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Legionella sheltonii sp. nov., a novel species isolated on a cruise ship during routine monitoring

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Abstract

Legionella-like isolates, HB10 and PATHC038^T, were isolated from a cold water distribution system on a cruise ship in 2017. The strains have been characterized by employing discriminatory genome typing, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry technique, fatty acid profiling, and biochemical tests thus identifying a new species in the genus *Legionella*. Colonies displayed phenotypic characteristics typical of the family *Legionellaceae*, including a requirement for L-cysteine and testing catalase positive. Fatty acid methyl ester analysis showed that the predominant fatty acids detected in the studied strains are C16:0 iso, C16:1 ω7c, C16:0 and C15:0 anteiso. The obtained MIC values showed antimicrobial susceptibility to the antimicrobial drugs ciprofloxacin and erythromycin (0.8 µg/ml and 2 µg/ml, respectively). The bacteria were also Gram-negative, rod-shaped, grew aerobically on BCYE agar and weakly grew on GVPC media at 36 °C with the ability to autofluoresce a blue-white color when placed under a long-wavelength UV light (365 nm). The whole genome sequencing performed displayed a G+C content of 38.2 mol %. The digital DNA-DNA hybridization analysis demonstrated a separation from the phylogenetically most related *Legionella cherrii*, with 54.7 % DNA-DNA relatedness. The identity percentage measured by average nucleotide identity between the PATHC038^T strain and its respective closest species, *L. cherrii,* was 93.9 % also confirming the distinctiveness of the novel species. The 16S rRNA gene, *mip* and *rpoB* sequences showed a similarity of 98.7 %, 98.4 % and

96.2 %, respectively, with *L. cherrii* NCTC 11976. Additionally, Average Amino Acid Identity (AAI) and Percentage of Conserved Proteins (POCP) analyses further supported their classification as a novel species. The results obtained in this study confirm the status of an independent species. The name proposed for this species is *Legionella sheltonii* sp. nov. with PATHC038^T (CCUG 76918T, ATCC TSD-370) as the type strain.

Keywords: *Legionella sheltonii* sp. nov.; MALDI-TOF MS; phylogenomics; AAI; POCP; virulence factors

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Abbreviations: BCYE medium (Buffered Charcoal Yeast Extract); Cys+ (with L-cysteine); Cys- (without L-cysteine); BMPA medium (Buffered Cefamandole-Polymixin-Anisomycin-Alphaketoglutarate); GVPC medium (BCYE with Glycine-Vancomycin-Polymyxin-Cycloheximide); CDC (Centers for Disease Control and Prevention); MALDI-TOF MS (Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry); WGS (Whole Genome Sequencing); ANI (Average Nucleotide Identity); ANIb (Average Nucleotide Identity Based on BLAST); GGDC (Genome-to-Genome Distance Calculator); GBDP (Genome BLAST Distance Phylogeny); VFDB (Virulence Factor Database); MIC (Minimum

Inhibitory Concentration); FAME (Fatty Acid Methyl Ester); AAI (Average Amino Acid Identity); POCP (Percentage of Conserved Proteins)

GenBank/EMBL/DDBJ/PIR accession numbers: The GenBank accession numbers for the 16S rRNA genes of the strains PATHC038^T and HB10 are PQ120583 and PQ120582, respectively. The GenBank accession numbers for the mip genes of the strains PATHC038^T and HB10 are PQ119488 and PQ119489, respectively. The GenBank accession number for the whole genome sequences of the strain PATHC038^T is GCF_026191355.1.

Note: Supplementary material is available with the online version of this article.

Introduction

The genus *Legionella*, the sole genus in the *Legionellaceae* family, comprises 66 validly published species [1, 2]. In the last six years, six new species of *Legionella* have been discovered [3–9]. The genus was first identified in 1976 during a CDC investigation of a pneumonia outbreak at the American Legion Convention in Philadelphia, Pennsylvania, USA [1, 10, 11].

Legionella species are Gram-negative bacilli measuring 0.3 - 0.9 µm in width and 2 - 20 µm in length. These non-spore-forming, strictly aerobic bacteria utilize amino acids as an energy source, requiring L-cysteine and iron salts for growth [1, 12]. The primary hosts of *Legionella* spp. are free-living amoebae (FLA), but approximately 30 species are opportunistic pathogens in humans, capable of causing legionellosis, a spectrum of respiratory illnesses including Pontiac fever and the more severe Legionnaires' disease (LD), which imposes significant costs on healthcare systems [1, 13]. Although exposure to *Legionella* in building water sources may not always cause disease, it can nevertheless lead to increased LD risk if *Legionella* growth is not effectively controlled in exposure sources that produce aerosols. Proactive prevention efforts, including species identification and source control, are key to minimizing risks associated with Legionnaires' disease [10].

The pathogenicity of *Legionella* spp. in humans corresponds with its co-evolution with FLA which offer a protective niche for intracellular replication, shielding the bacteria from environmental stressors, including temperature fluctuations, antimicrobial agents, and osmotic stress. Legionellosis primarily occurs when *Legionella*-contaminated aerosols are inhaled, enabling bacterial replication in alveolar macrophages, which mimic the amoebic hosts in aquatic environments [14]. A key virulence factor of *Legionella* is the Dot/Icm type IVB secretion system, a conserved apparatus consisting of 27 proteins that translocate bacterial effectors into host cells, promoting intracellular replication. Among intracellular pathogens, the species of *Legionella* have some of the most extensive and diverse effector repertoires, underscoring the complexity of their pathogen-host interactions [13, 14].

