Neobacillus Paridis sp. nov., an Endophyte of *Paris Polyphylla* Smith Var. *Yunnanensis*

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Abstract

A novel endophytic strain, designated as YIM B02564^T, was isolated from the root of *Paris polyphylla* Smith var*. yunnanensis* obtained from Yunnan Province, southwest China. By using a polyphasic approach, cells of the strain were characterized as facultative anaerobic, Gram-positive and rod-shaped. The growth conditions of the strain were found to occur at 20–55 ℃ (optimum, 30 ℃), pH 6.0–9.0 (optimum, pH 7.0). Strain YIM B02564^T can tolerate 2% NaCl concentration. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain YIM B02564^T belonged to the genus *Neobacillus* and the 16S rRNA gene sequence similarity values of strain YIM B02564^T to the type strains of members of this genus ranged from 95.6%–97.8%. The DNA G+C content of strain YIM B02564^T calculated from the whole genome sequence was 41.6 mol%. Values of the ANI and the dDDH between strain YIM B02564^T and its closely related *Neobacillus* species were below 77.87% and 21.50%. Strain YIM B02564^T contained MK-7 as the major menaquinone, iso-$C_{15:0}$ and anteiso-$C_{15:0}$ as the major fatty acids. The major polar lipids were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, an unidentified aminophospholipid and four unidentified lipids. It contained *meso*-diaminopimelic acid in the cell-wall peptidoglycan. On the basis of polyphasic analysis, strain YIM B02564^T could be differentiated genotypically and phenotypically from recognized species of the genus *Neobacillus*. The isolate therefore represents a novel species, for which the name *Neobacillus paridis* is proposed. The type strain is YIM B02564^T (= JCM 34668^T = CGMCC 1.18655^T).

Introduction

Genus *Neobacillus*, within the family *Bacillaceae* of phylum *Firmicutes*, was separated from the genus *Bacillus* by Patel and Gupta (2020). Based on comprehensive phylogenomic and comparative genomic analysis, 12 species were reclassified as *Neobacillus* species. Subsequently, three species were transferred from *Bacillus* to *Neobacillus* (Gupta et al. 2020). Also one species was newly published with valid name (*Neobacillus endophyticus*; Jiang et al. 2021). Members of the genus *Neobacillus* are rod-shaped, endospore-forming, Gram-positive or Gram-variable staining, aerobic or facultatively anaerobic bacteria (Patel and Gupta 2020), which was widely distributed in various environments such as soil (Logan et al. 2000; Heyrman et al. 2004; Kämpfer et al. 2016), human gut (Bittar et al. 2015) and plant roots (Jiang et al. 2021). At the time of writing, the genus *Neobacillus* consisted of 16 validly published species. And in this study, we report the characterisations of a novel strain YIM B02564^T, and suggest that this strain represents a novel species of the genus *Neobacillus*.

Methods And Materials

Bacterial isolation and Maintenances

Healthy root of *Paris polyphylla* var. *yunnanensis* was collected from Shilin County, Yunnan Province, southwest China for further sterilizing and pulverizing before distribution on R2A medium which was

described by Yang et al. (2016). During 10 days' incubation at 25 °C, the colonies obtained were re-streaked on the same medium until pure colonies were obtained and stored both on R2A slants at 4 °C and in 15% (v/v) glycerol at -80 °C for long-term preservation.

