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Legionella qingyii sp. nov., isolated from water samples in China

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Abstract

Three *Legionella*-like strains, designed km488^T, km489 and km521, were isolated from freshwater samples in China. Cells were Gram-stain-negative, rod-shaped and non-spore-forming. Growth was observed on BCYEα agar, but not on BCYEα agar without L-cysteine, chocolate agar with PolyViteX or Columbia blood agar. The major fatty acids (>5 %) of strains km488^T, km489 and km521 were $C_{16:0}$, anteiso-$C_{15:0}$, iso-$C_{16:0}$ and anteiso-$C_{17:0}$. The *mip* gene sequences (574 nt) showed the isolates were almost identical with more than 99.7 % sequence similarities, and closely matched to *L. gormanii* ATCC 33297^T with 95.4–95.6 % sequence similarities. Phylogenetic analyses based on concatenated gene (16S rRNA, *mip*, *rpoB* and *rnpB*) sequences indicated that the isolates formed a distinct cluster along with *L. gormanii* within the genus *Legionella*. Matrix-assisted laser desorption ionization time-of-flight analyses also demonstrated a clear separation between the isolates and other closely and distantly related *Legionella* species. DNA–DNA hybridization studies demonstrated that the isolates were closely related (92.0–95.0 % DNA-DNA relatedness) but differentiated from their phylogenetic neighbours (<70 % DNA–DNA relatedness). The whole genome of km488^T was sequenced, and showed a G+C content of 37.8 mol%. Based on the findings from this polyphasic taxonomic study, the isolates are considered to represent a single novel species, for which the name *Legionella qingyii* sp. nov. is proposed. The type strain is km488^T (KCTC 15636^T=CCTCC AB 2018025^T=NRBC 113223^T).

Members of the genus *Legionella* are generally considered as fastidious facultative intracellular agents that can cause a type of pneumonia termed Legionnaires' disease [1, 2]. They are found widely in natural and man-made water, moist soil and single-celled protozoa as natural hosts and reservoirs [3–5]. With the development of co-culture techniques, species of the genus *Legionella* are increasingly being reported both from environmental and from clinical samples. At the time of writing, more than 61 species and three subspecies have been characterized in the genus *Legionella* [6], of which around 30 species have been reported as human pathogens [7–9]. To determine the diversity of the genus *Legionella* in China, water samples were collected from different ecological niches from 2002 to 2015. According to our earlier protocol [10], the samples were pre-enriched at 35 °C for 72 h, then concentrated by centrifugation and treated with acid buffer solution (pH 2.2) for 10 min at room temperature, and finally cultured on a 90 mm Petri dish containing buffered charcoal yeast extract medium supplemented with α-ketoglutaric acid (BCYEα) agar supplemented with dyes, glycine, vancomycin and polymyxin (DGVP) at 35 °C under a 5 % CO_2 atmosphere for 7 days. From among the strains isolated, three bacteria, designated strains km488^T, km489 and km521, with nearly identical 16S rRNA gene and *mip* gene sequences, were characterized using a polyphasic taxonomic approach.

Strains km488^T and km489 were isolated from freshwater samples collected from Majiagou River of Harbin city (126° 39′ E 45° 33′ N), Heilongjiang Province, China, in August 2013. Strain km521 was isolated from a lake water sample collected from South Lake Park of Shenyang city (122° 53′ E 41° 45′ N), Liaoning Province, China, in August 2013. Those isolates were subcultured at 35 °C under a 5 % CO_2 atmosphere and stored at −80 °C in a glycerol suspension (30 % in distilled water, w/v).

For phylogenetic characterization, genomic DNA from strains km488^T, km489 and km521 was extracted using a TIANamp Bacteria DNA kit (Tiangen Biotech) according to the manufacturer's instructions. PCR was performed to

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Abbreviations: ANI, average nucleotide identity; BCYEα, buffered charcoal yeast extract medium supplemented with α-ketoglutaric acid; DDH, DNA–DNA hybridization; G+C, guanine-plus-cytosine; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.
The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequences studied are given in Table S2. The GenBank/DDBJ/EMBL accession number for the whole genome shotgun project of *Legionella qingyii* km488^T is QHJG00000000 (QHJG01000001-QHJG01000099).
Five supplementary figures and three supplementary tables are available with the online version of this article.