While *Legionella* spp. are ubiquitous in natural aquatic habitats, engineered water systems can also provide optimal conditions for their proliferation by hosting biofilms of other bacterial species and maintaining stable temperature and flow conditions [13, 15, 16]. A notable example are internal seawater systems (ISS) on maritime vessels, which play a critical role in ship operations, providing cooling and potable water, and essential services like firefighting and sanitation. However, these systems can serve as reservoirs for microorganisms, including *Legionella* spp., which can lead to biofouling, compromised system functionality, and contamination with potentially harmful pathogens [17]. Effective monitoring not only ensures compliance with safety standards but also enhances understanding of *Legionella* ecology, prevalence, and adaptive strategies in diverse environments[18].

In our previous work, genomic studies on 39 *Legionella* isolates revealed four strains potentially representing new species [19]. Two of these strains, including PATHC038^T, were related to *L. cherrii*, while the remaining two were closest to *L. pneumophila*. Moreover, MALDI-TOF MS analysis identified PATHC038^T with low confidence, prompting further investigation into its taxonomic status [20, 21]. This study builds on previous findings by conducting a comprehensive characterization of two strains recovered from a cruise ship's ISS during routine monitoring, PATHC038^T and HB10. MALDI-TOF, fatty acid composition and additional phylogenetic analyses were conducted for both *Legionella* strains (HB10 and PATHC038^T) as well as for closely related species. Based on the obtained results, we propose the classification of these strains as a novel species, *Legionella sheltonii* sp. nov., with PATHC038^T (=CCUG 76918T =ATCC TSD-370) as the type strain.

Materials and methods

Bacterial isolation

The *Legionella* sp. strains HB10 and PATHC038^T were obtained from the same sampling location within a three-week interval during a routine *Legionella* surveillance program, which involved the testing and retesting of positive sampling points. These strains were isolated from

the cold-water distribution system, specifically from the water tank used for washing the deck of a cruise ship with an international itinerary. Laboratory culture of both strains followed the methods outlined in HRN ISO 11731:2000 (ISO 11731:1998) [22] and HRN EN ISO 11731-2:2008 (EN ISO 11731-2:2008; ISO 11731-2:2004 [23]), as well as the procedures described in the "Procedures for the Recovery of *Legionella* from the Environment" (January 2005, USDHHS, Public Health Service, CDC, Atlanta, GA) [24]. The samples were cultivated on buffered charcoal yeast extract (BCYE) media and glycine-vancomycin-polymyxin-cycloheximide (GVPC) agar and incubated at 36 °C with 3 % CO_2 for 10 days. Presumptive *Legionella* colonies detected solely on BCYE media were further confirmed using BCYE agar Cys+ and BCYE Cys- agar in accordance with the ISO 11731 method (HAA 1550).

MALDI-TOF MS analysis of strains

Strains HB10 and PATHC038^T were analyzed using MALDI-TOF MS with full extraction, following the procedure previously described [20]. A bacterial colony was suspended in 300 µl of deionized water using an inoculating loop. After vortexing, 900 µl of absolute ethanol (Kemika, Croatia) was added to the suspension. The mixture was centrifuged at 13 000 rpm for 2 minutes. After decanting of the supernatant, the pellet was resuspended in an equal volume of 70 % formic acid (Sigma Aldrich, Germany) and 100 % acetonitrile (Fisher Chemical, Spain), and centrifuged again at 13 000 rpm for 2 minutes. The supernatant was spotted onto a 96-spot polished steel target plate (Bruker Daltonik, Germany), dried at room temperature, and overlaid with 1 µl of 10 mg/ml alpha-4-cyano-4-hydroxycinnamic acid (CHCA, Bruker Daltonik, Germany) in 50 % acetonitrile and 2.5 % trifluoroacetic acid. Once dried, spectra were acquired in the positive linear ion mode within a mass range of 2 - 20 kDa. The mass spectra were matched to the Bruker Biotyper database (version 11) using MBT Compass HT software (version 5.0, Bruker Daltonik). Species identification was determined based on log scores, where a score of 2.00 - 3.00 indicated high-confidence identification, 1.70 - 1.99 indicated low-confidence identification, and a score of 0 - 1.69 was considered unreliable. To further assess their taxonomic relationships, a dendrogram was constructed

using MALDI Biotyper Compass Explorer 4.1 software (Bruker Daltonik) based on the mass spectra of the investigated strains and selected reference *Legionella* strains from the Bruker library. Correlation was used as the distance measure and linkage was performed using the complete method.

Genomic features of strain PATHC038^T

DNA extraction, sequencing, de novo hybrid assembly, and genome annotation of *Legionella* strain PATHC038^T were performed following the methods outlined in our previous study [19]. In this study, genomic DNA was subjected to Illumina Nextera library preparation, and sequencing was carried out using the Illumina MiSeq system [25] to generate reads of 150 bp paired-end reads. Long-read sequencing was also performed using the MinION Nanopore system [26]. The sequencing data underwent quality control and trimming, after which reads were assembled using the Unicycler hybrid assembly tool (v0.4.1) with default settings. Genome annotation was performed using Prokka (Galaxy v1.14.6). The completeness and contamination of the genome assembly were assessed using checkM (v1.0.18, kBASE), with yielding results of 99.8 % completeness and 1.4 % contamination, both within the acceptable ranges defined by Parks et al. (2015) [27] (≥ 90 % for completeness and ≤ 5 % for contamination). The presence of plasmid-derived sequences was evaluated by performing a BLASTn search against the NCBI non-redundant (nr) database, identifying plasmid p3A2 from *Legionella anisa* strain UMCG_3A (CP029565.1) with 91.6 % identity and 40 % query coverage.

