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1 **Whole genome-based population biology**
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3 **of *Listeria monocytogenes***
4
5

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34

35 **Abstract**

36 *Listeria monocytogenes* (*Lm*) is a major human foodborne pathogen. Numerous *Lm*
37 outbreaks have been reported worldwide, associated with high case fatality rate,
38 reinforcing the need for strongly coordinated surveillance and outbreak control. We
39 developed a universally applicable genome-wide strain genotyping approach and
40 investigated the population diversity of *Lm* using 1,696 isolates from diverse
41 sources and geographical locations. We define, with unprecedented precision, the
42 population structure of *Lm*, demonstrate the occurrence of international circulation
43 of strains, and reveal the extent of heterogeneity in virulence and stress resistance
44 genomic features among clinical and food isolates. Using historical isolates, we show
45 that the evolutionary rate of *Lm* from lineage I and lineage II is low ($\sim 2.5 \times 10^{-7}$
46 substitutions per site per year, as inferred from the core genome) and that major
47 sublineages (corresponding to so-called 'epidemic clones') are estimated to be at
48 least 50 to 150 years old. This work demonstrates the urgent need of monitoring *Lm*
49 strains at the global level and provides the unified approach needed for global
50 harmonization of *Lm* genome-based typing and population biology.

51

52 **Introduction**

53 Pathogens know no border and can cause multi-country outbreaks and
54 pandemics,^{1,2} emphasizing the importance of international coordination for
55 infectious diseases surveillance.³ Microbiological surveillance programs rely on the
56 continuous monitoring of circulating genotypes in space and time, enabling the
57 rapid detection of common-source clusters and the implementation of control
58 measures.⁴ Despite outstanding exceptions,⁵⁻⁷ most pathogens are so far
59 monitored only at the national level. The lack of international coordination implies
60 that outbreaks affecting multiple countries are either not detected or not
61 controlled optimally.^{3,4} International and cross-sector surveillance of pathogens
62 requires strain subtyping methods that combine high resolution, reproducibility
63 and exchangeability, so that epidemiologically relevant groups of matching isolates
64 can be rapidly recognized across space and time.⁴ Besides, harmonized and
65 universally shared strain nomenclatures, which must be rooted in the
66 microorganism population biology, are a prerequisite for rapid detection and
67 efficient communication on emerging strain types.

68 The foodborne pathogen *Listeria monocytogenes* (*Lm*) causes listeriosis, a human
69 systemic infection characterized by septicemia, central nervous system and
70 maternal-fetal invasion, with high hospitalization and fatality rates.⁸ Less severe
71 manifestations include gastroenteritis⁹ and may often remain undiagnosed. In the
72 PulseNet program,⁶ the microbiological typing golden standard, pulsed-field gel
73 electrophoresis (PFGE), has been standardized internationally, but naming of
74 profiles is not coordinated between the different international PulseNet networks.
75 Furthermore, PFGE does not reflect evolutionary relationships and certain profiles

76 are highly prevalent leading to insufficient discriminative power. In contrast,
77 multilocus sequence typing (MLST) based on seven genes provides highly
78 standardized genotypes and nomenclature,¹⁰⁻¹² but lacks the discriminatory power
79 required for epidemiological surveillance of most bacterial pathogens. Advances in
80 high-throughput sequencing technologies have established whole genome
81 sequencing (WGS) as a powerful epidemiological typing tool^{1,13,14} that has been
82 applied to investigate outbreaks and *Lm* contamination of food production
83 plants.¹⁵⁻²⁰ However, these studies were restricted to local or national levels and a
84 relatively small number of isolates.

85 Wide-range transmission of *Lm* strains can occur through international food
86 trade²¹ and the major MLST-defined clonal complexes (CCs) of *Lm* are distributed
87 globally.¹¹ However, the rate of evolution of *Lm* genomes and the speed at which
88 strains can spread over large distances are currently unknown. Further, a global
89 view of the relationships between genotype and virulence potential of *Lm* strains
90 remains to be established.

91 To enable population biology studies of global *Lm* collections and for prospective
92 international epidemiological surveillance, a harmonized protocol to translate
93 genomic sequence into its corresponding nomenclatural genotype needs to be
94 established. Although single-nucleotide polymorphism (SNP)-based approaches
95 can provide maximal discrimination,²⁰ they are difficult to standardize and can be
96 difficult to interpret.^{18,22} In contrast, genome-wide MLST approaches rely on well-
97 defined standard sets of hundreds of genes that can be validated *a priori* for strain
98 genotyping.^{14,19,23,24}

99 Here we developed a core genome MLST (cgMLST) method for *Lm* and applied it to
100 a large number of strains from a wide spectrum of geographic, temporal and
101 epidemiological origins. This enabled us to decipher the population structure and
102 evolutionary rate of *Lm*, to demonstrate international transmission of major
103 sublineages, and to develop a unified genome-based nomenclature of *Lm* strains
104 accessible through an open bioinformatics platform, allowing international
105 collaboration on research and public health surveillance based on high-throughput
106 genome sequencing.

107

108 **Results**

109 ***Universal Lm cgMLST***

110 A core genome MLST (cgMLST) scheme of 1,748 loci was defined based on a high
111 level of conservation of this set of genes among 957 genomes of diverse origins
112 (**Supplementary Information 2.1**). Using an independent set of 650
113 prospectively collected isolates to estimated typeability⁴, each of these genes could
114 be detected in 644 genomes (99.1%) on average, resulting in half of the genomes
115 having 8 or fewer uncalled alleles (average±standard deviation of uncalled alleles
116 15±20; **Supplementary Information 2.1**). These results demonstrate the
117 universal applicability of this cgMLST scheme for *Lm* strain genotyping.

118 Reproducibility of allele calls based on genomic sequences obtained from
119 independent cultures and sequencing protocols of the reference EGD-e strain was
120 absolute (error rate <0.029%, *i.e.* <1 error in 3,496 allelic comparisons). cgMLST
121 genotyping was also reproducible irrespective of assembly pipeline for coverage
122 depths ≥40 (with per-site Phred quality score ≥20, *i.e.* corresponding to ≥99% base
123 accuracy) and *de novo* assembly allele calls were identical to assembly-free
124 methods (**Supplementary Information 2.4**). Altogether, the cgMLST scheme
125 developed herein constitutes an extremely robust genotyping method, even when
126 applied on a very wide variety of *Lm* strains sequenced from diverse sources and
127 geographical locations.

128

129 ***Definition of cgMLST types***

130 To provide a definition of cgMLST types (CTs) that would be maximally useful for
131 surveillance purposes, we compared the genetic heterogeneity between

132 epidemiologically related isolates on the one hand, and between isolates with no
133 documented epidemiological link on the other hand. Pairwise allelic mismatches
134 revealed two distinct distributions (**Fig. 1A**). First, most isolates sampled during
135 investigations of single outbreaks had seven or fewer allelic mismatches (**Fig. 1A**).
136 Among these, pairs of isolates from vertical maternal-neonatal transmission cases
137 had no allelic differences (not shown). Second, taking into account the entire
138 dataset (**Fig. 1A**), a sharp discontinuity was observed, with few pairs of isolates
139 having between 7 and 10 allelic mismatches, showing that isolates with no
140 documented epidemiological link differed most generally by more than 10
141 mismatches. Clustering efficiency was optimal when using a cut-off value of 7.3
142 allelic mismatches (*i.e.* 0.414% of mismatched loci; **Supplementary Information**
143 **2.7**). Therefore, we propose to define CTs as groups of cgMLST profiles that differ
144 by up to 7 allelic mismatches out of 1,748 loci (*i.e.*, in case of uncalled alleles,
145 0.400% of mismatched loci among those that are called in both profiles), from at
146 least one other member of the group.

