

Session 4.1 – Tipificación basada en perfil alélico o gene-by-gene

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- Typing resolution
- Concepts: homology, core, accessory and pan-genome
- Schema definition
- e.g. *Listeria monocytogenes*

Definitions of terms commonly used to classify genetic relatedness among bacterial strains

Simar et al., Current Opinion 2021

Table 2. Definitions of terms commonly used to classify genetic relatedness among bacterial strains

Term	Definition
Clade	A group of organisms that contains a single ancestor and its descendants; a monophyletic group [6]
Clone	A group of isolates that are genetically indistinguishable [though not necessarily identical] based on a particular molecular typing method and are presumed to be descendants of a common ancestor [7]
Sequence type (ST)	Organisms that possess identical allelic profiles of fragments of predetermined housekeeping genes [4]
Clonal group	All isolates that belong to a particular ST [8,9]
Clonal complex	A cluster of bacterial organisms that originate from a common ancestor and generally share at least 6/7 alleles of their associated ST with another member of the group [8]
Strain	Isolate(s) that are distinct from other isolates of the same genus and species based on phenotypic and/or genotypic features [9]

Outbreak definition and Typing methods: DNA-based methods

A disease **OUTBREAK** is the occurrence of disease cases in excess of normal expectancy.

Bacterial identification and characterization at subspecies level is commonly known as **Microbial Typing**. Currently, these methodologies are fundamental tools in Clinical Microbiology and bacterial population genetics studies to track outbreaks and to study the dissemination and evolution of virulence or pathogenicity factors and antimicrobial resistance

Several typing methods have been used in outbreak detection and epidemiological surveillance ranging from **phenotypic methods to fragment based methods and sequence based methods**.

WHAT IS MOLECULAR TYPING?

Molecular typing is a way of identifying specific strains of microorganisms, such as bacteria or viruses, by looking at their genetic material. It is mainly used in outbreak investigation as pinpoint the **source of foodborne outbreaks**. It can also be used to identify which microorganisms are: Most virulent and cause serious diseases, resistant to antibiotics, or able to survive and multiply.

Typing methods: DNA-based methods

PFGE *Gold standard*

Is a rather time-consuming and labour-intensive technique.

Discriminatory power of PFGE profiles is limited as only nucleotide changes in the restriction enzyme recognition sites are detected.

Relatedness of strains may be over- or underestimated

Epidemiologically unrelated isolates may be assigned to one 'pseudo'-cluster whereas even highly related strains fall into distinct clusters.

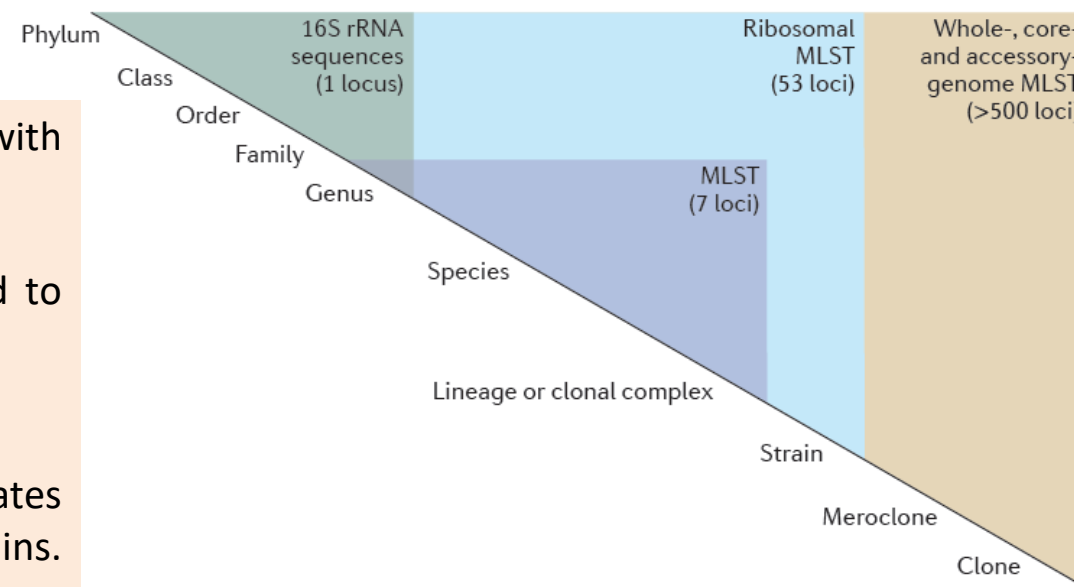
Sequence data for taxonomy and typing Maiden et al., Nat Rev Microbiology 2013

Different levels of sequence information can be associated with different taxonomic levels.

The need for higher-resolution characterization of isolates has led to the development of a wide range of strain-typing methods

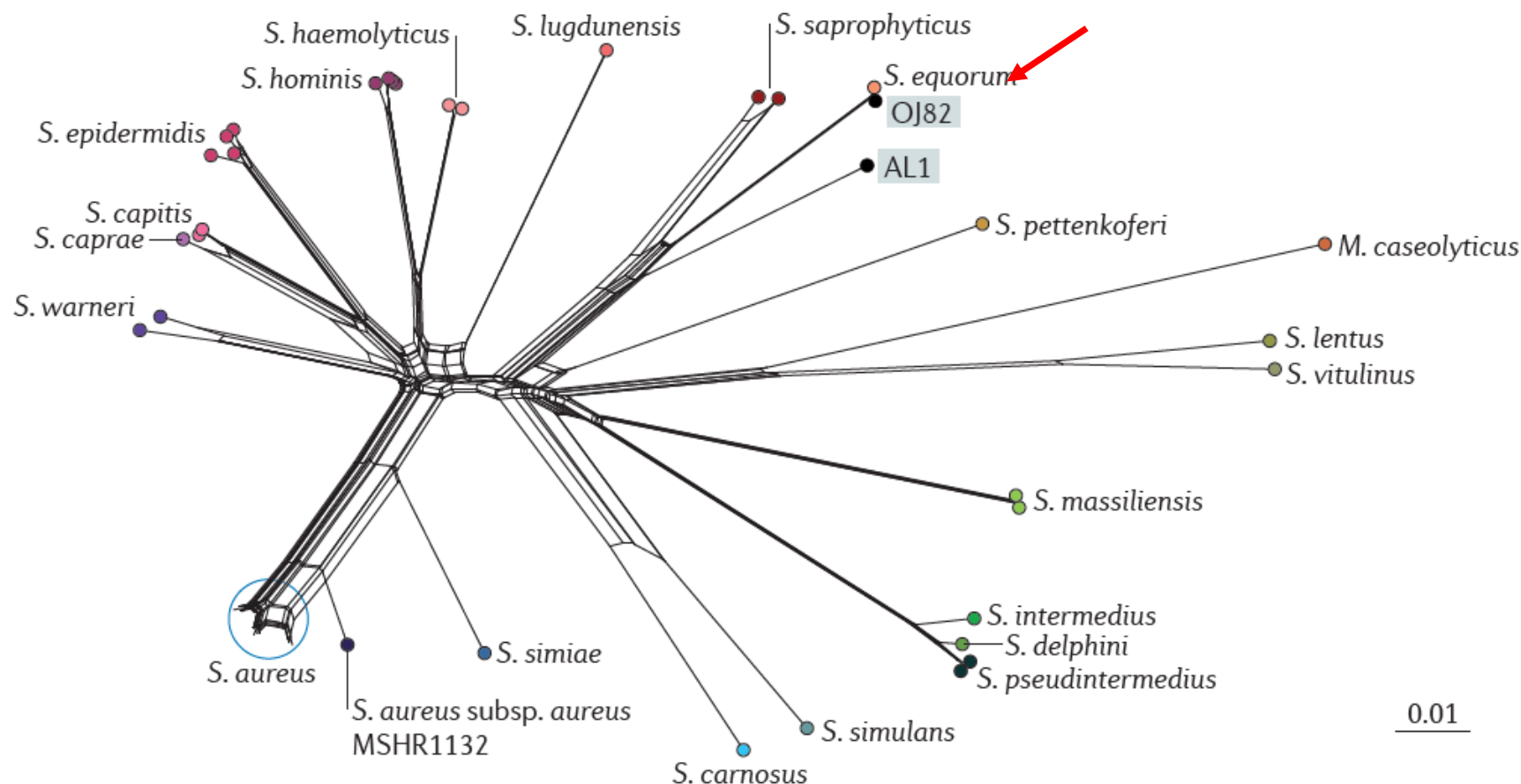
The highest discrimination is required for studies of bacterial isolates from one patient or from very closely related transmission chains. wgMLST

Progressively lower resolution is required for studies of isolates with more distant common ancestors and, therefore, with more genetic differences. rMLST, cgMLST



Ribosomal multilocus sequence typing-based analysis of *Staphylococcus* spp. whole-genome sequence data

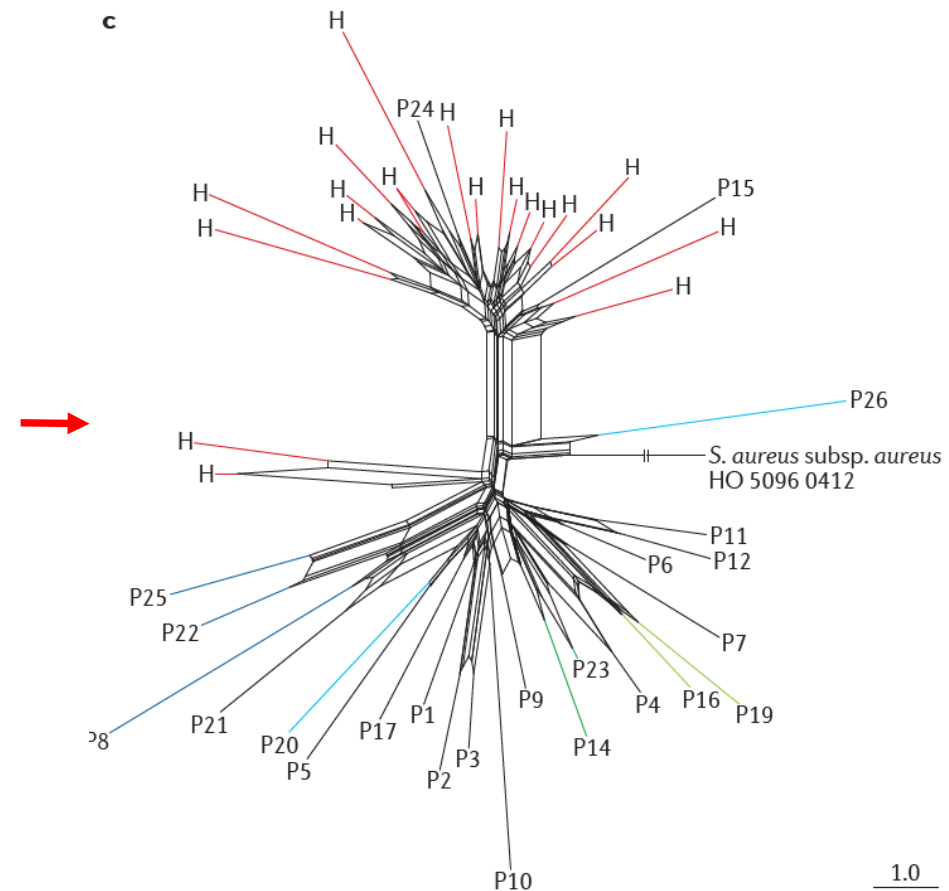
Resolution of 52 staphylococcal isolates on the basis of nucleotide sequence diversity at 51 ribosomal multilocus sequence typing (rMLST) loci, permitting the determination of the species assignment of two recently described isolates, ***Staphylococcus* sp. OJ82** and ***Staphylococcus* sp. AL1**. *Staphylococcus* sp. OJ82 probably corresponds to *Staphylococcus equorum*, whereas *Staphylococcus* sp. AL1 is related to, but distinct from, *S. equorum* and *Staphylococcus saprophyticus*.



