylethanolamine (PE), diphosphatydilglycerol (DPG), phosphatydilglycerol (PG), phosphatidylcholine (PC), aminolipid (AL) and nine unknown lipid (L) (Fig. S4).

The dominant respiratory quinone of strains BT290^T and BT689^T was ubiquinone 10 (Q-10).

Regarding the chemotaxonomic characteristics of BT290^T and BT689^T, both strains could be differentiated from other *Microvirga* species. Based on phenotypic, phylogenetic and biochemical features, it is concluded that strains BT290^T and BT689^T represent two novel species of the genus *Microvirga*, for which the name *Microvirga terrestris* and *Microvirga arvi*, respectively, are proposed.

Description of Microvirga terrestris **sp. nov.**

Microvirga terrestris (ter.res'tris. L. fem. adj. *terrestris* of the earth).

Cells are Gram-stain-negative, aerobic, rod-shaped, 0.5 - 1.4 µm in diameter and about 1.3 - 2.4 µm in length, non-spore forming and non-motile. Colonies are irregular, convex and light-pink-colored on Reasoner's 2A (R2A) agar plates after growth for three days at 25°C. Growth is observed at temperatures ranging from 10 to 30°C (optimum 25°C). The pH range for growth is 6.0 - 9.0 (optimum pH 8.0) on R2A agar. Normal cell growth occurs at 10 - 30°C (optimum 25°C) and pH 6.0 - 9.0 (optimum 8.0). Cells grow on R2A agar and MacConkey (MAC) agar (weakly) but not on Luria-Bertani agar (LB), Tryptic Soy Agar (TSA) and Nutrient Agar (NA). Cells are positive for oxidase and catalase activity. The major respiratory quinone of strain BT290^T is ubiquinone 10 (Q-10). The dominant cellular fatty acids are $C_{16:0}$ and $C_{18:1}$ $\omega 7c$. The major polar lipids are phosphatidylethanolamine (PE), diphosphatydilglycerol (DPG), phosphatidylcholine (PC) and phosphatydilglycerol (PG). Positive for D-glucose (API 20NE). Positive for acid phosphatase (API ZYM). The whole-genome sequence of strain BT290^T has been deposited in GenBank under the accession number NZ_JADQDN000000000 (6.10 Mb). The genome-based G+C content is 62.3 mol %. The GenBank accession number for the 16S rRNA gene sequence of strain BT290^T is MT795754 (1,417 bp). The type strain BT290^T (= KCTC 72367^T=NBRC 114844^T) was isolated from a soil sample collected in Jeongseon province (37° 22' 45" N, 128° 39' 53" E), South Korea.

Description of Microvirga arvi **sp. nov.**

Microvirga alba (ar'vi. L. gen. n. *arvi* of a field).

Cells are Gram-stain-negative, aerobic, rod-shaped, 0.6 - 0.7 µm in diameter and about 1.1 - 2.0 µm in length, non-spore forming and non-motile. Colonies are irregular, convex and white colored on Reasoner's 2A (R2A) agar plates after growth for three days at 25°C. Growth is observed at temperatures ranging

from 10 to 30°C (optimum 25°C). The pH range for growth is 6.0 - 9.0 (optimum pH 8.0) on R2A agar. Cells grow on R2A agar, Luria-Bertani agar (LB) (weakly) and MacConkey (MAC) (weakly) agar but not on Tryptic Soy Agar (TSA) and Nutrient Agar (NA). Cells are positive for oxidase and catalase activity. The major respiratory quinone of strain is ubiquinone 10 (Q-10). The dominant cellular fatty acids are $C_{18:1}$ *ω7c* and $C_{16:0}$. The major polar lipids are phosphatidylethanolamine (PE), diphosphatydilglycerol (DPG), phosphatidylcholine (PC) and phosphatydilglycerol (PG). Positive for nitrate reduction to NO_2 (weakly), nitrate reduction to N_2, D-glucose and l-malate (weakly) (API 20NE). Positive for alkaline phosphatase and acid phosphatase (API ZYM).

The whole genome sequence of strain BT689^T has been deposited in GenBank under the accession number NZ_JAFEMD000000000 (4,42 Mb). The genome-based G+C content is 62.4 mol%. The GenBank accession number for the 16S rRNA gene sequence of strain BT689^T is MT795749 (1,451 bp). The type strain BT689^T (= KACC 22016^T = NBRC 114858^T) was isolated from a soil sample collected in Uijeongbu city (37° 44' 55" N, 127° 2' 20" E), South Korea.

