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Listeria thailandensis sp. nov.

Alexandre Leclercq[1,2,3*], Alexandra Moura[1,2,3*], Guillaume Vales[1,2], Nathalie Tessaud-Rita [1,2], Christine Aguilhon[4], Marc Lecuit[1,2,3,5#]

[1] Institut Pasteur, National Reference Centre and WHO Collaborating Centre for *Listeria*, Paris, France
[2] Institut Pasteur, Biology of Infection Unit, Paris, France
[3] Inserm U1117, Paris-France
[4] bioMérieux, Marcy l'Etoile, France
[5] Paris Descartes University, Sorbonne Paris Cité, Institut Imagine, Necker-Enfants Malades University Hospital, Division of Infectious Diseases and Tropical Medicine, APHP, Paris, France

*These authors share first authorship

Corresponding author:

E-mail: marc.lecuit@pasteur.fr ; Phone: +33 01 40 61 37 87

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Abstract

During a screening of *Listeria* spp. in food samples in Thailand, a *Listeria*-like bacterium was recovered from fried chicken and could not be assigned to any known species. Phylogenetic analysis based on the 16S rRNA gene and on 243 *Listeria* core genes placed the novel taxon within the *Listeria aquatica, Listeria floridensis, Listeria fleishmannii* and *Listeria costaricensis* clade (*Listeria sensu lato*), with highest similarity to *L. floridensis* (98.9%) and *L. costaricensis* (98.8%). Whole-genome sequence analyses based on the average nucleotide BLAST identity (ANI<86%), the pairwise amino acid identity (AAI>64%) and on the percentage of conserved proteins (POCP>77%) with currently known *Listeria* species, confirmed that the strain constituted a new taxon within the *Listeria* genus. At the phenotypical level, it differs from other *Listeria* species by the production of acid from D-tagatose and inositol. The name *Listeria thailandensis* sp. nov. is proposed for the novel species, and is represented by the type strain CLIP 2015/00305^{T} (=CIP 111635^{T}=DSM 107638^{T}).

The GenBank/EMBL/DDBJ accession numbers for the draft genome and the 16S rRNA gene sequence of strain CLIP 2015/00305^{T} are ERP109849 and MK014484, respectively.

Main text

The genus *Listeria* currently comprises 19 species which has been proposed to be subdivided in two major groups [1–3]: (i) *Listeria sensu stricto*, constituted by the species *Listeria monocytogenes* [4], *L. innocua* [5], *L. welshimeri* [6], *L. seeligeri* [6], *L. ivanovii* [7] and *L. marthii* [8], and (ii) *Listeria sensu lato*, constituted by the species *L. grayi* [9, 10], *L. rocourtiae* [11], *L. fleischmannii* [12, 13], *L. weihenstephanensis* [14], *L. floridensis* [15], *L. aquatica* [15], *L. cornellensis* [15], *L. riparia* [15], *L. grandensis* [15], *L. booriae* [16], *L. newyorkensis* [16], *L. costaricensis* [17] and *L. goaensis* [18]. Of those, *L. monocytogenes* and *L. ivanovii* are reported to be pathogenic to human and other animals [19] and constitute a major concern for the food industry and public health authorities.

In 2015, a *Listeria*-like bacterium was recovered from a food retailer in Thailand. The isolate was obtained from a fried chicken sample during a screening of *Listeria* spp. in food, using the enzyme linked fluorescent immunoassay VIDAS LIS and LPT methods (bioMérieux, Marcy l'Etoile, France). The isolate CLIP 2015/00305^T (originally designated as bioMérieux S.A. R&D Industrie) was received at the World Health Organization Collaborating Centre for *Listeria*, Paris, France, for characterization.

Species identification by MALDI-TOF mass spectrometry using either the MicroFlex LT system with MBT library DB-5989 (Bruker Daltonics, Bremen, Germany) [20] or the VITEK 2 MS system (bioMérieux, Marcy l'Etoile, France) were inconclusive. Similarly, the API *Listeria* (bioMérieux API web database, version 2.0), VITEK 2 (bioMérieux, Marcy l'Etoile, France) biochemical systems also failed to provide any reliable identification (profiles 2731 and 510200021533601, respectively).