0.01

Gene-by-gene typing-based analysis of *Staphylococcus* spp. whole-genome sequence data

Resolution of **multidrug-resistant *S. aureus* (MRSA)** isolates from an **outbreak in a special-care baby unit⁴¹**, using a gene-by-gene comparison to a reference genome (*S. aureus* subsp. *aureus* HO 5096 0412). Twenty isolates obtained from a health care worker are indicated with the letter H and shown in red, whereas patient isolates are indicated with a letter P. Groups of isolates from patients who were members of the same family are shown in the same colour.



Variability between bacterial genomes of the same species

GENOME EVOLUTION

Vertical transfer: is the passing of genetic material by descent

Horizontal transfer: is the movement of genetic material among bacteria that do not necessarily share a mother cell.

- transformation: the uptake of DNA by a cell
- conjugation: transfer facilitated by conjugative elements
- phage-mediated transduction

DNA replication:

- Mutations caused by polymerase errors

- Point mutations: SNPs, single nucleotide insertion / deletion.
- Large insertions / deletions
- Genome rearrangements
- Transfer of exogenous DNA
- Plasmids or phages

Concepts

Homology: share a common ancestor, either by descent or recombination. No such thing as “significantly homologous”. A sequence either is or is not homologous.

Infer homology from knowledge of evolutionary relationships and from degrees of similarity between sequences, features or other data.

Orthologues: sequences have common ancestor and have split due to speciation event.

Paralogues: genes arise by gene duplication

Concepts

Core genome: the number of shared features in a pool of genomes. Shared genes among multiple strains are mostly related to house-keeping genes or central metabolic processes, most of the structural information and main genotypic features. **Orthologues** in all genomes of bacteria belonging to the same taxa

Accessory genome or adaptative genome: includes genes conferring adaptive advantages to the strain in order to survive in a specific environment. In most cases, these factors are linked to antibiotic resistance, virulence, capsular serotype, adaptation, and might reflect the organisms predominant lifestyle.

Concepts

Pangenome: The term “pan-genome” refers to pan (from Greek παν, whole) and genome (genome) referring to the inclusion of the core and the dispensable genome.

Phylogenetic approaches

Uelze et al., One Health Outlook 2020

Method	Approach	Reference	Primary result	Secondary result
cgMLST	Alignment to scheme of core genes	Set of allele sequences for set of core genes	Allele distance matrix	Minimum-spanning tree
wgMLST	Alignment to scheme of core and accessory genes	Set of allele sequences for set of core and accessory genes	Allele distance matrix	Minimum-spanning tree
SNP	Mapping to reference	Closely related reference genome	Core SNP alignment, SNP distance matrix	Neighbor-joining tree Maximum-likelihood tree
split K-mer based SNP detection	Pairwise K-mer comparison	No reference	Core SNP alignment, SNP distance matrix	Neighbor-joining tree
MinHash	Pairwise MinHash comparison and clustering	No reference	MinHash distances, clustering information	Neighbor-joining tree

Features of molecular strain typing methods for bacterial organisms

Method	Type of markers used for differentiation	Discriminatory power	Reproducibility	Bioinformatic knowledge needed	Cost
Pulsed-field gel electrophoresis (PFGE)	Number of bands depending on restriction enzyme	•	••	•	••
Multilocus sequence typing (MLST)	7–8 housekeeping genes	••	•••• ^a	••	••
Core genome MLST (cgMLST)	Hundreds to thousands of core genes	•••	•••• ^a	•••	•••
Whole genome MLST (wgMLST)	Hundreds to thousands of core plus accessory genes	•••	••••	•••	•••
Reference-based single nucleotide polymorphism (SNP) calling	Depends on organism of interest plus reference choice	••••	•••	••••	••••
Reference-agnostic/k-mer based SNP calling	Depends on organism of interest	••••	••••	••••	••••

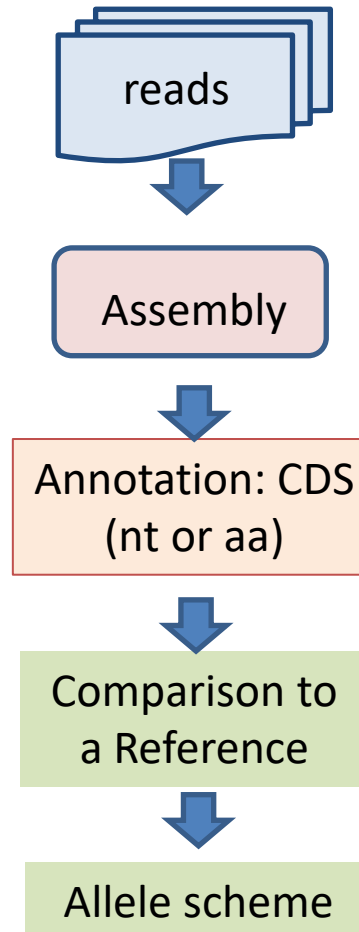
• low, •• medium, ••• high, •••• very high.

^aGenerally high, but depends on organism of interest and chosen reference.

Available cgMLST schemes

Provider	Website	Publically accessible	Species
Enterobase	http://enterobase.warwick.ac.uk/	Yes	<i>Salmonella</i> , <i>Escherichia/Shigella</i> , <i>Clostridioides</i> , <i>Vibrio</i> , <i>Yersinia</i> , <i>Helicobacter</i> , <i>Moraxella</i>
Pasteur Institute	https://bigsdbs.pasteur.fr/	Yes	<i>Klebsiella pneumoniae/quasipneumoniae/variicola</i> , <i>Listeria</i> , <i>Bordetella</i> , <i>Corynebacterium diphtheriae</i> , <i>Yersinia</i> , <i>Leptospira</i> , <i>Elizabethkingia anopheles/meningoseptica/miricola</i>
Ridom	https://cgMLST.org/ncs	Yes	<i>Acinetobacter baumannii</i> , <i>Brucella melitensis</i> , <i>Clostridioides difficile</i> , <i>Enterococcus faecalis</i> , <i>Enterococcus faecium</i> , <i>Escherichia coli</i> , <i>Francisella tularensis</i> , <i>Klebsiella pneumoniae/variicola/quasipneumoniae</i> , <i>Legionella pneumophila</i> , <i>Listeria monocytogenes</i> , <i>Mycobacterium tuberculosis/bovis/africanum/canettii</i> , <i>Mycoplasma gallisepticum</i> , <i>Staphylococcus aureus</i>
Applied Maths	http://www.applied-maths.com/applications/wgmlst	No	<i>Acinetobacter baumannii</i> , <i>Bacillus cereus</i> , <i>Bacillus subtilis</i> , <i>Burkholderia cepacia</i> complex, <i>Brucella</i> spp. <i>Campylobacter coli</i> - <i>C. jejuni</i> , <i>Citrobacter</i> spp., <i>Clostridium difficile</i> , <i>Cronobacter</i> spp., <i>Enterobacter cloacae</i> , <i>Enterococcus faecalis</i> , <i>Enterococcus faecium</i> , <i>Enterococcus raffinosus</i> , <i>Escherichia coli / Shigella</i> , <i>Francisella tularensis</i> , <i>Klebsiella aerogenes</i> , <i>Klebsiella oxytoca</i> , <i>Klebsiella pneumoniae</i> , <i>Legionella pneumophila</i> , <i>Listeria monocytogenes</i> , <i>Micrococcus</i> spp., <i>Mycobacterium bovis</i> , <i>Mycobacterium leprae</i> , <i>Mycobacterium tuberculosis</i> , <i>Neisseria gonorrhoeae</i> , <i>Pseudomonas aeruginosa</i> , <i>Salmonella enterica</i> , <i>Serratia marcescens</i> , <i>Staphylococcus aureus</i> , <i>Staphylococcus epidermidis</i> , <i>Staphylococcus pseudointermedius</i> , <i>Streptococcus pyogenes</i>
INNUENDO/ chewBBACA	http://chewbbaca.online/	Yes	<i>Acinetobacter calcoaceticus/baumannii</i> complex, <i>Legionella pneumophila</i> , <i>Streptococcus pyogenes</i> , <i>Escherichia coli</i> , <i>Yersinia enterocolitica</i> , <i>Campylobacter jejuni</i> , <i>Salmonella</i>

General analytical process for cgMLST / wgMLST



Gene-by-gene: Defining a schema

Sequence-clustering algorithms:

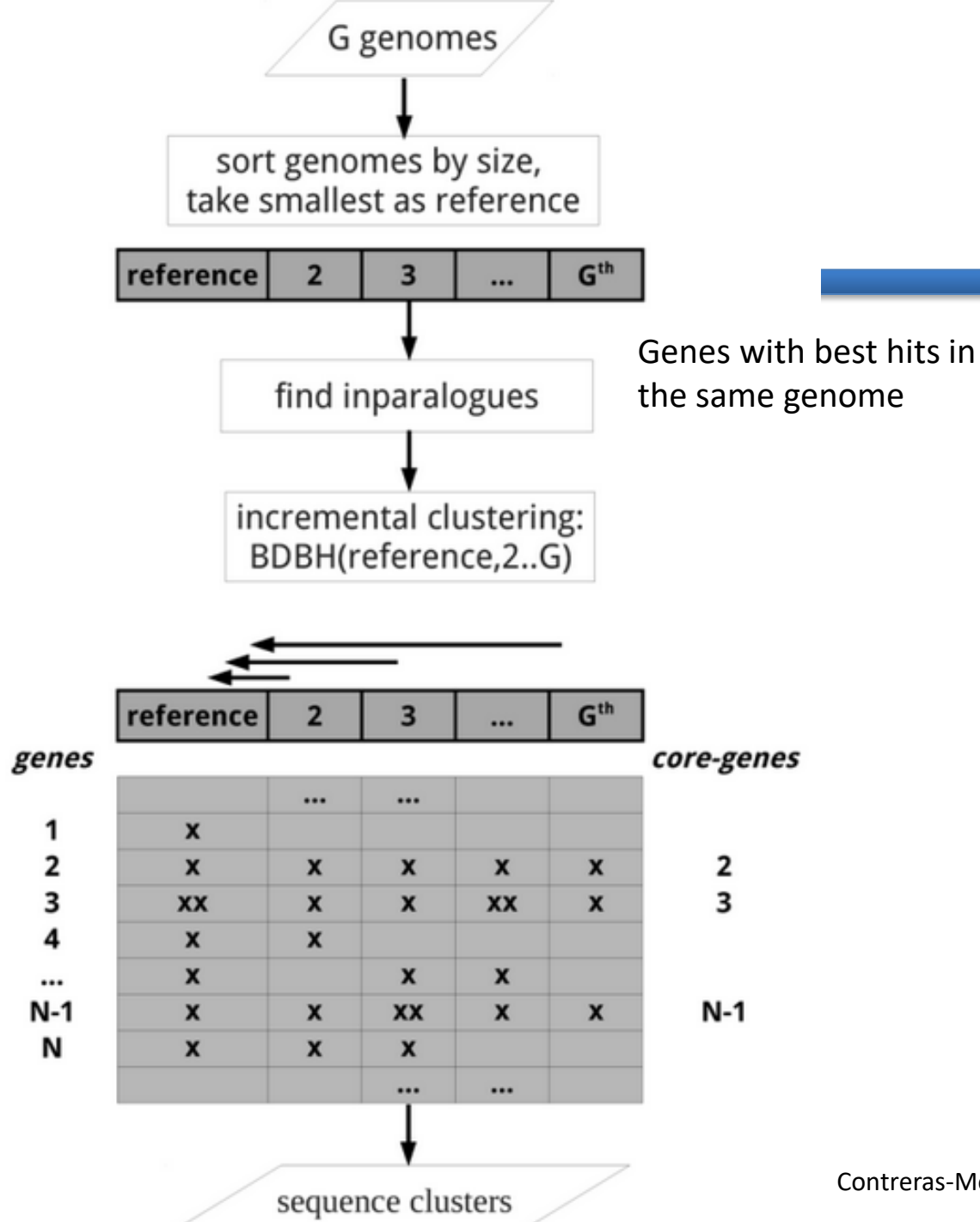
BDBH:
OMCL
COGtriangles

name	option	
BDBH	default	Starting from a reference genome, keep adding genomes stepwise while storing the sequence clusters that result of merging the latest bidirectional best hits, as illustrated in Figure 3.
COGS	-G	Merges triangles of inter-genomic symmetrical best matches, as described in PubMed= 20439257 . Note that a single sequence might occasionally be included in several COGS clusters with option -x.
OMCL	-M	OrthoMCL v1.4, uses the Markov Cluster Algorithm to group sequences, with inflation (-F) controlling cluster granularity, as described in PubMed= 12952885 .

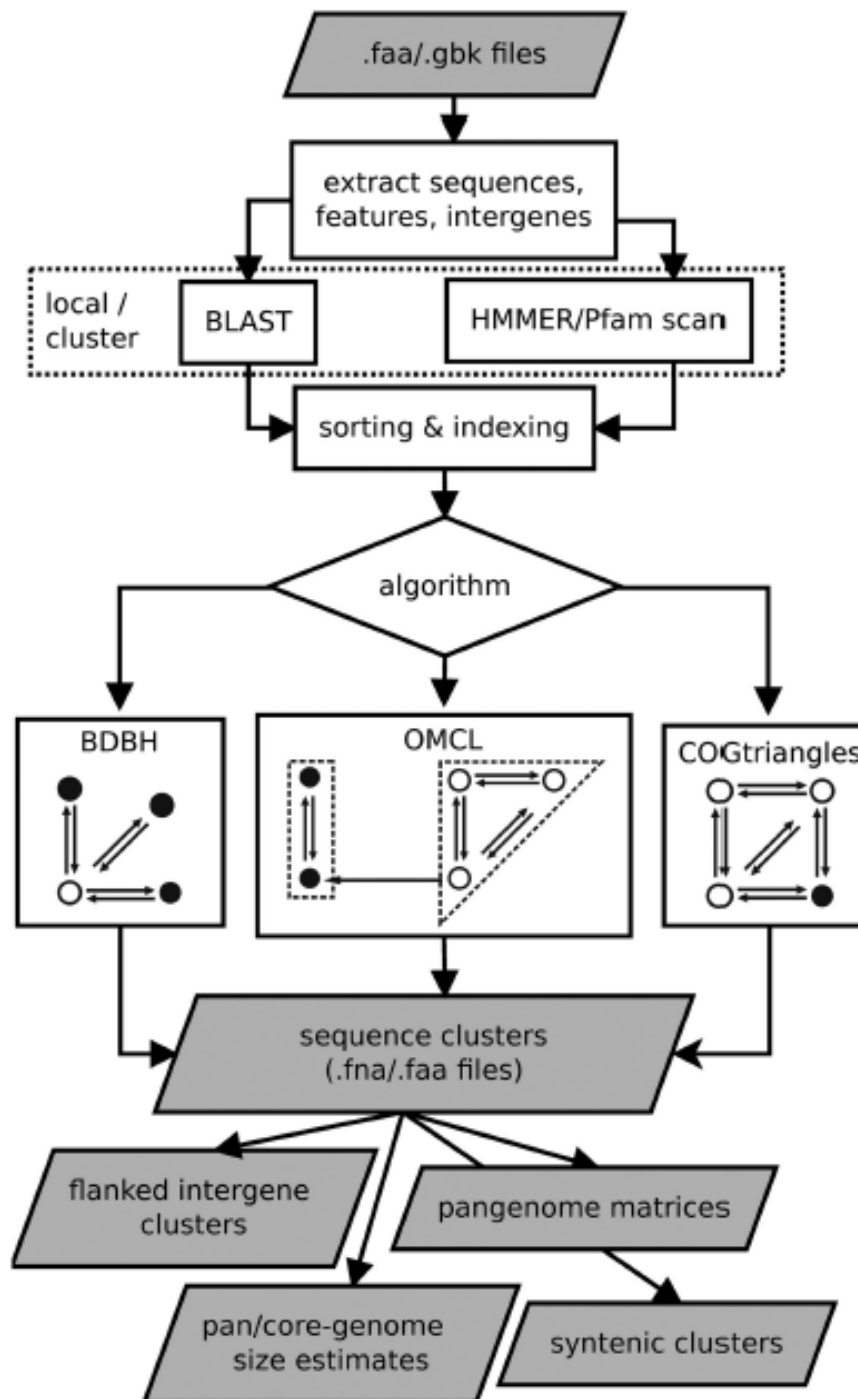
BLAST

- % of coverage in the pairwise alignments query/subject
- % of sequence identity in query/subject pairs
- Genome uses as reference genome

BDBH

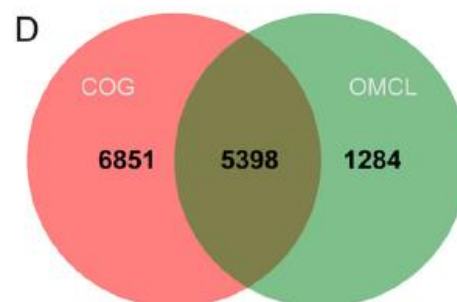
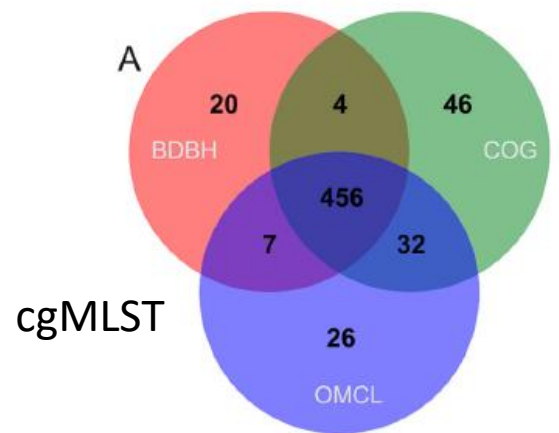


Get_Homologues

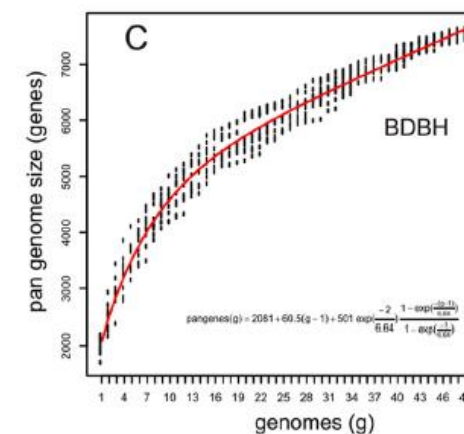
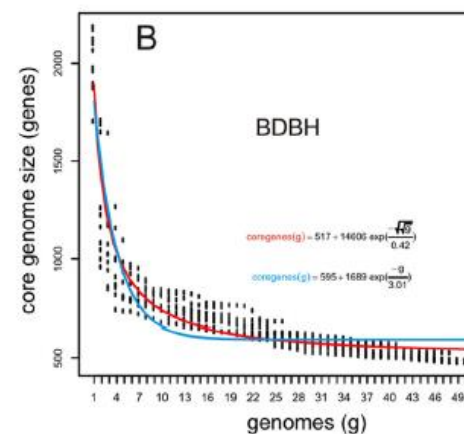


Get_Homologues

Contreras-Moreira, Appl Environ Microbiol 2013



50 Streptococcus proteomes from 14 species
BLAST: minimum pairwise alignment coverage of 75%



Reasons why schemas are different

Any collection of isolates is a subset of the entire population for the species of interest, and if the subset of isolates has limited genetic diversity then the number of “core” genes shared by all isolates in that sample will be higher than in a dataset which is genetically more diverse.