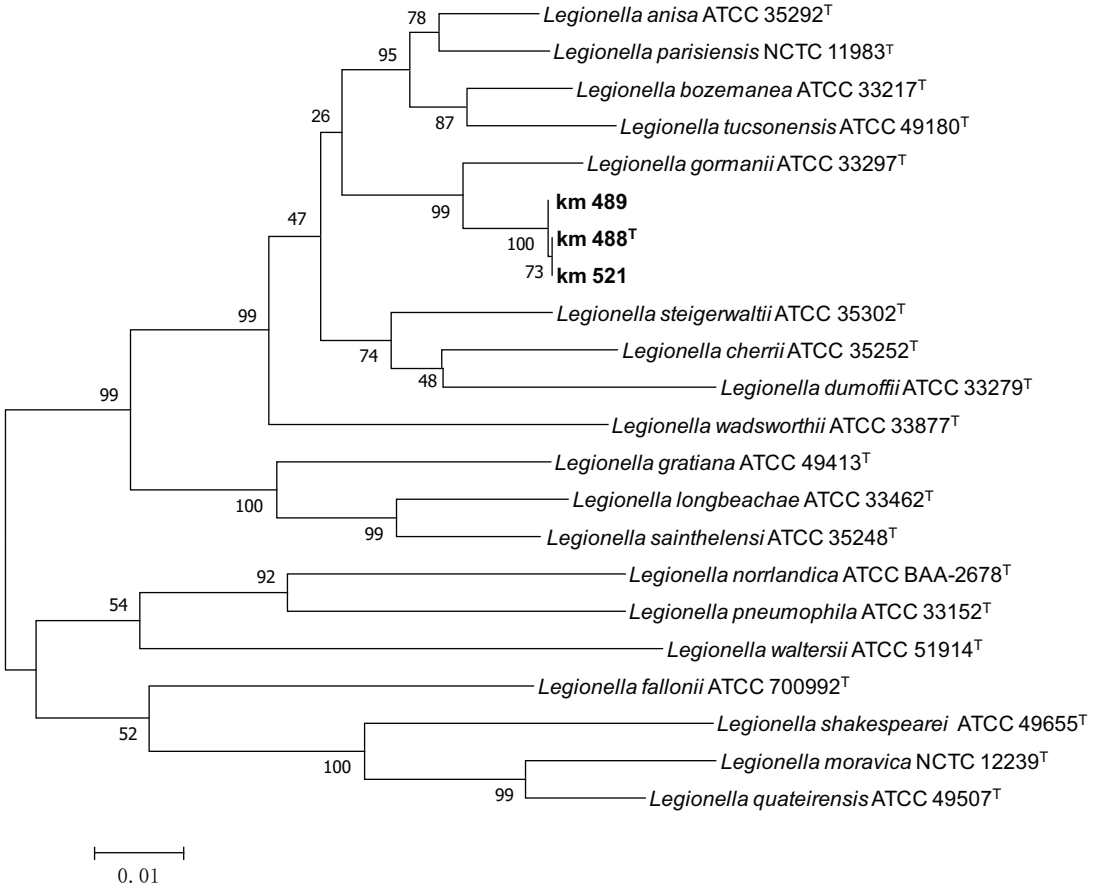

Fig. 1. Maximum-likelihood dendrogram based on the consensus sequence of the 16S rRNA, *mip*, *rpoB* and *rnpB* genes of sequenced type strains of the genus *Legionella*. Bootstrap values based on 1000 calculations are shown. Bar, 0.01 substitutions per site. Accession numbers for all sequences included in the phylogenetic analysis are listed in Table S2.

amplify the 16S rRNA gene [11], macrophage infectivity potentiator (*mip*) gene [12], RNA polymerase beta subunit (*rpoB*) gene [13] and RNase P RNA (*rnpB*) gene [14]. After purification, the 16S rRNA gene amplicon was cloned via a pMD18T Vector Cloning Kit (TaKara) according to the manufacturer's instructions. Sequencing of the screened 16S rRNA gene clones and other positive PCR products was performed with an Applied Biosystems automatic sequencer (ABI 3730XL). Comparative sequence analysis was performed using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic dendrograms of the four genes were generated with the maximum-likelihood [15] and neighbour-joining methods [16] by using the software package MEGA version 7.0 [17].