The genome of isolate CLIP 2015/00305^T was obtained after DNA extraction (DNeasy Blood & Tissue kit, Qiagen, København, Denmark), library preparation (Nextera XT DNA Sample kit, Illumina, California, USA), and sequencing using the NextSeq 500 (2 x 150 bp) platform (Illumina), according to the manufacturer's protocol, as previously described [21].

Phylogenetic analyses were performed based on the 16S rRNA gene sequence comparisons and on the concatenated deduced amino acid sequences of 243 core genes present in all *Listeria* species [17], defined using Roary v.3.11 [22] and a BLASTP identity cut-off of 80%. Sequences were aligned using MUSCLE v.3.8 [23]. Maximum likelihood phylogenetic trees were inferred by using IQ-Tree v.1.5 [24] and visualized in MEGA v.7.0 [25].

Whole-genome pairwise average nucleotide and amino acid identity comparisons (ANI and AAI, respectively) against the *Listeria* species reference genomes were determined using the enveomics package [26]. Dendrograms based on the unweighted pair group method with arithmetic mean (UPGMA) method were obtained from the distance matrixes using Bionumerics v.7.6 (Applied Maths, Sint-Martens-Latem, Belgium).
The percentage of conserved proteins (POCP) was calculated as described [27]. Multiplex and in silico PCR-serogrouping were determined as described previously [28, 29]. The draft genome assembly was obtained using CLC Assembly Cell 4.3.0 (Qiagen, Hilden, Germany) as previously described [21], and annotated with Prokka v.1.12 [30].

The draft assembly of CLIP 2015/00305^{T} obtained from high-quality reads (final coverage of 108 X) contained 15 contigs, with a total length of 2.70 Mb and a N50 length of 554,335 bp. Data obtained followed the quality standards for its use for taxonomic purposes [32]. The genome of CLIP 2015/00305^{T} contained 2,689 predicted protein-coding sequences, 1,107 (41%) of which with no known function.
16S rRNA gene phylogenetic analysis showed highest similarity with *L. floridensis* (98.9%) and *L. costaricensis* (98.8%; Fig. 1), in the borderline of the proposed species cut-off based on 16S sequence similarity (98.7-99.0%; [33, 34]). Maximum likelihood phylogeny based on the amino acid sequences of *Listeria* core genes (Fig. 2) placed the novel taxon within the *L. aquatica, L. floridensis, L. fleishmannii* and *L. costaricensis* clade (*Listeria sensu lato*), with highest similarity to *L. aquatica*.
ANI analysis revealed that CLIP 2015/00305^{T} shared less than 90% genome sequence identity with all known *Listeria* species (Fig. 3), thus lower than the proposed genomic species cut-off of 95% [35]. Highest ANI values were obtained *L. aquatica* (two-way ANI of 85.3±4.4%, based on 8082 genome fragments). Consistently, analyses based on the deduced proteomes also showed highest identities *L. aquatica* (two-way AAI of 91.4±9.4%, based on 2180 orthologous proteins; POCP of 83.1%). Both AAI and POCP pairwise analyses were higher than proposed cut-offs for genus delineation of 60% and 50%, respectively [35, 36], confirming that the CLIP 2015/00305^{T} represents a novel *Listeria* taxon.

In silico and multiplex PCR-serogrouping were positive for the *prs* gene (serogroup L, typical of *Listeria* species with the exception for *L. monocytogenes* [28]).

Phenotypic analyses of isolate CLIP 2015/00305^T were carried out on BHI agar plates, Agar *Listeria* according to Ottaviani and Agosti (ALOA; bioMérieux, Craponne, France) after incubation for 24h at 30 and 37 °C. The presence of a capsule was tested using the India ink test [37]. Gram staining, catalase and oxidase activities, respiratory characteristics and endospore formation were tested as described in the Bacteriological Analytical Manual (BAM) [38]. Growth characteristics were determined on BHI agar and broth at 4 °C for 10 days and at 22, 30, 37 and 42 °C for 7 days. Growth was considered positive if there was an increase in cell number of at least 1.0 log (cfu.ml^{-1}) over 14 days. Motility was tested by stab-inoculation in mannitol-mobility semi-solid agar (Bio-Rad, Marnes-La-Coquette, France) and incubation at different growth temperatures (4 °C, 22 °C and 37 °C) for 10 days in aerobic conditions. *L. monocytogenes* ATCC 35152^T and *L. booriae* CIP 111022^T were used as positive and negative controls, respectively. Nitrate reduction and Voges-Proskauer tests were performed as described in the BAM [38]. Haemolysis was tested using either Columbia agar plates containing 5% defibrinated horse blood (bioMérieux) or the Christie, Atkins, Munch-Petersen (CAMP) test on Columbia agar containing 5% defibrinated sheep blood (bioMérieux) as described in the BAM [38]. Biochemical tests performed with API *Listeria* strips (bioMérieux), the API50CH system (bioMérieux), RAPIDEC Lmono (bioMérieux) and VITEK 2 version 07.01 (bioMérieux) with Gram positive (GP) card as recommended by the manufacturer [39]. API *Listeria* tests were recorded after incubation at 37 °C for 24h. API50CH tests were recorded after 2, 5, 10 and 15 days of incubation at 37 °C. Susceptibility to a wide range of antibacterial agents was determined with the disk diffusion method on Mueller–Hinton agar plates (Bio-Rad), using the interpretative criteria and recommendations from the French Microbiology Society and the European Committee on Antibiotic Susceptibility Testing [40, 41].