More generally, the **size of the core genome is dependent on the size of the data set**, with the core genome decreasing in size as more genomes are added to the analysis

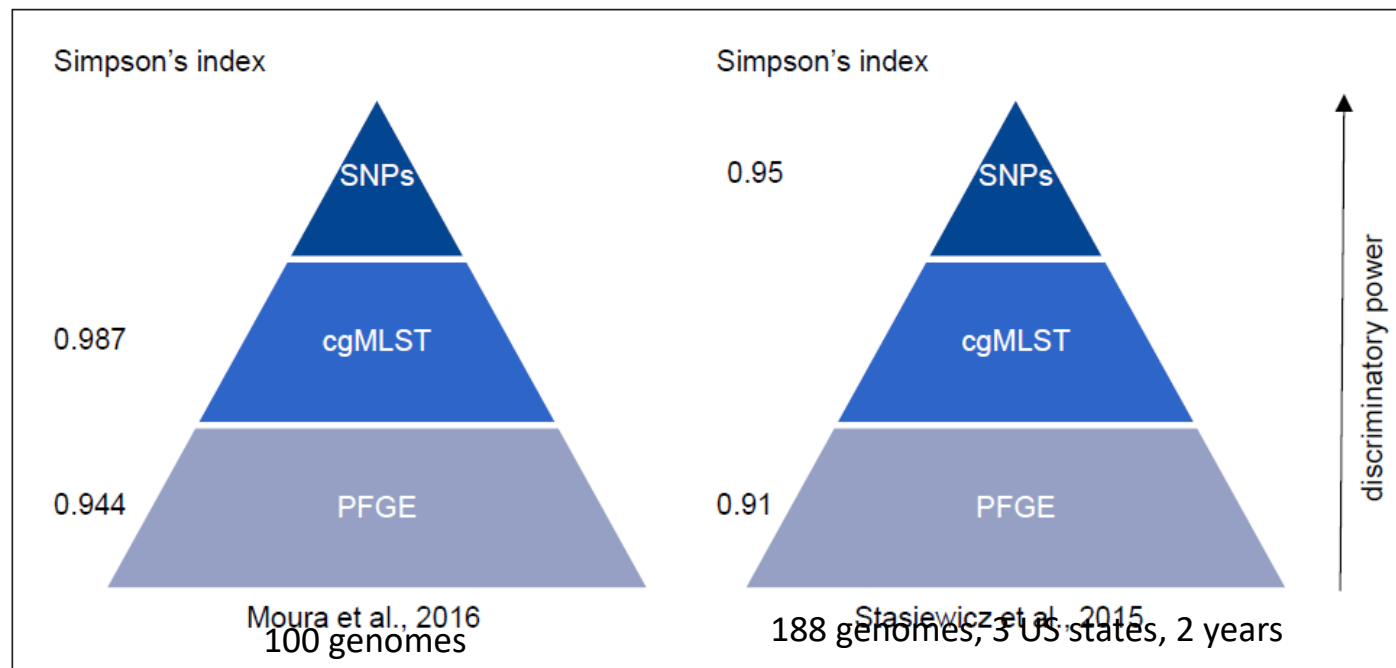
Technical reason:

Incomplete or “draft” genomes. This is acceptable for most studies, but analyses of these genomes may exclude a gene from a list of core genes simply because it contains a sequence gap or is otherwise incomplete at that locus in the assembly of one or a few genomes

BLAST parameters

Discriminatory power of typing methods

Listeria monocytogenes



The Simpson's index is used to quantify the probability that two unrelated strains are assigned to different typing groups

Core-genome schemas for *Listeria monocytogenes*

Ruppitsch et al., 2015

SeqSphere+

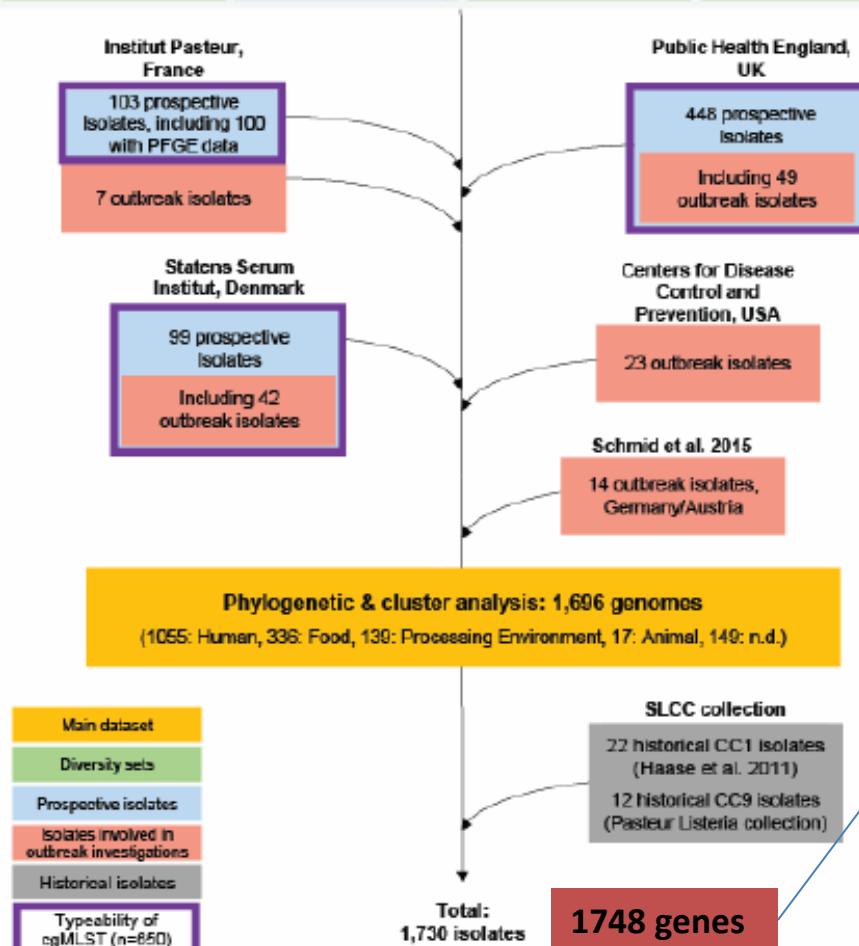
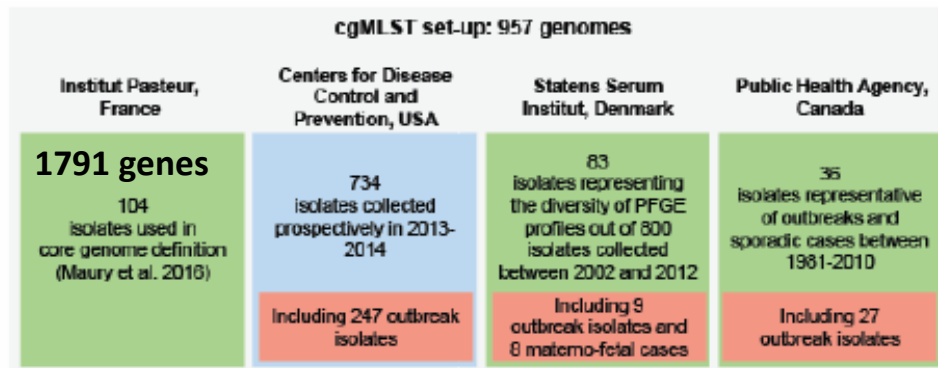
Pightling et al, 2015

Bioinformatics pipeline that takes raw sequence reads as input and calculating a core genome profile by comparing it to an expandable database to compile a phylogeny

Moura et al., 2016

1748 loci

All 4
lineages



BLASTN

Minimum nucleotide identity 70%
Alignment length coverage 70%

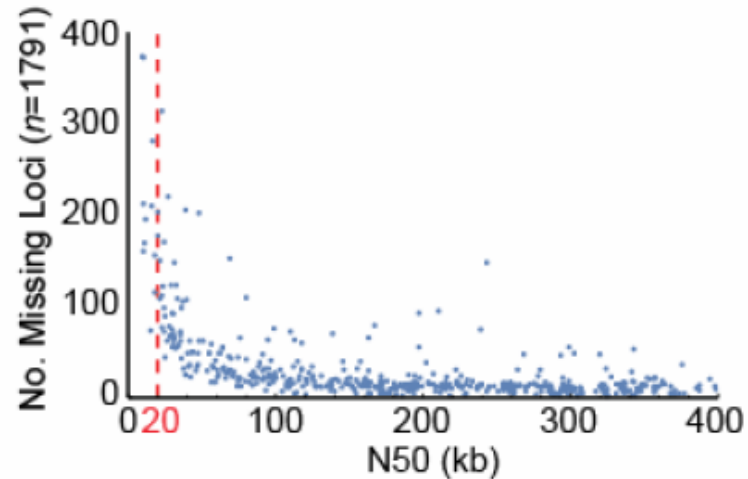
62% of its CDS

Moura et al., Nature Microbiology 2016

Moura core-genome schema for *Listeria monocytogenes*

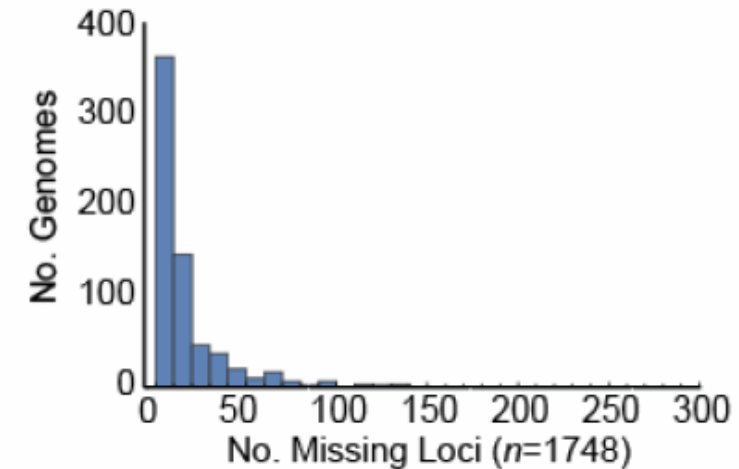
Validation of the cgMLST scheme with a set of 650 genomes

A



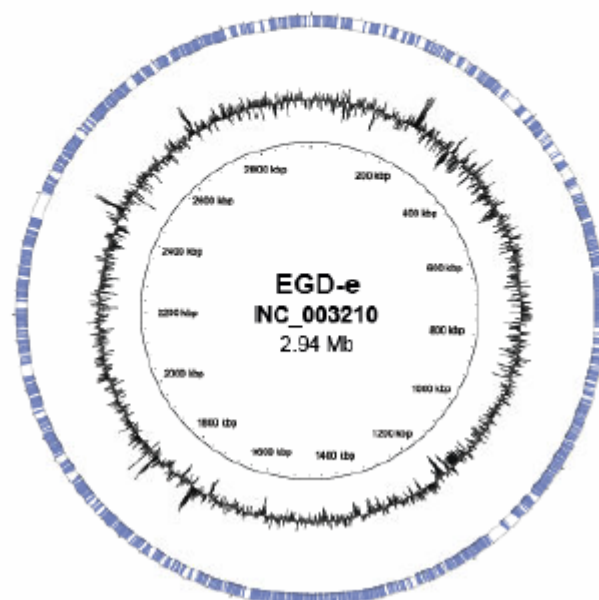
Impact of the N50 assembly size in the number of missing loci. Cut-off N50 of 20kb