Several genome-based typing analyses were performed to assess the relationship between strain PATHC038^T and 32 other type strains in the genus. Average nucleotide identity (ANI) was calculated using an alignment-free approach with the fastANI tool [28], as well as through BLASTn pairwise comparisons (ANIb) using the JSpeciesWS server [29]. The genome-to-genome distance was estimated by in silico DNA–DNA hybridization (dDDH) using the Genome-to-Genome Distance Calculator (GGDC) [30]. The Average Amino Acid Identity (AAI) was calculated from proteome assemblies using the AAI matrix tool available through the

Kostas lab website [31]; a matrix of AAI values for each pair of the 33 species in our database is available in Table S1. The Percentage Of Conserved Proteins (POCP) [32] was calculated using the POCP-nf pipeline [33] with the following parameters: e-value: 1e-5; sequence identity: 0.4; alignment length: 0.5. A matrix of POCP values for each pair of the species in our database can be found in Table S1.

To examine the virulence profile of PATHC038^{T}, the functionally annotated genome was queried against the Virulence Factor Database (VFDB) [34], with a threshold identity of 60 %. The identified virulence factors were further categorized according to VFDB.

Gene sequence comparisons, phylogenetic, and phylogenomic analyses of strains

In order to make gene sequence comparisons and perform phylogenetic analyses of strains, a dataset composed of 16S rRNA [35], macrophage infectivity potentiator (*mip*), gyrase subunit A (*gyrA*), ribosomal polymerase B (*rpoB*), and RNase P (*rnpB*) gene sequences [36–41] from 33 *Legionella* species was selected. Selected gene sequences were obtained from the NCBI WGS sequences (Table S2), with the addition of 16S rRNA gene and *mip* sequences of the strain HB10 obtained in this study.

PCR was carried out to amplify the 16S rRNA and mip gene sequences according to [42] and [37], respectively. PCR products were visualized by gel electrophoresis on 1 % agarose gel stained with Xpert Green DNA stain (GRISP Lda., Porto, Portugal), purified and sent to Macrogen Inc. for Sanger sequencing.

Sequences were aligned by each locus using MAFFT v7.450 [43, 44] available as Geneious Prime 2023.0.4 plugin. Concatenation of gene alignments of 16S rRNA gene (1545 bp), *mip* (705 bp), *gyrA* (2631 bp), *rpoB* (4107 bp) and *rnpB* (405 bp) was done using Geneious Prime 2023.0.4. Phylogenetic analyses were conducted using Maximum Likelihood (ML) analysis in IQTREE v1.6.12 [45, 46] and a Bayesian Inference (BI) analysis in MrBayes 3.2.6 (Geneious plugin; [47]). The best model (GTR+F+I+G4) was selected by ModelFinder implemented in IQ-TREE considering separately the corrected Akaike, and Bayesian Information Criterion (cAIC,

BIC) [47]. The phylogenetic analyses were performed with both the single and concatenated (16S rRNA gene, *mip*, *gyrA*, *rpoB*, *rnpB*) sequence alignments. ML analysis was executed by applying the ultrafast bootstrap approximation with 1000 replicates. BI analysis was executed for 5 000 000 generations, sampling trees and other parameters every 10 000 generations. The default number of chains (four) and heating parameters were used. Posterior probabilities (BPP) were calculated after burning the first 25 % of the posterior sample. The clade containing *Legionella septentrionalis* km711^T*, Legionella oakridgensis* Oak Ridge-10^T *and Legionella nagasakiensis* JCM 15315^T was selected as the outgroup for phylogenetic analyses based on previously published phylogenies [48, 49]. Phylogenetic trees were visualized and annotated using iTOL v6.5.4 [50, 51] and FigTree 1.4.3 [52, 53].

The genome sequence data of strain PATHC038^T and reference type strains (Table S2) were uploaded to the Type (Strain) Genome Server (TYGS) for a whole genome-based taxonomic analysis [54]. For the phylogenomic inference, all pairwise comparisons among the set of genomes were conducted using Genome BLAST Distance Phylogeny (GBDP) and accurate intergenomic distances inferred under the algorithm 'trimming' and distance formula *d5* [55]. A total of 100 distance replicates were calculated. The phylogenomic tree was inferred with FastME 2.1.6.1 from genome sequences of *L. sheltonii* sp. nov., strain PATHC038^T and closely related species of the genus *Legionella*. A second GBDP phylogenomic analysis was inferred using the amino acid sequences of the entire proteome, allowing for a better-resolved phylogeny of more distantly related taxa. Digital DDH values and confidence intervals were calculated using the recommended settings of the GGDC 4.0 [30, 55]. A balanced minimum evolution phylogenomic tree with branch support from whole-proteome-based GBDP distances of *L. sheltonii* sp. nov., strain PATHC038^T and closely related species of the genus *Legionella* was inferred via FASTME 2.1.6.1 including Subtree Pruning-Re-grafting (SPR) postprocessing [56]. Branch support was inferred from 100 pseudo-bootstrap replicates each. The trees were rooted at the midpoint [57], rerooted to a known outgroup, and visualized using iTOL [51].