According to its phenotypic and biochemical profile (Table 1), CLIP 2015/00305^T clustered together with *L. aquatica* (Fig. 4).

Cells of CLIP 2015/00305^T were Gram-stain-positive facultative anaerobic rods, oxidase negative and catalase positive. Colonies on BHI were opaque, flat, with a diameter of 0.5 to 1.0 mm. On Palcam agar, colonies were approximately 2mm in

diameter, grey-green in colour with a black sunken centre and a black halo against a cherry-red medium background. On ALOA agar, colonies were blue, due to β-glucosidase activity, and not surrounded by a white halo, denoting the absence of phosphatidylinositol-specific phospholipase C (PI-PLC) activity (Table 1).

CLIP 2015/00305^T showed growth between 22 °C and 42 °C but not at 4 °C. No capsule or spores were detected. Motility was also absent.

Voges-Proskauer and nitrate reductase tests were positive (Table 1).

The CAMP test was negative and no haemolysis was detected in either horse or sheep bloods. The lack of haemolysis was consistent with the absence of *Listeria* pathogenicity islands (LIPI-1 to -4) [42, 43] within the draft genome of CLIP 2015/00305^T, combined with absence of internalins *inlABCDEFGHJK* genes and genes coding bile resistance, suggesting that this novel taxon is not pathogenic.

CLIP 2015/00305^T was sensitive to penicillin G, ampicillin, amoxicillin, imipenem, kanamycin, streptomycin, gentamicin, rifampicin, erythromycin, levofloxacin, moxifloxacin, tetracycline, chloramphenicol, fusidic acid, trimethoprim and vancomycin. However, CLIP 2015/00305^T showed resistance to nalidixic acid, fosfomycin, sulphonamides, clindamycin, ciprofloxacin and cefotaxime, as other *Listeria* species [44]. Within the draft genome of CLIP 2015/00305^T, genes conferring resistance to nalidixic acid (*norB*), fosfomycin (*fosX*), sulphonamides (*sul*), lincomycin (*lmrB*) and quaternary ammonium compounds (*qacA*) used in food industry for cleaning and disinfection were present.

Thus, on the basis of the molecular findings described above as well as the phenotypic distinctiveness of strain CLIP 2015/00305^T, we propose that this strain should be classified as a member of a novel species of the genus *Listeria* for which the name *Listeria thailandensis* sp. nov. is proposed.

DESCRIPTION OF *LISTERIA THAILANDENSIS* SP. NOV.

Listeria thailandensis (thai.land.en'sis N.L. fem. adj. *thailandensis*, “from Thailand”, the country where the type strain was originally isolated).