Declarations

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

Ethical Approval: This article does not contain any studies with human participants or animals.

Author Contributions:

All authors contributed equally to this manuscript.

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Tables

Table 1. Different characteristics of *Microvirga terrestris* and *Microvirga arvi* and closely related species of genus *Microvirga*.

Taxa: 1, strain BT290^T (data was obtained in this study); 2, strain BT689^T (data was obtained in this study); 3, *M. aerilata* 5420S-16^T (data was taken Weon et al. 2010). All strains were negative for production of acid from glucose, production of indole, *N*-acetyl-*β* glucosaminidase, arginine dihydrolase, *α*-chymotrypsin, cystine arylamidase, *α*-fucosidase, *α*-galactosidase, *β*-galactosidase (ONPG), *β*-galactosidase (PNPG), *α*-glucosidase (starch hydrolysis), *β*-glucosidase (esculin hydrolysis), *β*-glucosidase, *β*-glucuronidase, lipase (C14), *α*-mannosidase, urease, valine arylamidase and *N*-acetyl-D-glucosamine. All strains were positive for acid phosphatase.

+, positive; -, negative; w, weak positive

Characteristic	1	2	3
Isolation source	soil	soil	air
Size (μm long)	1.3 - 2.4	1.1 - 2.0	1.6 - 3.3
Size (μm wide)	0.5 - 1.4	0.6 - 0.7	1.2 - 1.5
Optimum growth temperature (˚C)	10 - 30 °C	10 - 30 °C	10 - 35 °C
Colony color	light pink	white	light pink
Oxidase			+
Catalase			+
Nitrate reduction			
Nitrate reduction to NO_2	-	w	-
Nitrate reduction to N_2	-	+	-
Enzyme activity			
alkaline phosphatase	-	+	+
esterase (C4)	-	-	+
esterase (C8)	-	-	+
leucine arylamidase	-	-	+
naphtol-AS-BI-phosphohydrolase	-	-	+
protease (gelatin hydrolysis)	-	-	w
trypsin	-	-	+
D-glucose	w	-	-
G+C content	62.3 mol%	62.4 mol%	61.5 mol%

Table 2. Cellular fatty acid profiles of *Microvirga terrestris* sp. nov., and *Microvirga arvi* sp. nov., and closely related species of genus *Microvirga*.

Taxa: 1, strain BT290^T (data was obtained in this study); 2, strain BT689^T (data was obtained in this study); 3, *M. aerilata* 5420S-16^T (data was taken Weon et al. 2010). All strains were grown on R2A agar at 28 °C. For unsaturated fatty acids, the location of the double bond was presented by counting the number from the methyl (*ω*) end of the carbon chain. ND, not detected; TR, trace amount (<1%).

Fatty acids	1	2	3
Saturated			
14:0	1.6	1.3	TR
16:0	17.7	10.8	9.8
17:0	ND	TR	ND
17:0 cyclo	TR	TR	ND
18:0	5.7	6.6	2.1
19:0 10-methyl	TR	TR	TR
Unsaturated			
15:1 *ω8c*	ND	ND	TR
18:1 *ω7c*	58.2	61.8	71.8
18:1 *ω7c* 11-methyl	1.2	2.3	ND
18:1 *ω9c*	ND	TR	ND
19:0 cyclo *ω8c*	2.2	8.2	ND
18:0 3OH	TR	1.0	ND
Summed Feature 2 (16:1 iso I / 14:0 3OH)	2.4	2.1	3.7
Summed Feature 3 (16:1 *ω6c* / 16:1 *ω7c*)	8.2	3.2	10.4

Figures

Figure 1

Transmission electron micrograph of strains BT290^T (a) and BT689^T (b) from cultures grown on R2A agar for three days at 25 °C. Bars are 500 nm.