The sequencing results allowed the determination of partial sequences for the 16S rRNA (1517 nt), *mip* (574 nt), *rpoB* (153 nt) and *rnpB* (286 nt) genes (Table S1, available in the online version of this article). In *mip* gene sequence analyses, the isolates were nearly identical, sharing more than 99.7 % sequence similarity, and were most closely related to *Legionella gormanii* ATCC 33297^T at 95.4–95.6 % sequence similarity. According to the previous description of 3.6–30.5 % interspecies variation of *mip* gene sequences [12], the three new isolates thus represented a possible novel species in the genus *Legionella*. Phylogenetic analyses based on concatenated gene (16S rRNA, *mip*, *rpoB* and *rnpB*) sequences also showed that the isolates formed a distinct cluster along with *L. gormanii* within the genus *Legionella* (Fig. 1). The topologies of the individual gene trees also supported the consensus assignment of these three strains and *L. gormanii* as different taxa. (Figs S1–S4)

Growth characteristics were observed on BCYEα agar [18], BCYEα agar without L-cysteine, chocolate agar with PolyViteX and Columbia blood agar. Growth at different temperatures (25, 28, 30, 32, 35, 37 and 42 °C) was tested on BCYEα agar. Pure cultures grown on BCYEα agar medium at 35 °C for 3 days were used for the observation of cellular morphology and other biochemical tests. Morphological features were investigated by using transmission electron microscopy (TEM-1230; JEOL). Spore formation was observed by phase-contrast microscopy and cell motility was tested under a dark field microscope. Gram staining was carried

Table 1. Differential characteristics between the novel strains and related members of the genus *Legionella*

Strains: 1, km488^T; 2, km489; 3, km521; 4, *L. gormanii*; 5, *L. anisa*; 6, *L. dumoffii*; 7, *L. parisiensis*; 8, *L. bozemanae*; 9, *L. tucsonensis*; +, positive; W, weakly positive; −, negative; NA, no data; BW, blue-white. All strains were positive for catalase, β-lactamase, gelatinase, alkaline phosphatase, leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase. All strains were negative for oxidase, glucose fermentation, hippurate hydrolysis, urease, nitrate, lipase, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-gucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase.

Characteristic	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Origin	**Water, river**	**Water, river**	**Water, lake**	**Soil, creek bank**	**Potable and environmental water**	**Pleural fluid**	**Water, cooling tower**	**Blood, human**	**Pleural fluid, human**
Autofluorescence	+(BW)	+(BW)	+(BW)	+(BW)	+(BW)/−	+(BW)	+(BW)	+(BW)	+(BW)
Esterase (C4)	+	+	+	+	+	−	+	+	+
Esterase lipase	W	W	W	W	W	+	W	W	W
Valine arylamidase	+	+	+	+	+	+	−	−	+
Cystine arylamidase	W	W	W	W	W	W	W	+	+
Trypsin arylamidase	−	−	−	−	−	−	−	+	−
Major fatty acids (>5 %)	$C_{16:0}$, anteiso-$C_{15:0}$, iso-$C_{16:0}$, anteiso-$C_{17:0}$	$C_{15:0}$, $C_{16:0}$, anteiso-$C_{15:0}$, iso-$C_{16:0}$, anteiso-$C_{17:0}$, $C_{17:0}$ cyclo	$C_{15:0}$, $C_{16:0}$, anteiso-$C_{15:0}$, iso-$C_{16:0}$, anteiso-$C_{17:0}$	$C_{16:0}$, anteiso-$C_{15:0}$, iso-$C_{16:0}$, anteiso-$C_{17:0}$, $C_{17:0}$ cyclo	$C_{16:0}$, iso-$C_{14:0}$, anteiso-$C_{15:0}$, iso-$C_{16:0}$, anteiso-$C_{17:0}$, $C_{17:0}$ cyclo	$C_{16:0}$, anteiso-$C_{15:0}$, iso-$C_{16:0}$, anteiso-$C_{17:0}$, $C_{17:0}$ cyclo	$C_{16:0}$, anteiso-$C_{15:0}$, iso-$C_{16:0}$, $C_{17:0}$ cyclo	$C_{16:0}$, anteiso-$C_{15:0}$, iso-$C_{16:0}$, anteiso-$C_{17:0}$, $C_{17:0}$ cyclo	anteiso-$C_{15:0}$, iso-$C_{16:0}$, anteiso-$C_{17:0}$
G+C content (mol%)*	37.8	NA	NA	38.0	38.2	39.5	38.0	37.9	37.4