B

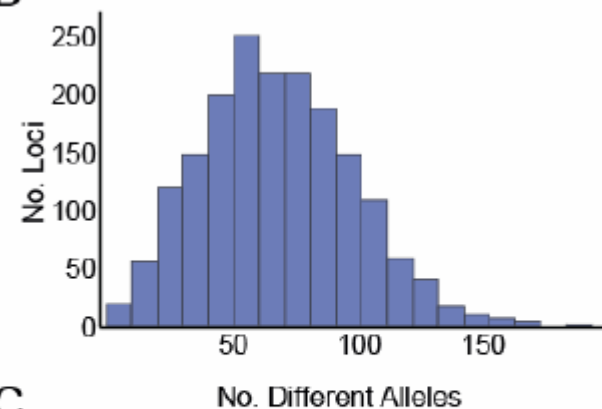


Distribution of the number of missing loci per genome

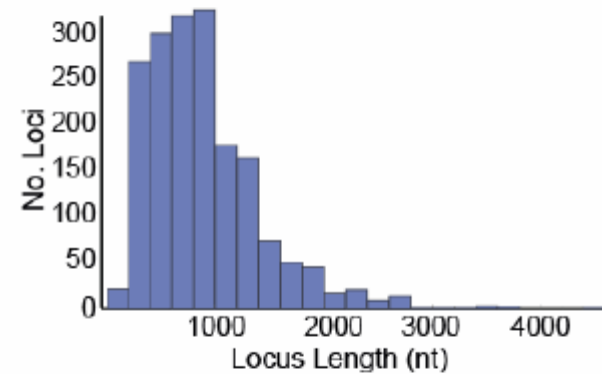
A



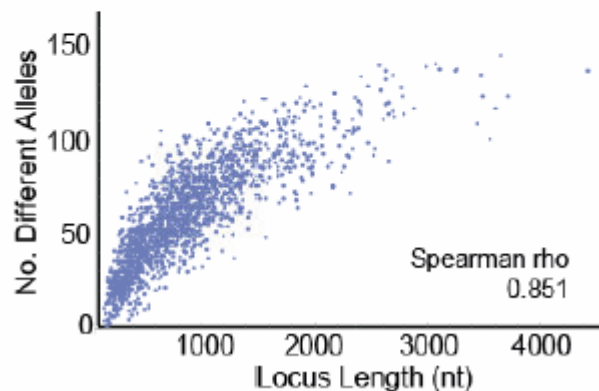
B



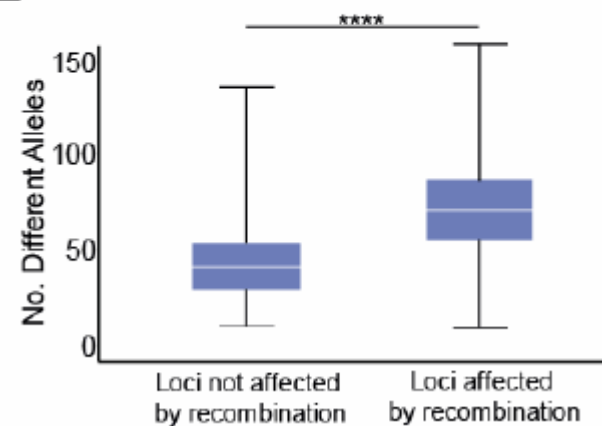
C



D

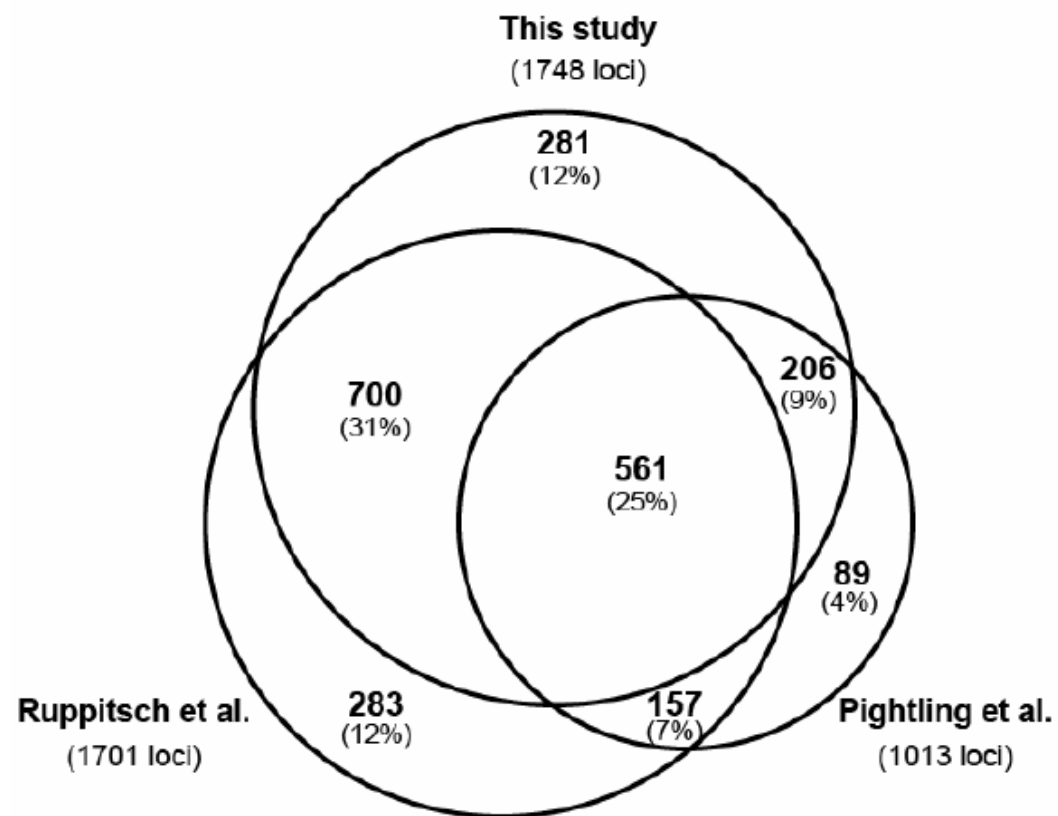


E

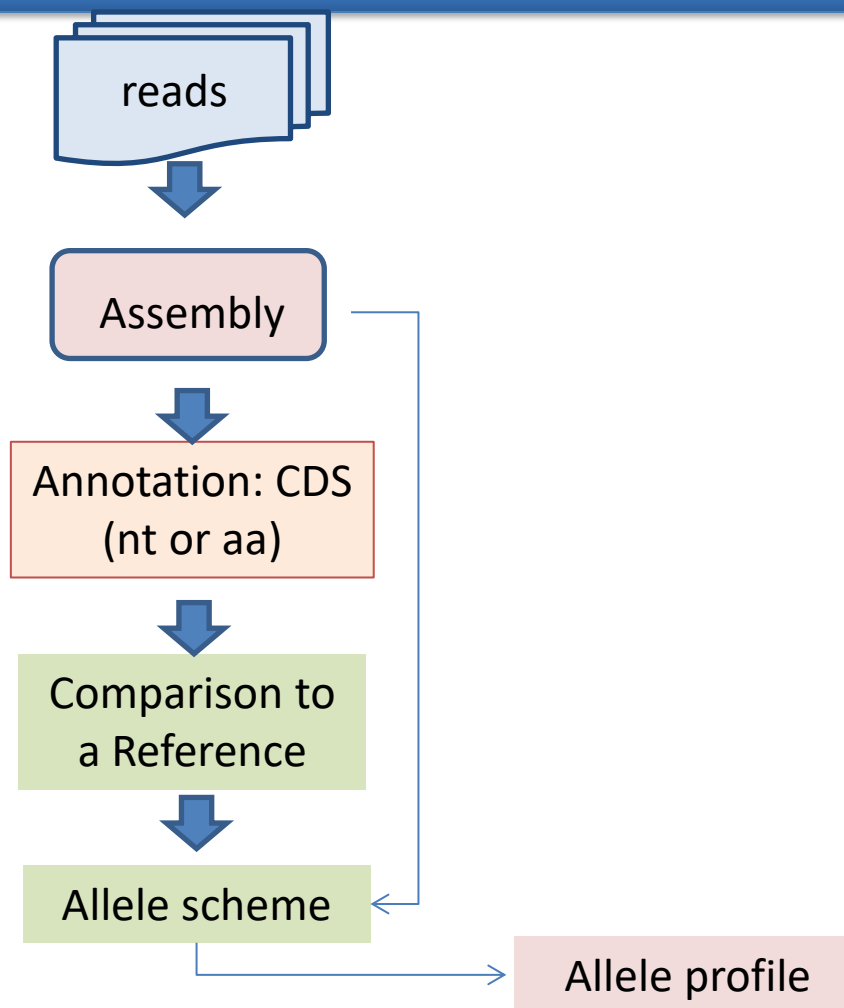


1370 Distinct allelic profiles in 1696 genomes

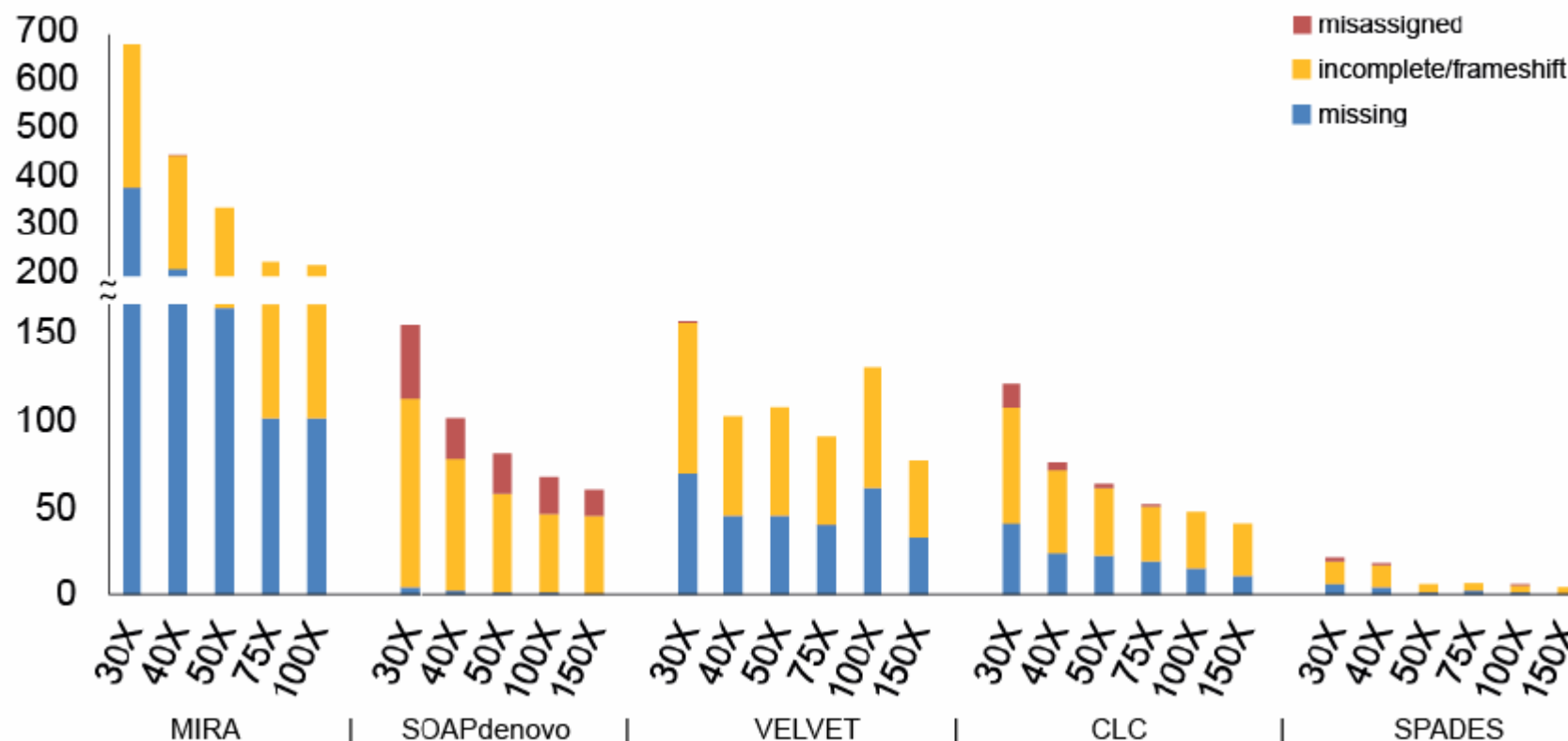
Comparison to other genome-based MLST schemes



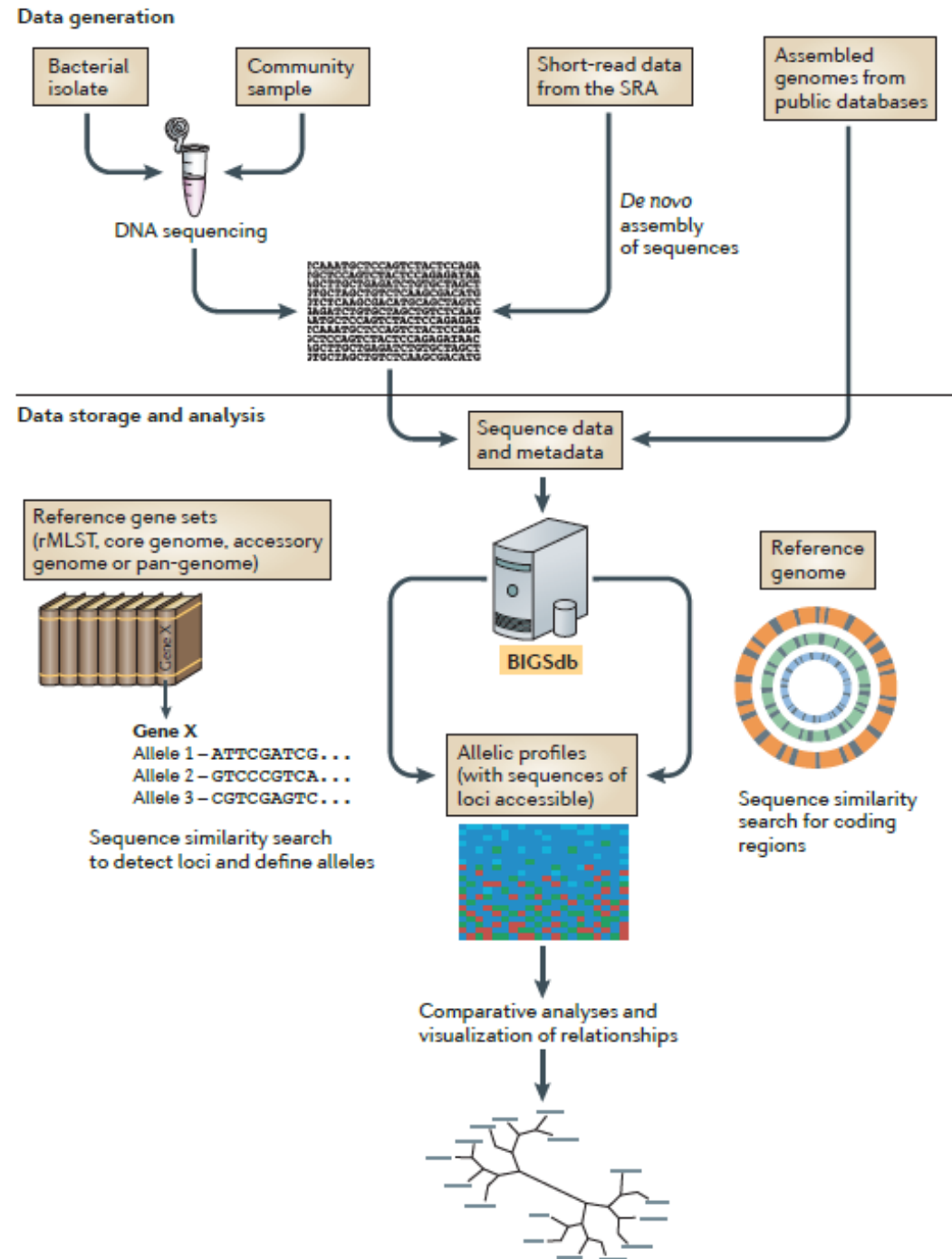
General analytical process for cgMLST / wgMLST



Reproducibility of cgMLST allelic calls, sequencing depth & assembly strategie



BIGSdb-1m



Retrospective validation of whole genome sequencing-enhanced surveillance of listeriosis in Europe, 2010 to 2015

2,726 Lm isolates from human cases from 27 EU/EEA countries, 2010-2015

1,069 isolates -> public health laboratories

1,657 isolates -> commercial sequencing provider

MiSeq (2x150, 2x250, 2x300) NextSeq (2x150), HiSeq (2x100), Ion Torrent PGM

Trimming

- Removal of any adaptor sequences
- Removal of leading bases and trailing bases with $Q < 25$
- Window of 20 bases has average $Q < 25$
- Removal reads with length $< 36b$

Retrospective validation of whole genome sequencing-enhanced surveillance of listeriosis in Europe, 2010 to 2015

Assembly: Spades 3.7.1 or Velvet 1.1.04. Minimum contig length 300nt. Assembly with the highest N50 was retained.

Allelic profile: two subsets of isolate pairs, $AD \leq 7$ (closely related isolates likely to share a common epidemiological link), $AD \leq 150$ (sublineages where isolates are still likely to have common phenotypic properties that may be relevant, e.g. source attribution)
Moura (Bionumerics: reads (kmers) or BLASTN), 1748 loci
Ruppitsch (SeqSphere +3.4.1), 1701 loci

Retrospective validation of whole genome sequencing-enhanced surveillance of listeriosis in Europe, 2010 to 2015

Conclusions (I):

- The average coverage up to around 55x before trimming and 45x after trimming (Illumina)
- Assembly-based allele calling outperforms reads-based allele calling -> more loci were detected (increase typeability) and the average distances between isolates were slightly smaller.