Cells are straight, Gram-stain-positive, non-motile and non-spore-forming rods. Facultative aerobic, catalase-positive and oxidase-negative. Capsule is not formed. Colonies are opaque, not pigmented, with a flat shape and entire margin on BHI. On ALOA, colonies are blue centred without white halo, typical of *Listeria* species. Growth occurs at 22-42 °C, with optimal growth at 30-37 °C. Negative for haemolysis and nitrite reduction. Positive for Voges-Proskauer and nitrate reduction tests. Acid is produced from aesculin, N-acetylglucosamine, amygdalin, arbutin, D-cellobiose, D-fructose, gentiobiose, D-glucose, glycerol (weakly positive), D-mannose, L-rhamnose, D-ribose, salicin, dulcitol, D-trehalose, D-tagatose, methyl α-D-glucoside, D-arabitol, inositol and D-xylose. Acid is not produced from D-adonitol, D-arabinose, L-arabitol, D-galactose, L-fucose, D-lactose, D-maltose, D-saccharose, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, methyl β-D-xylopyranoside, potassium gluconate, potassium 5-ketogluconate, L-arabinose, glucose 1-phosphate, xylitol, starch, erythritol, D-fucose, glycogen, inulin, D-mannitol, D-melezitose, D-melibiose, potassium 2-ketogluconate, D-raffinose, D-sorbitol, L-sorbose, D-turanose, D-lyxose, L-xylose. It can be differentiated from other species of the genus *Listeria* by the production of acid from D-tagatose and inositol.
The type strain CLIP 2015/00305^T (=CIP 111635^T = DSM 107638^T) was isolated in March 2015 from a fried chicken sample in Thailand. The genomic DNA G+C content of the type strain is 40.3 mol%.

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Conflicts of interest

The authors declare no conflict of interests.

Abbreviations:

AAI	Average amino acid identity
ANI	Average nucleotide identity
BAM	Bacteriological analytical manual
BHI	Brain hearth infusion
BLAST	Basic local alignment search tool
CAMP	Christie, Atkins, Munch-Petersen test
N50	Minimum contig length covering 50% of the genome
POCP	Percentage of conserved proteins
UPGMA	Unweighted pair group method with arithmetic mean

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Legends to figure

FIGURE 1. Phylogenetic analysis of the 16S rRNA gene based on the maximum likelihood method. Distance estimation was obtained by the model of Kimura 2-parameter [45]. Selected members of *Brochothrix* genus were used as outgroup. Positions containing gaps and missing data were eliminated, resulting in a total of 1072 positions. Branch lengths represent the number of nucleotide substitutions per site and bootstrap percentages of 1,000 replicates are shown. GenBank accession numbers are provided in brackets. The newly *Listeria thailandensis* isolate is highlighted in bold**.**

FIGURE 2. Maximum likelihood phylogenetic analysis based on the concatenated amino acid sequences of 243 core genes present in all *Listeria* species. Distance estimation was obtained by the Whelan and Goldman (WAG) model [46]. Branch lengths represent the number of amino acid substitutions per site and bootstrap percentages of 1,000 replicates are shown. GenBank accession numbers are provided in brackets.

FIGURE 3. UPGMA clustering based on the genomic average nucleotide difference (ANI). The vertical dashed bar represents the proposed 95% ANIb species cut-off that correlates with the 70% DNA-DNA hybridization threshold [35]. Scale bar represents the percentage of similarity.

FIGURE 4. UPGMA analysis based on the 33 biochemical characteristics of *Listeria* species shown in Table 1. Unknown data and traits with variations between different isolates and/or studies were ignored in the pairwise analyses. Scale bar represents the percentage of similarity of phenotypical profiles.

Table 1. Biochemical characteristics of species of the genus *Listeria* based on observations made in this study and on previously published studies [15, 17, 18].