*Data from genome information.

out by using a Baso Gram Staining Kit for microscopy. Autofluorescence was measured by excitation at a wavelength region near 366 nm by using a Woods lamp [19]. Formation of a brown, water-soluble pigment was observed on cultures on tyrosine-containing yeast extract agar [19]. Biochemical tests for gelatinase, urease, nitrate reduction, catalase, hippurate hydrolysis and glucose fermentation were performed as described previously [19]. Oxidase was tested by using a test paper containing tetramethyl-phenylenediamine dihydrochloride (bioMérieux), and β-lactamase activity was tested via cefinase discs (bioMérieux). Other enzyme activities were determined using API ZYM kits (bioMérieux) as described previously [20]. Tests using related type strains of the genus *Legionella* based on phylogenetic analysis of the concatenated genes (Fig. 1), namely *L. gormanii* ATCC 33297^T, *L. bozemanae* ATCC 33217^T, *L. anisa* ATCC 35292^T, *L. tucsonensis* ATCC 49180^T, *L. parisiensis* ATCC 35299^T and *L. dumoffii* ATCC 33279^T, were also performed for comparison.

Cells of strains km488^T, km489 and km521 were Gram-stain-negative, non-spore-forming and motile via flagella. Transmission electron microscopy revealed that cells of strain km488^T were 0.5–0.7×1.6–3.2 µm in size (Fig. S5). Growth was observed at 25–42 °C (optimum 35 °C) on BCYEα agar, but not on BCYEα agar without L-cysteine, chocolate agar with PolyViteX or Columbia blood agar. Colonies were circular, with an approximate diameter of 1 mm on BCYEα agar after 7 days of incubation at 35 °C. Blue-white autofluorescence was observed under the Woods lamp. A brown diffusible pigment was observed on TS-BYE agar after incubation at 35 °C for more than 3 days. Strains km488^T, km489 and km521 were positive for catalase, gelatinase and β-lactamase activities but negative for oxidase, urease, nitrate, hippurate and glucose fermentation. In API ZYM tests, they were positive for alkaline phosphatase, esterase, leucine arylamidase, valine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase activities, and weakly positive for esterase lipase, lipase and cysteine arylamidase. Differential characteristics between the novel strains and related type strains are listed in Table 1.

For determination of chemotaxonomic characteristics, cells were harvested from cultures grown on BCYEα agar for 72 h at 35 °C under a 5 % CO_2 atmosphere. Cellular fatty acid methyl ester profiles were determined by GC (7890B; Agilent) according to the standard protocol of the Microbial Identification System (MIDI) with TSBA 6 database (version 6.2). The major fatty acids (>5 %) of strains km488^T, km489 and km521 were $C_{16:0}$, anteiso-$C_{15:0}$, iso-$C_{16:0}$ and anteiso-$C_{17:0}$ (Table 2). This fatty acid methyl ester profile is very similar to that of the genus *Legionella*, which was

Table 2. Cellular fatty acid compositions of strains km488^T, km489, km521 and closely related strains of the genus *Legionella*

Strains: 1, km488^T; 2, km489; 3, km521; 4, *L. gormanii* ATCC 33297^T; 5, *L. dumoffii* ATCC 33279^T; 6, *L. anisa* ATCC 35292^T; 7, *L. parisiensis* ATCC 35299^T; 8, *L. bozemanae* ATCC 33217^T; 9. *L. tucsonensis* ATCC 49180^T. All data are from this study. Values are percentages of the total fatty acids. –, Not detected.