16S rRNA gene sequencing and phylogenetic analysis

Genomic DNA of strain YIM B02564^T was extracted using a genomic DNA extraction kit (Tiangen, China) whose 16S rRNA was subsequently PCR-amplified with the forward primer 27F (5'-AGA GTT TGA TCC TGG CT-3') and reverse primer 1492R (5'-GGT TAC CTT GTT ACG ACT T-3'). Amplified products were purified and cloned into vector pClone007 (TsingKe, China). The final sequence for analysis had a size of 1514 bp and was submitted to the GenBank database. 16S rRNA sequence similarities to closely related type strains were calculated using EZBioCloud server (https://www.ezbiocloud.net/) (Yoon et al. 2017). The sequences of closely related strains retrieved from GenBank and EZBioCloud were aligned using Clustal Omega (Sievers et al. 2011) and aligned sequences were used to constructed the phylogenetic tress with Maximum-likelihood (ML) (Felsenstein 1981) method by Mega X software (Kumar et al. 2018). Phylogenetic distances were calculated with Kimura's two-parameter model (Kimura 1980). The bootstrap values were calculated based on 1000 replications (Felsenstein 1985). The sequence of *Lysinibacillus halotolerans* LAM612^T, *Lysinibacillus boronitolerans* NBRC 103108^T and *Lysinibacillus alkalisoli* Y2A20^T were selected as outgroups.

Whole genome sequencing and analysis

Using Illumina platform to perform the whole-genome sequencing of strain YIM B02564^T, which was based on the China General Microbiological Culture Collection Center (CGMCC) as part of the Global Catalogue of Microorganisms (GCM) 10K project (Shi et al. 2021). The paired-end reads were *de novo*-assembled using SOAPdenovo 2.04 (Li et al. 2015). The DNA G+C content of strain YIM B02564^T was calculated from the whole genomic sequence. Average nucleotide identity (ANI) values, average amino acid identity (AAI) values and digital DNA-DNA hybridization (dDDH) values were respectively calculated using FastANI (Jain et al. 2018), online AAI calculator (http://enve-omics.ce.gatech.edu/aai/), and DSMZ Genome-to-Genome Distance Calculator web server (http://ggdc.dsmz.de/distcalc2.php) with formula 2 (Meier-Kolthoff et al. 2013). The closely related strains with YIM B02564^T were obtained from NCBI GenBank Database for phylogenomics analysis. Functional annotation was conducted using PROKKA (Seemann 2014). OrthoFinder (Emms and Kelly 2015) was used to infer the orthologous genes which were to aligned by Clustal Omega (Sievers et al. 2011). Gblocks was used to select conserved blocks from the concatenated alignments. Based on the conserved blocks, a maximum-likelihood tree was constructed by IQ-TREE (Nguyen et al. 2015).

Morphology and physiology and biochemical analysis

After growth for 3 days in R2A medium at 30 °C, transmission electron microscope (JEM-2100, JEOL) was used to observe the cell morphology of the strain. The presence of endospores was examined

according to Schaeffer–Fulton stain method, and then observed by light microscope (DM2000, Leica). Motility was assayed using the soft (0.4%, w/v) agar stabbing technique (tube method) for 5 days. By incubating inoculated R2A plates in a bacteria incubator at 30 °C for 7 days, the best growing condition for this new species was determined. Therefore, the species was incubated at different temperature (10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60°C) and pH values (4.0–10.0, at intervals of 1.0 pH units). The buffer systems were prepared using Na-citrate, KH_2PO_4/NaOH and $NaHCO_3$/Na_2CO_3 for pH 4.0–5.0, pH 6.0–8.0 and pH 9.0–10.0, respectively (Tang et al. 2010). Growth of strain YIM B02564^T at different NaCl concentration (0–5.0%, w/v, at intervals of 0.5%) was tested in R2A plates. The growth of the strain under anaerobic conditions was assessed by incubating inoculated R2A plates in anaerobic workstation (whitley A35) at 30 °C for 7 days. The Gram reaction was carried out using 3% (w/v) KOH for cell lysis (Cerny 1978). Catalase activity was examined by investigating bubble production with 3% (v/v) H_2O_2. Oxidase activity was detected using API oxidase reagent (bioMérieux) according to the manufacturer's instructions. API ZYM, API 20NE, API 50CH (BioMérieux, France) and Biolog GEN microplates (BIOLOG Inc., Hayward, CA, United States) were used to view the other biochemical characters and utilization of various substrates according to the manufacturer's protocols.