Characteristics	Lth	Lmo	Lin	Lse	Liv	Lws	Lma	Lgy	Lro	Lwp	Lcn	Lri	Lgd	Lfc	Laq	Lfo	Lny	Lbo	Lco	Lgo
Motility	-	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	+	-
Nitrate reduction	+	–	–	–	–	–	–	v	+	+	+	+	+	+	+	–	+	+	+	-
Voges–Proskauer	+	+	+	+	+	+	+	+	–	–	–	–	–	–	v	–	–	–	+	-
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
Haemolysis	-	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	-	+ (α)
D-Arylamidase	-	–	+	+	v	v	–	+	–	–	–	–	–	–	–	–	–	–	-	-
α-Mannosidase	-	+	+	–	–	+	+	v	+	–	–	+	–	–	+	–	–	+	-	-
Phosphatidylinositol-specific phospholipase C (PI-PLC)	-	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	-	-
Acidification of:																				
D-Arabitol	+*	+	+	+	+	+	+	+	–	+	–	–	v	+	–	–	–	+	+	+
D-Galactose	-	v	–	–	v	–	–	+	+	–	–	+	–	–	–	+	+	+	+	-
D-Glucose	+	v!	v!	+	v!	+	v!	+	+	+	+	+	+	+	+	+	+	+	+	+
Glycerol	(+)	v	+	+	+	+	–	v	+	+	v	v	–	+	v	–	+	+	+	(+)
L-Fucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
D-Lactose	-	+	+	+	+	+	+	+	+	v!	(+)	+	–	+	–	+	+	+	+	+
D-Maltose	-	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+
L-Rhamnose	+	+	v	–	–	v	–	–	+	+	–	+	–	+	+	+	v	+	+	+
D-Ribose	+	–	–	–	+	–	–	+	+	–	+	v	+	+	+	–	+	v	+	-
D-Saccharose	-	+	+	+	+	+	–	–	–	–	–	–	–	v	–	–	–	–	+	-
Methyl α-D-glucoside	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	-
Methyl α-D-mannose	-	–	–	nd	–	nd	–	+	–	–	–	–	–	v	–	–	–	–	+	(+)
Potassium 5-ketogluconate	-	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	-
D-Xylose	+	–	–	+	+	+	–	–	+	+	+	+	+	+	+	+	+	+	+	+
L-Arabinose	-	–	–	–	–	–	–	–	–	–	v	+	–	–	+	+	+	+	-	-
Glucose 1-phosphate	-	–	–	–	v	–	–	–	–	–	–	–	–	–	–	–	–	–	-	-
Inositol	+	–	–	–	–	–	–	–	–	–	–	v	–	v	v	–	–	–	-	-

Inulin	-	v!	v!	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	-	-
D-Lyxose	-	v	v	–	–	v	–	v	–	–	–	–	–	–	v	+	–	–	-	-
D-Mannitol	-	–	–	–	–	–	–	+	+	+	–	v	–	v	–	–	+	+	-	-
D-Melezitose	-	v	v	v	v	v	–	–	–	–	–	–	–	v	–	–	–	–	-	-
D-Melibiose	-	v!	v	–	–	–	v	–	+	–	–	v	–	v	–	–	–	+	-	-
L-Sorbose	-	v!	v!	–	v!	–	v!	v!	–	–	–	–	–	v	–	–	–	–	-	-
D-Tagatose	+	–	–	–	–	+	–	–	–	–	–	–	–	–	+	–	–	–	-	-
D-Turanose	-	–	v	–	–	–	+	–	–	–	–	–	–	v	–	–	–	–	-	-

Notation: +, positive; (+), weakly positive; −, negative; v, variable (between replicates and/or between strains); v!, variable between studies (possibly due to differences in incubation times and temperatures between studies); *, based on API Listeria (negative based on API 50 CH); nd, not determined or not recorded.

Strains: Lth, *L. thailandensis* sp. nov. strain CLIP 2015/00305^T (this study); Lmo, *L. monocytogenes* strain 10403S (data from McLauchlin & Rees, 2009 and Bertsch *et al.*, 2013); Lin, *L. innocua* strain FSL S4-378 (data from McLauchlin & Rees, 2009 and Bertsch *et al.*, 2013); Lse, *L. seeligeri* (data from McLauchlin & Rees, 2009 and Bertsch *et al.*, 2013); Liv, *L. ivanovii* strain ATCC BAA-678 (data from McLauchlin & Rees, 2009; Bertsch *et al.*, 2013); Lws, *L. welshimeri* (data from Bille *et al.*, 1992; McLauchlin & Rees, 2009; Bertsch *et al.*, 2013); Lma, *L. marthii* strain FSL S4-120^T (data from den Bakker *et al.*, 2014); Lgy, *L. grayi* strains ATCC 19120^T, ATCC 25401^T (data from den Bakker *et al.*, 2014); Lro, *L. rocourtiae* strain CIP 109804^T (data from den Bakker *et al.*, 2014); Lwp, *L. weihenstephanensis* strain DSM 24698^T (data from den Bakker *et al.*, 2014); Lcn, *L. cornellensis* strains TTU A1-0210^T, FSL F6-0970 (data from den Bakker *et al.*, 2014); Lri, *L. riparia* strains FSL S10-1204^T, FSL S10-1219 (data from den Bakker *et al.*, 2014); Lgd, *L. grandensis* strain TTU A1-0212^T (data from den Bakker *et al.*, 2014); Lfc, *L. fleischmannii* strains DSM 24998T, ATCC BAA-2414^T, FSL F6-1019, FSL S10-1186, FSL S10-1203 and FSL S10-1220 (data from den Bakker *et al.*, 2014); Laq, *L. aquatica* strains FSL S10-1188^T and FSL S10-1181 (data from den Bakker *et al.*, 2014); Lfo, *L. floridensis* strain FSL S10-1187^T (data from den Bakker *et al.*, 2014); Lny, *L. newyorkensis* strains FSL M6-0635^T and A5-0209; Lbo, *L. booriae* strains FSL A5-0279^T and FSL A5-0281; Lco, *L. costaricensis* strain CIP 111400^T (data from Núñez-Montero *et al.*, 2018); Lgo, *L. goaensis* strain DSM 29886 (data from Doijad *et al.*, 2018).

