Mycobacterium tuberculosis complex lineage 5 exhibits high levels of within-lineage genomic diversity and differing gene content compared to the type strain H37Rv

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Abstract

Pathogens of the *Mycobacterium tuberculosis* complex (MTBC) are considered monomorphic, with little gene content variation between strains. Nevertheless, several genotypic and phenotypic factors separate the different MTBC lineages (L), especially L5 and L6 (traditionally termed *Mycobacterium africanum*), from each other. However, genome variability and gene content especially of L5 and L6 strains have not been fully explored and may be potentially important for pathobiology and current approaches for genomic analysis of MTBC isolates, including transmission studies.

We compared the genomes of 358 L5 clinical isolates (including 3 completed genomes and 355 Illumina WGS (whole genome sequenced) isolates) to the L5 complete genomes and H37Rv, and identified multiple genes differentially present or absent between H37Rv and L5 strains. Additionally, considerable gene content variability was found across L5 strains, including a split in the L5.3 sublineage into L5.3.1 and L5.3.2. These gene content differences had a small knock on effect on transmission cluster estimation, with clustering rates influenced by the selection of reference genome, and with potential over-estimation of recent transmission when using H37Rv as the reference genome.

Our data show that the use of H37Rv as reference genome results in missing SNPs in genes unique for L5 strains. This potentially leads to an underestimation of the diversity present in

the genome of L5 strains and in turn affects the transmission clustering rates. As such, a full capture of the gene diversity, especially for high resolution outbreak analysis, requires a variation of the single H37Rv-centric reference genome mapping approach currently used in most WGS data analysis pipelines. Moreover, the high within-lineage gene content variability suggests that the pan-genome of *M. tuberculosis* is at least several kilobases larger than previously thought, implying a concatenated or reference-free genome assembly (*de novo*) approach may be needed for particular questions.

Keywords: gene content, *M. africanum*, Lineage 5, H37Rv, reference genome, genomic diversity, within-lineage variability

Data summary

Sequence data for the Illumina dataset are available at European Genome-phenome Archive (EGA; https://www.ebi.ac.uk/ega/) under the study accession numbers PRJEB38317 and PRJEB38656. Individual runs accession numbers are indicated in Table S8.

PacBio raw reads for the L5 Benin genome are available on the ENA accession SAME3170744. The assembled L5 Benin genome is available on NCBI with accession PRJNA641267. To ensure naming conventions of the genes in the three L5 genomes can be followed, we have uploaded these annotated GFF files to figshare at https://doi.org/10.6084/m9.figshare.12911849.v1.

Custom python scripts used in this analysis can be found at https://github.com/conmeehan/pathophy.

Introduction

Tuberculosis (TB) is caused by pathogenic bacteria of the *Mycobacterium tuberculosis* complex (MTBC) that consists of 9 human-adapted lineages and several animal-adapted

67 lineages (Mireia Coscolla and Gagneux 2014; Ngabonziza et al. 2020; M Coscolla et al. 2020;
68 Brites et al. 2018). This group is highly clonal with no horizontal gene transfer (Boritsch et al.
69 2016; Chiner-Oms et al. 2019). Strains of particular lineages are primarily defined by large
70 sequence polymorphisms (LSP, the presence or deletion of genomic regions) such as the TbD1
71 region (MTBC specific deletion 1), other regions of difference (RD) (Boritsch et al. 2016; Brosch
72 et al. 2002; Gordon et al. 1999; Behr et al. 1999; Gagneux et al. 2006), and signature single
73 nucleotide polymorphisms (SNPs)(Coll et al. 2014). RDs are particular long sequence genomic
74 region deleted in some groups of MTBC strains but present in others; so that MTBC strains
75 can be classified in groups using that LSP (Gordon et al. 1999; Brosch et al. 2002; Mostowy et
76 al. 2002; Gagneux et al. 2006). Broadly, the lineages of the MTBC occur within three major
77 clades: (1) L1-L4 and L7 form one group, (2) L5, L6, L9 and the animal lineages form another,
78 and (3) L8 sits within its own clade (Coscolla and Gagneux 2014; Ngabonziza et al. 2020;
79 Coscolla et al. 2020), based on the presence/absence of specific RDs, especially TbD1
80 (Firdessa et al. 2013; Brosch et al. 2002; Nebenzahl-Guimaraes et al. 2016). L5 and L6 are also
81 called *M. tuberculosis* var. africanum (or historically *M. africanum* West-african 1 and 2
82 respectively) (Riojas et al. 2018; Niemann et al. 2004) and are restricted to West Africa where
83 they cause up to 40% of human TB (de Jong, Antonio, and Gagneux 2010). Reasons for the
84 geographical restriction of L5 and L6 remain unclear, however, adaptation to particular
85 human subpopulation has been suggested (Intemann et al. 2009; Thye et al. 2011; Comas et
86 al. 2013; Gagneux et al. 2006).