Chemotaxonomic characteristics

Several standard methods were applied to analyze the chemotaxonomic characteristics of strain YIM B02564^T. Biomass for fatty acid analysis was harvested from cultures grown on TSA medium at 25 ℃ for 2 days. The fatty acids were extracted using a standard MIDI protocol and identified by using the Sherlock Microbial Identification System (Sherlock version 6.1; MIDI database: TSBA6) following the manufacturer's instructions (Sasser 2001). Determination of the cell-wall diaminopimelic acid was performed according to the method described by Staneck and Roberts (1974). Menaquinone and polar lipids were extracted following the method described by Minnikin et al. (1984). Menaquinone was analyzed by a reversed-phase HPLC system (Agilent Technologies 1260 Infinity) with a C18 column (25 cm×4.6 mm, 5 μm). Identification and analysis of polar lipids were performed by a two-dimensional TLC procedure on silica gel G60 plates and then 5% molybdatophosphoric acid, 0.2% ninhydrin and molybdenum blue were used to detect the lipids, aminolipids and phospholipids, respectively.

Results And Discussion

Molecular phylogenetic analysis

The complete 16S rRNA gene sequence (1514 bp) was obtained and submitted to GenBank under the accession number MW911620. The 16S rRNA gene sequence similarity values showed that strain YIM B02564^T had the highest similarity to *Neobacillus fumarioli* (97.8%), followed by *Neobacillus mesonae* (97.4%), *Neobacillus soli* (97.4%), *Neobacillus endophyticus* (97.3%) and *Neobacillus drentensis* (97.3%). The values between YIM B02564^T and *Neobacillus* species were less than the threshold for recognizing a novel species (98.6%) (Kim et al. 2014). The ML phylogenetic tree based on 16S rRNA gene sequences also showed that strain YIM B02564^T was closely related to members of the genus *Neobacillus* and

formed a cluster with *N. fumarioli* and *N. endophyticus* (Fig. 1). Given all that, the phylogenetic analyses based on 16S rRNA gene sequences clearly suggested that the isolate can be grouped as a species of the genus *Neobacillus*.

The draft genome of strain YIM B02564^T had a total length of 4,629,313 bp with N50 length of 179,987 bp. 12 rRNA genes, 98 tRNA genes and 4449 protein-coding genes were predicted in the annotated result of strain YIM B02564^T. The G+C content of strain YIM B02564^T calculated from the genome was 41.6 mol%. To confirm the phylogenetic relationship of strain YIM B02564^T, a maximum-likelihood (ML) phylogenomic tree was constructed on the basis of 698 orthologous genes. YIM B02564^T formed a branch with *N. mesonae* FJAT-13985^T, and formed a broader distinct cluster which also contained *N. fumarioli* NBRC 102428^T and *N. endophyticus* BRMEA1^T (Fig. 2). The dDDH estimate values for YIM B02564^T were 21.50% with *N. mesonae* FJAT-13985^T, 18.90% with *N. fumarioli* NBRC 102428^T and 19.80% with *N. endophyticus* BRMEA1^T, which were clearly lower than the standard cut-off value (70%) (Wayne et al. 1987). Additionally, The ANI values between YIM B02564^T and its closely related species *N. mesonae*, *N. fumarioli* and *N. endophyticus* were 77.87%, 76.05% and 75.62%, respectively. AAI values ranged from 63.39% to 77.80% between YIM B02564^T and other species in *Neobacillus* (Table S1). ANI and AAI values were also significantly lower than the threshold of 95%–96% for describing prokaryote species (Richter and Rosselló-Móra 2009; Konstantinidis and Tiedje 2005). Detailed information for ANI, AAI and dDDH values are given in Table S1. The eleven specific conserved signature indels (CSIs) in protein sequences of strain YIM B02564^T were identical with the description of genus *Neobacillus* (Patel and Gupta 2020). Based on the analysis above, strain YIM B02564^T can represent a novel species of the genus *Neobacillus*.

