Hymenobacter Humicola sp. nov., Isolated from Soil in South Korea.

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Abstract

Two novel bacterial strains, designated as BT186^T and BT505, were isolated from a soil sample collected in South Korea and characterized. Both strains were Gram-stain-negative, rod-shaped, aerobic, circular, convex, and had red-colored colonies. 16S rRNA sequence analysis indicated that strains BT186^T and BT505 belong to a distinct lineage within the genus *Hymenobacter* (family *Hymenobacteraceae*, order *Cytophagales*, class *Cytophagia*, phylum *Bacteroidetes*, kingdom *Bacteria*). Both strains were closely related to *Hymenobacter norwichensis* DSM 15439^T (98.3% 16S rRNA gene similarity), *Hymenobacter aquaticus* JCM 31653^T (96.8%), and *Hymenobacter perfusus* LMG26000^T (96.5%). The strain BT186^T was found to have the MK-7 as the major respiratory quinone. The major polar lipid of strain BT186^T was identified to be phosphatidylethanolamine (PE). The major cellular fatty acid profiles of strain BT186^T were $C_{16:1}$ $\omega5c$ (24.3%), iso-$C_{15:0}$ (20.3%) and summed feature 3 ($C_{16:1}$ $\omega6c$ / $C_{16:1}$ $\omega7c$) (19.9%). Characterization based on polyphasic analysis indicated that strains BT186^T and BT505 represent novel species of the genus *Hymenobacter* and the name *Hymenobacter humicola* sp. nov. is proposed. The type strain of *Hymenobacter humicola* is BT186^T (= KCTC 72338^T= NBRC 114968^T).

Introduction

The genus *Hymenobacter* was first described by Hirsch et al. (1999) and then corrected by Buczolits et al. (2006) and Han et al. (2014) allocated to the family *Hymenobacteraceae* (Munoz et al. 2017), order *Cytophagales* and class *Cytophagia*. At the time of writing (April 2021), the genus comprises 89 species with validly published names (https://lpsn.dsmz.de/genus/hymenobacter). Recently, novel strains of genus *hymenobacter* have been discovered from different sources including cold environments, such as air sampling in maritime Antarctica (Roldan et al. 2020), abandoned lead-zinc mine (Feng et al. 2020), arctic station (Dahal et al. 2020), regoliths in Antarctica (Sedlacek et al. 2020), lake sediments (Wang et al. 2020), the bark of ginkgo tree (Cha et al. 2020), ubiquitous weedy grass *Setaria viridis* (Chhetri et al. 2020) and soil (Jang et al. 2021; Lee et al. 2021).

In the present study, we conducted phylogenetic analysis, phenotypic, genotypic, and chemotaxonomic characterization to determine the taxonomic position of strains BT186^T and BT505. The results suggested that strains BT186^T and BT505 represent novel species of the genus *Hymenobacter*, for which the name *Hymenobacter humicola* sp. nov. is proposed.

Materials And Methods

Strain isolation

Two novel strains designated as sBT186^T and BT505, respectively, were isolated from a soil sample collected at Jungangro (35° 59' 0" N, 126° 43' 0" E) located in Gunsan City, South Korea. After one week of incubation at 25°C on Reasoner's 2A (R2A) agar medium (Difco), single colonies were picked and

subcultured using the same medium at least two times to obtain pure colonies. The new bacterial cultures were routinely subcultured on R2A agar at 25 °C, maintained at 4 °C, and stored in 10% (w/v) glycerol suspension at −80 °C before use.