226 **Intracellular growth of strain PATHC038^T within *Acanthamoeba castellanii* ATCC 30234**

227 *Acanthamoeba castellanii* was obtained from the American Type Culture Collection
228 (ATCC 30234) and cultivated in peptone-yeast-glucose medium (PYG = ATCC medium 712)
229 at 25 °C. *Legionella pneumophila* ATCC 33152^T was used as a positive control.

230 The number of *A. castellanii* ATCC 30234 cells (10^6 cells per ml) was determined using a
231 Neubauer chamber. The amoeba cells were transferred to six-well plates and incubated
232 overnight at 27 °C in 3 ml of media. On the following day, the amoeba cells were infected with
233 the PATHC038^T strain and the control bacteria, *L. pneumophila* ATCC 33152^T, at a multiplicity
234 of infection (MOI) of 1000, centrifuged at 240 × g for 3 min at room temperature to synchronize
235 the infection and then incubated at 27 °C for 6 h. After incubation, the amoeba cells were
236 washed three times with ATCC glucose-free medium to remove extracellular bacteria. This was
237 considered as the initial incubation time (time zero). At multiple points after infection (3, 6, 9,
238 12, and 15 days post-infection), the amoeba cells were treated with 0.1 % Triton X-100 (Sigma,
239 St. Louis, MO, USA) for 10 min to lyse the amoeba cells. The number of replicated bacteria in
240 the amoeba cells was determined by spread plating onto BCYE agar plates and incubation at
241 36 °C for 96 h.

242 **Physiology, metabolism and antimicrobial susceptibility of strains**

243 API 20E [58, 59] and API 20NE [60] tests (bioMérieux, France) were used to determine the
244 physiological and biochemical properties of *Legionella sheltonii* sp. nov. strains; tests were
245 conducted according to the manufacturer's instructions [5, 60].

246 To assess the antibiotic susceptibility of the *L. sheltonii* sp. nov. strains, we determined the
247 minimum inhibitory concentration (MIC) of erythromycin and ciprofloxacin against the isolated
248 *Legionella* using the Etest® (Biomerieux, France) on BCYE agar according to the EUCAST
249 instructions [61]. A suspension of the bacteria grown on the BCYE media was prepared in
250 0.9 % NaCl (bioMérieux, France) with a McFarland density of 0.5 (1.5×10^8 CFU/ml). The
251 density of the suspension was measured with a densitometer (Vitek Systems ATB 1550,

bioMérieux, France). The suspension was inoculated in duplicate onto BCYE agar plates. Etest® strips (bioMérieux, France) containing ciprofloxacin (CI 0.002 - 32 µg/ml) and erythromycin (EM 0.016 - 256 µg/ml) were then applied to the plates using sterile forceps and incubated for 48 hours at 35 °C in a humidified atmosphere. The MIC values were read at the intersection of the ellipse with the Etest® strip.

To investigate its optimal cultivation conditions, the strain PATHC038^T was aerobically subcultured on BCYE medium, BCYE without L-cysteine (BCYE Cys-) medium; GVPC medium; buffererd cefamandole-polymyxin-anisomycin-alphaketoglutarate (BMPA) medium (Thermo Fisher Scientific, Diagnostic, B.V., Landsmeer, Netherlands) and modified wadowsky yee (MWY) selective agar (Thermo Fisher Scientific, Diagnostic, B.V., Landsmeer, Netherlands). Plates were incubated for ten days in a variety of conditions, including the presence or absence of 3 % CO_2 and a temperature range of 32 °C to 38 °C.

Cell fatty acid composition of strains

The fatty acid composition of strains HB10 and PATHC038^T, *L. cherrii*, *L. dumoffii*, *L. bozemanae*, *L. gormanii*, *L. anisa*, *L. pneumophila* SG1 was determined by in-situ transesterification of dry cell biomass according to the protocol previously developed for the fatty acid analysis of microalgae [62]. Cell biomass was cultivated at 36 °C on BCYE agar plates during 3 - 4 days. Grown biomass was carefully scraped from the agar surface, transferred to a dry Eppy tube and dried to constant weight at 50 °C. The dried biomass was then ground into a fine powder using a mortar and pastel and used for total fatty acid composition. Ten milligrams of dried bacterial biomass (< 5 % of moisture) were placed in a 1.5 ml GC vial and mixed with 20 µL of the tridecanoic acid methyl ester standard (concentration 10 mg/ml; Sigma Aldrich #91558 - 5ML), 200 µl of chloroform: methanol (2:1, vol/vol) and 300 µl of 0.6 M HCl: methanol. The vials were sealed with the PTFE/silicone/PTFE crimp caps, and the content was mixed. The vials were incubated in the preheated water bath at 85 °C for one hour. The vials were occasionally well-mixed during heating. After the transesterification reaction was completed, the vials cooled to room temperature. Next, 1.0 ml

of HPLC-grade hexane was added to each vial, the contents were mixed well, and the vials were left undisturbed at room temperature for 1 h to allow the phases to separate. 400 µl of upper hexane phase were transferred into a new 1.5 ml GC vial, and 10 µl of internal standard (pentadecane, 1 mg/ml) was added.

The fatty acid composition of total bacterial biomass was determined using Shimadzu GC-2010 Plus Capillary gas chromatograph (Shimadzu; Kyoto, Japan) equipped with a flame ionization detector (FID) and high-cyanopropyl capillary column (ZB-FAME 30 m × 0.25 mm × 0.2 µm; Phenomenex; Torrance, CA, USA).

