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Identification of *Listeria* Isolates by Using a Pragmatic Multilocus Phylogenetic Analysis

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Abstract: Species identification of *Listeria* isolates remained a tedious process still based on culturing methods that, in recent years, have led to the description of many species that are not even part of the genus *Listeria*. It is advisable to provide new precise techniques since this taxon includes two pathogens that are usually transmitted through the food chain, *Listeria monocytogenes* and *L. ivanovii*. The approach, so-called multilocus phylogenetic analysis (MLPA) that uses several concatenated housekeeping gene sequences, provides accurate and affordable classification frameworks to easily identify *Listeria* species by simple Sanger sequencing. Fragments of seven housekeeping genes (*gyrA*, *cpn60*, *parE*, *recA*, *rpoB*, *atpA*, and *gyrB*) from 218 strains of all *Listeria* species currently described were used to build an MLPA of the concatenated sequence, a total of 4375 bp. All isolates subjected to identification were clustered within the species of *Listeria* sensu stricto, *L. monocytogenes*, *L. innocua*, and *L. welshimeri*, and some reference strains were reclassified as *L. ivanovii* and *L. seeligeri*. Housekeeping-gene sequencing has been demonstrated to represent a pragmatic tool that can be firmly considered in food control.

Keywords: *Listeria*; *L. monocytogenes*; multilocus phylogenetic analysis; MLPA

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1. Introduction

Listeria is a ubiquitous bacterium that can be found in a wide range of environments, from the soil, water, or decaying vegetation to food or animal digestive tracts [1,2]. This is due to its ability to withstand and multiply at a broad range of pH (pH 4.5–9.2), temperature (−1 °C to 45 °C), and high salt concentrations (10% NaCl) [3]. The *Listeria* genus is formed by facultative anaerobic, Gram-positive, non-spore forming, rod-shaped bacteria with low G + C content [4]. Only two species, *L. monocytogenes* and *L. ivanovii*, are considered pathogens, although rare cases of infections caused by other species have been reported [5–7]. *Listeria ivanovii* is particularly pathogenic in ruminants but rarely affects humans [8]. *Listeria monocytogenes* is the main culprit of listeriosis, which, although not the most common zoonosis in the EU, is the deadliest, with a hospitalization rate of over 90% and a fatality rate of over 10% [9]. It affects particularly vulnerable individuals like the elderly, pregnant women, infants, and immunocompromised individuals. It was not until the 80s when several large common-source outbreaks of listeriosis highlighted the significance of food as the primary path of transmission for human infection by *L. monocytogenes*; to date, it is generally considered that most cases involve foodborne transmission [10]. Outbreaks occur generally in developed countries due to processed, refrigerated, ready-to-eat products of animal origin that have a long shelf life and are consumed without further listericidal treatments. The ability of *Listeria* to grow at a wide range of conditions allows it to proliferate in food-processing environments, making it challenging to ensure food safety [11]. *L. monocytogenes* is divided into four phylogenetic lineages with distinct evolutionary and phenotypic characteristics [12–16]. Most of the strains isolated worldwide belong to lineages I and II [17]. Lineage I strains are more frequently associated with human

clinical cases than those from lineage II and are considered more virulent, while lineage II strains are more frequently isolated from food and environmental sources [18,19]. Lineage III and IV strains are predominantly isolated from animals [20]. Therefore, the assignation of isolates to *L. monocytogenes* lineages may help to ascertain virulence levels.

The genus currently comprises twenty-seven recognized species (*L. monocytogenes*, *L. grayi*, *L. innocua*, *L. seeligeri*, *L. welshimeri*, *L. ivanovii* (2 subspecies), *L. marthii*, *L. rocourtiae*, *L. fleischmannii* (2 subspecies), *L. weihenstephanensis*, *L. aquatica*, *L. cornellensis*, *L. floridensis*, *L. grandensis*, *L. riparia*, *L. booriae*, *L. newyorkensis*, *L. costaricensis*, *L. goaensis*, *L. thailandensis*, *L. valentina*, *L. cossartiae* (2 subspecies), *L. farberi*, *L. immobilis*, *L. portnoyi*, *L. rustica*, and *L. ilorinensis*), twenty-one of which have only been described since 2010 [21–33]. Recently, a strain isolated from soil collected in the Great Smoky Mountains National Park (US) was proposed as a new species named "Listeria swaminathanii" but has not been validly published yet [34,35]. All *Listeria* species so far described have been divided into two large clades due to genomic and phenotypic differences: *Listeria* sensu stricto (*L. monocytogenes*, *L. innocua*, *L. seeligeri*, *L. welshimeri*, *L. ivanovii*, *L. marthii*, *L. cossartiae*, *L. farberi*, *L. immobilis*, and "L. swaminathanii" [28]), and *Listeria* sensu lato, comprising species for which three new genera, "Murraya", "Mesolisteria" and "Paenilisteria", have been proposed [36,37]. During the last decades, some species of *Listeria* have also been subjected to misclassification. For instance, J. Rocourt et al., in 1987 [38], established a new taxa, *Jonesia denitrificans*, from a previously classified strain of *Listeria*, *Listeria denitrificans*. Also, J. Rocourt et al., in 1992 [39], using molecular techniques, established that *Listeria murrayi* and *Listeria grayi*, are, in fact, the same species, which led to the reclassification of this species as *Listeria grayi*.