Morphology, physiology, and biochemical analysis

Cell morphologies of new isolates were observed using transmission electron microscopy (JEOL, JEM1010) using the negative staining method. The Gram-staining reaction was performed using a kit, following the manufacturer's instructions (bioMérieux). Catalase activity was determined by adding 3% (w/v) H_2O_2 solution and oxidase activity was examined using 1% (w/v) tetramethyl-p-phenylenediamine diamine. (Cappuccino and Sherman 2002). The bacterial growth of strains BT186^T and BT505 was tested on Reasoner's 2A (R2A) agar, Luria-Bertani (LB) agar, Tryptic Soy Agar (TSA) Nutrient Agar (NA), and MacConkey (MAC) agar, respectively. Growth at 10, 15, 20, 25, 30 and 30 °C was assessed under various pH conditions (5 to 9, 1 pH intervals) and different effect of NaCl concentrations (1%to 5% [w/v %], 1% intervals). Enzyme activities, assimilation of carbon sources, acid production from substrates and other physiological characteristics were determined by inoculating API 20NE, API ZYM and API ID32GN strips performed according to the manufacturer's instructions (bioMérieux).

Phylogenetic analysis

For phylogenetic analysis, the 16S rRNA genes were amplified using two universal primers 9F and 1512R (Weisburg et al. 1991). The PCR products were purified and sequenced using universal primers 337F, 518R, 785F, and 926R (Macrogen). The 16S rRNA gene sequences were identified by EzBioCloud server (https://www.ezbiocloud.net/). To determine the taxonomic positions of strains BT186^T and BT505, the 16S rRNA sequences of related taxa of the genus *Hymenobacter* were obtained from EzBioCloud (Yoon et al. 2017) and compared with those of strains BT186^T and BT505 using the EzEditor2 program (Jeon et al. 2014). Phylogenetic trees were constructed using the MEGAX program (Kumar et al. 2016) with the neighbor-joining (NJ) (Saitou and Nei 1987), maximum-likelihood (ML) (Felsenstein 1981), and maximum-parsimony (MP) algorithms (Fitch 1971). The stability of the tree topologies was evaluated by bootstrap analysis based on the 1,000 resampling method (Felsenstein 1985). Evolutionary distances were calculated according to the Kimura two-parameter model (Kimura 1983).

Genome sequencing

The genomic DNA of strain BT186^T was extracted using a genomic DNA extraction kit according to the manufacturer's instruction (Solgent). Then, the DNA library was prepared using the Nextera DNA Flex Library Prep Kit (Illumina) according to the manufacturer's protocol. Whole-genome sequencing (WGS) was performed using iSeq 100 and assembled by using the SPAdes software version 3.10.1 (Algorithmic Biology Lab, St. Petersburg Academic University of the Russian Academy of Sciences). The whole-genome sequence of strain BT186^T was deposited in GenBank (www.ncbi.nlm.nih.gov) database and annotated using the National Center for Biotechnology Information Prokaryotic Genome Annotation

Pipeline (PGAP) (Tatusova et al. 2016). The average nucleotide identity (ANI) and digital DNA-DNA hybridization (dDDH) values based on whole-genome sequences were calculated by using the EzBioCloud (https://www.ezbiocloud.net) and Genome-to Genome Distance Calculator (GGDC), respectively, with the recommended formula 2 (Table S1) (Meier-Kolthoff et al. 2013).

Chemotaxonomic characterization

To analyze the polar lipid, fatty acid, and lipoquinone components of strain BT186^T, cells were grown on R2A agar at 25 °C for three days. After then, cells were harvested and freeze-dried. The 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 as previously described (Komagata and Suzuki 1987; Minnikin et al. 1984). The cellular fatty acids methyl esters (FAME) of strain BT186^T were analyzed using the Sherlock Microbial Identification System V6.01 (MIS, database TSBA6, MIDI Inc) according to the protocol described by Sasser (1990). The respiratory quinone was extracted using the Sep-Pak Vac cartridges (Waters) and analyzed by high-performance lipid chromatography (HPLC) follow the previous methods (Hiraishi et al. 1996).