Fatty acid	1	2	3	4	5	6	7	8	9
Saturated straight-chain									
$C_{14:0}$	2.0	0.4	0.8	–	–	–	1.7	–	–
$C_{15:0}$	3.3	**6.6**	**7.8**	2.3	1.5	2.1	1.7	2.2	–
$C_{16:0}$	**8.3**	**8.0**	**16.4**	**9.6**	**8.4**	**7.8**	**15.9**	**10.4**	1.8
$C_{17:0}$	4.8	5.0	4.1	3.6	2.8	2.3	2.3	3.6	1.6
$C_{18:0}$	1.9	1.2	1.3	1.5	0.9	1.4	2.3	1.7	1.3
$C_{19:0}$	–	1.6	1.0	–	–	–	–	–	–
$C_{20:0}$	–	0.8	–	0.7	–	0.7	1.0	0.75	–
Saturated branched-chain									
iso-$C_{14:0}$	2.9	2.7	1.6	4.3	2.5	**5.5**	3.3	3.1	3.6
iso-$C_{15:0}$	–	0.7	0.6	–	–	–	–	–	–
anteiso-$C_{15:0}$	**25.9**	**26.3**	**23.2**	**22.1**	**29.6**	**24.4**	**22.6**	**28.7**	**35.6**
iso-$C_{16:0}$	**14.5**	**12.4**	**5.6**	**19.4**	**13.9**	**23.7**	**14.4**	**14.5**	**16.6**
iso-$C_{17:0}$	–	0.9	0.8	0.7	–	1.4	–	1.5	0.6
anteiso-$C_{17:0}$	**13.8**	**11.7**	**6.9**	**9.9**	**13.9**	**7.0**	–	**10.3**	**11.6**
Unsaturated/hydroxy									
$C_{15:1}\omega 6c$	1.8	3.1	3.5	2.1	1.0	1.9	–	1.1	1.7
$C_{16:1}\omega 5c$	–	0.9	1.5	–				–	0.8
$C_{17:0}$ cyclo	4.8	**6.6**	4.7	**9.6**	**10.4**	**8.8**	**7.5**	**11.8**	4.2
$C_{18:1}\omega 7c$	–	–	0.5	–	–	–	–	–	–

characterized as branched-chain fatty acids and a few hydroxyl fatty acids [21].

To differentiate the proposed novel species from related *Legionella* strains, matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) fingerprints of these three strains were analysed along with selected *Legionella* strains. The fresh biomass of all strains was obtained after incubation on BCYEα agar at 35 °C under a 5 % CO_2 atmosphere for 3 days. Preparation of the sample and protein extraction were performed as described previously [22]. Calibration and qualification were performed using *Escherichia coli* DH5α, and the measurements were carried out using an Autof ms 1000 (Autobio) according to the manufacturer's instructions. The dendrogram based on whole-cell MALDI-TOF mass spectra (Fig. 2) also demonstrated a clear separation between the new isolates and closely and distantly related *Legionella* species.

DNA–DNA hybridization (DDH) studies with closely related strains were performed by the fluorometric DDH method in microdilution wells as previously described [23, 24]. Hybridization analysis showed that the mean value between strains km488^T, km489 and km521 was 93.5 ±1.5 %, which indicated that the strains were members of the same species. Relatedness values between the novel species and *L. gormanii* ATCC 33297^T, *L. dumoffii* ATCC 33279^T, *L. cherrii* ATCC 35252^T, *L. anisa* ATCC 35292^T and *L. parisiensis* ATCC 35299^T were only 26.35 % (±0.30), 23.42 % (±0.23), 23.23 % (±1.85), 18.16 % (±1.60) and 14.92 % (±0.29), respectively. All those values were well below the 70 % cut-off for species delineation and supported the suggestion that the isolates represented a novel species [25].