Current methods for the identification of *Listeria* isolates according to EN ISO 11290-1:2017 [40] are based on classical cultures, which are tedious, take a long time to perform, and may show wrong or unconclusive results. Commonly used methods for *Listeria* detection are antibody-based lateral flow or enzyme immunoassays (EIA) as well as polymerase chain reaction (PCR)-based methods [10]. PCR is highly specific and sensitive and is more reliable than phenotypic methods [10] but does not characterize differences in the DNA sequence. Multilocus phylogenetic analysis (MLPA) is a highly discriminative approach to characterizing microbial isolates, which has been demonstrated to be very useful for species identification. This method uses the concatenated nucleotide sequences of several housekeeping genes to construct a phylogeny, which allows for a robust hierarchical classification of the species [41–44]. MLPA technology cannot be confused with MLST (multilocus sequence typing), which analyzes allelic differences for intra-species typing [45–48]. The concatenation of housekeeping genes allows for a more reliable evaluation of the evolution of the species, minimizing the weight of recombination events in the final phylogenetic tree [49]. Various studies have stated the capacity of MLPA as a tool to provide a robust phylogenetic frame and resolve the phylogeny of closely related species [42,48,50–55] and its capacity as a pragmatic technique for species identification.

In the present study, an MLPA of the genus *Listeria* that uses seven housekeeping gene fragments (*gyrA*, *cpn60*, *parE*, *recA*, *rpoB*, *atpA*, and *gyrB*; a total of 4375 bp) of representative strains belonging to all *Listeria* species described so far, has been developed. This MLPA scheme has been demonstrated to be a practical tool to rapidly identify *Listeria* isolates and may help in the control of *Listeria* pathogens in food and related environments.

2. Materials and Methods

2.1. Bacterial Strains

Listeria strains used in this study were received from the Colección Española de Cultivos Tipo (CECT, Valencia, Spain) (Table S1). Strains were maintained on TSA plates at 4 °C and stored in 30% glycerol in 0.9% NaCl with 10% TSB at −80 °C.

2.2. DNA Extraction and Purification

A single colony isolated from a fresh plate culture was resuspended in 100 µL TE buffer by vortexing. The tube was incubated at 95 °C for 5 min, vortexed, and kept for

5 min in dry ice. This process was repeated three times consecutively. The sample was centrifuged at 9500 rpm for 10 min, and 30 µL of the supernatant was transferred to a fresh tube and stored at −20 °C.

2.3. MLPA Genes and Primers

The MLPA was performed with housekeeping genes whose sequences were available on public databases. Most of them are involved in DNA/RNA processing functions. PCR and sequencing primers were designed for the seven housekeeping genes: DNA gyrase subunit A (*gyrA*); chaperonin-60 (*cpn60*); DNA topoisomerase IV subunit B (*parE*); homologous recombination factor (*recA*); DNA-directed RNA polymerase subunit beta (*rpoB*); F0F1 ATP synthase subunit alpha (*atpA*); and the DNA gyrase subunit B (*gyrB*) (Table 1). Primers were designed based on preliminary alignments of available gene sequences from different species of *Listeria* and related genera.

Table 1. Name of the genes used in this MLPA study, length of the amplified fragments, and primer sequences.

Locus	Encoded Protein	Amplified Fragment	Primers Code	Sequence (5′–3′)
gyrA	DNA gyrase subunit A	971 bp	Lis-gyrA-3F Lis-gyrA-13R2	GATGGACATGGTAACTTTG CGCATATCTAAAATGGCTTG
cpn60	chaperonin-60	931 bp	Lis-cpn60-2F Lis-cpn60-11R	GAATTAGAAGACCCATTTGA GTTTTGCTAAACGTTCTTGT
parE	DNA topoisomerase IV subunit B	928 bp	Lis-parE-0F′ Lis-parE-9R′	AATGATGATTCTATTCAGGTGC CGCGAATATCGCTACCYTC
recA	homologous recombination factor	757 bp	Lis-recA-1F Lis-recA-8R	TGAAAAACAATTCGGTAAAGG TGARATACCTTCWCCGTACA
rpoB	DNA-directed RNA polymerase subunit beta	1021 bp	Lis-rpoB-23F Lis-rpoB-33R	AGGATGCGATCATCATGAG GCTTCGTAAGTTTTCACACG
atpA	F0F1 ATP synthase subunit alpha	947 bp	Lis-atpA-2F Lis-atpA-11R	TATGGCCCAAAACTTAGAA TTTTCATTGCTTTAATTTGCG
gyrB	DNA gyrase subunit B	646 bp	Lis-gyrB-4F Lis-gyrB-10R	GCGGCGGCGGATATAAAGTA CCTTCACGAACATCTTCACC

2.4. PCR Amplification and Sequencing

Fragments of the selected genes, with lengths ranging from 640 to 1020 bp, were amplified by PCR using a peqSTAR 2X Thermocycler. PCR reaction mixtures contained 12.5 µL HotStart Mix Y (Peqlab Biotechnology GmbH, Erlangen, Germany), 1 µL of each primer (10 µM), 1 µL DNA, and 9.5 µL sterile ultrapure water, in a total volume of 25 µL. DNA amplification was performed with denaturation at 94 °C for 3 min, 35 cycles of amplification that consisted of denaturation at 94 °C for 15 s, annealing at 45 °C for 30 s, extension at 72 °C for 1 min, and a final extension step at 72 °C for 5 min. Amplified fragments were analyzed by electrophoresis on 1% agarose-TAE gels. PCR products were purified using the peqGOLD Cycle-Pure Kit (Peqlab Biotechnology GmbH, Germany), following the manufacturer's instructions. PCR fragments were sequenced by GATC (Eurofins Genomics) using the forward primers (5 µL of purified PCR product + 5 µL of 5 µM primer). Gene sequences from additional *Listeria* isolates and 16S rRNA coding-gene sequences from reference strains were downloaded from NCBI (National Center for Biotechnology Information) Nucleotide and Whole-genome shotgun contigs databases using BLAST (Basic Local Alignment Search Tool, NCBI) to identify sequences from our seven selected genes.

2.5. Single Gene and Multilocus Sequence Analysis

Pairwise sequence comparisons were performed for each individual gene sequence to obtain single-gene phylogenetic trees. For each analyzed strain, sequence fragments of the seven selected genes, beginning and ending at the same positions, were concatenated. Matrices of nucleotide differences and phylogenetic trees were obtained from the alignment of the concatenated sequences. Alignments were obtained with the MEGA5 program [56] by Clustal W [57]. Evolutionary distances were obtained using the Kimura 2-parameter method [58], and phylogenetic trees were constructed by the neighbor-joining [59] with the MEGA5 program [56] with 1000 bootstrap replicates. The same protocol was followed to obtain a reference 16S rDNA phylogenetic tree using downloaded sequences from type strains when available.