Results And Discussion

Morphology, physiology, and biochemical characteristics

Cells of strains BT186^T and BT505 were rod-shaped, Gram-stain-negative, aerobic, and non-motile. Colonies of strains BT186^T and BT505 were convex, smooth, circular, and red-colored. Cells of strains BT186^T and BT505 could survive at 10-30°C (optimum 25°C) and pH 6.0-8.0 (optimum 6.0) on R2A agar plate. Distinct features between the newly isolated strains and reference strains were provided in Table 1. The negative reactions of strain BT186^T on API kits were given as supplementary tables (Table S2).

Phylogenetic analysis and whole-genome sequence analysis

Based on the 16S rRNA gene sequence similarities, strains BT186^T and BT505 were affiliated with the family *Hymenobacteraceae* and showed high sequence similarities with the genus *Hymenobacter*. The level of 16S rRNA gene sequence similarity between the strains BT186^T and BT505 was 100%, indicating that they represent an identical species. Both strains were closely related to *H. norwichensis* DSM 15439^T (98.3% 16S rRNA sequence similarity), *H. aquaticus* JCM 31653^T (96.8%), and *H. perfusus* LMG26000^T (96.5%). The 16S rRNA gene sequence similarities of strains BT186^T and BT505 with the closely related type strains were less than 98.3% and with other *Hymenobacter* species were less than 96.4%. These values were below 98.7% of which value is recently used as the threshold for differentiating among bacterial species (Chun et al. 2018). The other *Hymenobacter* species exhibited sequence similarities lower than 97.0%. According to the phylogenetic tree based on the neighbor-joining, maximum-likelihood (Fig. S1), and maximum-parsimony (Fig. S2) algorithm, strains BT186^T and BT505 were placed within the genus *Hymenobacter* (Fig. 1).

The draft genome of strain BT186^T was 6,019,942 bp (24.7×) and consisted of 4,860 protein-coding genes, 54 RNA genes (7 rRNA genes, 44 tRNA genes), and 25 pseudogenes. The genome sequence of strain BT186^T has been deposited in GenBank under the accession numbers NZ_JAFLQZ000000000. The DNA G+C content of strain BT186^T was 57.5 mol%. This value was within the range of the G+C contents for the genus *Hymenobacter* (55-71 mol%) (Feng et al. 2019). The digital DNA-DNA hybridization values between strain BT186^T and other related type strains of genus *Hymenobacter* were less than 30.2% (Table S1), which are below the cutoff (70%) point (Meier-Kolthoff et al. 2013). Average nucleotide identity (ANI) values between strain BT186^T and other related type strains of genus *Hymenobacter* were less than 85.6%, respectively (Table S1). These values are below the ANI species threshold (95 - 96% ANI value) as described by Richter and Rossello-Mora (2009).

Chemotaxonomic characterization

The fatty acid profiles of strain BT186^T and four reference strains were presented in Table 2. The fatty acid profiles of strain BT186^T were similar to those of the most closely related three *Hymenobacter* strains (*H. norwichensis* DSM 15439^T, *H. aquaticus* JCM 31653^T, and *H. perfusus* LMG26000^T). The major cellular fatty acids of strain BT186^T were iso-$C_{15:0}$, summed feature 3 ($C_{16:1}\omega6c$ / $C_{16:1}\omega7c$), and $C_{16:1}\omega5c$. Strain BT186^T has larger amounts of iso-$C_{16:1}$ H (1.8%) and $C_{17:1}\omega6c$ (3.9%) than those of other strains. In contrast, other closely related *Hymenobacter* species (*H. norwichensis* DSM 15439^T, *H. aquaticus* JCM 31653^T, *H. perfusus* LMG26000^T) have smaller amounts of corresponding fatty acids. Strain BT186^T did not contain anteiso-$C_{17:0}$ and $C_{17:2}$ 2OH while other closely related two *Hymenobacter* species (*H. norwichensis* DSM 15439^T, *H. perfusus* LMG26000^T) contained those fatty acids. The polar lipids of strain BT186^T consisted of phosphatidylethanolamine (PE), five unknown aminophospholipids (APL), three unknown lipids (L), an unknown phospholipid (PL), and one unknown aminolipid (AL) (Fig. S3).

