Brenneria Yuansilingia Sp. Nov., Isolated From Symptomatic Bark of Willow Canker

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Abstract

Two Gram-negative, facultative anaerobic strains are isolated from the symptomatic tissue of willow bark canker. The cells grow at 4–41 ℃, pH 5.0–10.0, with optimal growth occuring at 30 °C and pH 7.0. Two novel strains share the highest 16S rRNA gene sequence similarity with *Brenneria nigrifluens* LMG 2694^T (98.8%). In the phylogenetic trees based on sequences of 16S rRNA gene and four housekeeping genes (*gyr*B, *rpo*B, *atp*D and *inf*B), two novel strains form a distinct branch from *Brenneria species*, indicating that two novel strains should belong to a novel species in the genus *Brenneria*, which is confirmed by the results of average nucleotide identity analysis and Digital DNA–DNA hybridization. Two novel strains have 81.9–85.0 % average nucleotide identity values with its closely relatives, *B. nigrifluens* LMG 2694^T, *B. alni* DSM 11811^T, *B.corticis* CFCC11842^T, *B. populi* CFCC 11963^T and *B. corticis* gBX10-1-2^T, respectively, lower than the proposed species boundary cut-off for ANI (95-96 %). The main fatty acids are $C_{16:0,}$ $C_{16:1}$ *ω7c and* $C_{18:1}$ *ω7c.* The DNA G+C content is 53.5 -53.7 %. Based on these data, two novel strains represent a novel species within the genus *Brenneria.* The name *Brenneria yuansilingia* sp. nov. is proposed. The type strain is hezel4-2-4^T (=CFCC 15597^T = LMG 31719^T).

Introduction

The genus *Brenneria* was first described by Hauben (1998) to accommodate six species of *Erwinia*. At the time of writing, the genus includes eight validly published species with two subspecies, i.e. *B. salicis* (type species)*, B. alni*, *B. corticis* (Li et al. 2019a), *B. goodwinii* (Denman et al. 2012), *B. nigrifluens*, *B. populi* (Li et al. 2015, including two subspecies), *B. roseae* (Brady et al. 2014, including two subspecies), and *B. rubrifaciens*. All *Brenneria* species with validly published name are associated with tree diseases, and three of them cause serious tree diseases. *B. alni*, is the causal agent of bark canker of black alder, Italian alder in Italy (Surico et al., 1996), and Caucasian alder in Iran (Moradi-Amirabad and 250 Khodakaramian, 2020). *B. nigrifluens* causes shallow bark canker of walnut, which has widely been isolated from the USA (Wilson et al., 1957), Spain (Lopez et al., 1994), Italy (Morone et al., 1998), France (Ménard et al., 2004), Iran (Yousefikopaei et al., 2007), Serbia (Popović et al., 2013) and Hungary (Végh et al., 2014). *B. rubrifaciens* are causal agent of walnut tree bark canker (McClean et al. 2008). *B. rubrifaciens* is the causal agent of shallow bark canker of walnut, which has been reported in the USA (Wilson et al., 1967), Spain (González et al., 2002) and Iran (Amirsardari et al., 2017). *B. salicis* is an important pathogen of willow causing willow water mark disease in many countries such as in Europe including Belgium (Rijckaert et al. 1984), England (Day 1924), and Netherlands (Lindeijer 1931), and Japan (Sakamoto et al. 1999).

Recently, two novel strains named hezel4-2-4^T and L3-3C-1 are isolated from samples of willow (*Salix matsudana*) bark canker colletected from Heze City, Shandong Province and Zhongwei city, Ningxia Hui Autonomous Region, China, respectively. This disease caused by *Lonsdalea populi* is often found in the branches and trunks of willow trees leading to tree or branch death (Li et al. 2019b). In the present study, the taxonomic status of two novel strains will be determined by using 16S rRNA gene analysis, multilocus

sequence analysis (MLSA), genome sequencing, average nuclide identity (ANI), digital DNA:DNA hybridization (dDDH), fatty acid analysis, and physiological and biochemical characterization.