Whole genome sequencing on the randomly selected isolate km488^T was performed using Nextseq Technology (Illumina) with the mate pair strategy. Minimum coverage was 600 and quality was 30. Automated cluster generation and sequencing runs were performed using a 2×300 bp library. Illumina reads were trimmed using Trimmomatic [26], then assembled using Velvet software [27]. Contigs obtained were combined using SSPACE [28] and clustering by Cd-hit software [29]. The draft genome was annotated using the Prokaryotic Genomes Annotation Pipeline in the NCBI. The G+C content of the genomic DNA and average nucleotide identity (ANI) were calculated by the online Ribocon GmbH - Version: 3.0.20 software (http://jspecies.ribohost.com/jspeciesws/#analyse). This Whole Genome Shotgun project of strain km488^T has been deposited at DDBJ/ENA/GenBank under accession QHJG00000000 (QHJG01000001 -QHJG01000098). The version described in this paper is version QHJG01000000. Sequences of related taxa were obtained from the GenBank database.

The genome of strain km488^T was 5 241 559 bp long, with G+C content of 37.8 mol% computed from the draft genome sequence, which was consistent with the G+C content of the

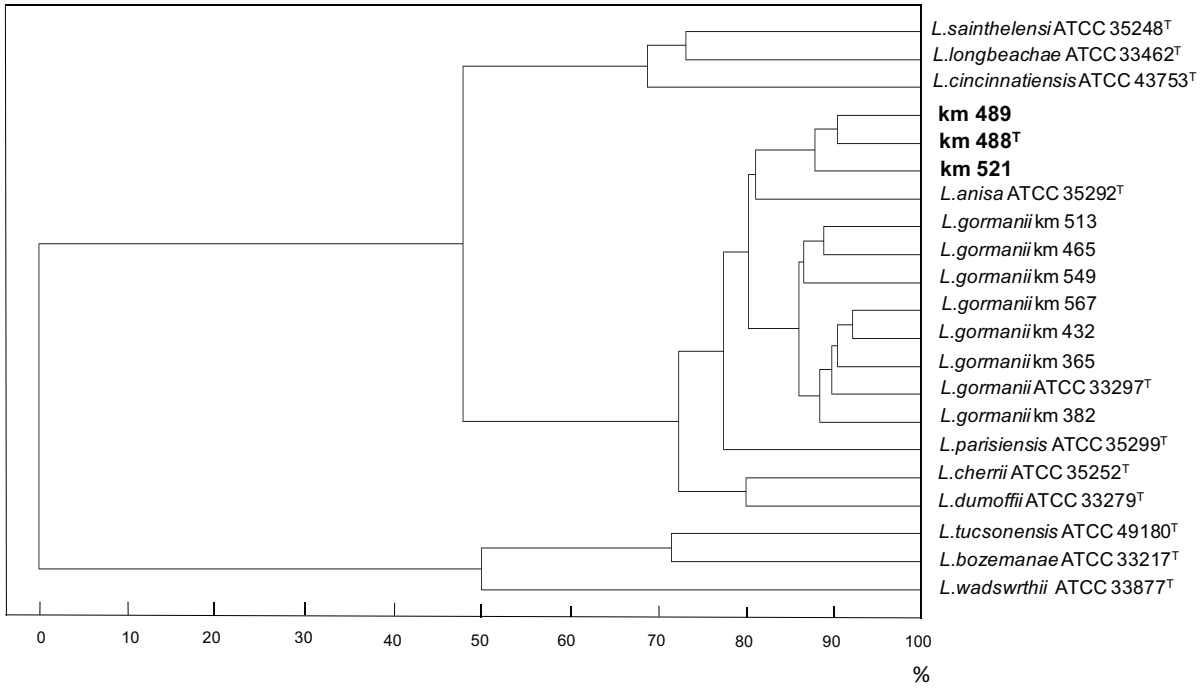

Fig. 2. Dendrogram based on whole-cell MALDI-TOF mass spectra of strains km488^T, km489 and km521 and related strains of other species of the genus *Legionella*.