Ranges of intra-species and inter-species nucleotide substitutions were calculated by determining the maximum and minimum values (excluding identical sequences) of nucleotide substitutions from pairwise comparisons of strains for each *Listeria* species (intra-species) or between strains of different *Listeria* species (inter-species), including all isolates. Nucleotide substitutions corresponded to the percentage of base differences per position between each pair of compared sequences. All positions containing gaps were excluded from the analysis. Intra-species and inter-species phylogenetic depth was calculated by summing the nucleotide substitution percentages from pairwise comparisons and dividing by the total number of comparisons.

3. Results

Fragments from the seven selected genes, with lengths ranging from 458 bp to 833 bp, were amplified by PCR with primers listed in Table 1, using DNA extracted from 16 *Listeria* isolates from foods and 30 CECT *Listeria* strains listed in Table S1. Additionally, 172 sequences of other isolates from all *Listeria* species previously identified using BLAST were downloaded from the NCBI database. The fragments of the seven genes for each isolate were concatenated in the same order (*gyrA—cpn60—parE—recA—rpoB—atpA—gyrB*), giving rise to concatenated sequences of ca. 4375 bp. Sequences from each gene were aligned, and nucleotide differences for each pairwise comparison were obtained using the MEGA5 program [56] by Clustal W [57]. Percentages of sequence similarity values were calculated. The fragments of the seven genes, concatenated in the same order (*gyrA—cpn60—parE—recA—rpoB—atpA—gyrB*, 4375 bp), showed a sequence similarity ranging from 77.51% to 99.89%, which corresponded to 5–981 nucleotide differences and there was a total of 1805 variable positions (41.23% of the sequence). Sequences of the seven concatenated gene fragments were identical for *L. ivanovii* subsp. *londoniensis* CECT 5374^{T} and CECT 5375; *L. monocytogenes* CECT 936 and L56; *L. monocytogenes* L69 and L114; *L. monocytogenes* CECT 911, L103, L130 and L15; *L. seeligeri* CECT 941 and CECT 5339; *L. welshimeri* CECT 5371 and Lwell6, and *L. welshimeri* L79 and Lwell7. Those identical sequences were excluded from calculations of intra-species and inter-species nucleotide substitution ranges and intra-species and inter-species phylogenetic depths. Phylogenetic trees were obtained by the neighbor-joining method for the 16S rDNA sequences (Figure 1) and the concatenated housekeeping-gene sequences (Figure 2).

The phylogenetic trees obtained from the concatenated protein-coding gene sequences showed four large clusters, which is in agreement with the previous phylogenetic studies based on whole-genome sequencing data [21–23,34,37]. All species considered *Listeria* sensu stricto appeared in a cluster that included *L. monocytogenes*, *L. innocua*, *L. seeligeri*, *L. welshimeri*, *L. ivanovii*, *L. marthii*, *L. cossartiae*, *L. farberi*, *L. immobilis*, and non-yet validated, "L. swaminathanii". The rest of the *Listeria* species, most described since 2010, were distributed into three clusters clearly separated from each other and, altogether, were previously so-called *Listeria* sensu lato [37]. This result was in agreement with the previous proposal of three additional genera: "Murraya"; "Mesolisteria"; and "Paenilisteria" [21]. The phylogenetic tree obtained from the reference 16S rDNA sequences was in agreement with this distribution of species into four large clusters.

All isolates sequenced in the present study belonged to *Listeria* sensu stricto, which included the pathogenic species *L. monocytogenes* and *L. ivanovii*, and thus, this group was analyzed in greater depth. For this purpose, additional sequences of *Listeria* sensu stricto were selected and retrieved for the NCBI data. The concatenated fragments of the seven genes (*gyrA—cpn60—parE—recA—rpoB—atpA—gyrB*; a total of 4375 bp) showed a sequence similarity ranging from 88.71% to 99.98%, which corresponded to 1–492 nucleotide differences, and there was a total of 1093 variable positions (24.98% of the sequence). For each *Listeria* sensu stricto species, ranges of intra-species and inter-species nucleotide differences were determined, and intra-species and inter-species phylogenetic depth values were calculated (Figure 3), as detailed in Materials and Methods. Ranges of intra- and inter-species nucleotide similarities (%) for the concatenated sequence only overlapped for the species *L. monocytogenes* and *L. cossartiae*. Overall, a similar picture was obtained from each single-gene phylogeny (Figures S1–S7), indicating that the obtained clusters all showed similar phylogenetic relationships, or, in other words, the genes evolved in concert. The MLPA tree derived from the alignment of the concatenated sequence (Figure 4) showed clearly defined clusters for the *Listeria* sensu stricto species with bootstrap values of 100%, except for *L. monocytogenes* that was divided into four subclusters also sustained by 100% bootstrap, which corresponded to the four genetic lineages previously described.