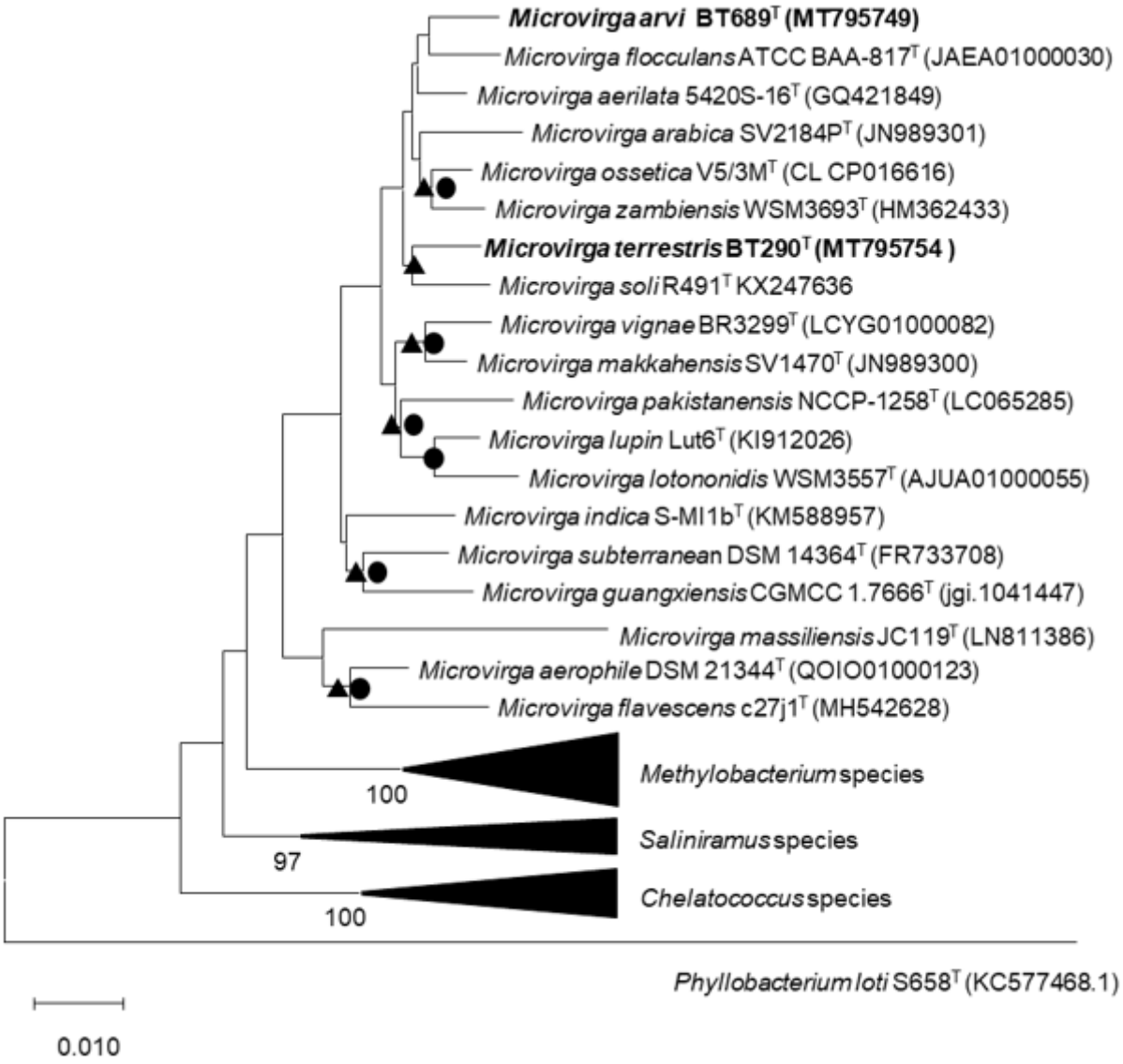

Figure 2

Neighbor-joining phylogenetic tree reconstructed from a comparative analysis of 16S rRNA gene sequences showing the relationships of strains BT290^T and BT689^T with closely related validly published species. Bootstrap values (based on 1,000 replications) greater than 70 % based on neighbor-joining method is shown at the internodes. Circles indicate that the corresponding nodes were also recovered in the maximum-parsimony tree. Triangles indicate that the corresponding nodes were recovered in the maximum-likelihood tree. *Phyllobacterium loti* S658^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position. The compact triangles represent species of the genus *Methylobacterium*, *Saliniramus* and *Chelatococcus*.

Supplementary Files

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