147

148 ***Comparison of cgMLST and PFGE genotyping***

149 PFGE is the current reference method for *Lm* epidemiological surveillance and
150 outbreak investigation.⁶ Among the 100 *Lm* isolates used for cgMLST and PFGE
151 comparison, only 36 distinct *AscI-ApaI* combined PFGE profiles (Simpson's
152 diversity index = 0.944, 95% confidence interval CI = [0.926, 0.963]) were
153 identified, whereas cgMLST distinguished 68 CTs (Simpson's index = 0.987; 95% CI
154 = [0.981, 0.994]). This indicates that cgMLST greatly improves discrimination
155 among *Lm* isolates as compared with PFGE ($p < 0.001$; **Supplementary**

156 **Information 2.5).** Consistent with this, PFGE did not subtype any CT, whereas
157 multiple PFGE types could be subdivided using cgMLST (adjusted Wallace index of
158 concordance = 0.215; 95% CI = [0.156, 0.304]). Retrospective analysis indicated no
159 epidemiological link among isolates that were grouped by PFGE but not by cgMLST
160 (NRC and InVS, France). These results are consistent with previous work that
161 reported improved discrimination of genome sequence typing over PFGE,^{18,19} and
162 our collective unpublished experience covering more than one year with WGS for
163 real-time surveillance of listeriosis in Denmark, France, the United Kingdom and
164 the United States. Implementation of cgMLST in *Lm* surveillance therefore shows
165 great promise to improve the definition of clusters of cases, thus facilitating
166 investigations of contamination sources.

167

168 ***Phylogenetic structure and nomenclature of *Lm* sublineages***

169 A unified nomenclature of *Lm* subtypes is critically needed for real-time exchange
170 of information on the emergence and geographic dispersal of strains. To provide
171 an optimized subtype definition, we analyzed the phylogenetic structure of *Lm*.
172 The four major phylogenetic lineages of *Lm* were clearly separated (**Fig. 2A**).
173 cgMLST-based clustering of isolates into lineages and their sublineages was highly
174 concordant with the sequence-based phylogenetic tree (**Fig. 2B**). Whereas the
175 strains of lineages III and IV (which are rarely isolated in the context of
176 surveillance) were scattered into multiple rare sublineages, lineages I and II were
177 strongly structured into major sublineages, each comprising multiple closely-
178 related isolates (**Fig. 3A**). Two atypically divergent sublineages within lineage II

179 were identified (sublineages SL842 and SL843, **Fig. 2A**), showing that lineage II is
180 more diverse than previously reported.

181 The observed trimodal distribution of allelic mismatches among all pairs of
182 isolates (**Fig. 1A**) was consistent with phylogenetic structure: isolates belonging to
183 distinct major phylogenetic lineages differed by 1,500 loci or more out of 1,748
184 loci, isolates from different sublineages within a given lineage typically showed
185 between 1,000 and 1,400 allelic differences, and most isolates within the same
186 sublineage were up to 150 allelic mismatches distant. Moreover, clustering
187 efficiency was optimal between 140 and 150 allelic mismatches (**Supplementary**
188 **Information 2.7**). Therefore, a threshold of 150 allelic mismatches (8.58%
189 dissimilarity) was chosen to define sublineages. This cut-off value led to the
190 identification of 163 sublineages. Remarkably, the flat rarefaction curve obtained
191 for sublineages within lineages I and II suggests that this study has captured most
192 of the phylogenetic sublineages of these two epidemiologically major lineages (**Fig.**
193 **1B**). In contrast, the almost linear rarefaction curve of CT richness indicates that
194 the 1,013 CTs sampled represent only a small fraction of those expected to be
195 uncovered upon further sampling (**Fig. 1B**), underlining the fine subtyping power
196 of cgMLST and its ability to subdivide *Lm* biodiversity into a multitude of
197 epidemiologically relevant genotypic groups.

198 We next analyzed the correspondence of sublineages with classical 7-genes MLST
199 nomenclature.¹⁰⁻¹² Whereas 156 sequence types (STs) have been previously
200 defined in the Institut Pasteur MLST database (now in BIGSdb-*Lm*,
201 <http://bigsdb.pasteur.fr/listeria>), 63 new ones were identified, revealing a
202 significant amount of novel diversity of *Lm* strains. MLST-defined CCs were

203 mapped onto the cgMLST-based phylogenetic structure (**Supplementary**
204 **Information 2.7**), largely revealing a one-to-one correspondence with cgMLST
205 sublineages. Therefore, the MLST nomenclature was mapped onto sublineages
206 where possible (**Supplementary Table 4**). As expected, frequent sublineages
207 corresponded to previously recognized major MLST clones.^{10-12,25,30} As a result, the
208 sublineage cgMLST-based nomenclature can be easily matched with the widely
209 used MLST nomenclature, which remains a valuable tool for first line identification
210 of sublineages.³¹

211

212 ***Evidence for international spread of *Lm* strains***

213 To investigate international transmission of *Lm* strains, we first mapped the
214 geographic origin of isolates onto the phylogeny (**Fig. 3A**). All sublineages
215 represented by more than 50 isolates were recovered from at least four distinct
216 countries (**Fig. 3B**). Using a stochastic mapping approach to reconstruct ancestral
217 states, we estimated the average number of cross-country transmission events as
218 ranging from 13 to 48 in the 10 most frequent sublineages (**Fig. 3C and 3D**). These
219 results show that subsequent to the evolutionary origin of major sublineages,
220 geographical shifts have occurred repeatedly. When normalizing the number of
221 geographical transitions by taking into account the number of isolates and
222 evolutionary time (jointly approximated by total tree length), large differences in
223 cross-country transition rates were apparent (**Fig. 3D**). Interestingly, the most
224 food-associated sublineages SL9 and SL121²⁵ had among the highest geographical
225 transition rates.

226 To investigate international transmission at a more recent epidemiological time-
227 scale, we searched for internationally distributed CTs. Interestingly 9 CTs,
228 comprising a total of 34 isolates, included isolates from at least two countries (**Fig.**
229 **4, Supplementary Table 6**). These results demonstrate the international
230 distribution of genotypic groups of *Lm* isolates that exhibit levels of genetic
231 divergence typical of those observed within documented outbreaks and
232 transmission events.

233

234 ***Temporal accumulation of variation within *Lm* lineages and outbreaks***

235 Phylogenetic analysis of the most prevalent sublineage (SL1) (**Supplementary**
236 **Table 5**) showed that the root-to-tip distances were significantly associated
237 ($p < 0.0001$, F-test) with the isolation year of isolates (**Fig. 5B**). The inferred slope of
238 the linear regression indicated an accumulation of 0.23 allelic mismatches per
239 cgMLST profile (i.e., 1.58 Mb) per year. BEAST analysis of the concatenated
240 multiple sequence alignments confirmed the existence of a temporal signal
241 (**Supplementary Information 2.8**) and estimated an evolutionary rate of 2.6×10^{-7}
242 subst/site/year (0.41 subst/1.58Mb/year), i. e. 1 substitution on the core genome
243 every 2.5 years (95% HPD=[1.9-3.4]). We also estimated independently the rate of
244 SL9, as a representative of major lineage II. Remarkably, the SL9 rate was 2.4×10^{-7}
245 subst/site/year (0.38 subst/1.58Mb/year), indicating a highly similar rate in SL1
246 and SL9. These results demonstrate measurable evolution of *Lm* genomes over a
247 few decades and provide an estimate of the short-term rate of accumulation of
248 genetic variation in representative sublineages of the two major lineages of *Lm*.
249 Based on the hypothesis that the substitution rate is conserved in *Lm*, we

250 estimated that the root of the other major sublineages was 50 to 150 years old
251 (**Supplementary Information 2.8**). Note that these estimates must be taken with
252 care: the rate may vary in some sublineages, and it is likely that our sampling has
253 missed some divergent branches, implying that our estimates are minimal ages.
254 Nevertheless, our current age estimates suggest an expansion of major sublineages
255 in modern times. Whether the dissemination of *Lm* was driven by an increase in
256 the intensity of exchange of people, animals and food in recent times is an
257 intriguing possibility.

258 *Lm* can survive for long periods of time in various sources, where genetic
259 diversification from a single population founder can occur.^{15,20,32} Consistently, we
260 observed that allelic divergence within outbreak sets and international clusters
261 was positively associated with the time span between the first and last isolate
262 collected ($p < 0.05$, F-test; **Fig. 5C**), with an accumulation of 0.28 allelic mismatch
263 per year, highly consistent with the phylogenetic tree-based evolutionary rate
264 estimate. These results illustrate the importance and possibility of taking the
265 temporal dimension into account when interpreting genomic data in the context of
266 persistent contaminations.²⁰

267 The phylogeny of SL1 (**Fig. 5A**) showed that outbreaks strains were dispersed in
268 multiple branches, suggesting that all SL1 isolates have the potential to cause
269 outbreaks. Moreover, it demonstrated that the multiple outbreaks caused by this
270 sublineage, previously called 'epidemic clone ECI',³⁰ are actually independent
271 epidemiological events. The most recent common ancestor of SL1 was estimated to
272 have existed around 1876 (95% HPD=[1861-1891]), reinforcing the idea that
273 extant SL1 isolates do not derive from a single recent epidemic.