Materials And Methods

Isolation and cultivation of bacteria

Bark canker samples of willows were collected from Heze City, Shandong Province, China, in September 2018. The bacterial strains were isolated according to the method described by Li et al. (2014). In brief, the collected bark was sterilized in 4% sodium hypochlorite solution for 3 minutes. After washing three times in sterile water, the bark tissues were cut into small pieces and ground using sterile mortar by the pestle. Then the ground tissue was transferred into a conical flask with 10 ml sterile water and shaking 10 mins at 30 ℃. The resulting suspension was serially diluted to 10^{-1}–10^{-4} with sterile water and spread onto nutrient agar plates. After incubation at 30 ℃ for 48 h, single colonies were streaked out on nutrient agar plate. The purified strains were preserved at -80 ℃.

Morphological, physiological and biochemicalcharacterization

Cell morphology of strain hezel4-2-4^T was observed using transmission electron microscopy. The growth conditions at different temperatures (4, 10, 20, 30, 37 and 41 ℃) and concentrations of NaCl (0–10 % (w/v), at intervals of 1 %) were determined in tryptic soy broth (TSB; Difco) (Bakir et al. 2008). The TSB adjusted to pH 4.0–11.0 (interval of 1.0) was used to determine the pH growth range (Li et al. 2017). The pH values were adjusted using the following buffers: citric acid/ sodium citrate (pH 4.0–5.0), Na_2HPO_4 /NaH_2PO_4 (pH 6.0– 8.0), Na_2CO_3 /$NaHCO_3$ (pH 8.0–9.0) and Na_2HPO4 /NaOH (pH 10.0–11.0). Anaerobic growth was observed after incubation of the novel strain on the same medium at 30 ℃ for one week in anaerobic jars (candle-jar method; Gerhardt et al. 1981). The catalase and oxidase activities were determined according to the method described by Smibert & Krieg (Smibert & Krieg 1994). Other physiological and biochemical characteristics were tested by using API 20E, API 50CHB/E test kits (bioMérieux) and Biolog Gen III according to the manufacturer's instructions.

Phylogenetic analysis

The 16SrRNA gene was amplified and sequenced by using primers 27F/1492R (5'-AGAGTTTGATCCTGGCTCAG-3'/5'-GGTTACCTTGTTACGACTT-3') (Lane 1991). An almost complete 16S rRNA gene sequence (1443 bp) was obtained for subsequent similarity analysis. The 16S rRNA gene sequence similarities were carried out by EzTaxon-e (http://eztaxon-e.ezbiocloud.net; Kim et al. 2012). After alignment and trimming, a 1302 bp-long sequence (corresponding to nucleotide positions 81–1382 in the *Escherichia coli*) was used for phylogenetic tree reconstruction by using Mage 7.0 Kumar et al. 2016based on maximum likelihood (Kimura 2-parameter model with Gamma distributed with invariant sites G+I). *Cronobactersakazakii* was used as the out group, and the resulting trees were evaluated using 1000 resamplings.

MLSA based on partial sequences of four housekeeping genes (*gyr*B, 743 bp; *rpo*B, 637 bp; *inf*B, 615 bp; *atp*D, 638bp) was performed on two novel and reference strains(Brady et al. 2008). Sequences of the four housekeeping genes for two novel strains were retrieved from its genomic sequence. Sequences of the reference strains were downloaded from GeneBank and the accession numbers were listed in table S1. The concatenated data of four housekeeping genes were aligned and used to reconstruct maximum likelihood method tree using Mage7.0 (Kumar et al. 2016). The general time reversible model with gamma distributed with invariant sites (G+I) was used and the resulting trees were evaluated using 1000 resamplings.

Genome analysis

Genome sequencing of two novel strains were carried out as described by Li et al. (2016). In brief, the library was constructed by the 300 to 500-bp DNA fragmentation performed using a TruSeq™ DNA Sample Prep Kit. Draft genomes were assembled using SOAPdenovo v2.04. The Glimmer 3.02 software were used to coding sequences prediction (Lagesen et al. 2007). The G+C contents were deduced from the genomic data. Virulence genes of hezel4-2-4^T were predicted using the Pathogen Host Interactions (PHI) (Martin et al. 2015).