Samples were injected in split mode (split ratio 1:15) with AOC-20i injector using helium as a carrier gas. The injector and detector temperatures were 250 and 260 °C, respectively. Separation was performed at the following temperature increments: 100 °C to 140 °C for 3 °C/min, 140 °C to 190 °C for 3 °C/min, then up to 260 °C for 30 °C/min and holding at that temperature for 2 min. Shimadzu LabStation Software was used for instrument control, data acquisition and data analysis (integration, retention times and peak areas). Identification of FAME was obtained by co-chromatography with FAME standards (Supelco FAME Mix, C4–C24; and methyl branched fatty acids C14:0 iso, C15:0 anteiso, C16:0 iso C17:0 anteiso obtained from Larodan (Solna, Sweden).

Cell morphology of strains by transmission electron microscopy (TEM)

TEM analysis was performed to observe the morphological and structural appearance of strains PATHC038^T and HB10. The strains were cultured on BCYE for 5 days at 37 ˚C in the presence of 3 % CO_2. The samples were prepared for TEM on carbon-coated Cooper grids (SPI Supplies, USA) and stained with 2 % phosphotungstic acid. The bacterial suspension (10 µL) was applied to the grid for 2 min and then removed from the grid using filter paper. Subsequently, 10 µL of acid was added to the grid for 1 min, and then drained using filter paper. After the grids were air-dried 10, fields of each grid were randomly observed under a TEM device Morgagni 268D Philips (FEI Company, USA).

Results and Discussion

In our earlier study, the MALDI-TOF MS analysis of the strain PATHC038^T identified it as *L. cherrii* with low confidence, suggesting that it could represent a distinct species [19–21]. This hypothesis was further tested by repeating the MALDI-TOF MS analysis on two strains collected from the same source, PATHC038^T and HB10 (Table S3). The MALDI-TOF MS dendrogram demonstrates that *Legionella sheltonii* forms a distinct and separate cluster, clearly differentiated from all other *Legionella* species examined (Figure S1). The substantial distance level separating *L. sheltonii* from other clusters indicates a significant difference in protein profiles, supporting its classification as a unique and separate species within the genus *Legionella*.

Furthermore, the initial core genome analysis also indicated that the strain PATHC038^T strain might represent a distinct evolutionary line (Svetlicic et al., 2023)[19]. In this study, we further characterize these two strains using a variety of morphological, biochemical, and phylogenetic analyses and providing comprehensive evidence that they meet criteria for the description of a novel *Legionella* species.

The genome assembly and annotation of strain PATHC038^T indicate a high-quality genome with minimal contamination. The genome assembly was found to be highly complete (99.8 %) and has a relatively low contamination level (1.4 %) (Table 1), ensuring a reliable dataset for further analyses. Comparative analysis of plasimd sequences revealed the presence of a plasmid similar to that found in *Legionella anisa* strain UMCG_3A, confirming the potential for plasmid acquisition or transfer. These genomic findings align with previous results reported by Svetlicic et al. (2023)[19].

328 **Table 1.** General characteristics of the genome assembly of *Legionella sheltonii* sp. nov., strain
329 PATHC038^T compared to those of its closest related species *L. cherrii* (strain NCTC 11976^T) and type
330 species *L. pneumophila* strain Philadelphia-1^T. Assemblies were obtained from NCBI database
331 (accession numbers GCF_026191355.1, GCF_900635815.1 and GCF_001941585.1, respectively).

Chararacteristic	***L. sheltonii* sp. nov., strain PATHC038^T**	***L. cherrii* strain NCTC 11976^T**	***L. pneumophila* strain Philadelphia-1^T**
Total length (Mb)	4.3	3.7	3.4
No of contigs	63	1	1
G+C content (mol%)	39.0	39.0	38.5
Contig N50 (kb)	235.7	3729.5	3409.1
Completeness (%)	99.8	95.3	99.4
Contamination (%)	1.4	0.0	0.4
Plasmid	1	1	1
Number of coding sequences	3757	3110	2981
No of rRNAs	6	9	9
No of tRNAs	43	43	44

332

333 Genome-based typing analyses showed that strain PATHC038^T exhibits the closest
334 relationship to *L. cherrii*. However, the ANI (average nucleotide identity) and dDDH (digital
335 DNA-DNA hybridization) values were below the generally accepted thresholds for species
336 identification [63] (ANI < 95 – 96 % and dDDH < 70 %) (Table S4, S5, S6). Although the AAI
337 (average amino acid identity) and POCP (percentage of conserved proteins) values are
338 generally used for genus-level delineation [64], they were also calculated to further
339 demonstrate the uniqueness of the new species. While the AAI (Table S1, Figure S2)
340 expectedly follows the ANI values, POCP values (Figure S3) range from approximately 55
341 upward, ilustrating a high proteome diversity within the genus *Legionella* [32]. Notably, the
342 POCP value for the *L. sheltonii* sp. nov. and *L. cherrii* pair is 86.5 %, meaning that only around
343 86 % of their proteins are shared, further supporting *L. sheltonii* sp. nov. as a distinct
344 evolutionary lineage with a high number of species-specific functions.