274

275 ***Biological features of Lm sublineages and CTs***

276 Important genomic differences among sublineages are shown in **Fig. 6**. PCR-
277 serogroup distribution across the phylogenetic tree was consistent with previous
278 knowledge, with major PCR-serogroups being strong markers of the main divisions
279 of *Lm* diversity.^{10,33,34} In contrast, PCR-serogroup variant IVb-v1³⁵ was found in
280 various branches. Likewise, and as expected, serogroup L³⁵ was present in lineages
281 III and IV, but also in lineage I. These results underline that caution is needed when
282 interpreting molecular serotyping data for *Lm* epidemiological purposes.

283 The screening for virulence and stress resistance genes showed important
284 differences among *Lm* lineages and sublineages (**Fig. 6**). As expected, the major
285 pathogenicity island LIPI-1 was highly conserved. A complete LIPI-3³⁶ was almost
286 exclusively detected within lineage I. The recently described LIPI-4²⁵ was nearly
287 universally present in SL4 and closely related sublineages (**Fig. 6**), and was also
288 found in few other isolates, including in lineages III and IV. *inlA* alleles encoding
289 truncated InlA variants, which are associated with hypovirulence,³⁷ were observed
290 in most isolates of lineage II sublineages SL9, SL31, SL121, SL199 and SL321 (**Fig.**
291 **6 and Supplementary Information 2.9**) and were significantly associated with
292 food and food-production isolates ($p < 0.0001$). The presence of a non-disrupted
293 form of the *comK* gene, involved in intracellular survival switch and biofilm
294 formation^{38,39}, was dispersed across multiple sublineages and far more frequent in
295 lineage I than in lineage II (79% vs 38%, respectively, $p < 0.0001$, Fisher's exact
296 test). Finally, genes that confer resistance to benzalkonium chloride, a major
297 disinfectant applied on food-industry surfaces,⁴⁰ were significantly associated

298 ($p < 0.0001$, Fisher's exact test) with lineage II and particularly frequent in SL121,
299 consistent with persistence of this clonal complex in food processing plants.⁴¹
300 Taken together, these results demonstrate the strong heterogeneity among *Lm*
301 sublineages with regards to genomic features involved in either pathogenesis or
302 food contamination.

303

304 **Discussion**

305 Listeriosis surveillance is currently organized almost exclusively at national levels,
306 thereby limiting our capacity to trace sources of infections involving international
307 transmission through food trade or human travel. An efficient global laboratory
308 surveillance system would consist of three parts: standardized methods and
309 databases, open sharing of data between public health laboratories, and rapid
310 communication about outbreaks. Here, we have addressed these issues by
311 developing a genome-wide genotyping system validated on a large international
312 collection of *Lm* strains. Furthermore, we have set up an openly accessible
313 database and analysis tool (BIGSdb-*Lm* at <http://bigsdbs.pasteur.fr/listeria>), which
314 provides a unified nomenclature that will ease global communication on *Lm*
315 genotypes. Real-time incorporation of genotypic variation of future *Lm* isolates
316 uncovered through prospective genomic surveillance will enable global
317 coordination of epidemiological surveillance.

318

319 Although alternative sets of cgMLST loci (**Supplementary Information 2.3**) have
320 been recently proposed for *Lm* typing,^{19,24} the scheme developed here in the
321 context of a global collaboration contains more genes, was validated using isolates
322 from diverse origins, and was extremely reproducible when comparing the results
323 from independent allele calling approaches. We also show that cgMLST has a far
324 greater discriminative power than PFGE when applied to the prospective
325 surveillance of isolates. It is worth noting that although we already identified 1,013
326 CTs, they represent only a small fraction of existing CTs that will be revealed by
327 future genomic surveillance (**Fig. 1B**), indicating that referenced CTs should

328 rapidly surpass the number of PFGE types distinguished during 20 years of
329 PulseNet surveillance (4,119 unique *ApaI/AscI* combinations among 21,158
330 isolates with PFGE as of December 21, 2015). The largely improved refinement of
331 *Lm* genotyping using cgMLST is expected to (i) reduce in size clusters accurately
332 detected by PFGE, (ii) erase clusters falsely inferred from PFGE, and (iii) allow
333 detecting earlier, clusters that would likely remain ignored when belonging to
334 hyper-prevalent PFGE profiles. Together, these highly significant improvements of
335 *Lm* typing will strongly reduce and even eliminate unnecessary epidemiological
336 investigations, which is a major drawback of the lack of discrimination of the
337 current standard PFGE, and will help to identify the food source of clusters of
338 human cases by refining the definition of cases in case-control studies.

339

340 The analysis of a large and geographically diverse collection of *Lm* genomes also
341 allowed us to determine the population structure of this species with
342 unprecedented precision. The sharp discontinuities observed within the
343 phylogenetic diversity of *Lm* allowed to identify and define sublineages
344 unambiguously, which will constitute the basis of a universal genome-based
345 nomenclature. This nomenclature has the advantage of being congruent with the
346 previously widely adopted 7-genes MLST nomenclature and the corresponding
347 major clinically- and food-associated CCs.^{10,25} In addition, by including a large
348 number of sets of epidemiologically related isolates, we could also define cgMLST
349 types relevant for epidemiological purposes using a statistically optimized cut-off.
350 As cgMLST dissimilarity is highly congruent with phylogenetic relationships, *Lm*
351 strains can be assigned with high confidence to sublineages and types based on

352 their cgMLST profile. Because this does not require a multiple sequence alignment
353 step, this approach is considerably faster than sequence-based identification, and
354 easier to interpret by microbiologists, epidemiologists and public health
355 professionals. Thus, CT classification is poised to become a universal tool for
356 cluster detection and international communication during regional or global *Lm*
357 outbreaks.

358

359 By applying genomic sequencing to a large collection of *Lm* isolates from diverse
360 geographic origins, we were able to clearly demonstrate repeated international
361 transmission of multiple sublineages of *Lm*. Further, we identified international
362 groups of genetically highly related isolates, suggestive of recent cross-country and
363 intercontinental transmissions. These results provide a unique population-level
364 based snapshot of *Lm* international transmission and suggest that cross-country
365 outbreaks that were recognized up to now^{17,42} are only the tip of the iceberg of *Lm*
366 long distance dissemination.⁴³ Given the retrospective nature of our analyses, we
367 were not able to identify the epidemiological links among isolates of these
368 international clusters, but these observations suggest that their detection in real
369 time would allow tracing back to common sources, and firmly establish the
370 importance of monitoring in real time the diffusion of *Lm* genotypes at the
371 international level. The cgMLST collaborative approach here developed makes this
372 goal achievable and paves the way for future research aimed at better
373 understanding the routes and contributing factors of *Lm* dissemination.

374

375 We calibrated the short-term evolutionary rate of *Lm* genomes, and could
376 therefore provide a quantitative estimate of the widely recognized view that *Lm*
377 genomes are highly stable.^{12,44,45} Because cgMLST types diversify slowly (roughly
378 0.2 alleles per year), greater discrimination may be needed to decipher short-term
379 patterns of transmission.^{17,18} Therefore, to fully harness the power of genomic
380 sequencing for *Lm* epidemiology, multi-approach strategies can be applied,
381 including the use of pan-genomic MLST and reference-based SNP-calling. However,
382 in contrast to MLST, genome-wide SNP-based approaches do not rely on
383 predefined genomic loci and require *ad-hoc* reference sequences, thus being more
384 complex to standardize. In this context, the genotyping method and publicly shared
385 nomenclature developed herein will represent a pivot element of collaborative
386 approaches to control the burden of *Lm* infections at the global scale.