Phenotypic and chemotaxonomic characteristics

StrainYIM B02564^T was found to grow on NA, TSA and R2A medium. Colonies grown on TSA medium ware found to be circular, cream-colored and smooth after 3 days of cultivation. Cells of strain YIM B02564^T was facultative anaerobic, Gram-positive, oxidase-negative, catalase-positive, rod-shaped (0.4–0.7 μm wide and 2.0–5.0 μm long; Fig. S1) and motile. No endospores were observed in the cells. The optimum growth condition of strain YIM B02564^T occurred at 30 °C, pH 7.0 and the peak tolerance to NaCl concentration was 2%. The other results of physiological and biochemical analyses are summarized in the species description, and the properties comparation of strain YIM B02564^T with other related species are listed in Table 1.

The cell-wall peptidoglycan of strain YIM B02564^T contained glutamic acid, alanine acid and *meso*-diaminopimelic acid. The predominant menaquinone was MK-7. The major cellular fatty acids (>10 % of the total fatty acids) were iso-$C_{15:0}$ (27.6%) and anteiso-$C_{15:0}$ (23.9%). The minor fatty acids (>0.5 %) were iso-$C_{13:0}$ (0.6%), iso-$C_{14:0}$ (5.7%), $C_{14:0}$ (1.5%), $C_{16:1}$ $\omega 7c$ alcohol (1.1%), iso-$C_{16:0}$ (5.2%), $C_{16:1}$ $\omega 11c$ (2.9%), $C_{16:0}$ (9.4%), iso-$C_{17:1}$ $\omega 10c$ (0.9%), iso-$C_{17:0}$ (2.4%), anteiso-$C_{17:0}$ (5.2%), $C_{17:0}$ (0.6%), $C_{18:1}$ $\omega 9c$

(1.4%), $C_{18:0}$ (0.5%), summed feature 3 (2.3%) and 4 (2.8%). The overall fatty acid profile of strain YIM B02564^T was similar to those of the closely related reference type strains, but there were some differences in components (Table 2). The major polar lipids were diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine. One unidentified aminophospholipid and four unidentified lipids were also detected (Fig. S2). All these properties are consistent with the general chemotaxonomic features of the genus *Neobacillus* and support that strain YIM B02564^T represents a novel species of this genus, for which the name *Neobacillusparidis*sp. nov. is proposed.

Description of *Neobacillus paridis* sp. nov.

Neobacillusparidis (pa'ri.dis. L. gen. n. *paridis* of *Paris*, a plant genus, from which the type strain was isolated).

Cells are facultative anaerobic, Gram-positive, oxidase-negative, catalase-positive, rod-shaped, motile and free of spores. Colonies on TSA medium at 30 °C are circular, cream-colored and smooth. Growth is achieved between 20 °C and 55 °C (optimum 30 °C) and with a highest tolerance of 2% NaCl concentration and pH in the range of 6.0–9.0 (optimum 7.0). With the API 20NE test, cells are positive for nitrate reduction and hydrolysis of esculin, but negative for indole production, D-glucose fermentation, arginine dihydrolase, hydrolysis of urea□gelatin and 4-nitrophenyl *β*-D-galactopyranoside. API ZYM test show positive reactions for esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and *α*-glucosidase. API 50CH test indicate that production of acid from D-glucose, D-mannose, *N*-acetyl glucosamine, D-maltose; and weak production of acid from aesculin. For the utilization of carbon sources (Biolog GEN□ systems), the following substrates are utilized for growth: dextrin, D-maltose, D-trehalose, D-cellobiose, gentiobiose, sucrose, D-turanose, stachyose, D-raffinose, *α*-D-lactose, D-melibiose, *β*-methyl-D-glucoside,D-salicin, *N*-acetyl-*β*-dmannosamine, *N*-acetyl-D-galactosamine, *N*-acetyl neuraminic acid, *α*-D-glucose, D-fructose, 3-methyl glucose, D-fucose, L-fucose, L-rhamnose, *myo*-inositol, glycerol, D-glucose-6-phosphate, L-arginine, L-aspartic acid, L-glutamic acid, D-sorbitol, D-arabitol, *myo*-Inositol, glycerol, D-glucose-6-phosphate, L-arginine, L-aspartic acid, L-glutamic acid, pectin, D-galacturonic acid, L-galactonic acid lactone, D-gluconic acid, D-glucuronic acid, glucuronamide, mucic acid, quinic acid, p-hydroxy phenylacetic acid, D-Lactic acid methyl ester, L-lactic acid, *α*-keto-glutaric acid, L-malic acid, bromo-succinic acid, *γ*-amino-butryric acid, *α*-hydroxy-butyric acid, *β*-hydroxy-D, L-butyric acid, *α*-keto-butyric acid, acetoacetic acid, propionic acid, acetic acid and formic acid. The other tests by using API ZYM, API 50CH and Biolog GEN□ are negative. The cell-wall peptidoglycan contains *meso*-diaminopimelic as diagnostic diamino acid. The major menaquinone is MK-7 and the major components in the polar lipid profile are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, an unidentified aminophospholipid and four unidentified lipids. The predominant cellular fatty acids are iso-$C_{15:0}$ and anteiso-$C_{15:0}$.