345 The five-gene phylogenetic tree (Figure S4) was reconstructed with most branches receiving
346 maximal support (BI-PP/ ML-BP = 1 / 100). This analysis showed that strains PATHC038^T and
347 HB10, representing *L. sheltonii* sp. nov., along with their sister species *L. cherrii,* formed a

348 monophyletic group with *L. steelei* IMVS3376^T, *L. steigerwaltii* NCTC 11991^T and *L. dumoffii*
349 NY 23^T. This monophyletic cluster nested within a larger group that includes *L. bozemanae*,
350 *L. parisiensis*, *L. anisa*, *L. resiliens*, *L. tucsonensis*, *L. gingyii*, *L. gormanii*, *L. wadsworthii* type
351 strains. Single gene (16S rRNA gene, *mip*, *gyrA*, *rnpB* and *rpoB*) phylogenies yielded similar
352 results, encompassing the same *Legionella* species (Figures S5 - S9). Sequence comparisons
353 of 16S rRNA and mip genes between PATHC038^T and HB10 revealed 100 % identity. The
354 newly proposed species showed the highest sequence identity percentages with
355 *Legionella cherrii* (strain NCTC 11976^T) across all five genes: 98.7 % for 16S rRNA gene,
356 98.4 % for *mip*, 94.2 % for *gyrA*, 96.2 % for *rpoB* and 99.2 % for *rnpB* (Table S7).

357 Further phylogenomic trees inferred from genome sequences (Figure S10) and whole
358 proteomes (Figure 1) provide additional taxonomic support. The nodes of the genome-based
359 tree are largely unresolved, which is expected due to high interspecies diversity. In contrast,
360 the proteome-based tree closely mirrored the five-gene phylogeny with most receiving
361 maximum support. *L. sheltonii* sp. nov. and *L. cherri* are reconstructed as sister species, yet
362 the significant GBDP (Genome Blast Distance Phylogeny) distance between them, further
363 supports their cassification as separate evolutionary entities.

364 Figure 1.

365 To characterize the virulence genes of the new species, the annotated genome was compared
366 against the VFDB database. In total, 125 virulence genes were identified, with the majority
367 associated with the Dot/Icm type IV secretion system (48 genes) and flagellar motility (35
368 genes), followed by genes involved in adherence and iron uptake (Figure 2).

369 Figure 2.

370 Comparison of the virulence genes in strain PATHC038^T with the only one available genome
371 of *L. cherrii* as well as with genomes of two closely related fluorescent species, *L. anisa*, and
372 *L. bozemanae*, revealed that 100 virulence genes were shared across all four strains, while
373 121 genes were specifically shared between *L. cherrii* and PATHC038^T, as shown in the Venn

diagram (Figure S11). These results underscore the close relationship between PATHC038^T and fluorescent species of *Legionella*, while also illustrating that each member of this group carries a small subset of species-specific virulence genes. A detailed list of the identified virulence genes is provided in Table S8.

In this study we also examined the viability of strain PATHC038^T within *Acanthamoeba castellanii* ATCC 30234 over a 15-day period, during which the number of bacteria decreased from 7×10^4 to 6×10^3, suggesting that the strain remains viable for at least 15 days. In contrast, *L. pneumophila* ATCC 33152^T showed a decrease from 3×10^3 to 3×10^2 over the same period showing that PATHC038^T has significantly better persistence in *A. castellanii* than *L. pneumophila* (Figure 3). We observed a similar general trend in 3 independent replicates. The data suggest that the abundance of the two species generally increases in the initial phase (up to about day 3), followed by a slower increase, or a phase where a plateau is almost reached, between day 6 and 12. However, there is a slight tendency for the species to be more abundant after 9 days than after 6 days in most replicates.

Figure 3.

This could be due to ongoing replication or delayed responses related to environmental conditions or host cell dynamics. These results indicate that PATHC038^T maintains the viability within *A. castellanii* more effectively than the reference strain *L. pneumophila*. The studied strain may be better adapted to the intracellular environment of *A. castellanii*, or the differences in persistence may be due to genetic variations between PATHC038^T and *L. pneumophila*.

While *L. pneumophila* is the most extensively studied species, research has shown that *L. dumoffii* exhibits delayed multiplication in *A. castellanii* at 72 hours post-infection, whereas *L. steigerwaltii*, *L. gormanii*, *L. bozemanii*, and *L. micdadei* show decreasing counts in amoebae [65]. This ability to replicate within amoebae is primarily attributed to the conserved Icm/Dot Type IV secretion system, which enables *Legionella* species to manipulate host cellular pathways, ensuring their survival and replication [66]. *In silico* genome analysis

showed that the vast majority of 125 virulence genes detected in PATHC038^T belong to the very same secretion system.

Not all *Legionella* species are capable of replicating inside amoebae [67], which highlights the significance of our findings regarding the replication of PATHC038^T in amoebae. The results described in our study represent a first step in defining the cell biology of the interaction between *L. sheltonii* sp. nov., strain PATHC038^T and freshwater amoebae, as well as the environmental implications thereof.

The results of the biochemical properties of the *L. sheltonii* sp. nov. strains we tested are listed in Table S9. While positive reactions for gelatinase, catalase, and oxidase are common within the *Legionella* genus [67], the absence of urease activity, nitrate reduction, hippurate hydrolysis, and acid production from carbohydrates distinguishes this species from many established *Legionella* species. A key point of comparison arises with *L. cherrii*, most closely related species of the studied strains, which is known to be urease-negative, aligning with the new species. However, *L. cherrii* typically demonstrates variable nitrate reduction (some strains positive, some negative) and is generally positive for hippurate hydrolysis [68]. The new strains' consistent negative reactions for both nitrate reduction and hippurate hydrolysis set them apart from *L. cherrii*. Furthermore, while comprehensive carbohydrate utilization data for *L. cherrii* may be limited in some earlier publications, the general trend within the *Legionella* genus is the inability to ferment carbohydrates [67], a characteristic shared by the new species (Table S10).