- Velvet including k-mer optimisation performed slightly worse than SPAdes, but both produced near-equivalent results.
- cgMLST analysis to share assembled genomes rather than sequence read data (fastq , for SNPs analysis or to verify the analyses in some cases, e.g. multi-country outbreaks)
- Important: individual differences in ADs between Moura and Ruppitsch CG schemes can be relatively large, since only 1,261 loci are common to both schemes.

Retrospective validation of whole genome sequencing-enhanced surveillance of listeriosis in Europe, 2010 to 2015

Conclusions (II):

- The $AD \leq 7$ cutoff is useful for cluster detection for both schemas, in general, although there are exceptions when there are epidemiologically linked isolates with more allele differences than the cutoff (more than one strain or specific sublineages may have higher average mutation rates -> sublineages specific cutoff ??)
- WGS-enhanced surveillance of listeriosis: many clusters found involved more than one country. Earlier detection of clusters.
- The molecular typing results must also be combined with epidemiological and food exposure investigations.

External quality assessment scheme for *Listeria monocytogenes* typing – 5th 2018 vs 7th 2021

5th 2018 **Table 1. Number and percentage of laboratories submitting results for each method**

	Serotyping				Cluster analysis			
	Conventional only	Molecular only	Both	Total	PFGE-only	WGS-only	Both	Total
Number of participants	1	12	5	18	3	8	4	15
Percentage of participants	6%	67%	28%	90%*	20%	53%	27%	75%*

Thirteen of the 20 participants (65%) completed both parts (serotyping and cluster analysis) of the EQA.

** Percentage of total number of participating laboratories (20)*

7th 2021 **Table 1. Number and percentage of laboratories submitting results for each method**

	Serotyping				Cluster analysis			
	Conventional only	Molecular only	Both	Total	PFGE only	WGS only	Both	Total
Number of participants	1	10	3	14	2	12	1	15
Percentage of participants	7	71	21	82*	13	80	6	88*

Twelve of the 17 participants (71%) completed both parts (serotyping and cluster analysis) of the EQA.