387

388 **Methods**

389 ***Bacterial isolates***

390 A total of 1,696 *Lm* genomes were included in the main dataset (1,055 human
391 isolates, 475 isolates from food and food-processing environments, and 166
392 isolates from other or unknown sources; **Supplementary Table 1**;
393 **Supplementary Figure 1**), comprising isolates collected between 1960 and 2015,
394 mostly from North America and Europe. This set included the 104 genomes
395 representative of the clonal diversity of *Lm* used for core genome definition,²⁵
396 genomic sequences from isolates collected in the context of *Lm* surveillance
397 programs in Canada ($n=36$ isolates), Denmark ($n=224$), France ($n=112$), the United
398 Kingdom ($n=448$) and the United States ($n=758$), and 14 genomes from a German-
399 Austrian outbreak.¹⁷ This collection included (i) prospectively collected isolates as
400 well as (ii) isolates collected in the frame of outbreak investigations or mother-
401 child transmission cases (**Supplementary Table 1**; **Supplementary Figure 1**). In
402 addition, 34 historical isolates (**Supplementary Table 5**), were included for
403 analysis of *Lm* evolutionary rates. DNA extraction, library preparation and Illumina
404 sequencing using MiSeq, NextSeq or HiSeq instruments were performed locally in
405 each reference center. Sequence assembly was performed using BioNumerics v.7.5
406 (Applied Maths NV, Sint-Martens-Latem, Belgium) or CLC Assembly Cell 4.3.0
407 (Qiagen, Aarhus, Denmark). Provenance data and genomic assembly details of the
408 1,696 isolates are listed in **Supplementary Table 1**.

409

410 ***Validation of a universal cgMLST scheme for Lm genotyping***

411 A previously defined *Lm* core genome with 1,791 loci²⁵ was further refined by
412 removing genes present in less than 95% of 957 high-quality genome sequences
413 (**Supplementary Figure 1**), genes with close paralogs and genes belonging to the
414 seven MLST scheme (**Supplementary Information 2.1**). This filtering procedure
415 led to a final subset of 1,748 core genes, here referred as the *Lm* cgMLST scheme
416 (**Supplementary Tables 2 and 3**). The levels of diversity, selection and
417 recombination were quantified for each cgMLST locus (**Supplementary**
418 **Information 2.2**). The robustness of cgMLST genotyping was tested using both
419 assembly-free and *de novo* assembly-based methods, to control that allelic profiles
420 generated by the two approaches are consistent and to exclude potential assembly
421 artefacts. The performance of different assemblers was also tested at different
422 sequencing coverage depths (**Supplementary Information 2.4**).

423

424 ***Comparison of cgMLST and PFGE genotyping***

425 To compare cgMLST with PFGE for *Lm* strain typing, we analyzed in parallel 100
426 isolates (57 human isolates, 33 food isolates and 10 isolates from food production
427 environments) prospectively collected between January and April 2015 in the
428 frame of the French listeriosis surveillance system by the National Reference
429 Center for *Listeria* (Institut Pasteur, France). PFGE restriction profiles were
430 obtained using the enzymes *AscI* and *ApaI* according to PulseNet standardized
431 procedures (<http://www.cdc.gov/pulsenet/PDF/listeria-pfge-protocol-508c.pdf>)
432 and were analyzed using BioNumerics. PFGE and cgMLST typing results were

433 compared using Simpson's index of diversity and the adjusted Wallace index of
434 concordance (see **Supplementary Information 2.5** for details).

435

436 ***Phylogenetic and clustering analyses***

437 The phylogenetic relationships of the 1,696 isolates were inferred based either on
438 the allelic profiles or on the recombination-purged multiple sequence alignments
439 of the 1,748 loci (see **Supplementary Information 2.6** for details). Single-linkage
440 clustering analysis was performed from the *p*-distances among allelic profiles
441 (cgMLST allelic distances, *i.e.* proportion of mismatched loci among those that are
442 called in both strains). Clustering efficiency (*i.e.* optimizing both compactness
443 within clusters and separateness among clusters) was assessed with Dunn's index
444 (**Supplementary Information 2.7**) using different allelic mismatch thresholds.

445

446 ***Phylogeography and temporal analysis***

447 Geographical transitions within major sublineages were inferred from FastME
448 v.2.07 trees using discrete trait transition modelling based on 100 simulations with
449 the make.simmap tool in the phytools R package.^{26,27} Once the ancestral states
450 were estimated, the total number of character changes was computed from the
451 resulting set of trees, using the count.simmap function within the same R
452 package.²⁷

453 To estimate the evolutionary rate of sequences and cgMLST profiles, 22 historical
454 isolates belonging to MLST clonal complex CC1 and 12 isolates from clonal
455 complex CC9, collected between 1921 and 1974, were analyzed jointly with the
456 isolates from sublineages SL1 and SL9 (see below) from the main dataset

457 **(Supplementary Information 2.8, Supplementary Tables 1 and 5).**
458 Phylogenetic analyses were performed using FastME on p-distances estimated
459 from either concatenated multiple sequence alignments or cgMLST profiles. Linear
460 regression of the root-to-tip distances against the year of isolation was carried out
461 using Path-O-Gen v1.4 (<http://tree.bio.ed.ac.uk/software/pathogen/>). The rate of
462 evolution of SL1 and SL9 genomes were independently estimated from the
463 concatenated multiple sequence alignments of the 1,748 loci using BEAST v.2.3.1²⁸.
464 For this analysis, Gubbins²⁹ was used to detect recombination within the
465 alignments. Isolates with recombinant regions were discarded from the alignments
466 **(Supplementary Information 2.8)**. Subsequently, the mean of the rates of SL1
467 and SL9 (2.5×10^{-7} substitutions per site per year) was used to estimate the age of
468 all major sublineages using BEAST v.2.3.1²⁸. Details of the temporal analysis
469 methods are given in **Supplementary Information 2.8**. Genetic divergence as a
470 function of the time span between the first and last isolate of outbreak sets and
471 international clusters was evaluated using regression analysis.

472

473 ***Determination of PCR serogroups, virulence and resistance genes profiles***

474 To investigate the biological differences among sublineages, the PCR-serogroup
475 and the presence of 76 loci involved in virulence or resistance were deduced *in*
476 *silico* from genomic sequences using the BIGSdb platform²³ for each of the 1,696
477 genomes (see **Supplementary Information 2.9** for details).

478

479 ***Online implementation of an open bioinformatics platform for Lm strain*** 480 ***nomenclature and genome analysis***

481 To make the cgMLST-based nomenclature sharable and expandable, the *Lm*
482 cgMLST scheme was implemented in an integrative database and analysis platform
483 (BIGSdb-*Lm*) powered by the BIGSdb v.1.10²³ bioinformatics tool. To unify *Lm*
484 genotyping resources, the classical 7-gene MLST scheme was transferred into the
485 BIGSdb-*Lm* platform. Openly accessible predefined schemes for molecular
486 serogrouping and for virulence and resistance gene analyses were also
487 incorporated in the BIGSdb-*Lm* platform. BIGSdb-*Lm* is publicly accessible at
488 <http://bigsdbs.pasteur.fr/listeria>.

489

490 ***Accession numbers***

491 FASTQ data files were deposited in NCBI-SRA and EBI-ENA public archives under
492 the project's accession numbers PRJEB12738 (Institut Pasteur), PRJEB14476
493 (Statens Serum Institut), PRJNA248549 (Public Health England) and PRJNA212117
494 (Centers for Disease Control and Prevention). The accession numbers of all isolates
495 are indicated in Supplementary Table 1.

496

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- 619

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634

635 **Author contributions**

636 This study was designed by SB, ML, PGS and BP. Selection of isolates was carried out by
637 EMN, CN, VCF, AL, AR, KG, TD and LSK. DNA preparation and sequencing was performed
638 by HBD, VCF, AL, CT, HC, SS, ZK, JTB, AR, CN, KG, MW and VE. PFGE analysis was performed
639 by HBD, VCF, AL and AM. Sequence analysis was carried out by AM, HP, TC, LK, HC, JTB.
640 Definition of core genome was done by MMM, EPCR, MTouchon. Validation and
641 reproducibility of cgMLST loci was performed by AM, HP and EL. Phylogenetic and
642 clustering analyses were carried out by AM and AC. Online database implementation was
643 done by LJ, AM and SB. Epidemiological data analysis was performed by MTourdjman, AL,
644 AM, TD, KG, EMN and CT. AM and SB wrote the manuscript, with contributions and
645 comments from all authors.