The type strain is YIM B02564^T (= JCM 34668^T = CGMCC 1.18655^T), isolated from a root of *P. polyphylla* Smith var*. yunnanensis* collected from Shilin County, Yunnan Province, southwest China. The

GenBank accession numbers for the 16S rRNA gene sequence of *Neobacillusparidis* YIM B02564^T is MW911620. The whole genome sequences have been deposited at GenBank and GCM Type Strains Genome Database under accession JAESWB000000000 and GCM60020047, respectively.

Abbreviations

R2A, Reasoner's 2A; NA, nutrition agar; TSA, tryptic soy agar; ML, maximum-likelihood; GCM, the Global Catalogue of Microorganisms; CSI, conserved signature indel; MK, menaquinone; AAI, average amino acid identity; ANI, average nucleotide identity; dDDH, digital DNA-DNA hybridization.

Declarations

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Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Code availability Not applicable

Authors' contributions P-CZ and L-LY analyzed the data and wrote the manuscript. P-CZ, C-JL, ZZ, R-FM, J-RL and X-WJ performed the experiments. L-LY and X-YZ designed the experiment and directed the classification. L-LY takes full responsibility for the final submission. All the authors reviewed and approved the final version of the paper.

Compliance with ethical standards

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

Consent for publication The manuscript is submitted with the consent of all authors.

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Tables

Table 1. Characteristics that differentiate strain YIM B02564^{T} from closely related species of the genus *Neobacillus.*

Characteristics	1	2	3	4
Respiration	Facultatively anaerobic	Aerobic	Aerobic	Facultatively anaerobic
Cell size (μm)	0.4–0.7×2.0–5.0	0.5–0.8×4.0–8.0	0.6–1.2×2.2–2.7	0.7–1.3×1.5–6.1
Colonies Morphology	Circular, cream-coloured, smooth	Irregular, butyrous, brownish-cream, opaque	Pale yellow, flat, opaque, circular/ slightly irregular margins	Circular, cream-coloured, smooth, glossy colony
Motility	Motile	Feebly motile	Motile	Weakly motile
pH range for growth (optimum)	6.0–9.0 (7.0)	4.0–6.5 (5.5)	5.7–9.0 (7.0)	6.0–8.0 (7.0)
Temperature range (optimum, °C)	20–55 (30)	25–55 (50)	20–45 (30)	15–50 (25–30)
Highest NaCl tolerance (%, w/v)	2	6	2	0
Nitrate reduction	+	–	–	+
β-Galactosidase	–	–	+	+
Hydrolysis of aesculin	+	–	+	+
API 50CH (acid production):				
Glycerol	–	v	–	–
D-Ribose	–	v	+	–
D-Galactose	–	v	–	–
D-Glucose	+	+	–	+
D-Fructose	–	+	–	+
D-Mannose	+	+	–	–
L-Sorbose	–	–	–	+
D-Mannitol	–	+	–	–
Methyl *a*-D -glucopyranoside	–	v	–	–
N-Acetyl gulcosamine	+	w	–	+
Amygdalin	–	–	w	–
Aesculin	w	–	+	–
D-Cellobiose	–	–	+	–
D-Maltose	+	v	+	+
D-Lactose	–	v	+	–
D-Melibiose	–	v	+	–
D-Sucrose	–	+	+	+
D-Trehalose	–	w	+	+
D-Melezitose	–	v	–	+
D-Raffinose	–	v	+	w
Starch	–	–	–	+
Glycogen	–	–	–	w
D-Turanose	–	v	–	w
G+C content (mol%)*	41.6	40.4	40.3	38.5