**: percentage of total number of participating laboratories (17).*

Fifth external quality assessment scheme for *Listeria monocytogenes* typing

Annex 7. Reported sequencing details

Sequencing performed	Protocol (library prep)	Commercial kit	Sequencing platform
In own laboratory	Commercial kits	Nextera XT DNA library Preparation Kit*	HiSeq2500
In own laboratory	Commercial kits	NEBNext® Fast DNA Fragmentation & Library Prep Set for Ion Torrent, New England 'Biolabs**	Ion Torrent PGM
Externally	Commercial kits	Illumina	HiSeq 2500
In own laboratory	Commercial kits	Ion Xpress™ Plus Fragment Library Kit for AB Library Builder™ System	IonTorrent S5XL
In own laboratory	Commercial kits	Nextera XT	MiSeq
In own laboratory	Commercial kits	NEXTERA	MiSeq
In own laboratory	Commercial kits	SureSelect QXT Library Prep Kit (Agilent)	MiSeq
In own laboratory	Commercial kits	Nextera XT DNA Library Preparation Kit	MiSeq
In own laboratory	Commercial kits	Nextera XT	MiSeq
In own laboratory	Commercial kits	Nextera XT***	Miniseq
In own laboratory	Commercial kits	Nextera XT Library Prep kit (96 samples)***	NextSeq
In own laboratory	Commercial kits	Illumina Nextera XT library Prep Kit	MiSeq

* 5ng input DNA (as opposed to 1ng)

Altered PCR protocol to favour longer fragment sizes

Adjustment of extension temperature (and final extension) from 72° to 65°C

'Manual' normalisation using library concentration and fragment size as opposed to bead-based normalisation.

** Shearing carried out for 15 minutes at 25°C instead of 20 minutes because 400bp sequencing protocol was used

*** Half volume for all reagents.

Table 7. Results of raw reads submitted by participants evaluated by EQA provider QC pipeline summarised by laboratory

Parameters	Ranges*	Laboratory ID											
		19	35	56	70	105	108	129	135	141	142	144	146
No. of genera detected	{1}	1	1	1	1	1	1	1	1	1	1	1	1
Detected species	{Lm}	Lm	Lm	Lm	Lm	Lm-N	Lm	Lm	Lm	Lm	Lm	Lm	Lm
Unclassified reads (%)		1.5-2.5	0.6-2.5	0.6-2.2	1.5-2.9	0.7-50.8	1.1-1.8	0.6-1.7	0.5-1.0	0.9-1.8	0.8-1.4	0.2-1.3	0.4-2.0
Length at 25 x min. coverage (Mbp)	{>2.8 ^ <3.1}	2.9-3.0	2.9-3.0	1.8-2.7	2.9-3.0	0.1-3.0	2.9-3.0	2.9-2.9	2.9-3.0	1.0-3.0	2.9-3.0	2.9-3.0	2.9-3.0
Length [0-25] x min. coverage (Mbp)	{<0.25}	0	0	0	0	0-0.9	0	0-0.1	0	0.0-1.8	0	0	0
No. of contigs at 25 x min. coverage	{>0}	14-21	12-25	876-1056	17-45	14-193	57-146	15-47	17-24	19-85	13-17	11-17	17-25
No. of contigs [0-25] x min. coverage#	{<1000}	0	0	0	0-4	0-517	0-5	0-24	0	0-165	0-2	0	0-1
Average coverage	{>50}	160-224	40-175	61-104	51-100	8-94	30-70	50-244	153-221	24-126	40-58	75-128	140-200
No. of reads (x 1000)		1741-2457	250-1120	707-1278	528-1035	345-622	285-689	530-2704	1898-2835	158-883	261-385	525-881	2148-3169
No. of trimmed reads (x1000)		1721-2428	248-1110	691-1235	524-1028	342-609	521-617	523-2677	1878-2800	150-865	295-380	534-870	2148-3169
Maximum read length		151	301	285-365	151	301	241-319	151	126	301	251	251	101
Mean read length		140-142	215-251	217-229	143-146	204-241	186-200	139-145	123-124	218-235	245-234	210-227	97-100
Read insert size		267.9-305	333-394	NA	288-391	199-363	NA	244-450	326-351	279-358	361-399	280-327	204-360
Insert size StdDev		100-106	158-199	NA	100-149	67-158	NA	108-196	175-188	102-130	157-174	93-125	85-169
N50 (kbp)		238-551	274.4-558	1.4-3.4	162.3-318	1.3-407	34.0-87	125-551	295-482	22-263	262-556	353-558	286-510
N75 (kbp)		143.3-257.3	139-263	0.9-1.9	78-238	0.8-262	23-45	61-258	142-258	11-236	198-262	183-263	144-262

* Indicative QC range

Lm: L. monocytogenes

N: Neisseria

Number of contigs with coverage < 25 (Figure 10B)



Fifth external quality assessment scheme for *Listeria monocytogenes* typing

Table 4. Results of SNP-based cluster analysis

Lab ID	SNP-based						
	Approach	Reference	Read mapper	Variant caller	Assembler	Distance within cluster	Distance outside cluster
Provider	Reference-based	ST6 (REF4)	BWA	GATK		0-3	38-71
19*	Reference-based	ST6 ID 2362	BWA	GATK		0-4	43-81
56	Assembly-based			ksnp3	SPAdes	0-57#	561-591 (6109)
105	Reference-based	ST6 J1817	Bowtie2	VARSCAN 2		0-2#	22-42 (1049)
108	Reference-based	In-house strain resp ST	CLC assembly cell v4.4.2	CLC assembly cell v4.4.2		0-2	37-72
142*	Reference-based	<i>Listeria</i> EGDe (cc9)	CLC Bio	CLC Bio		0-1219	1223-2814 (8138)
146	Reference-based	ST6 ref. CP006046 ST1 ref. F2365 ST213/ST382 no ref.	BWA	In-house		0-358	

* Additional analysis

Only three isolates included due to data quality not meeting laboratory's own QC thresholds

✕ Reported distance to ST6 (non-ST6) isolates (Annex 9).

Fifth external quality assessment scheme for *Listeria monocytogenes* typing

Table 5. Results of allele-based cluster analysis

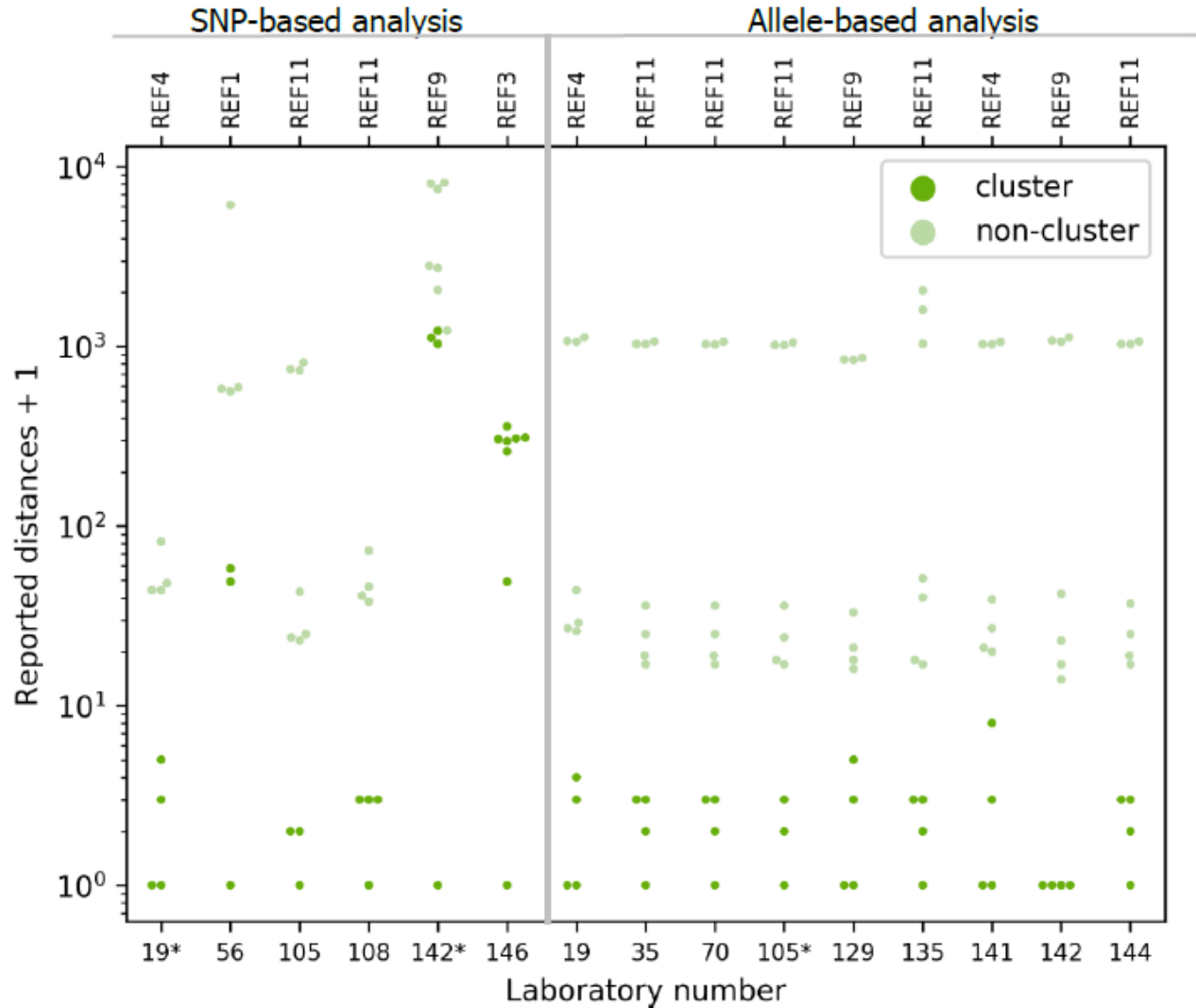
Lab ID	Allele based analysis					
	Approach	Allelic calling method	Assembler	Scheme	Difference within cluster	Difference outside cluster
EQA provider	BioNumerics	Assembly- and mapping-based	SPAdes	Applied Math (cgMLST/Pasteur)	0-3	24-1112
19	BioNumerics	Assembly- and mapping-based	SPAdes	Applied Math (cgMLST/Pasteur)	0-3	25-1120
35	SeqPhere	Assembly-based only	Velvet	Ruppitsch (cgMLST)	0-2	16-1065
70	SeqPhere	Assembly-based only	Velvet	Ruppitsch (cgMLST)	0-2	16-1062
105*	SeqPhere	Assembly-based only	SPAdes v 3.80	Ruppitsch (cgMLST)	0-1 [#]	23-812
129	SeqPhere	Assembly-based only	Velvet	In-house (cgMLST)	0-4	15-862
135	SeqPhere	Assembly-based only	CLC Genomics Workbench 10	Ruppitsch (cgMLST)	0-2	16-2042
141	SeqPhere	Assembly-based only	SPAdes 3.9.0	Ruppitsch (cgMLST)	0-7	19-1060
142	Inhouse	Assembly-based only	SPAdes	Pasteur (cgMLST)	0	13-1120
144	SeqPhere	Assembly-based only	Velvet	Ruppitsch (cgMLST)	0-2	16-1065

* Additional analysis

[#] Only three isolates included due to data quality not meeting laboratory's own QC thresholds (Annex 9).