Description of *Hymenobacterhumicola* sp. nov.

Hymenobacter humicola (hu.mi'co.la. L. fem. n. *humus* soil; L. suff. -cola (from L. masc./fem. n. *incola*) inhabiting; N.L. masc. n. *humicola* soil-inhabiting)

Cells are Gram-stain-negative, aerobic, rod-shaped, 0.9-1.8 μm in width and about 7.2-8.5 μm in length. Colonies are circular, smooth, convex, non-motile, and red-colored on Reasoner's 2A (R2A) agar plates after growth for three days at 25 °C. Growth is observed at various temperatures ranging from 10 to 30 °C (optimum at 25 °C). The pH range for growth is 6.0-8.0 (optimum pH 6.0) on R2A agar. Cells grow on R2A agar, NA, TSA, and LB agar but not on MAC agar. Cells are positive for oxidase and catalase. Positive for alkaline phosphatase, esterase (C4) (weakly), esterase (C8) (weakly), leucine arylamidase, valine arylamidase (weakly), and cystine arylamidase by API ZYM. Positive for *β*-glucosidase (Esculin hydrolysis), *β*-galactosidase (PNPG), D-glucose (weakly), L-arabinose (weakly), D-mannose (weakly), and D-maltose by API 20NE. Positive for D-sucrose, D-maltose, D, L-lactate, glycogen, D-mannitol, D-glucose,

D-melibiose, D-sorbitol, and L-arabinose by API ID32GN. The major polar lipid is phosphatidylethanolamine (PE). The major respiratory quinone is MK-7. The major cellular fatty acids of strain BT186^T are $C_{16:1}\omega5c$ (24.3%), iso-$C_{15:0}$ (20.3%) and summed feature 3 ($C_{16:1}\omega6c$ / $C_{16:1}\omega7c$) (19.9%).

The whole-genome sequence of strain BT186^T has been deposited in GenBank under the accession number NZ_JAFLQZ000000000 (6,019,942 bp). The genome-based G+C content is 57.5 mol%. The GenBank accession number for the 16S rRNA gene sequence of strain BT186^T is MW876235 (1,389 bp). The type strain BT186^T (= KCTC 72338^T= NBRC 114968^T) was isolated from a soil sample collected in Jungangro (35° 59' 0" N, 126° 43' 0" E), South Korea.

Declarations

Acknowledgements

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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.

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Tables

Table 1. Different characteristics of strain BT186^T and closely related species.

Taxa: 1, strain BT186^T; 2, *H. norwichensis* DSM 15439^T; 3, *H. aquaticus* JCM 31653^T; 4, *H. perfusus* LMG26000^T. Data of strain BT186^T was obtained in this study. Those of reference strains were obtained from previous papers (Buczolits et al.2006; Lee et al. 2017; Chung et al. 2010). All strains were positive for alkaline phosphatase and leucine arylamidase. All data were negative for *β*-glucuronidase, *α*-fucosidase, L-malate, *α*-mannosidase and lipase (C14).

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

Characteristic	1	2	3	4
Size (µm long)	7.2-8.5	3.0-4.0	2.6-3.1	3.5-5.0
Size (µm wide)	0.9-1.8	0.8	1.0-1.2	1.0-1.5
Colony color	red	brick red	reddish pink	red pink
Oxidase	+	+	+	-
Catalase	+	+	+	-
Nitrate reduction				
Nitrate reduction to NO_2	-	w	-	+
Nitrate reduction to N_2	-	-	-	+
Production of acid from glucose	-	w	-	+
Enzyme activity				
N-Acetyl-*β*-glucosaminidase	-	-	-	+
Acid phosphatase	-	w	w	+
Cystine arylamidase	w	-	w	+
Esterase (C4)	w	-	w	w
Esterase (C8)	w	w	w	+
α-Galactosidase	-	-	-	+
β-Galactosidase (PNPG)	+	-	-	+
α-Glucosidase (starch hydrolysis)	-	-	-	+
β-Glucosidase (Esculin hydrolysis)	+	w	-	+
β-Glucosidase	-	-	-	+
Naphtol-AS-BI-phosphohydrolase	-	w	w	+
Trypsin	-	-	-	+
Valine arylamidase	w	w	+	+
Assimilation				
L-Arabinose	w	-	-	-
D-Maltose	+	w	+	+
D-Mannose	+	w	-	+
D-Sucrose	+	-	-	+