Taxa: 1, YIM B02564^T; 2, *N. fumarioli* NBRC 102428^T (Logan et al. 2000); 3, *N. mesonae* DSM 25968^T (Liu et al. 2014); 4,*N. endophyticus* BRMEA1^T (Jiang et al. 2021).+, Positive; –, negative; w, weakly positive; v, variable. *, The DNA G+C contents were calculated based on their genome sequences in this study.

Table 2. Cellular fatty acid composition of strains YIM B02564^T and the type strains of related *Neobacillus* species.

Fatty acids	1	2	3	4
Saturated:				
$C_{14:0}$	1.5	0.5	0.6	0.3
$C_{16:0}$	9.4	1.4	4.8	2.1
$C_{17:0}$	0.6	–	0.9	–
$C_{18:0}$	0.5	0.1	1.0	1.5
Branched:				
iso-$C_{13:0}$	0.6	–	0.5	–
iso-$C_{14:0}$	5.7	0.7	3.1	3.1
iso-$C_{15:0}$	27.6	41.6	40.8	55.9
anteiso-$C_{15:0}$	23.9	7.1	23.3	11.7
iso-$C_{16:0}$	5.2	6.6	3.8	2.8
iso-$C_{17:0}$	2.4	5.2	6.2	1.9
anteiso-$C_{17:0}$	5.2	13.4	3.7	2.3
Unsaturated:				
$C_{16:1}$ $\omega 7c$ alcohol	1.1	4.3	1.0	4.7
$C_{16:1}$ $\omega 11c$	2.9	3.0	4.6	2.2
iso-$C_{17:1}$ $\omega 10c$	0.9	9.2	2.3	5.3
$C_{18:1}$ $\omega 9c$	1.4	0.2	0.7	1.1
Summed feature*				
3	2.3	–	–	1.7
4	2.8	5.8	0.9	3.2

Taxa: 1, YIM B02564^T; 2, *N. fumarioli* NBRC 102428^T; 3, *N. mesonae* DSM 25968^T; 4,*N. endophyticus* BRMEA1^T. Data for column 2 and 4 were taken from Logan et al. (2000); for column 3 were taken from Liu et al. (2014). *, Summed features represent groups of two or three fatty acids that cannot be separated using MIDI system. Summed feature 3, $C_{16:1}$ $\omega 7c$ and/or $C_{16:1}$ $\omega 6c$; Summed feature 4, iso-$C_{17:1}$ and/or anteiso-$C_{17:1}$. –, Not detected or trace <0.5.