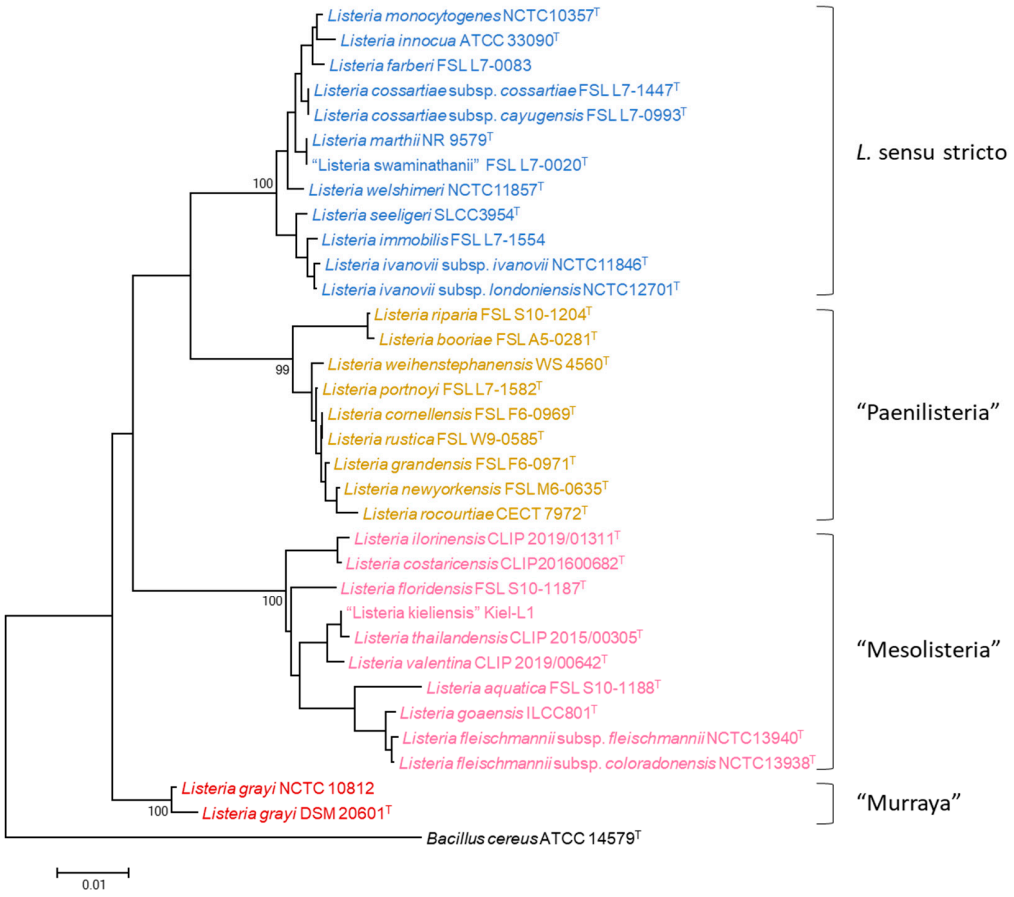

Figure 1. Neighbor-joining phylogenetic tree based on the analysis of 16S rRNA gene sequences (935 bp) of all described *Listeria* sensu stricto and *Listeria* sensu lato species, routed using *Bacillus cereus*. Numbers at nodes indicate bootstrap values (percentage of 1000 replicates). ^{T}—type strains.