Figure 7. Reported SNP distances or allelic differences for each test isolate to selected cluster representative isolate



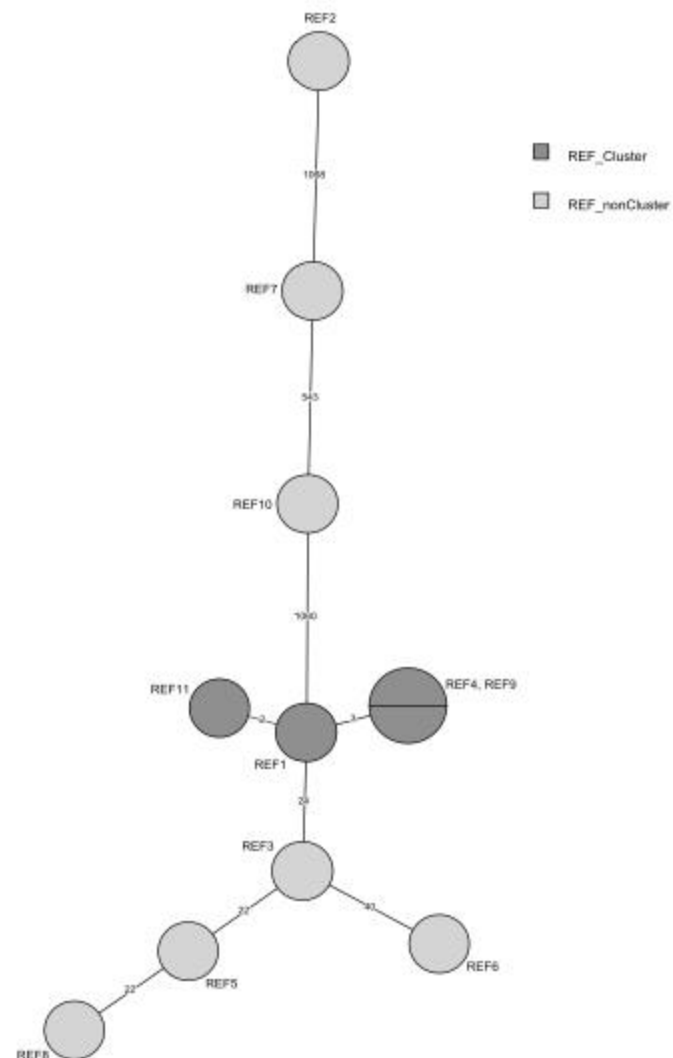
* Additional analysis

SNP: Single nucleotide polymorphism

Selected cluster representative marked as REF in dark green: Reported cluster of closely related isolates

Light green: Not reported as part of cluster.

Annex 4. EQA provider cluster analysis based on WGS-derived data



Minimum spanning tree of core genome multi locus sequence typing (cgMLST, [6]) profiles of *L. monocytogenes* EQA-5 isolates. Logarithmic scaling in BioNumerics.

Dark grey: Cluster isolates

Light grey: Outside cluster isolates

Fifth external quality assessment scheme for *Listeria monocytogenes* typing

Annex 5. Reported cluster of closely related isolates based on PFGE-derived data

Lab ID	Reported cluster	Corresponding REF isolates	Correct
Provider	REF1, REF4, REF9, REF11 (4 and 9 technical duplicates)		
19	3362# 2539 2691 2719	REF4, REF1, REF9, REF11	Yes
100	2080 2295 2405 2499	REF4, REF9, REF11, REF1	Yes
105	2073 2709 2805 2978	REF4, REF9, REF1, REF11	Yes
138	2141 2349 2778 2947	REF9, REF1, REF4, REF11	Yes
141	2022 2050 2092 2872	REF1, REF4, REF11, REF9	Yes
142	2385 2529 2794 2837	REF9, REF4, REF11, REF1	Yes
145	2027 2235 2287 2444 2514 2592 2680 2699 2904 2961 2967	REF5, REF9, REF1, REF3, REF11, REF4 REF7, REF2, REF8, REF6, REF 10	No

Writing error 2362

Fifth external quality assessment scheme for *Listeria monocytogenes* typing

Annex 8. Reported cluster of closely related isolates based on WGS-derived data

Lab ID	Reported cluster	Corresponding to REF isolates	Correct
Provider	REF1, REF4, REF9, REF11 (4 and 9 technical duplicates)		
19	#3562 3539 2691 2719	REF4, REF1 REF9, REF11	Yes
35	2251 2737 2783 2993	REF11, REF9, REF1, REF4	Yes
56	2341 2165 2612	REF9, REF1, REF11	Yes
70	2104 2216 2567 2767	REF4, REF1, REF11, REF9	Yes
105	2073 2805 2978	REF4, REF1, REF11	Yes
108	2098 2788 2582 2422	REF1, REF11, REF9, REF4	Yes
129	2079 2640 2912 2950	REF1, REF9, REF11, REF4	Yes
135	2161 2423 2673 2897	REF1, REF4, REF11, REF9	Yes
141	2022 2050 2092 2872	REF1, REF4, REF11, REF9	Yes
142	2385 2529 2794 2837	REF9, REF4, REF11, REF1	Yes
144	2143 2626 2727 2822	REF4, REF11, REF1, REF9	Yes
146	2068 2197 2377 2488 ##2353 2575 2655 2726	REF5, REF8, REF3, REF1, REF6, REF4, REF9, REF11	No

#Writing error 2362

##Writing error 2553

Fifth external quality assessment scheme for *Listeria monocytogenes* typing

Annex 9. Reported SNP distance and allelic differences

SNP distances

Isolate no.	ST	Provider	Laboratory ID					
			19 [*]	56	105	108	142 [*]	146
REF1 [‡]	6	3	4	0 [‡]	1	2	1030	306
REF2	1	9999	9999	9999	812	9999	7502	9999
REF3	6	41	47	579	23	45	2814	0 [‡]
REF4 ^{‡#}	6	0 [‡]	0 [‡]	9999	1	2	1219	309
REF5	6	40	43	561	24	37	2056	259
REF6	6	72	81	591	42	72	2732	358
REF7	213	9999	9999	9999	734	9999	8050	9999
REF8	6	39	43	9999	22	40	1223	48
REF9 ^{‡#}	6	0	0	57	9999	2	0 [‡]	296
REF10	382	9999	9999	6109	745	9999	8138	9999
REF11 [‡]	6	1	2	48	0 [‡]	0 [‡]	1114	304

Fifth external quality assessment scheme for *Listeria monocytogenes* typing

Allelic distances

Isolates no.	ST	Provider	Laboratory ID								
			19	35	70	105 ⁺	129	135	141	142	144
REF1 [‡]	6	3	3	1	1	1	4	1	7	0	1
REF2	1	1118	1120	1065	1062	812	862	2042	1060	1120	1065
REF3	6	25	25	16	16	23	15	16	19	16	16
REF4 ^{‡#}	6	0 [*]	0 [*]	2	2	1	0	2	0 [*]	0	2
REF5	6	26	26	18	18	24	17	17	20	13	18
REF6	6	44	43	35	35	42	32	50	38	41	36
REF7	213	1073	1070	1028	1026	734	842	1031	1024	1074	1028
REF8	6	28	28	24	24	22	20	39	26	22	24
REF9 ^{‡#}	6	0	0	2	2	9999	0 [*]	2	0	0 [*]	2
REF10	382	1060	1060	1027	1021	745	839	1592	1025	1063	1027
REF11 [‡]	6	3	2	0 [*]	0 [*]	0 [*]	2	0 [*]	2	0	0 [*]

* Additional analysis

‡ Closely related isolates

Technical duplicate isolate

⌘ Isolate used as cluster representative by participant

9999: Isolates not included in analysis by participant

ST: Sequence type

Criteria for wg/cgMLST and SNP typing schemes

Examples of relatedness criteria for wg/cgMLST and SNP typing schemes of representative clinically relevant bacteria

Organism	Relatedness threshold ^a		References
	wg/cgMLST (allele)	SNPs	
<i>Acinetobacter baumannii</i>	≤8	≤3	[25,26]
<i>Brucella</i> spp.	Epidemiologic validation in progress ^b		http://www.applied-maths.com/applications/wgmlst
<i>Campylobacter coli</i> , <i>C. jejuni</i>	≤14	≤15	[27,28]
<i>Cronobacter</i> spp.	Epidemiologic validation in progress ^b		http://www.applied-maths.com/applications/wgmlst
<i>Clostridium difficile</i>	Epidemiologic validation in progress ^b	≤4	[29], http://www.cgmlst.org/ncs , http://www.applied-maths.com/applications/wgmlst
<i>Enterococcus faecium</i>	≤20	≤16	[30]
<i>Enterococcus raffinosus</i>	Epidemiologic validation in progress ^b		http://www.applied-maths.com/applications/wgmlst
<i>Escherichia coli</i>	≤10	≤10	[31,32], https://enterobase.warwick.ac.uk/
<i>Francisella tularensis</i>	≤1	≤2	[33,34]
<i>Klebsiella oxytoca</i>	Epidemiologic validation in progress ^b		http://www.applied-maths.com/applications/wgmlst
<i>Klebsiella pneumonia</i>	≤10	≤18	[35,36]
<i>Legionella pneumophila</i>	≤4	≤15	[37]
<i>Listeria monocytogenes</i>	≤10	≤3	[38,39]
<i>Mycobacterium abscessus</i>		≤30	[40]
<i>Mycobacterium tuberculosis</i>	≤12	≤12	[41]
<i>Neisseria gonorrhoeae</i>	Epidemiologic validation in progress ^b	≤14	[42], http://www.applied-maths.com/applications/wgmlst
<i>Neisseria meningitidis</i>	Epidemiologic validation in progress ^b		http://www.cgmlst.org/ncs
<i>Pseudomonas aeruginosa</i>	≤14	≤37	[31,43]
<i>Salmonella dublin</i>	Epidemiologic validation in progress ^b	≤13	[44], https://enterobase.warwick.ac.uk/
<i>Salmonella enterica</i>	Epidemiologic validation in progress ^b	≤4	[45], http://www.cgmlst.org/ncs , http://www.applied-maths.com/applications/wgmlst , https://enterobase.warwick.ac.uk/
<i>Salmonella typhimurium</i>	Epidemiologic validation in progress ^b	≤2	[46], https://enterobase.warwick.ac.uk/
<i>Staphylococcus aureus</i>	≤24	≤15	[47,48]
<i>Streptococcus suis</i>		≤21	[49]
<i>Vibrio parahaemolyticus</i>	≤10		[50]
<i>Yersinia</i> spp.	0		[51]

Thanks for your attention!