ANI is a measure of similarity between two genome sequences and 95 % ANI value corresponds to the 70 % DDH (Goris et al. 2007). In this study, *B. alni* DSM 11811^T; *B. corticis* CFCC 11842^T; *B. goodwinni* LMG 26270^T; *B. nigrifluens* LMG 2694^T *B.populi* D9-5^T were selected as reference strains according to the results of phylogenetic trees based on 16S rRNA gene and MLSA data. The ANI values among two novel strains and five closely related species of *Brenneria* were determined by Ortho ANI (Lee et al. 2016). The dDDH based on genome sequences was performed by using Genome-to-Genome Distance Calculator (GGDC), a free web service at http://ggdc.dsmz.de/ (Meier-Kolthoff et al. 2013).

Fatty acid analysis

The cellular fatty acid analysis was carried out for two novel and reference strains. After culturing on TSA at 30 °C for 24 h, the cells were harvested during the exponential phase. The fatty acids were extracted and analyzed according to the protocol of the Sherlock Microbial Identification System (MIDI, version 6.0) (Sasser M. 1990).

Results And Discussion

Physiological and biochemicalcharacteristics

Cells are Gram-negative, facultative anaerobic, motile with flagella, short rods, approximately 0.6-0.7 × 1.5-2.0 μm (Fig S1). The physiological and biochemical results were listed in Table 1 and the species description given below.

Phylogenetic analysis

Two novel strains have 99.8% 16S rRNA gene sequence similarity, and show highest similarity with *Brenneria nigrifluens* LMG 2694 (98.8 %), followed by *B. corticis* gBX10-1-2 (97.8 %) and *B. roseae* (96.9 %). In the ML phylogenetic tree based on 16S rRNA gene (Fig.1), two novel strains form a distinct branch from the reference strains within *Brenneria* clade, and cluster together with *B. nigrifluens* ATCC 13208^T with 91 % bootstrap, and next to the branch of *B. corticis* CFCC 11842^T. Two novel strains and its closely relatives, *B. nigrifluens* ATCC 13208^T, *B. corticis* CFCC 11842^T, *B. alni* NCPPB 3934^T and *B. populi* strains formed one big cluster within *Brenneria* clade.

In the ML phylogenetic tree based on MLSA data (Fig.2), two novel strains form a separate branch, and cluster together with *B. alni* NCPPB 3934^T, which next to the branch formed by *B. corticis*, *B. nigrifluens, B. goodwinii* and *B. populi.* Phylogenetic trees based on 16S rRNA gene sequences and MLSA data suggest that two novel strains should belong to a novel species within the genus *Brenneria*.

Genomic analysis

The genome length of strain hezel4-2-4^T is 4.54 Mb size across 32 contigs (N50= 479,859 bp), including 3,936 coding genes, 66 pseudogenes, 17 rRNAs, 69 tRNAs and 8 other RNAs. The max length of those contigs is 889,800 bp, the DNA G+C content is 53.48 %. 547 genes are found to be associated with pathogen and host interactions according to database of the Pathogen Host Interactions. 269 genes of those genes are reduced virulence, and 118 genes do not affect pathogenicity of the trains (detailed in Fig S 2). To detect potential contamination of the novel strain genome, the 16S rRNA gene sequence determined by conventional Sanger sequencing is compared with the sequence retrieved from its genome. They share 100% sequence similarity.

Five closely related species, *B. nigrifluens* LMG 2694^T, *B.alni* DSM 11811^T and *B. corticis* gBX10-1-2^T, are selected for ANI analysis and dDDH with two novel strains. Two novel strains showed 81.9-85% ANI values with closely relatives (Table S2), which are lower than the proposed species boundary cut-off for ANI (95-96 %) (Richter M, Rosselló-Móra R, 2009), revealed that they represent a novel species within genus *Brenneria*. Two novel strains have 26.0-29.5 dDDH values with their closely related referenc strains (Table S2), lower than the species boundary DDH value (70 %) (Wayne et al. 1987).

Fatty acid analysis

The main fatty acids of two novel strains were $C_{16:0,}$ $C_{16:1}$ *ω7c,* $C_{18:1}$ *ω7c,* similar to closely related reference strains (Table2). The amount of $C_{16:1}$ *ω7c* of the novel strains was higher than their closely related reference strains, which could be used to differentiate them from its close relatives.