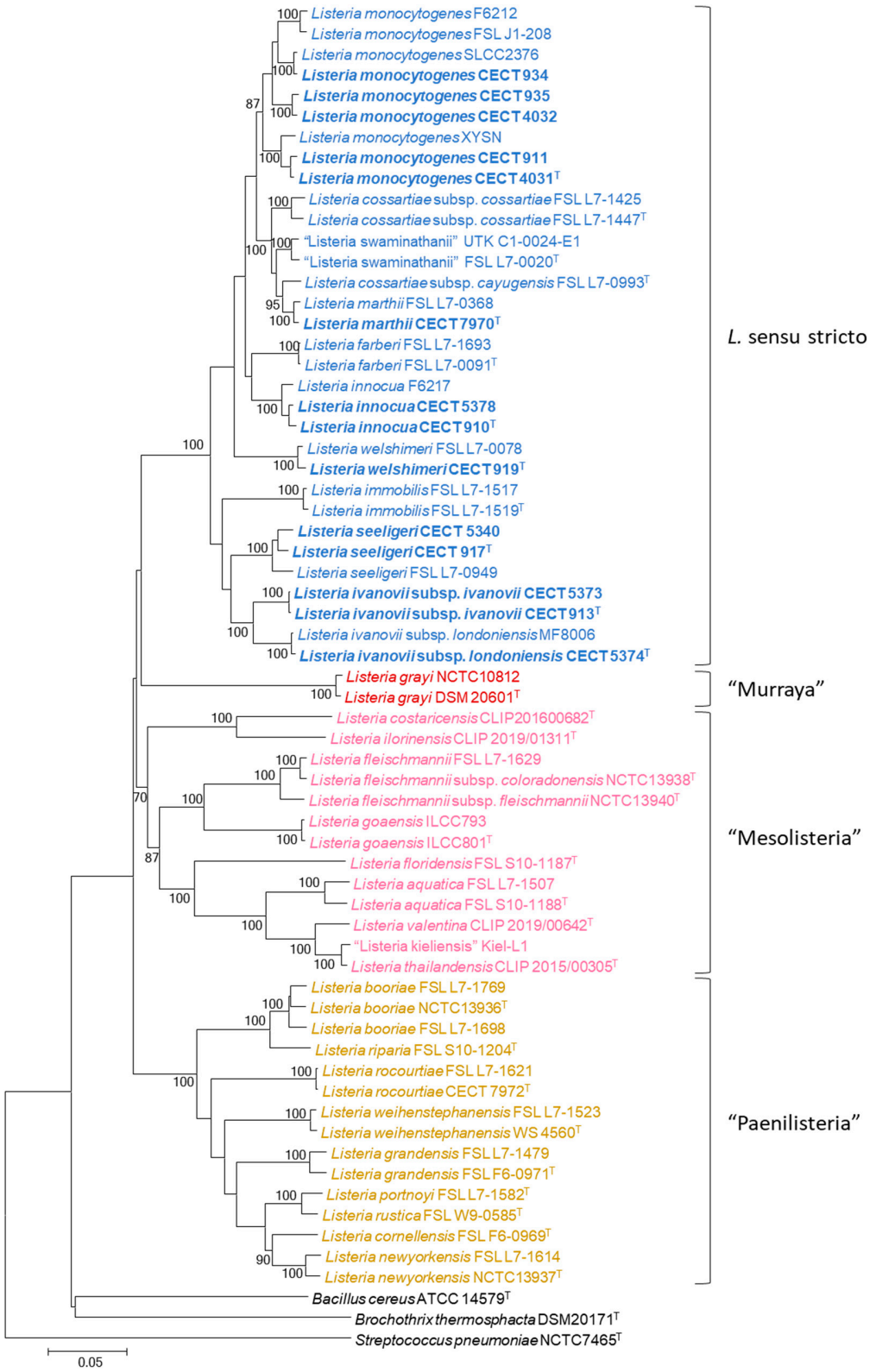

Figure 2. Neighbor-joining phylogenetic tree based on the MLPA from seven concatenated housekeeping genes (*gyrA*, *cpn60*, *parE*, *recA*, *rpoB*, *atpA* and *gyrB*; a total of 4375 bp) of strains of all described *Listeria* sensu stricto and sensu lato species, *Bacillus cereus*, *Brochothrix thermosphacta*, and *Streptococcus pneumoniae*. Numbers at nodes indicate bootstrap values (percentage of 1000 replicates). Strains sequenced in this study are shown in bold. ^{T}—type strains.

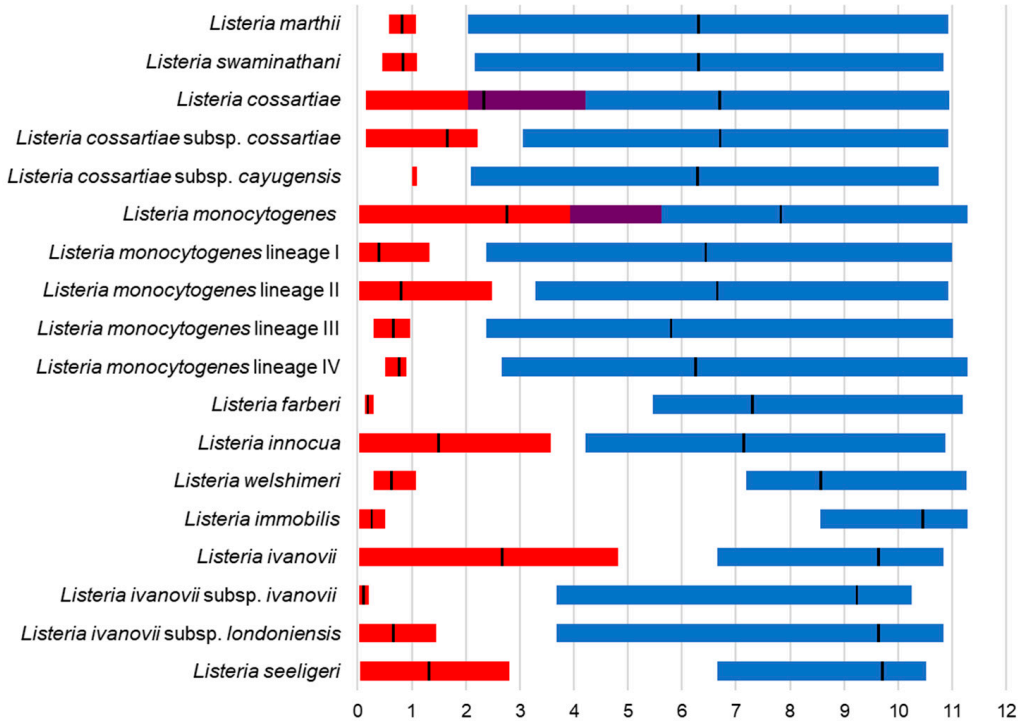

Figure 3. Graphical representation of the ranges of intra- and inter-species nucleotide substitution percentages in red and blue bars, respectively, and intra- and inter-species phylogenetic depth (black) for the concatenated seven-gene sequence, calculated for all *Listeria* sensu stricto species and subspecies and *L. monocytogenes* genetic lineages.

The concatenated sequence of the seven housekeeping genes was downloaded from the NCBI database for 169 *L. monocytogenes* strains, the genetic lineage of which had been previously determined [60]. The phylogenetic tree showed that all four *L. monocytogenes* lineages formed clearly defined clusters with bootstraps of 100%, confirming again that all strains were correctly clustered according to their lineage (Figure 5; only non-redundant sequences are shown). Ranges of intra- and inter-sequence similarity for each of the four lineages did not overlap (Figure 3). For *L. monocytogenes* strains of the four genetic lineages, the concatenated sequence showed an overall sequence similarity ranging from 94.37% to 99.98%, which corresponded to 1–245 nucleotide differences, and there was a total of 434 variable positions (9.92% of the sequence). *L. ivanovii* subsp. *ivanovii* and *L. ivanovii* subsp. *londoniensis* formed two different clusters that were quite divergent from each other. However, *L. cossartiae*, *L. marthii*, and "L. swaminathanii" were very closely related, finding that *L. cossartiae* subsp. *cayugensis* was closer to *L. marthii* than to *L. cossartiae* subsp. *cossartiae*.

The MLPA allowed for the identification of *Listeria* isolates of food origin obtained from the CECT (Figure 4). Isolates L56, L69, and L114 clustered in the MLPA with *L. monocytogenes* lineage I and L15, L49, L103, and L130 with *L. monocytogenes* lineage II. Isolates L51, L52, L123A, L138, Linn8, Linn16, and CECT 5372 clustered with *L. innocua* strains. CECT 5372 had been identified as *L. welshimeri* by the CECT. Isolates L79, Lwell6, and Lwell7 clustered with *L. welshimeri*. Strains CECT 939 and CECT 941, which were previously classified as *L. monocytogenes*, clustered with *L. seeligeri*. Nevertheless, CECT 939 has lately been discontinued. *L. ivanovii* CECT 5379 clustered with *Listeria ivanovii* subsp. *ivanovii*. *L. ivanovii* subsp. *ivanovii* CECT 5368 clustered with *Listeria ivanovii* subsp. *londoniensis*.