genus *Legionella* [19, 30]. The genome consisted of 99 scaffolds (composed of 216 contings). Of the 4129 predicted genes, 3955 were protein-coding genes, and 44 were RNAs (one 5S rRNA, one 16S rRNA, one 23S rRNA and 41 tRNA genes). ANI values between strain km488^T and *L. gormanii* ATCC 33297^T, *L. bozemanae* ATCC 33217^T, *L. anisa* ATCC 35292^T, *L. tucsonensis* ATCC 49180^T, *L. parisiensis* ATCC 35299^T and *L. dumoffii* ATCC 33279^T were 91.9, 85.6, 85.3, 85.2, 85.0 and 84.0 %, respectively (Table S3), which supported the suggestion that the three isolates represented a novel species, based on ANI values of 95 % corresponding to the traditional 70 % DNA–DNA reassociation as the current species definition [31].

Based on the above described phylogenetic, phenotypic, chemotaxonomic and genomic sequencing results, it was concluded that three strains represent a novel species of the genus *Legionella*, for which the name *Legionella qingyii* sp. nov. is proposed.

DESCRIPTION OF *LEGIONELLA QINGYII* SP. NOV.

Legionella qingyii (qing.yi'i. N.L. masc. gen. n. *qingyii* pertaining to the Chinese microbiologist Qingyi Zhu, who performed pioneering work on legionellosis in China).

Cells are Gram-stain-negative rods that require L-cysteine for growth. Motile with the presence of flagella. Grows at temperatures between 25 and 42 °C (optimal at 35 °C). Positive for catalase, gelatin liquefaction and β-lactamase. Negative for oxidase, urease, nitrate, hippurate and glucose fermentation. In API ZYM tests, positive for alkaline phosphatase, esterase, leucine arylamidase, valine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase activities, weakly positive for esterase lipase, lipase and cysteine arylamidase, but negative for trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, *N*-acetyl-β-glucosaminidase, β-glucosidase, α-mannosidase and α-fucosidase. Major cellular fatty acids are branch-chain.

The type strain, km488^T (=KCTC 15636^T=NRBC 113223^T =CCTCC AB 2018025^T), was isolated from lake water collected from China. The G+C content of the type strain is 37.8 mol% (genome).

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

References

1. Swart AL, Harrison CF, Eichinger L, Steinert M, Hilbi H. *Acanthamoeba* and *Dictyostelium* as Cellular Models for *Legionella* Infection. *Front Cell Infect Microbiol* 2018;8:61.
2. Burillo A, Pedro-Botet ML, Bouza E. Microbiology and epidemiology of legionnaire's disease. *Infect Dis Clin North Am* 2017;31:7–27.