Taxonomic conclusion

The phylogenetic trees based on 16S rRNA gene and MLSA data suggest that two novel strains should represent a novel species within the genus *Brenneria*. The results of fatty acids, ANI, dDDH are most useful data to demonstrate that two novel strains belong to a novel species of *Brenneria*. Moreover, there

are several physiological and biochemical characteristics which can differentiate two novel strains from their *Brenneria* reference strains. For example, the disability of utilization of dextrin, d-glucuronic acid, *α*-keto butyric acid, L-glutamic acid can distinguish two novel strains from their closely reference strains *Brenneria alni* DSM 11811^T, *B. corticis* CFCC 11842^T, *B. goodwinni* LMG 26270^T, *B. nigrifluens* LMG 2694^T and *B. populi* CFCC 11963^T. Based on those data, we propose that the two novel stains should be assigned to a novel species within genus *Brenneria*. The name proposed for them are *Brenneriayuansilingia* sp. nov.

Description of *Brenneria yuansilingia*sp. nov.

Brenneria yuansilingia Yuan.si.lin.gia N.L. gen. neut. n. *Yuansiling* named in honour of researcher Siling Yuan (1919–2017), a well-known forest pathologist in China

Cells are Gram-negative, facultative anaerobic, motile with flagella, short rods, approximately 0.6-0.7 × 1.5-2.0 μm. Colonies are circular, light cream, smooth with entire margin, and approximately 1.3-1.5 mm growth at 30 °C on TSA for 48 h. The cells grow at 4-41 ℃, pH 5-10 and optimal growth 30 °C and pH 7.0. Growth occurs in conditions of 0–7 % (w/v) salinity. Positive for oxidase and negative for catalase. Nitrite not produced by reduction of nitrate. Negative for the activities of arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, urease, *β*-galactosidase, tryptophane deaminase, gelatinase, and production of H_2S and indole. Citrate is not utilized. It is positive for acid production from glycerol, l-arabinose, d-ribose, d-glucose, d-fructose, d-mannose, l-rhamnose, inositol, d-mannitol, methyl-αd-glucopyranoside, *N*-acetyl-glucosamine, amygdalin, arbutin, aesculin, salicin, d-maltose, d-saccharose, d-trehalose, d-raffinose, gentiobiose, d-turanose, potassium gluconate, potassium 2-ketogluconate and potassium 5-keto-gluconate (API 50CHB/E). Cells are positive for assimilation of acetic acid, d-aspartic acid, d-fructose, d-fructose-6-PO4, d-galactose, d-gluconic acid, d-glucose-6-PO4, d-mannitol, d-mannose, d-saccharic acid, D-salicin, d-turanose, formic acid, glycerol, l-aspartic acid, l-rhamnose, l-serine, methyl pyruvate, mucic acid, myo-inositol, *N*-acetyl-d-glucosamine, pectin, *β*-methyl d-glucoside, quinic acid, sucrose, and variable for assimilation of gentiobiose (Biolog Gen III). The main fatty acids are $C_{16:0,}$ $C_{16:1}$ *ω7c,* and $C_{18:1}$ *ω7c*. The G+C content is G+C 53.5-53.7 %. Type strain hezel4-2-4^T (=CFCC 15597^T= LMG 31719^T) is isolated from bark tissue of a willow bark canker. The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and genome sequence of strain hezel4-2-4^T is MT036110, JABJXS000000000.

Declarations

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Author contributions

S. Sun and Y.·Li analyzed the data and prepared the figures, performed the genome analysis, and wrote the manuscript, Yunpeng Liu collected the willow samples used for strains isolation, and Luan revised the manuscript.

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Compliance with ethical standards

Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical statement

This article does not contain any studies with human participants or animals performed by any of the authors.

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Tables

Table 1. Phenotypic characteristics that distinguish the novel strain from reference species.

Taxa:1, hezel4-2-4^{T}; 2, L3-3C-1; 3, *B. alni* DSM 11811^{T}; 4, *B. corticis* CFCC 11842^{T}; 5, *B. goodwinni* LMG 26270^{T}; 6. *B. nigrifluens* LMG 2694^{T}; 7 *B. populi* CFCC 11963^{T}. +, positive; −, negative. * from WGS. All data come from in present study under the same experimental conditions except G+C content.