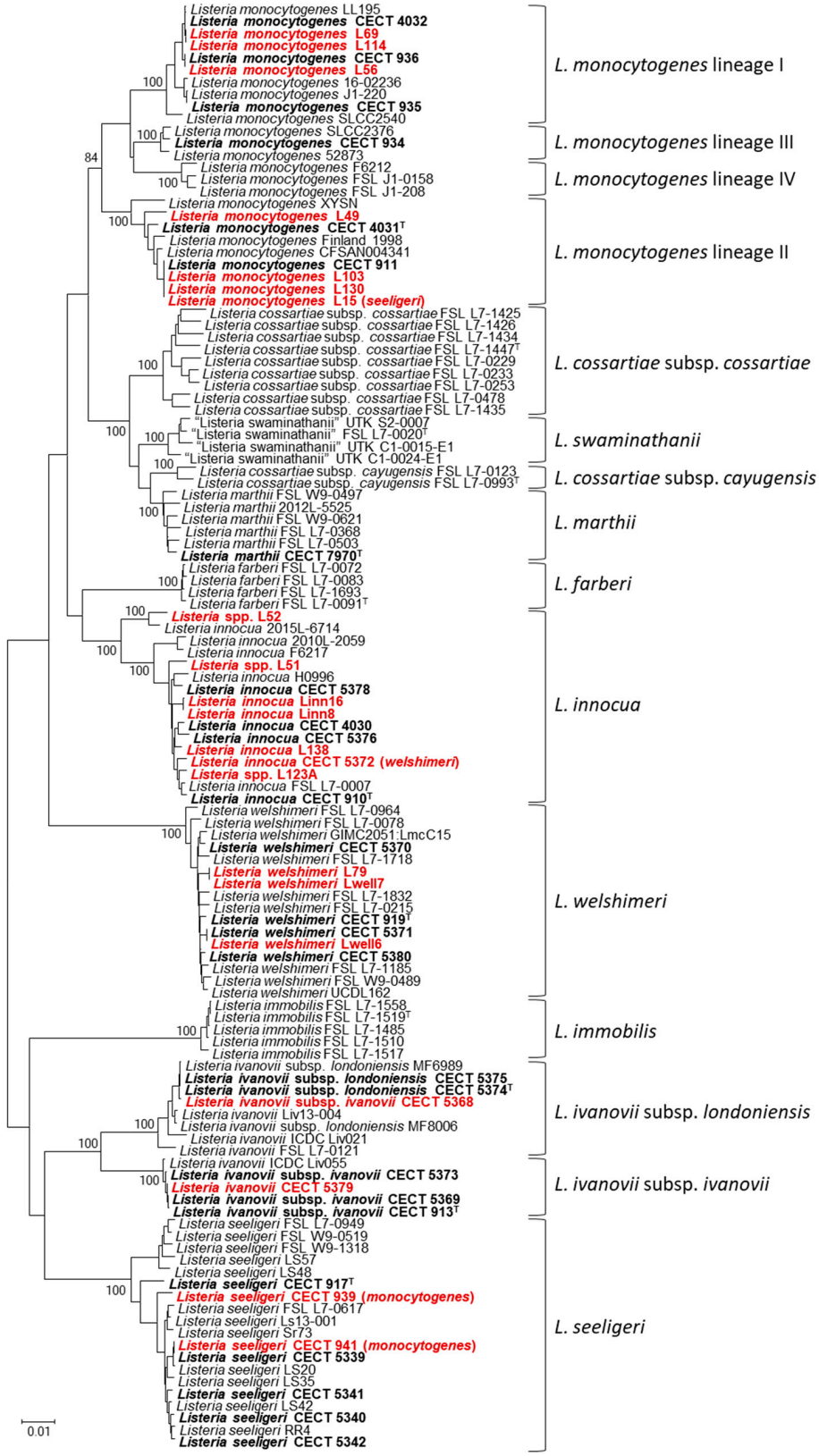

Figure 4. Neighbor-joining phylogenetic tree based on the MLPA from seven concatenated housekeeping genes (*gyrA*, *cpn60*, *parE*, *recA*, *rpoB*, *atpA*, and *gyrB*; a total of 4375 bp) of strains of all described *Listeria* sensu stricto, including non-characterized isolates. Numbers at nodes indicate bootstrap values (percentage of 1000 replicates). Strains sequenced in this study are shown in bold and *Listeria* isolates identified in this study are shown in red. ^{T}—type strains.