646

647 **Additional information**

648 Correspondence and requests for materials should be addressed to ML and SB.

649

650 **Declaration of interests**

651 HP and BP are co-developers of the BioNumerics software mentioned in the manuscript.

652 The remaining authors declare no competing interests.

653

654 **Legends to figures**

655 **Figure 1. Nomenclature of *Lm* cgMLST profiles.** A) Distribution of the number of
656 cgMLST allelic differences between pairs of isolates among the 1,696 genomes (blue) and
657 within 49 sets of epidemiologically related isolates (426 isolates in total; red). Dashed bars
658 represent cut-off values for cgMLST types (CT, 7 allelic mismatches) and sublineages (SL,
659 150 allelic mismatches). Inset: global dataset; Main figure: up to 200 allelic mismatches. B)
660 Rarefaction curves of the number of sublineages and cgMLST types identified, broken
661 down per main phylogenetic lineage (I-IV). Curves were estimated using 100 random
662 samples per point. Inset: zoom on the 0-50 X-axis values. Lineages III and IV were pooled
663 but must be sampled more extensively to determine the shape of the curve.

664

665 **Figure 2. Phylogenetic structure of the global *Lm* dataset.** A) Phylogeny of the four
666 phylogenetic lineages (I, red; II, orange; III, green; IV, blue). Representative isolates of the
667 four lineages were used to determine the location of the root, using *L. innocua* and *L.*
668 *marthii* as outgroups. The tree was obtained using FastME on the p-distance of the 1,748
669 concatenated alignments. B) Comparison of the phylogeny obtained from 1,748
670 recombination-purged sequence alignments (left) and from cgMLST allelic profile
671 distances (right). To reduce redundancy, only one strain per outbreak set was used. Scale
672 bars indicate the % of nucleotide substitutions (A right and B left) and the % of allelic
673 mismatches (B right). For practical reasons, bootstrap values (based on 500 replicates)
674 are shown only for long internal branches.

675

676 **Figure 3. International distribution of *Lm* sublineages.** A) Clustering of 1,696 *Lm*
677 isolates based on single-linkage analysis of the cgMLST profiles. Lineage branch colours
678 are as in Fig. 2. Light and dark grey alternation (inner circle) delimitates sublineages with
679 more than 10 isolates (main sublineages are labelled). Source country is represented in
680 the external ring using the colour key from panel C. B) Number of countries from which a
681 sublineage was isolated, as a function of number of isolates per sublineage. Disk size is a
682 function of number of isolates per sublineage. C) Inferred geographical origin of ancestral
683 nodes of the phylogeny of sublineage 1. Pie charts represent the likelihood proportion of
684 geographical origins. The tree was constructed using minimum evolution based on
685 cgMLST profiles. Bootstrap values above 50% (based on 500 replicates) are shown for the
686 major nodes. D) Absolute number of geographical transitions (left) and number of
687 geographical transitions normalized by total branch length (right) within the 10 most
688 frequent sublineages, as inferred by stochastic ancestral state reconstructions (numbers in
689 parentheses indicate the precise values inferred for each sublineage).

690

691 **Figure 4. International groups of isolates classified into the same cgMLST type.** The 9
692 groups of isolates are indicated by a specific colour. The genotype is indicated as a string
693 consisting of a succession of lineage (e.g., L1), sublineage (e.g., SL1), sequence type (e.g.,
694 ST1) and cgMLST type (e.g., CT288). Countries of isolation, isolation year range and total
695 number of isolates are given after the genotype string. The circles on the map indicate the
696 country where a particular CT was isolated and their size is related to the number of
697 isolates from that country. The details of each CT are given in Supplementary Information.

698 Abbreviations: L, lineage; ST, sequence type; SL, sublineage; CT, cgMLST type; US, United
699 States of America; CA, Canada; DK, Denmark; UK, United Kingdom; FR, France.

700

701 **Figure 5. Temporal analysis of cgMLST profiles evolution.** A) Best-fitting rooted
702 phylogeny of SL1 isolates ($n=195$), including the historical isolates. The tree was obtained
703 using FastME on cgMLST profiles. Coloured blocks represent the isolation time range
704 (1921-1950, pink; 1951-1980, purple; 1981-2010, blue; 2011-2015, green). Outbreak
705 reference strains are indicated by red dots. Outbreak identifier, country, year and cgMLST
706 type are provided on the right. The scale bar indicates the number of allelic substitutions
707 per locus. Statistical significance was assessed using F-test. B) Linear regression of
708 isolation year with root-to-tip cgMLST distance. C) Accumulation of cgMLST variation over
709 time, determined based on the international CTs ($n=9$) and outbreak sets ($n=49$).
710 Statistical significance was assessed using F-test.

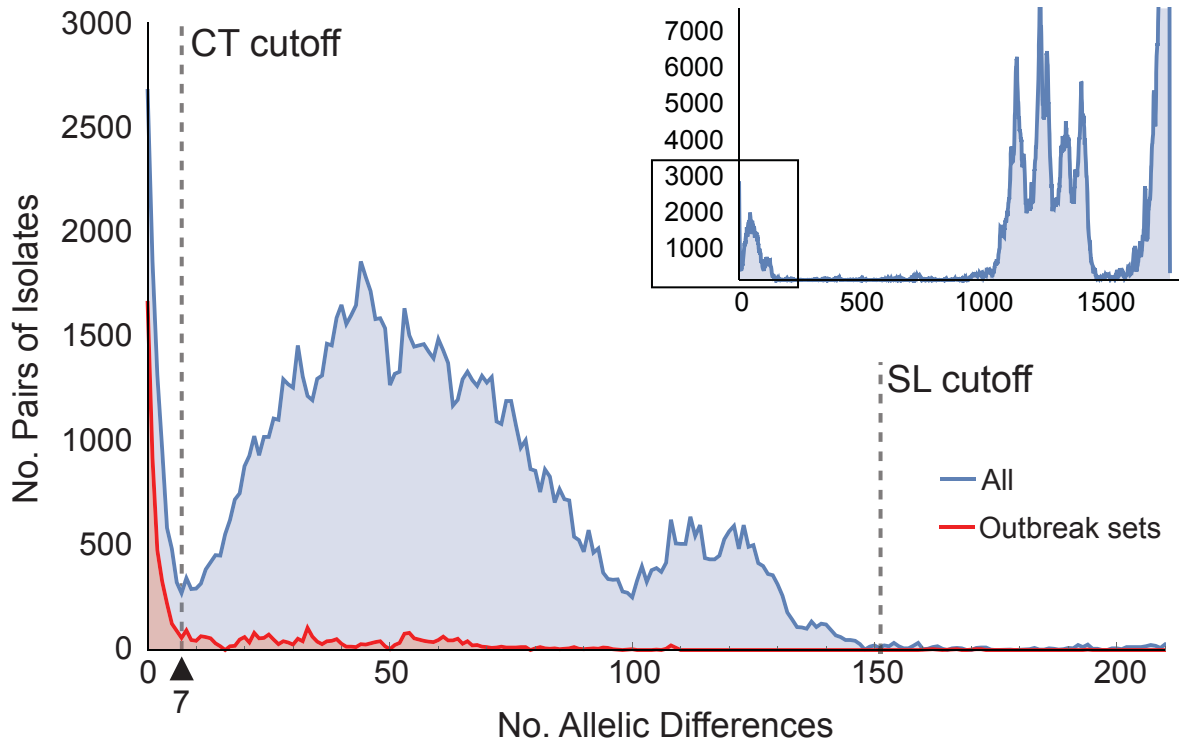
711

712 **Figure 6. Virulence and resistance profiles across the phylogeny of the 1,696 *Lm***
713 **isolates.** A) Cluster analysis based on cgMLST profiles. The dotted vertical bar indicates
714 the cgMLST mismatch cut-off for sublineages (SL). The 10 most frequent sublineages are
715 highlighted. B) Pattern of gene presence (color line) or absence (white). The first and last
716 columns corresponds to the serogroup and sample source, respectively, represented by
717 color codes (upper left key). The presence/absence gene matrix represents, from left to
718 right, genes involved in teichoic acid biosynthesis (*gltAB*, *tagB*, *gtcA*), genes located in the
719 pathogenicity islands LIPI-1 (*prfA*, *plcA*, *hly*, *mpl*, *actA*, *plcB*), LIPI-3 (*llsAGHXBYDP*) and
720 LIPI-4 (LM9005581_70009 to LM9005581_70014), genes coding for internalins
721 (*inlABCEFGHJK*), and other genes involved in adherence (*ami*, *dltA*, *fbpA*, *lap*, *lapB*),
722 invasion (*aut*, *aut_IVb*, *cwhA*, *lpeA*, *vip*), intracellular survival (*hpt*, *lplA1*, *oppA*, *prsA2*, *purQ*,
723 *svpA*), regulation of transcription and translation (*agrAC*, *cheAY*, *fur*, *lisKR*, *rsbV*, *sigB*, *stp*,
724 *virRS*), surface protein anchoring (*lgt*, *lspA*, *srtAB*), peptidoglycan modification (*oatA*,
725 *pdgA*), immune modulation (*IntA*), bile-resistance (*bsh*, *mdrM*, *mdrT*, *brtA*), resistance to
726 detergents (*qac*, *bcrABC*, *ermE*) and biofilm formation and virulence (*comK*).