The minimum inhibitory concentration (MIC) range determined for the *L. sheltonii* sp. nov. strains analyzed was 0.25 - 0.75 µg/ml for ciprofloxacin and 0.016 - 2 µg/ml for erythromycin. These values demonstrate the susceptibility of the tested *L. sheltonii* sp. nov. strains to the antimicrobial drugs ciprofloxacin and erythromycin, which are commonly used in the treatment of legionellosis (EUCAST) and consistent with studies on *Legionella* spp. in Greece [69]. Tests on BCYE agar yielded higher MIC values than the microbroth dilution method [70, 71]. A range of values for erythromycin, spanning from 0.016 to 2 µg/ml, can be regarded as extensive;

however, such discrepancies are frequently observed, particularly among *Legionella* spp. and related bacteria. These variations may arise from differences in testing methodologies (for instance, the E-test conducted on BCYE agar) or from experimental parameters such as inoculum density and the pH of the growth medium. Several studies have documented similar variations, highlighting the significant impact of testing methods and environmental conditions on MIC values [69, 71, 72]. Although the findings suggest a general susceptibility of the strain to erythromycin, additional testing utilizing alternative methodologies could further validate these findings.

The growth period of strain PATHC038^T on culture media was typically 4 days or more, after which smooth colonies that autofluoresce a blue-white color under a UV lamp (365 nm wavelength) start to appear on the inoculated media (Figure 4, Table S10). Of the 66 recognized species of *Legionella* [2], 12 can show a blue-white autofluorescence when subjected to long-wavelength ultraviolet light (365 nm), while three species exhibit a dark-red autofluorescence. In contrast, *L. pneumophila*, the representative species of the *Legionella* genus, does not display any autofluorescent characteristics [7]. As a result, the autofluorescence property is a useful method for distinguishing and identifying different *Legionella* species.

Figure 4.

The cultivation of *Legionella* species can be influenced by various factors, including the media used, incubation temperature, and the presence of CO_2. In this study, distinct growth patterns were observed across different media and incubation conditions (Table S11).

No growth was observed on BCYE Cys-, which was expected since it is known that *Legionella* species are cysteine auxotrophs [73]. Similarly, the lack of growth on MWY suggests that this medium, while selective for *Legionella*, might not have been optimal for this particular strain under investigation. MWY is designed to select for *Legionella* while suppressing other bacteria, but this selectivity can sometimes limit the growth of certain *Legionella* strains [74].

Weak growth was observed on GVPC agar which is a selective medium used to isolate *Legionella* from environmental samples that are expected to have high levels of competing microbial flora. The weak growth observed could be due to the selective agents in the media, which, while inhibiting other microorganisms, might also slightly hinder the growth of the target *Legionella* strain [75].

The strain PATHC038^T was most successfully subcultured on BCYE and BMPA media under all previously mentioned incubation conditions. BCYE is a widely recognized and frequently used medium for *Legionella* cultivation, providing the necessary nutrients, including cysteine, for optimal growth [76]. BMPA agar is also known to support *Legionella* growth, with pyruvate and activated charcoal further enhancing growth and recovery [77].

Regarding temperature, weak growth was observed at 32 °C and 38 °C, while optimal growth occurred at 34 °C and 36 °C, both with and without 3 % CO_2. *Legionella* species are known to grow within a temperature range, typically between 25 °C and 45 °C, with an optimal growth temperature at 35 - 37 °C [67]. The observed growth pattern of strain PATHC38^T aligns with these general characteristics, with the strain exhibiting a preference for the higher end of the optimal temperature range. The lack of a significant difference in growth with or without added CO_2 suggests that this strain does not have a strict requirement for elevated CO_2 levels for growth, although some *Legionella* species may benefit from it. Strain HB10 demonstrated the same culture requirements, colony morphology and the ability to autofluoresce as strain PATHC038^T.

The cellular fatty acid (CFA) profiles of *Legionella sheltonii* sp. nov., strains PATHC038^T and HB10, were compared with those of selected fluorescent species of the genus *Legionella*, including *L. cherrii*, *L. bozemanae*, *L. dumoffii*, *L. gormanii*, *L. anisa*, and a non-fluorescent *L. pneumophila* serogroup 1 (Table S12). This comparative analysis provided insights into the unique lipid composition of the novel species and its distinction from related taxa.

Strains PATHC038^T and HB10 exhibited similar CFA profiles, with C16:1 ω7c (15.5 - 17.6 %), iC16:0 + C15:1 ω6c (16.8 - 18.3 %), and aC15:0 (14.5 - 14.8 %) as predominant components. These profiles are characteristic of *Legionella* species [78] but, at the same time, show notable differences from other closely related species. Compared to *L. cherrii*, PATHC038^T and HB10 have significantly lower levels of C17:0 (4.3 - 4.7 % vs. 16.8 %) and lack C6:0, which is present in *L. cherrii* at 2.3 %. Additionally, *L. sheltonii* strains exhibit higher proportions of C14:0 (up to 1.3 %) and aC17:0 (9.9 - 10.8 %), which distinguish them from *L. cherrii* and other species analyzed. Among other fluorescent *Legionella* species, such as *L. bozemanae* and *L. gormanii*, the novel species shows reduced levels of C16:0 and C18:0, while maintaining higher proportions of branched fatty acids like aC15:0. Notably, *L. sheltonii* strains lack C24:0, a fatty acid found in *L. dumoffii* and *L. gormanii*, further supporting their unique fatty acid composition. Results of fatty acid profiling are shown in Table S12.