3. **Fields BS, Benson RF, Besser RE.** *Legionella* and Legionnaires disease: 25 years of investigation. *Clin Microbiol Rev* 2002;15:506–526.
4. **Newton HJ, Ang DK, van Driel IR, Hartland EL.** Molecular pathogenesis of infections caused by *Legionella pneumophila*. *Clin Microbiol Rev* 2010;23:274–298.
5. **Gomez-Valero L, Buchrieser C.** Genome dynamics in *Legionella*: the basis of versatility and adaptation to intracellular replication. *Cold Spring Harb Perspect Med* 2013;3:297–316.
6. **LPSN.** List of prokaryotic names with standing in nomenclature *Legionella*. Available at http://www.bacterio.net/legionella.html.
7. **Cunha BA, Burillo A, Bouza E.** Legionnaires' disease. *The Lancet* 2016;387:376–385.
8. **Palmer A, Painter J, Hassler H, Richards VP, Bruce T *et al.*** *Legionella clemsonensis* sp. nov.: a green fluorescing *Legionella* strain from a patient with pneumonia. *Microbiol Immunol* 2016;60:694–701.
9. **Relich RF, Schmitt BH, Raposo H, Barker L, Blosser SJ *et al.*** *Legionella indianapolisensis* sp. nov., isolated from a patient with pulmonary abscess. *Int J Infect Dis* 2018;69:26–28.
10. **Xiao-Yong Z, Chao-Hui H, Qing-Yi Z.** Comparative study on sampling methods for monitoring *Legionella* species in environmental water. *Afr J Microbiol Res* 2014;8:974–985.
11. **James G.** Universal bacterial identification by PCR and DNA sequencing of 16S rRNA gene [M]. In: Carter IWJ, Schuller M, James GS (editors). *PCR for Clinical Microbiology*. Verlag Ny: Springer; 2010.
12. **Ratcliff RM, Lanser JA, Manning PA, Heuzenroeder MW.** Sequence-based classification scheme for the genus *Legionella* targeting the *mip* gene. *J Clin Microbiol* 1998;36:1560–1567.
13. **Ko KS, Lee HK, Park MY, Lee KH, Yun YJ *et al.*** Application of RNA polymerase beta-subunit gene (*rpoB*) sequences for the molecular differentiation of *Legionella* species. *J Clin Microbiol* 2002;40:2653–2658.
14. **Rubin CJ, Thollesson M, Kirsebom LA, Herrmann B.** Phylogenetic relationships and species differentiation of 39 *Legionella* species by sequence determination of the RNase P RNA gene *rnpB*. *Int J Syst Evol Microbiol* 2005;55:2039–2049.
15. **Yang Z.** Phylogenetic analysis using parsimony and likelihood methods. *J Mol Evol* 1996;42:294–307.
16. **Saitou N, Nei M.** The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 1987;4:406.
17. **Kumar S, Stecher G, Tamura K.** MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol Biol Evol* 2016;33:1870–1880.
18. **Edelstein PH, Edelstein MA.** Comparison of the plating efficiencies and shelf lives of three different commercial buffered charcoal yeast extract media supplemented with alpha-ketoglutaric acid. *J Clin Microbiol* 2010;48:1882–1883.
19. **Brenner DJ, Steigerwalt AG, Gorman GW, Wilkinson HW, Bibb WF *et al.*** Ten new species of *Legionella*. *Int J Syst Bacteriol* 1985;35:50–59.
20. **Humble MW, King A, Phillips I.** API ZYM: a simple rapid system for the detection of bacterial enzymes. *J Clin Pathol* 1977;30:275–277.
21. **Lambert MA, Moss CW.** Cellular fatty acid compositions and isoprenoid quinone contents of 23 *Legionella* species. *J Clin Microbiol* 1989;27:465–473.
22. **He Y, Chang TC, Li H, Shi G, Tang YW.** Matrix-assisted laser desorption ionization time-of-flight mass spectrometry and database for identification of *Legionella* species. *Can J Microbiol* 2011;57:533–538.
23. **Ezaki T, Hashimoto Y, Yabuuchi E.** Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* 1989;39:224–229.
24. **Ezaki T, Hashimoto Y, Yamamoto H, Lucida ML, Liu SL *et al.*** Evaluation of the microplate hybridization method for rapid identification of *Legionella* species. *Eur J Clin Microbiol Infect Dis* 1990;9:213–217.
25. **Stackebrandt E, Goebel BM.** Taxonomic Note: A place for dna-dna reassociation and 16s rrna sequence analysis in the present species definition in bacteriology. *Int J Syst Evol Microbiol* 1994;44:846–849.
26. **Bolger AM, Lohse M, Usadel B.** Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 2014;30:2114–2120.
27. **Zerbino DR, Birney E.** Velvet: algorithms for de novo short read assembly using de Bruijn graphs. *Genome Res* 2008;18:821–829.
28. **Boetzer M, Henkel CV, Jansen HJ, Butler D, Pirovano W.** Scaffolding pre-assembled contigs using SSPACE. *Bioinformatics* 2011;27:578–579.
29. **Li W, Godzik A.** Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. *Bioinformatics* 2006;22:1658–1659.
30. **Dennis PJ, Brenner DJ, Thacker WL, Wait R, Vesey G *et al.*** Five new *Legionella* species isolated from water. *Int J Syst Bacteriol* 1993;43:329–337.
31. **Chun J, Oren A, Ventosa A, Christensen H, Arahal DR *et al.*** Proposed minimal standards for the use of genome data for the taxonomy of prokaryotes. *Int J Syst Evol Microbiol* 2018;68:461–466.