N-Acetyl-*D*-glucosamine	-	-	-	+
G+C content	57.5%	61.0%	61.9%	60.0%

Table 2. Cellular fatty acid profiles of strain BT186^T and closely related species.

Taxa: 1, strain BT186^T; 2, *H. norwichensis* DSM 15439^T; 3, *H. aquaticus* JCM 31653^T; 4, *H. perfusus* LMG26000^T. Data of strain BT186^T was obtained in this study. Those of reference strains were obtained from previous papers (Buczolits et al.2006; Lee et al. 2017; Chung et al. 2010).

For unsaturated fatty acids, the double bond location was presented by counting the number from the methyl (ω) end of the carbon chain. -, trace (< 1%) or not detected.

Fatty acids	1	2	3	4
Saturated				
13:0 iso	TR	ND	ND	TR
14:0	TR	1.6	1.5	TR
14:0 iso	TR	TR	ND	TR
15:0	ND	ND	ND	TR
15:0 iso	20.3	18.9	13	19.4
15:0 anteiso	4.7	6.8	6.3	19.8
15:0 2OH	TR	ND	ND	1
15:0 iso 3OH	2.3	3.1	1.9	2.1
16:0	1.1	2.6	19.8	TR
16:0 iso	1.6	TR	ND	1.1
16:0 N alcohol	ND	ND	ND	ND
16:0 3OH	TR	TR	1	TR
17:0 iso	1.7	2.3	ND	3.3
17:0 anteiso	ND	TR	ND	1.5
17:2 2OH	ND	TR	ND	TR
17:0 iso 3OH	3.6	5	3	3.6
18:0	ND	ND	12.5	ND
Unsaturated	ND	ND	ND	ND
15:1 iso G	TR	TR	2.5	TR
15:1 anteiso A	TR	TR	ND	TR
16:1 iso H	1.8	1.6	ND	1.3
16:1 *ω5c*	24.3	12.5	10.7	9.7
16:1 *ω7c* alcohol	TR	ND	ND	TR
17:1 iso *ω9c*	ND	TR	ND	ND
17:1 *ω6c*	3.9	TR	ND	ND
18:1 *ω9c*	ND	ND	1.1	ND
Summed Feature 1 (15:1 iso H / 13:0 3OH)	TR	ND	ND	1.6

Summed Feature 2 (16:1 iso I / 14:0 3OH)	ND	ND	1.0	ND
Summed Feature 3 (16:1 *ω6c* / 16:1 *ω7c*)	19.9	9.8	15.4	11.1
Summed Feature 4 (17:1 iso I / 17:1 anteiso B)	5.04	6.5	6.9	12.4
Summed Feature 9 (17:1 iso *ω9c* / 16:0 10-methyl)	ND	ND	ND	TR