Figures

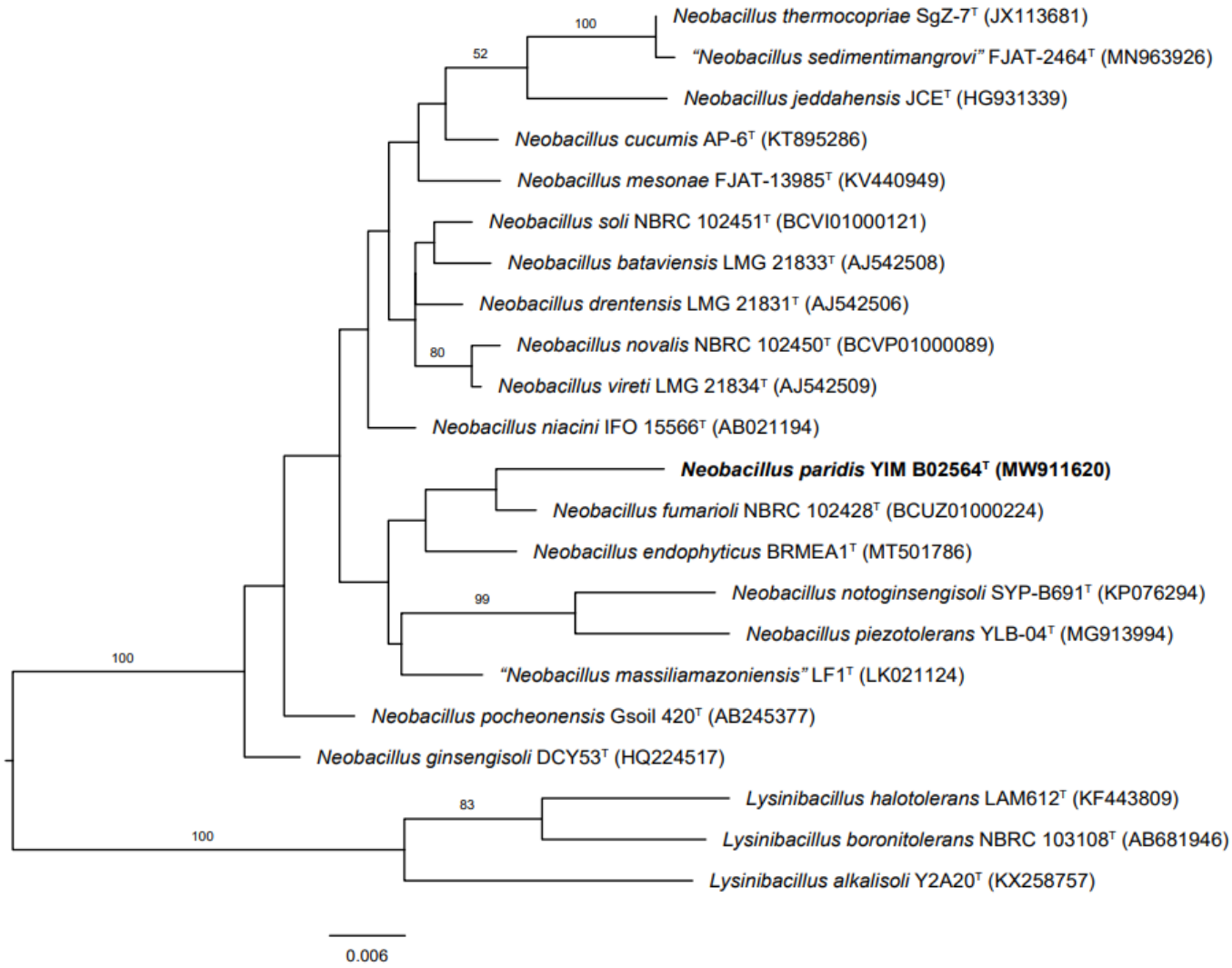

Figure 1

Phylogenetic tree reconstructed using the maximum-likelihood (ML) algorithm based on the 16S rRNA gene of strain YIM B02564T and closely related strains. Species with effective but invalid name are enclosed in double quotes. Bootstrap values (>50%) are indicated at the nodes. Scale bar, 0.006 changes per nucleotide position.