Characteristic	1	2	3	4	5	6	7
β-galactosidase	-	-	-	-	+	+	-
Acid production from (API 50CHB/E):							
Methyl *α*-d-glucopyranoside	+	+	-	-	-	+	+
d-Mannose	+	+	+	-	+	+	+
Gentiobiose	+	+	-	+	+	-	-
Inositol	+	+	-	+	+	-	-
d-Melibiose	-	-	-	+	+	-	+
Potassium gluconate	+	+	-	+	-	-	+
d-Raffinose	+	+	-	+	+	+	+
d-Sorbitol	-	-	-	+	+	-	+
d-Trehalose	+	+	+	-	+	+	-
d-Turanose	+	+	+	-	+	+	-
d-Arabinitol	-	-	-	+	-	-	-
l-Rhamnose	+	+	-	+	+	-	+
d-Maltose	+	+	+	+	+	-	+
Potassium 5-ketogluconate	+	+	+	+	+	-	-
Utilization of (Biolog GenIII):							
Dextrin	-	-	+	+	+	+	+
d-Arabitol	-	-	-	+	-	+	-
d-Cellobiose	-	-	+	+	-	+	+
d-Melibiose, D-sorbitol	-	-	-	+	+	+	+
d-Raffinose	-	-	+	+	+	+	+
d-Trehalose	-	-	+	-	+	+	-
d-Glucuronic acid, *α*-keto butyric acid, L-glutamic acid	-	-	+	+	+	+	+
Glucuronamide	-	-	-	-	-	+	-
α-Hydroxybutyric acid	-	-	-	+	-	-	+
N-Acetyl-D-galactosamine	-	-	+	-	-	+	-
Tween 40	-	-	-	-	-	+	-

Formic acid	+	+	-	+	+	-	+
Gentiobiose	+	-	-	-	+	+	-
G+C mol %	53.5*	53.7*	51*	56.2*	53.1*	55.9*	56.1*

Table 2. Fatty acid compositions of the novel and reference strains of *Brenneria*

Strains: 1, hezel4-2-4^T; 2. L3-3C-1; 3, *B. alni* DSM 11811^T; 4, *B. corticis* CFCC 11842^T; 5. *B. goodwinni* LMG 26270^T; 6, *B. nigrifluens* LMG 2694^T; 7 *B. populi* CFCC 11963^T. Values are percentages of the total fatty acid composition. The amount of fatty acids less than 0.5% are not shown. All data are from the present study.

Fatty acid	1	2	3	4	5	6	7
Saturated fatty acids							
$C_{12:0}$	4.6	3.3	6.1	3.1	6.1	3.7	5.6
$C_{13:0}$	-	-	1.4	1.5	-	-	
$C_{14:0}$	2.2	4.5	2.5	3.5	4.7	5.5	3.7
$C_{16:0}$	**22.7**	**25.8**	**30.8**	**21.5**	**31.6**	**36.1**	**27.1**
$C_{17:0}$	1.5	-	1.4	3.6	-	-	1.8
Unsaturated fatty acids							
$C_{16:1}$ $\omega 7c$	**34.7**	**33.0**	**19.8**	**13.4**	**10.9**	**14.8**	**16.4**
$C_{17:1}$ $\omega 8c$	1.4		1.2	6.2	-	-	
$C_{18:1}$ $\omega 7c$	**20.5**	**17.2**	**18.1**	**11.7**	**14.1**	**11.3**	**21.6**
Cyclopropane fatty acids							
$C_{17:0}$	3.9	**6.9**	**14.7**	**12.1**	**17.8**	**14.0**	**10.3**
$C_{19:0}$ $\omega 8c$	0.5	1.2	1.6	1.4	1.7	2.3	2.3
Hydroxy							
$C_{13:0}$ 3-OH	-	-	-	2.2	-	-	-
$C_{14:0}$ 3-OH	-	-	-	**8.6**	12.2	**11.3**	**9.9**
$C_{15:0}$ 3-OH	0.5	-	-	1.0	-	-	-
$C_{16:0}$-iso	-	-	-	6.6	-	-	-
$C_{18:0}$-iso	-	-	-	2.3	-	-	-
12:0 aldehyde	6.0	6.5	-	-	-	-	-