Figures

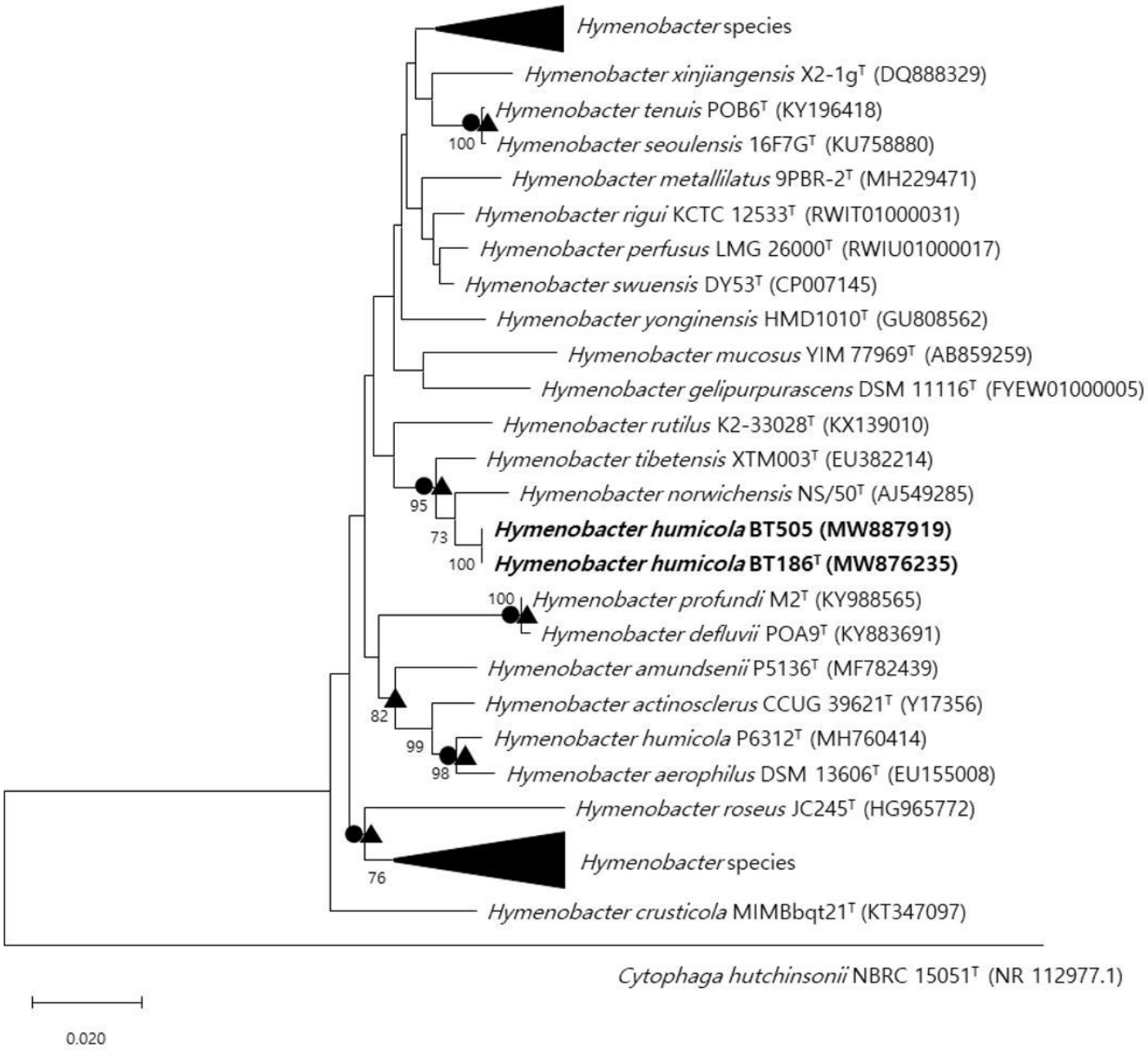

Figure 1

Neighbor-joining phylogenetic tree reconstructed from a comparative analysis of 16S rRNA gene sequences showing the relationships of strains BT186T and BT505 with closely related validly published species. Bootstrap values (> 70%) based on 1,000 replications are shown at the branch nodes. Circles indicate that the corresponding nodes were also recovered in the maximum-parsimony tree (Fig. S1). Triangles indicate that the corresponding nodes were also recovered in the maximum-likelihood trees (Fig. S2). Bar, 0.020 substitutions per nucleotide position. Cytophaga hutchinsonii NBRC 15051T were used as outgroup.

Supplementary Files

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

- Supplementarydata.docx