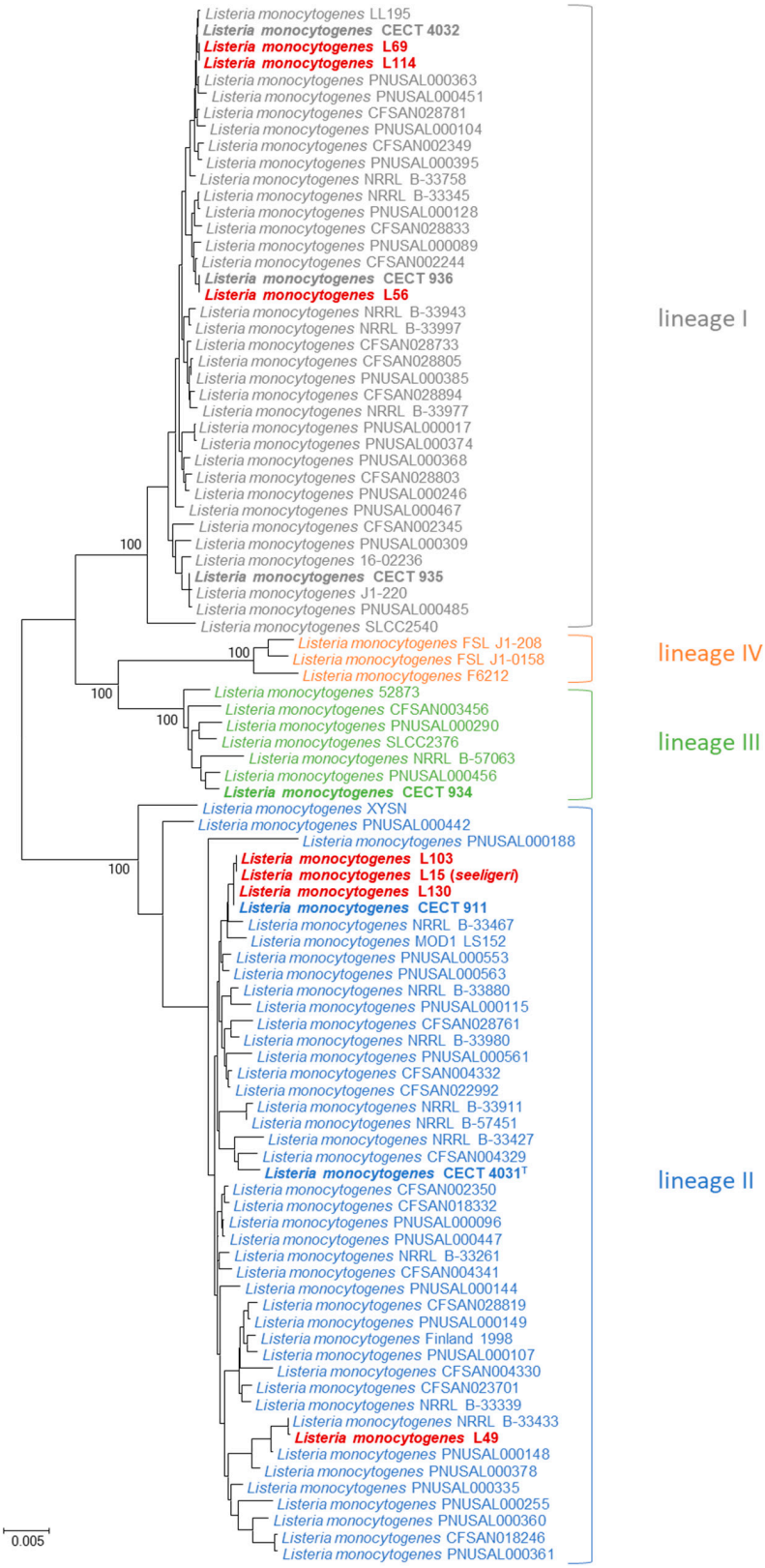

Figure 5. Neighbor-joining phylogenetic tree based on the MLPA from seven concatenated housekeeping genes (*gyrA*, *cpn60*, *parE*, *recA*, *rpoB*, *atpA*, and *gyrB*; a total of 4375 bp) of strains from the four *L. monocytogenes* genetic lineages. Numbers at nodes indicate bootstrap values (percentage of 1000 replicates). Strains sequenced in this study are shown in bold and *Listeria* isolates identified in this study are shown in red. ^{T}—type strains.

4. Discussion

The MLPA strategy to infer phylogenetic bacterial identification based on several housekeeping loci assumes a synchronized evolutionary pattern in genomes, where fundamental genes evolve in concert. Consequently, the concatenated multigene phylogeny can serve as a 'mirror' reflecting the overall relationships within the entire genomic content [42,44,48,50–54]. Sequences of housekeeping genes coding for proteins, particularly those involved in DNA processing (i.e., replication, transcription, translation, etc.), are phylogenetic markers suitable for species discrimination with a high resolution [55,61–63]. Mainly, this is because they are subjected to a degenerative code when translated into proteins, and, therefore, nucleotide changes can occur throughout the gene without consequences in the amino acid sequence. This mode of evolution contrasts with that of rRNAs, which exhibit a mosaic of discrete variation at some signature regions, making the protein-coding genes the best candidates for distinguishing closely related strains.

4.1. MLPA of the Genus Listeria

A comprehensive multilocus phylogenetic analysis (MLPA) of a diverse representation of the genus *Listeria* has been approached to provide a framework for simple, easy, and fast species identification from isolated colonies, as has been performed for other genera [64–66]. Both concatenated seven-gene MLPA and 16S rDNA phylogenetic trees showed that species of *Listeria* sensu stricto clustered together in a group separated from other species currently recognized as *Listeria* sensu lato (Figures 1 and 2). This result was in agreement with previous studies using genomic data [36,37] and proposed new genera, "Murraya", "Mesolisteria", and "Paenilisteria", to accommodate *Listeria* sensu lato species. "Murraya" only contained the species *Listeria grayi*. The group named "Mesolisteria" was formed by a very diverse species not able to grow at 4 °C [37], *L. costaricensis*, *L. ilorinensis*, *L. fleischmannii*, *L. goaensis*, *L. floridensis*, *L. aquatica*, *L. valentina*, *L. thailandensis*, and the non-validated species "L. kieliensis" [67], which was found highly related to *L. thailandensis*. And finally, the cluster containing *L. booriae*, *L. riparia*, *L. rocourtiae*, *L. weihenstephanensis*, *L. grandensis*, *L. portnoyi*, *L. rustica*, *L. cornellensis*, and *L. newyorkensis* corresponded to this previously proposed to be reclassified in the genus "Paenilisteria" [37]. The mere fact that the majority of species described in recent decades do not belong to the genus *Listeria* in the strict sense reaffirms the need to provide a framework for identifying isolates based on a tool as decisive as the MLPA. Additionally, this MLPA can also identify *L. monocytogenes* genetic lineages (Figure 5), being useful as an intra-species typing method, similar to MLST. The four genetic lineages have different characteristics but do not allow us to determine the serotype of the strains, as each lineage can correspond to different serotypes [12]. All cultures, isolates, and reference strains used in this study were identified by MLPA as species of *Listeria* sensu stricto (Figures 4 and 5) and, therefore, were subjected to a deeper analysis.