All species/strains are positive for aesculine and acid production from N-acetylglucosamine, amygdalin, arbutin, D-cellobiose, D-fructose, D-mannose and salicin.

All species/strains are negative for nitrite reduction and acid production from D-adonitol, D-arabinose, glycogen, methyl β-D-xylopyranoside, potassium 2-ketogluconate, and D-raffinose.

FIGURE 1

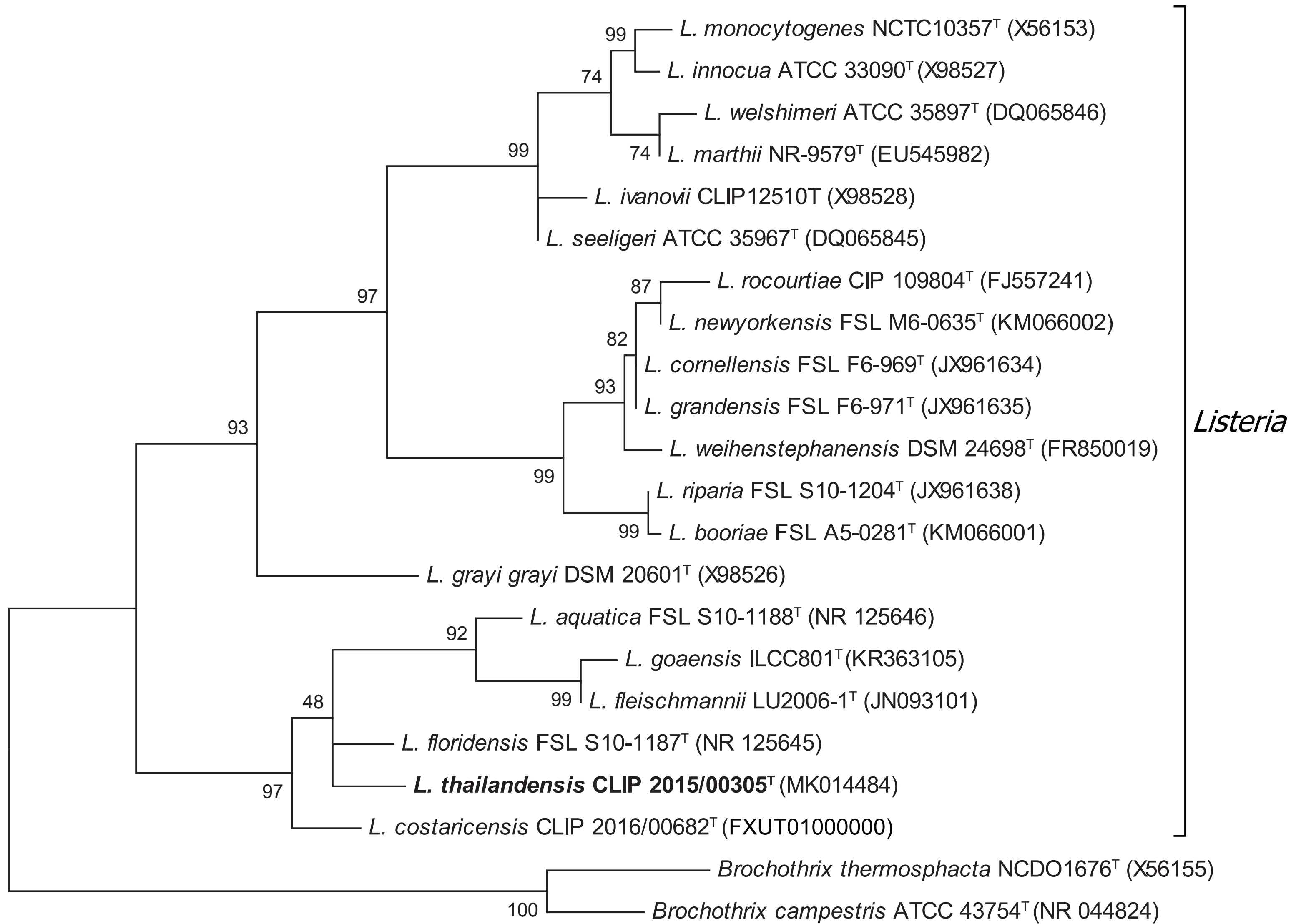

FIGURE 2

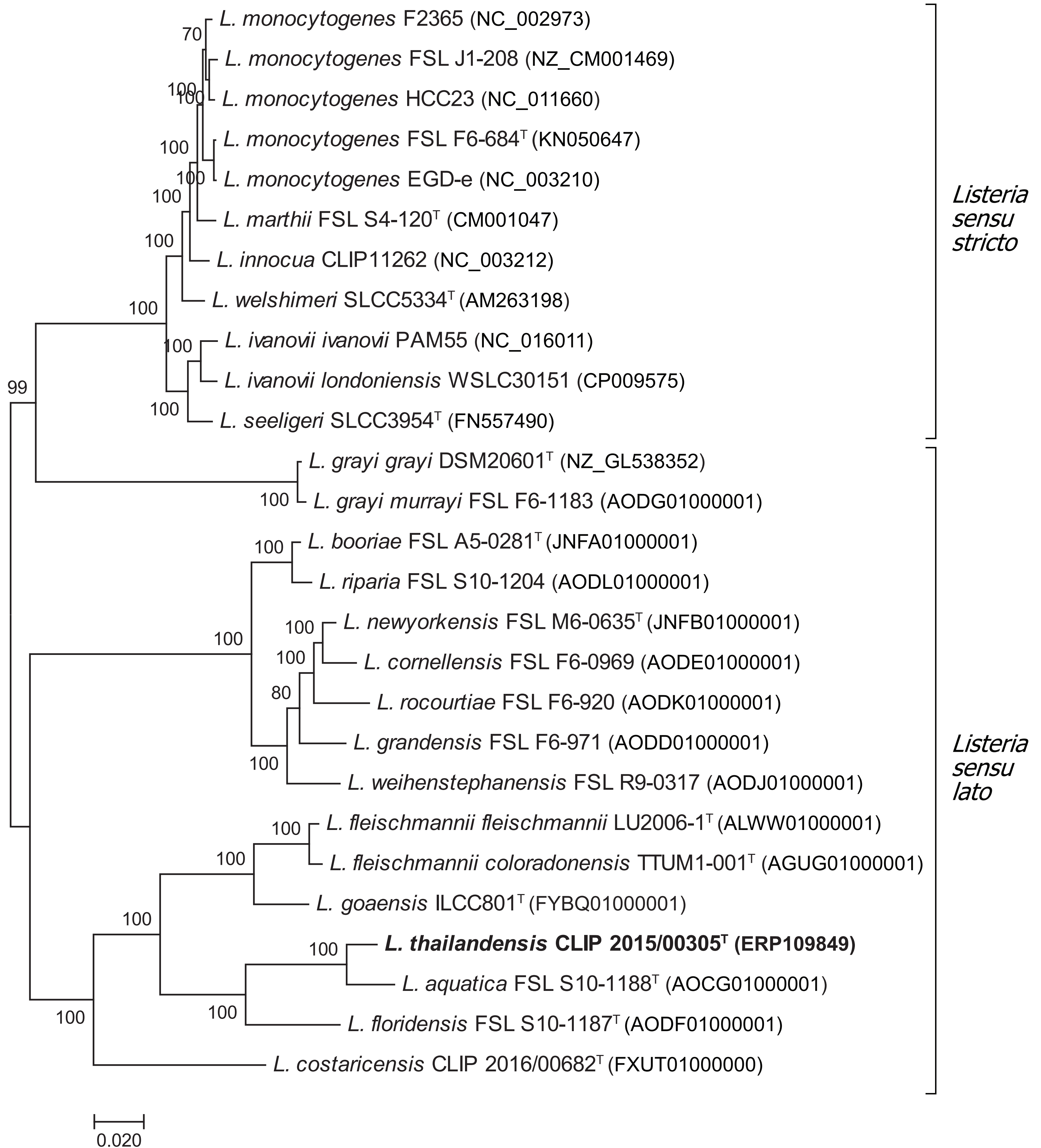

FIGURE 3

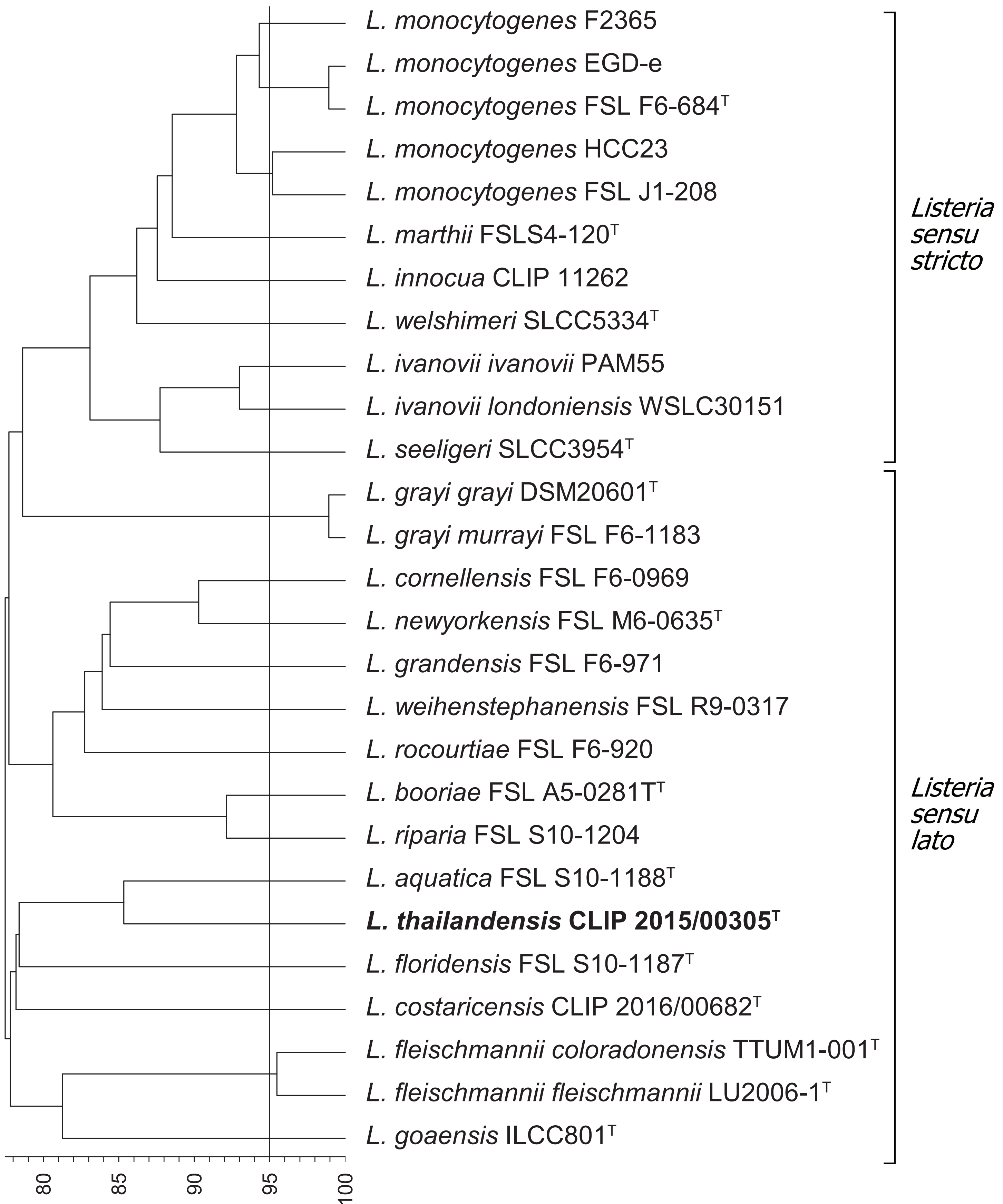

FIGURE 4