727

Figure 1

A



B

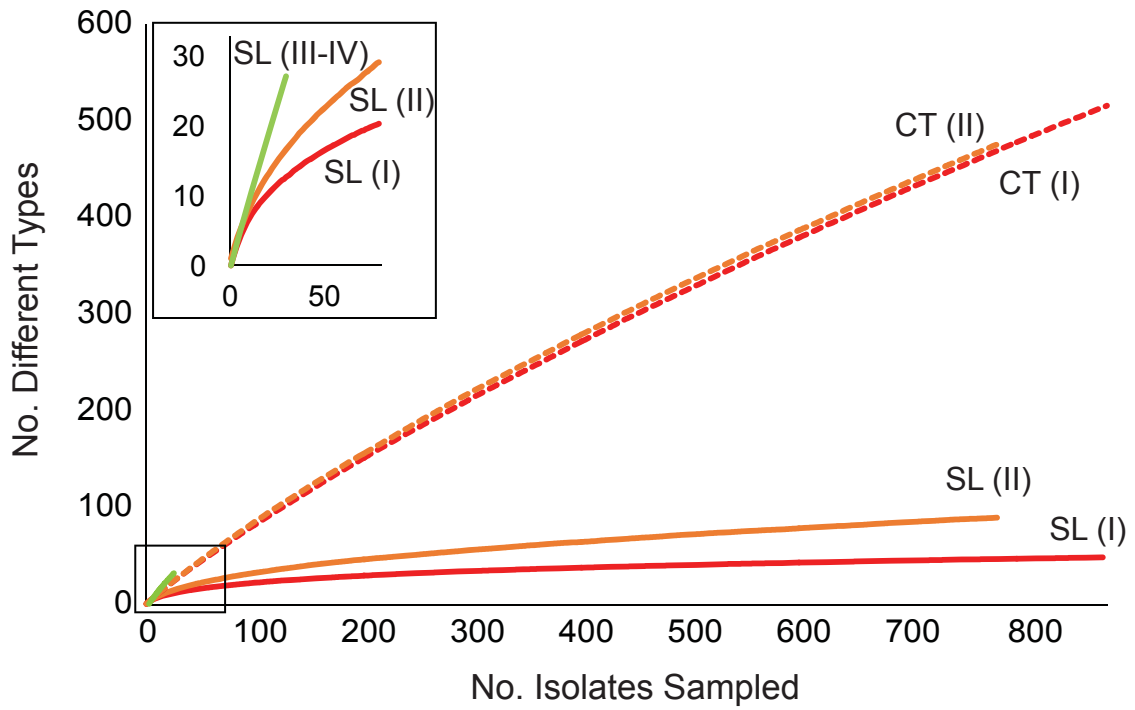
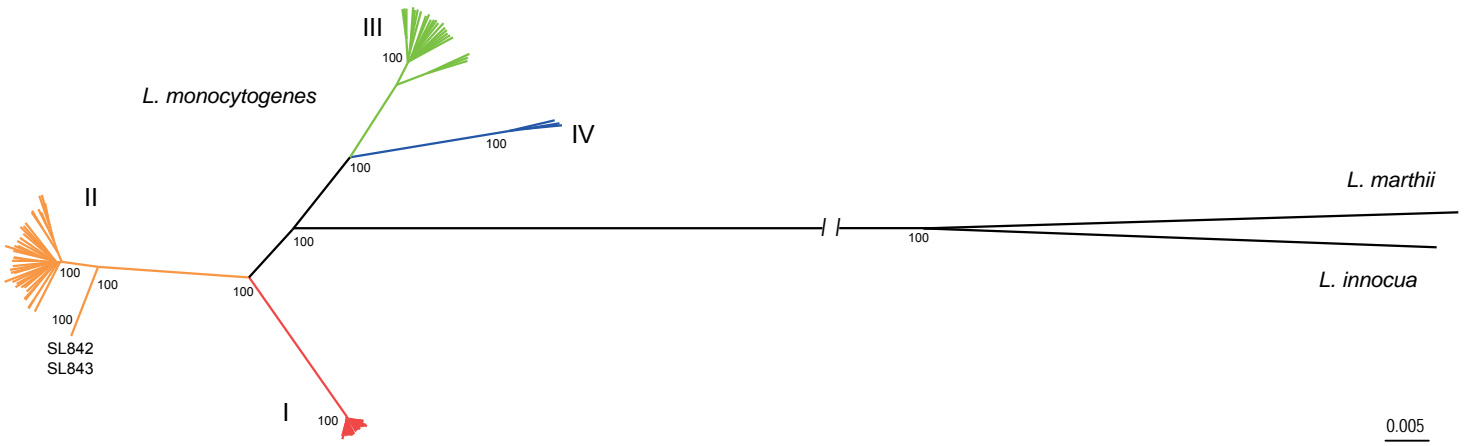