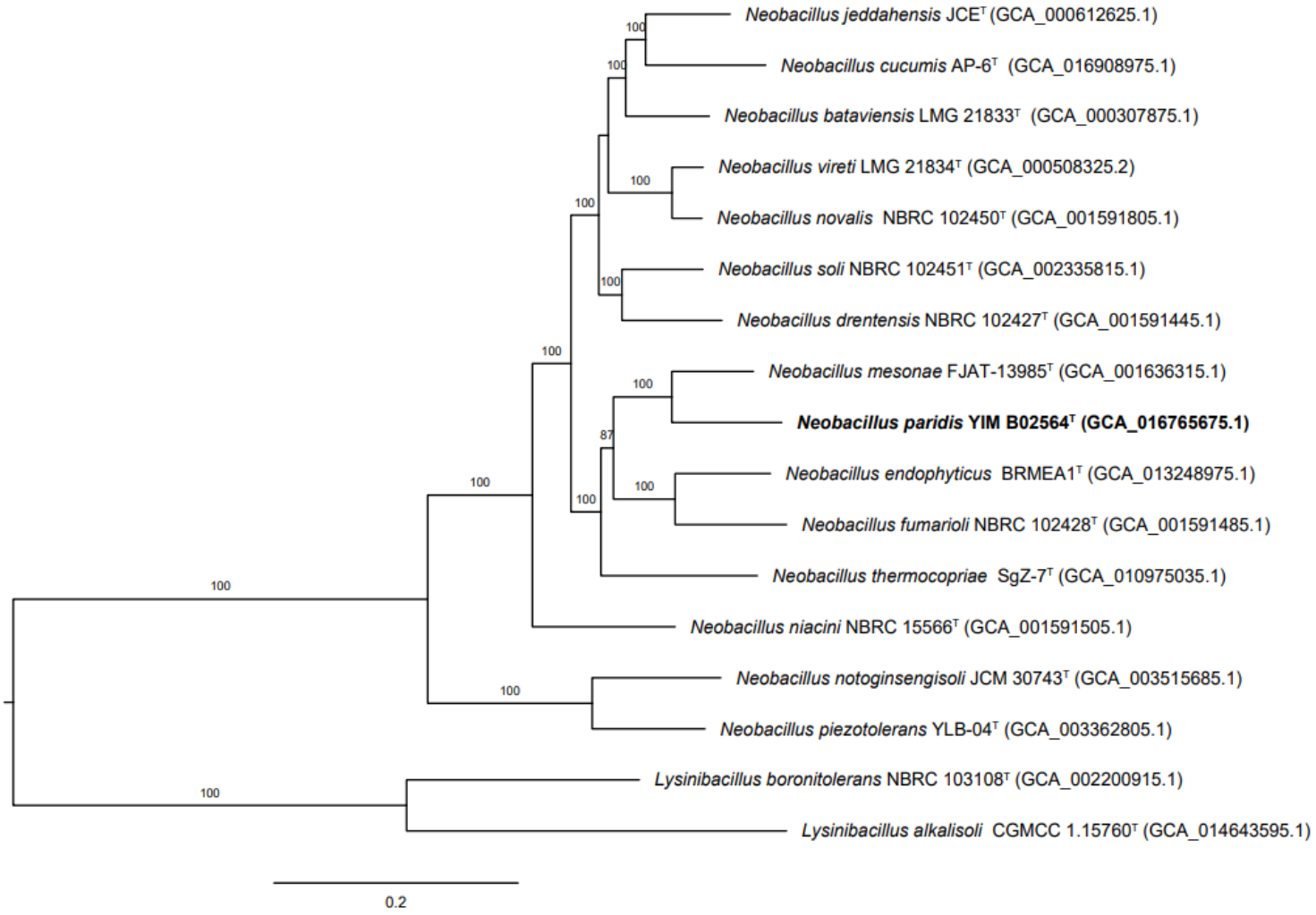

Figure 2

Phylogenomic tree showing the relationships between strain YIM B02564T and closely related strains. Bootstrap analysis was carried out using 100 replications. Percentage bootstrap values (>70%) are given at branching points. The scale bars denote the number of substitutions per site.

Supplementary Files

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

- supplementarymaterialv3clean.pdf