Figures

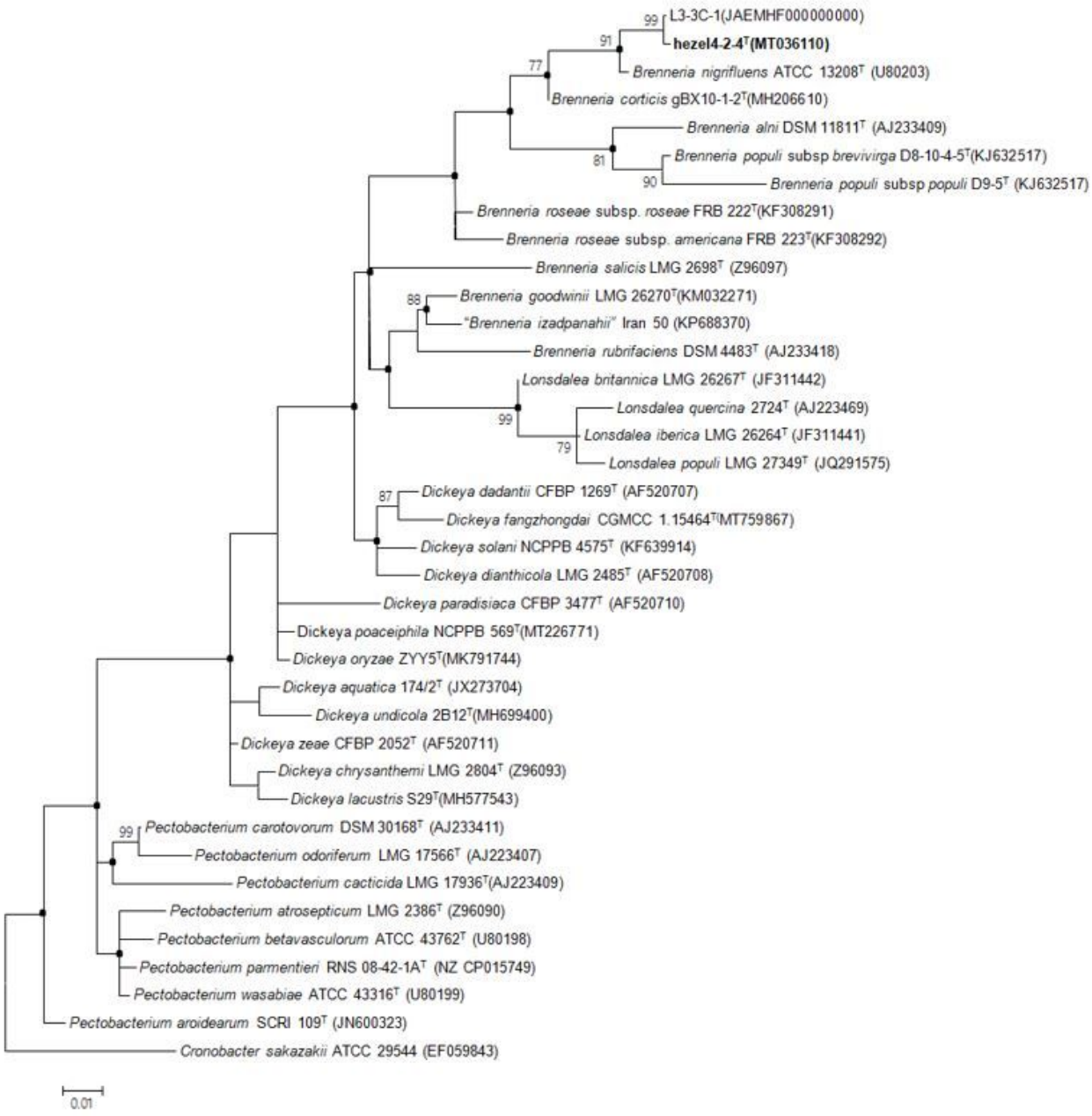

Figure 1

Maximum-likelihood tree based on nearly complete 16S rRNA gene sequences showing relationships the novel strain and reference strains. Phylogenetic analysis, performed using MEGA7.0, was based on the maximum-likelihood method, partial deletion of gaps/missing data treatment and site coverage cut-off 95 %. Bootstrap values >70 % (based on 1000 resamplings) are shown. Bar, 0.01 substitutions per nucleotide position.