When observed under a transmission electron microscope (TEM), strains appear rod-shaped with a length of 1 to 3 µm and a width of about 0.3 µm. The cell wall is slightly roughened. The new species has flagella (Figure 5, Table S10), thin, spiral-shaped structures that protrude from the bacterial surface and are used for movement. Observation of flagella is consistent with the detection of numerous flagellar motility genes in the genome of the strain PATHC038^T (Table S8). Several environmental factors can be linked to the presence of flagella, such as nutrient availability (e.g. amino acids and fatty acids), temperature, and medium viscosity [60]. Moreover, the most closely related species *L. cherrii*, *L. steelei*, *L. steigerwaltii* and *L. dumoffii* are also flagellated [79–81], a characteristic they share with the type species of the genus, *L. pneumophila* [82] (Table S10).

Figure 5.

In conclusion, genotypic, genomic, phylogenetic, biochemical, and phenotypic characteristics of the two studied strains, PATHC038^T and HB10, obtained by MALDI TOF MS, genome-based typing, phylogenetic analysis, biochemical testing, and fatty acid profiling undeniably indicate that a novel species in the genus *Legionella* has been discovered.

505 **Description of *Legionella sheltonii* sp. nov.**

506 *Legionella sheltonii* (shel.to'ni.i. N.L. gen. n. *sheltonii*, in honor of scientist and epidemiologist
507 Mr. Brian G. Shelton who dedicated his career to *Legionella* prevention, control and
508 remediation) is an aerobic, flagellated, unencapsulated, non-spore forming Gram-negative rod-
509 shaped bacterium. Colonies have the ability to autofluoresce a blue-white color when placed
510 under a 365 nm wavelength UV lamp. Growth is supported on BCYE, BMPA and weakly on
511 GVPC media, the optimal incubation conditions include the presence of 3 % CO_2 and a
512 temperature of 36 °C. Biochemical tests are positive for oxidase, catalase, and gelatinase
513 while negative for urease, hippurate hydrolysis and nitrate reduction. FAME analysis showed
514 that the predominant fatty acids detected are C16:0 iso, C16:1 ω7c, C16:0 and C15:0 anteiso.
515 Gene sequences of the newly proposed species showed highest similarity with those of
516 *L. cherrii* (NCTC strain 11976), including 98.7 % for 16S rRNA gene, 98.4 % for *mip*, 94.2 %
517 for *gyrA*, 96.2 % for *rpoB* and 99.2 % for *rnpB*. The POCP value for the *L. sheltonii* sp. nov.
518 and *L. cherrii* pair is 86.5 %.

519 The Whole Genome Shotgun Sequencing (WGS) project of the strain PATHC038^T has been
520 deposited at NCBI under the genome accession number GCF_026191355.1 with genome size
521 of 4.3 Mb and G+C content of 38.2 mol %. The species was isolated from a cold water
522 distribution system on a cruise ship with an international itinerary.

523 The GenBank accession numbers for the 16S rRNA genes of the strains PATHC038^T and
524 HB10 are PQ120583 and PQ120582, respectively. The GenBank accession numbers for the
525 mip genes of the strains PATHC038^T and HB10 are PQ119488 and PQ119489, respectively.
526 Type strain PATHC038^T culture collection deposit numbers are CCUG 76918T and ATCC
527 TSD-370.

528

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Author Contributions

RSK/MK performed sample preparation, data analysis, drafted the manuscript and prepared figures and tables. ES/NK performed data curation/analysis and prepared figures and tables. MS/MA performed TEM analysis to observe the morphological and structural appearance of strains. RSK/MK/KS/KB/KV designed the experiments, collected and isolated the bacterial strains, performed culture experiments and drafted the manuscript. SK performed MALDI-TOF MS analysis. OM/AP/MV performed sequence alignment and phylogenetic analysis. MIS performed fatty acid profiling. DK performed various biochemical tests. DF/GK/IM performed conceptualization, supervision and revision of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflicts 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.

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Figures:

Figure 1. Phylogenomic tree inferred from whole-proteome-based GBDP distances of *L. sheltonii* sp. nov., strain PATHC038^T and closely related species of the genus *Legionella*. The clade containing species *L. septentrionalis*, *L. oakridgensis*, and *L. nagasakiensis* was used as an outgroup. The branch lengths are scaled via GBDP distance formula *d5*. Branch values are GBDP pseudo-bootstrap support values > 60 % from 100 replications, with an average branch support of 96.7 %. Bootstrap values > 70 % are indicated.

Figure 2. Categorization of the *L. sheltonii* sp. nov., strain PATHC038^T virulence factors as per Virulence factor database. A total of 125 virulence genes were detected.

Figure 3. The intracellular growth kinetics of strain PATHC038^T and *Legionella pneumophila* ATCC 33152^T within *A. castellanii* ATCC 30234 at 3rd, 6th, 9th, 12th, and 15th day postinfection. The experiments were performed in triplicate and the error bars represent standard deviations. Student t-test ** $p < 0,01$; *** $p < 0,001$.

Figure 4. *Legionella sheltonii* sp. nov. colonies can autofluoresce in a bluish-white color when placed under a 365 nm wavelength UV lamp. PATHC038^T colonies on the 7th day of incubation. Bar 1 mm.

Figure 5. Transmission electron microscopy image of *Legionella sheltonii* sp. nov., strain PATHC038^T cells. One or a few flagella in the polar region aid *Legionella sheltonii* sp. nov. in motility. Bar 1 µm.