4.2. MLPA Clustering of Listeria Sensu Stricto

The clustering of *Listeria* species based on single-gene phylogenies of *gyrA*, *cpn60*, *parE*, *recA*, *rpoB*, *atpA*, and *gyrB* were consistent with each other. However, the topology of the phylogenetic trees at the deep branching points (or nodes) varied, likely due to differences in mutation rates between genes. For instance, the *parE*-based tree showed the type strain *L. monocytogenes* 4031^{T} more affiliated to the *L. cossartiae*, although it was branched at the expected position in all the other single-gene phylogenies. However, for a pragmatic taxonomy of the *Listeria* species, the MLPA approach, which grouped strains into well-defined clusters with high bootstrap support (close to 100%), was considered highly valuable. Although the concatenated sequence for some species displayed slight variations in nucleotide substitution ranges, intra-species phylogenetic depths, and inter-species phylogenetic distances (Figure 3), most species showed non-overlapping ranges of intra- and inter-species sequence similarities, indicating that they were well-delimited species, clearly separated from each other. These range values overlapped for the species *L. monocytogenes* and *L. cossartiae*, indicating that intra-species diversity was higher than

inter-species relationships; for example, some strains of *L. cossartiae* may be more related to other species. However, if the analysis of intra- and inter-species values is performed for the two subspecies of *L. cossartiae* (*L. cossartiae* subsp. *cossartiae* and *L. cossartiae* subsp. *cayugensis*) or the four lineages of *L. monocytogenes*, no overlapping was observed indicating that they were entities clearly separated from the other species of *Listeria* sensu stricto. Indeed, these lineages are quite divergent and can be considered different subspecies, as proposed by Orsi et al. in 2011 [12]. *L. monocytogenes* XYSN strain, which has been classified as belonging to the hybrid sub-lineage II [68], clustered borderline of lineage II but was distantly related. Moreover, the two sequences available of *L. cossartiae* subsp. *cayugensis* were phylogenetically closer to *L. marthii* than to *L. cossartiae* subsp. *cossartiae*. *L. ivanovii* subsp. *ivanovii* and *L. ivanovii* subsp. *londoniensis* are relatively divergent (3.67–4.82% inter-cluster nucleotide substitutions in the 4375 bp concatenate sequence) and form two well-delineated clusters, supported by 100% bootstrap values in our MLPA (Figure 4). These two subspecies of *L. ivanovii* could be close to being considered as different species. However, *L. marthii*, *L. cossartiae*, and "L. swaminathanii" were found to be very closely related and, perhaps, in future studies, should be reclassified into a single species.

4.3. Identification of Listeria Isolates

The MLPA performed in the present study allowed for the identification of *Listeria* isolates subjected to analysis (Figures 4 and 5). Isolates L56, L69, and L114 clustered in the MLPA with *L. monocytogenes* lineage I and L15, L49, L103, and L130 with *L. monocytogenes* lineage II. None were identified as *L. monocytogenes* lineages III and IV. This finding was in agreement with previous reports, indicating that most strains isolated worldwide belonged to lineages I and II [17], the first more frequently isolated from humans, while the second usually recovered from food and environmental samples [18,19]. The capacity of MLPA to discriminate between the four *L. monocytogenes* lineages seems of good value in ascertaining if they belong to different levels of virulence in future studies. We should remark that isolate L15 (*L. monocytogenes* lineage II) was received as *L. seeligeri* according to partial 16S sequencing. Isolates L51, L52, L123A, L138, Linn8, Linn16, and strain CECT 5372 clustered with the cluster corresponding to *L. innocua*. Strain L52, together with closely related *L. innocua* 2015L-6714, clustered at a border-line position of the cluster containing the rest of *L. innocua* strains; however, they clustered close to some strains of *L. monocytogenes* in *cpn60*, *rpoB*, and *gyrB* single-gene phylogenies. Strain CECT 5372, obtained from CECT as *L. welshimeri* clearly clustered within the *L. innocua* species. Isolates L79, Lwell6, and Lwell7 clustered with *L. welshimeri*. Strains CECT 939 and CECT 941, classified as *L. monocytogenes*, clustered with *L. seeligeri*. As a consequence, CECT 939 is no longer available. *L. ivanovii* CECT 5379, whose subspecies was not identified previously, clustered with *L. ivanovii* subsp. *ivanovii*. *L. ivanovii* subsp. *ivanovii* CECT 5368 clustered with *L. ivanovii* subsp. *londoniensis*. Almost the same identification results from MLPA can be obtained when using some of these single-gene phylogenies, for instance, when using *recA* or *gyrA* phylogenies. Whole-genome sequencing (WGS) is a powerful tool for new species description and phylogenetic analysis, but it is more expensive and time-consuming than MLPA for the identification of *Listeria* isolates. However, WGS also allows us to determine the serotype, antimicrobial-resistant genes, mobile genetic element, and virulence markers and perform clustering methods (MLPA, MLST) [10,69–71]. Once this MLPA framework shows all these genes evolve in concert (i.e., the same phylogenetic relationships were obtained), single-gene sequencing may be very useful as a fast, cheap, and simple identification approach for preliminary screening of isolates. The simultaneous sequencing of a second housekeeping gene, as confirmatory identification, is highly recommended.

5. Conclusions

Listeria currently comprises a complex group of species, some of which are highly virulent, often transmitted in the food chain, and of concern to public health. During the last decades, a number of new taxa have been wrongly described as belonging to

the genus *Listeria*. Therefore, unequivocal and fast identification of isolates represents a challenge, particularly in food control. Once *Listeria* spp. has been identified and isolated following the international norm EN ISO 11290-1:2017 [40], this MLPA approach could be employed for species identification, as it has demonstrated that simple housekeeping gene sequencing represented a powerful and affordable methodology for accurate species identification. All isolates identified in the present study belonged to species of *Listeria* sensu stricto; in particular, some of them were classified as *L. monocytogenes* lineages I and II, both considered virulent phylogenetic groups typically found in clinical and food samples.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/microbiolres15040142/s1, Figures S1–S7: Single-gene phylogenetic neighbor-joining trees of *Listeria* sensu stricto species; Table S1: List of *Listeria* strains used for sequencing.

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