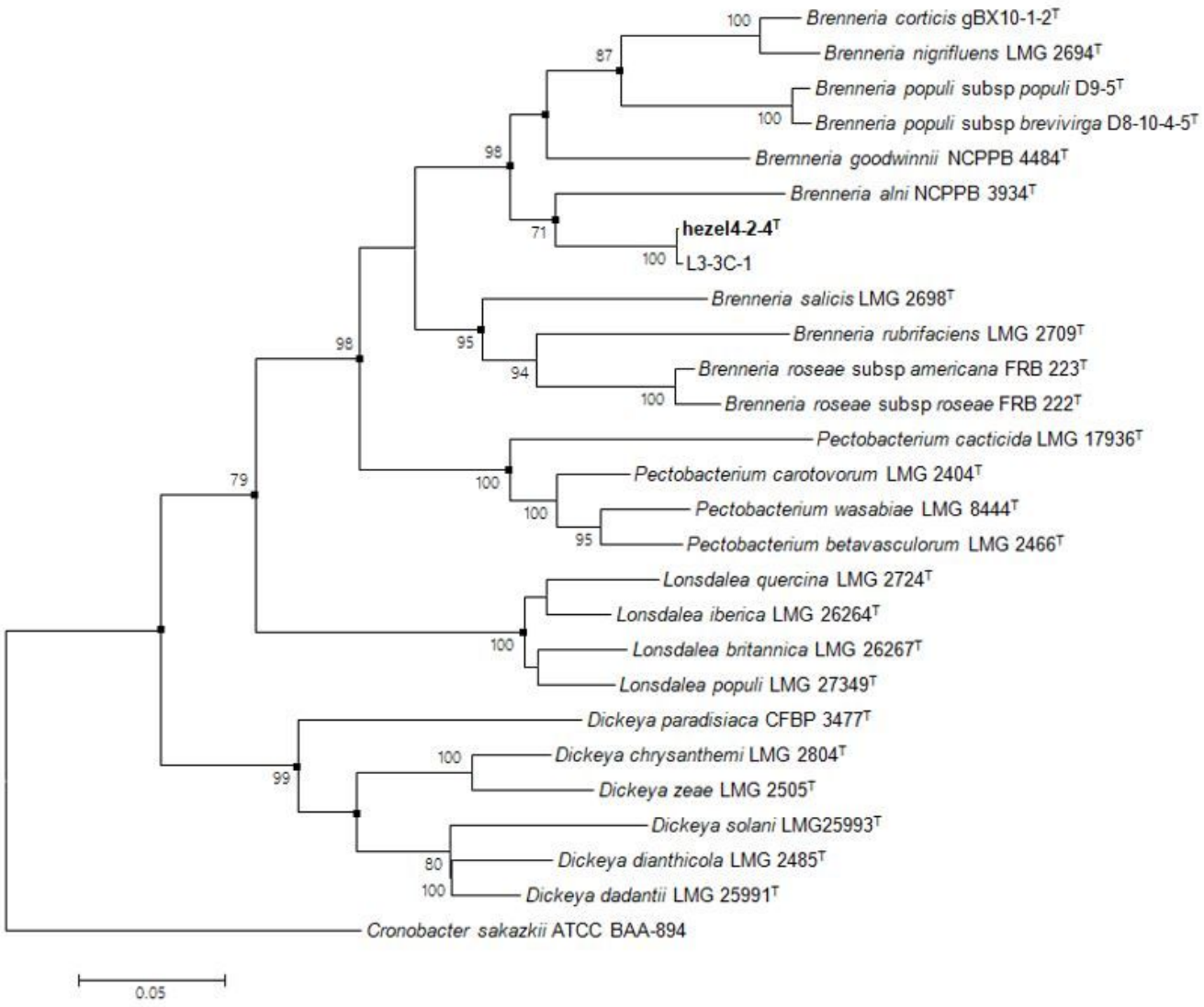

Figure 2

Maximum-likelihood tree based on concatenated partial gyrB, rpoB, atpD and infB gene sequences of the novel strain and of phylogenetically related species. Bootstrap percentage values based on 1000 resampled datasets are shown at nodes; only values above 70 % are given. The accession numbers are listed in Table S1. Bar, 0.05 nucleotide changes per site.

Supplementary Files

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

- Supplementaryfile2021311.doc