Figure 2

A



B

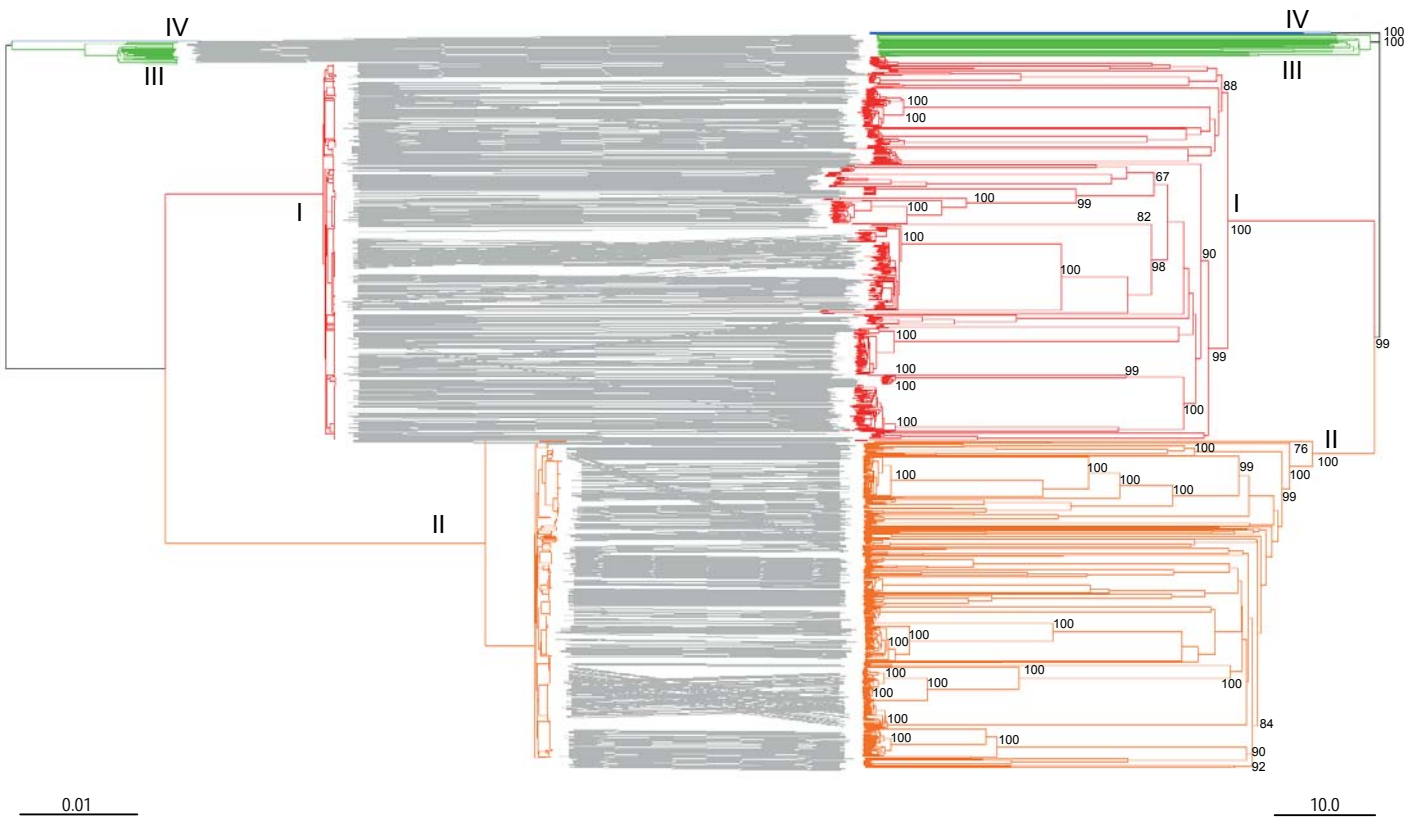
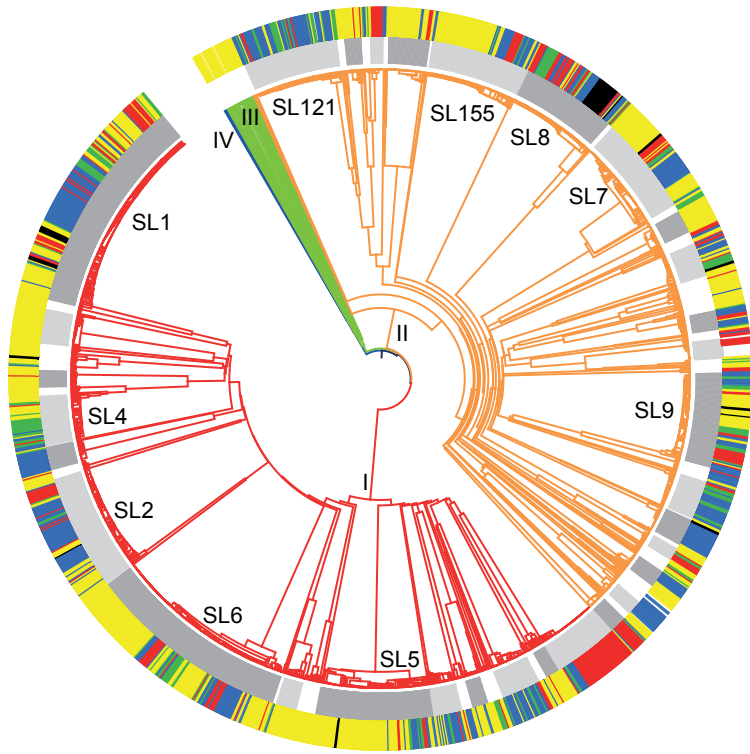
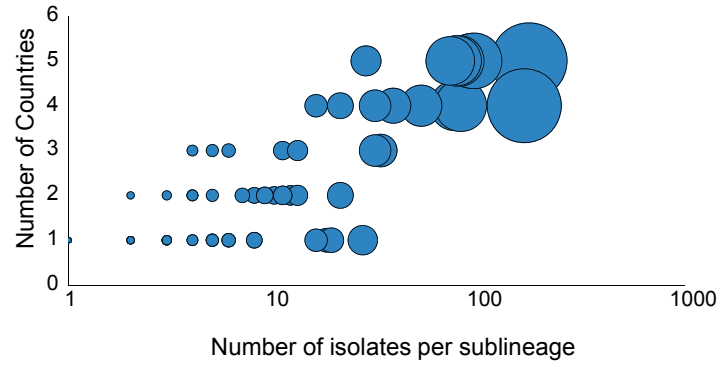


Figure 3

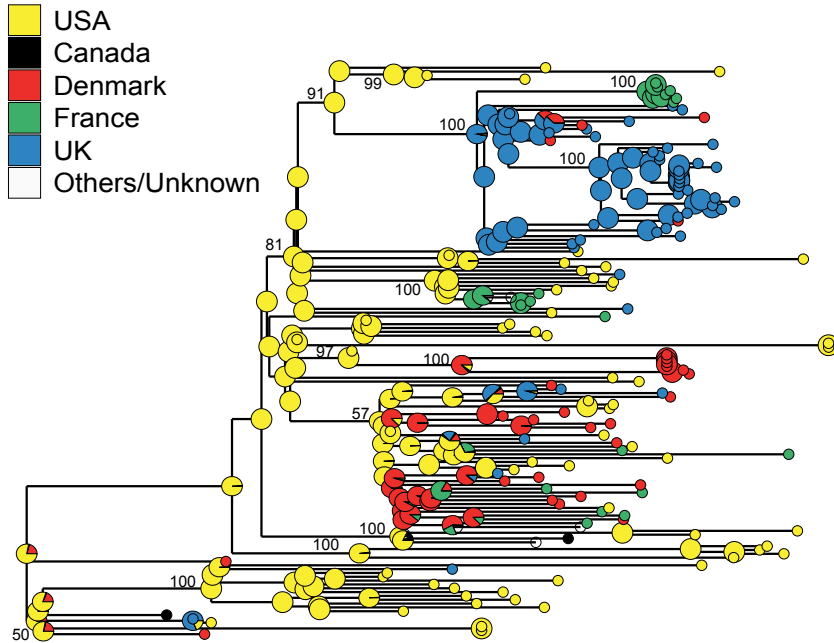
A



B



C



D

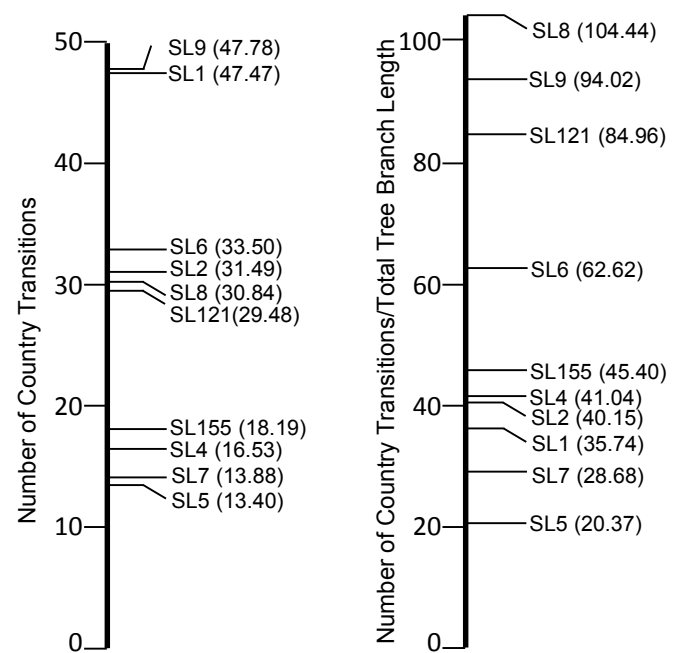


Figure 4

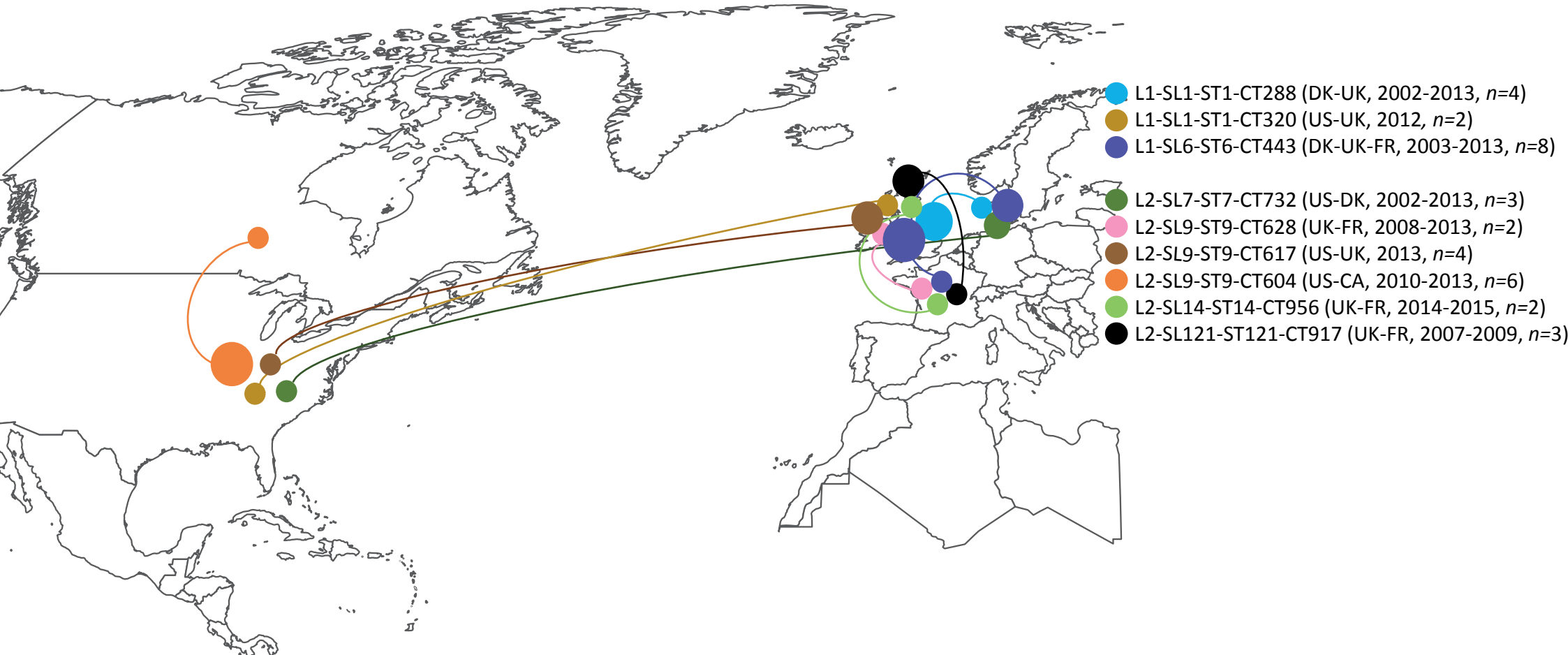
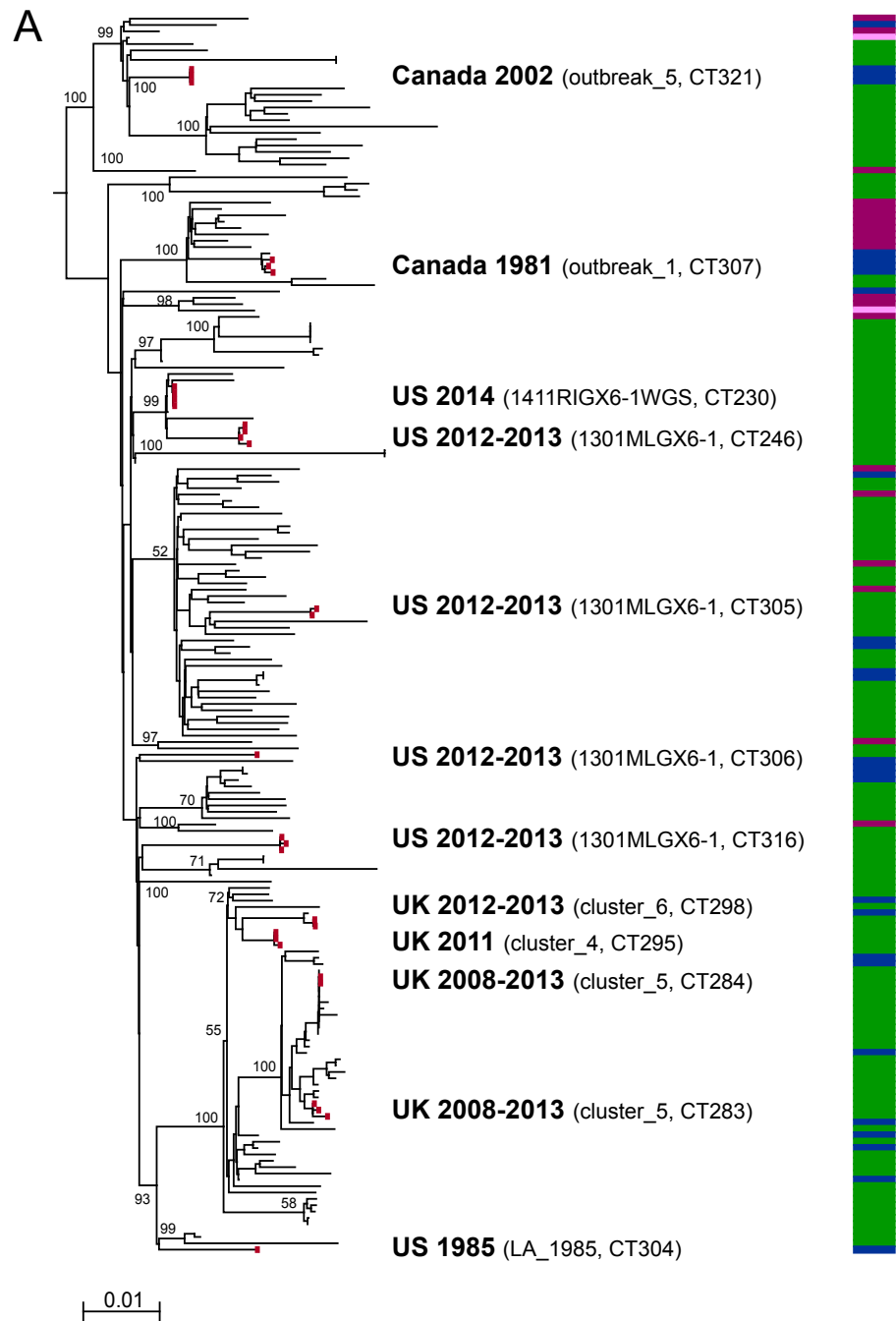
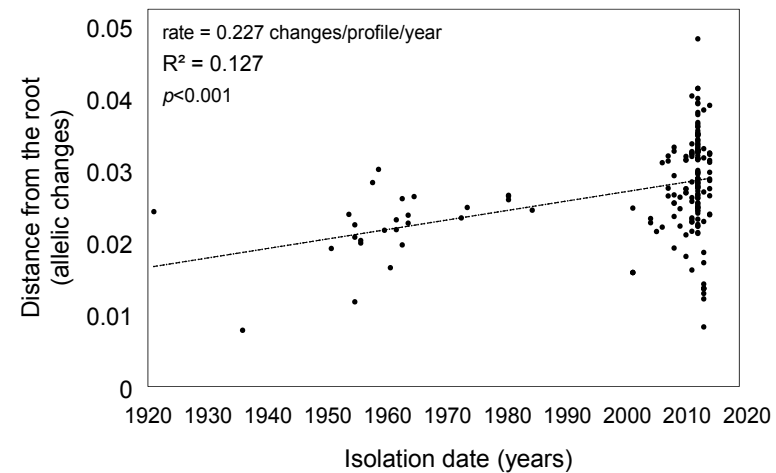


Figure 5



B



C

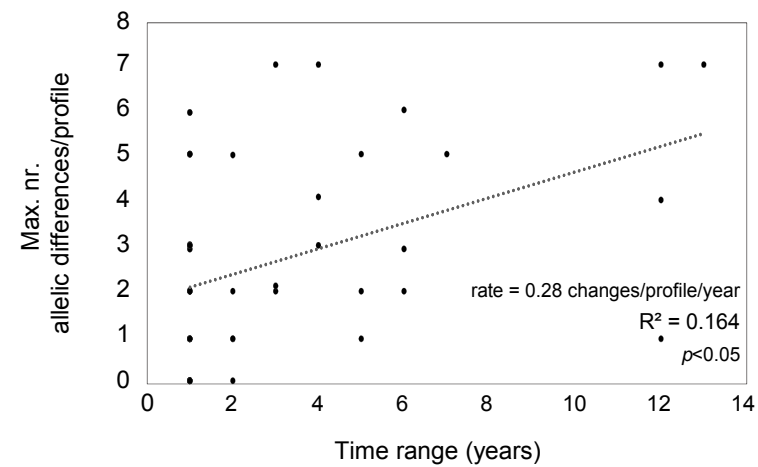


Figure 6