87 Several phenotypic and genotypic features separate L5 and L6 from the other human-adapted
88 lineages. Some TB diagnostics have a lower performance for L5 and L6 strains, compared to
89 other MTBC lineages (Sanoussi et al. 2018; Ofori-Anyinam et al. 2016) and these lineages are
90 less likely to grow in culture (Sanoussi et al. 2017), with dysgonic appearance on solid medium

91 (Leao et al. 2004; Pattyn et al. 1970; Sanoussi et al. 2018; Niemann et al. 2004). Mutations in
92 genes essential for growth in culture were identified for L6 strains (Gehre et al. 2013), yet for
93 L5 strains the reasons for the difficulty in growth remain unclear but may be related to the
94 presence of the TbD1 region and the absence of RD9 (Brosch et al. 2002). L6 strains and those
95 of the closely related animal strains/lineages such as *M. bovis* are reported to be less virulent
96 in humans than those from other human-adapted MTBC lineages in population-based studies
97 (de Jong et al. 2008; Magnus 1966; Reiling et al. 2013) and in genome studies of mutations in
98 virulence regulation genes/systems (Gonzalo-Asensio et al. 2014; Ofori-Anyinam et al. 2020).
99 Infection with an L6 strain progresses slowly to TB disease, and is associated with impaired
100 immunity in some settings but not all (e.g. HIV infection (de Jong et al. 2008)). In contrast,
101 little is known about the genomics, virulence and disease progression of L5 strains.

102 Whole genome sequencing (WGS) based on next generation sequencing (NGS) of MTBC
103 strains often involves data analysis by mapping of short sequence reads to a reference
104 genome, usually H37Rv (Cole et al. 1998; Camus et al. 2019; Meehan et al. 2019) or a
105 reconstructed ancestor with the same gene content as H37Rv (Comas et al. 2013; Goig et al.
106 2019, 2020). The resulting SNPs are then used for drug resistance determination, subtyping
107 and transmission analyses (Meehan et al. 2019). However, since H37Rv is a L4 strain, it might
108 not be representative of other MTBC lineages, including L5 strains. Thus, if H37Rv is used as
109 basis for genome analysis, several genes or larger genomic regions may be missed resulting
110 in an underestimation of genome diversity among strains of other lineages that may,
111 however, provide additional information e.g. for transmission analysis.

112 The members of the MTBC evolved from an environmental organism to an obligate pathogen
113 through genome reduction and acquisition of new genes (Gagneux 2018) and it is known that

114 some differences in gene content exist between lineages (Meehan et al. 2019; Bifani et al.
115 2000; Periwal et al. 2015; Kato-Maeda et al. 2001; O'Toole and Gautam 2017; Gagneux et al.
116 2006; Tsolaki et al. 2005). Furthermore, it was reported that genes had a higher genetic
117 diversity among L6 strains compared to L5 ones (Darko Otchere et al. 2018), but the difference
118 in gene content between these two lineages was not investigated. Also, little is known about
119 the gene differences between L4 and L5 and the potential limitations this may impose for in-
120 depth analysis of L5 genome studies (e.g. sub-lineage detection and transmission tracking).
121 Similarly, little is known about within-lineage diversity in terms of gene content.

122 To address these questions, we assessed the gene content diversity of L5 by analyzing in the
123 context of whole genome sequencing and reference selection. To this end, we analyzed 358
124 genomes of L5 strains including three completed genomes and compared them to H37Rv and
125 closely related lineages (L6, *M. tuberculosis* var. bovis (hereafter called *M. bovis*)(Riojas et al.
126 2018)). Our main focus was to determine particularities in the genomes of L5 strains and to
127 define the level of within-lineage gene content differences among strains of this lineage of
128 the MTBC.

129

130

131 ## Materials and Methods

132 ### Genomes

133 **Complete genomes (PacBio, completed long reads)**. Three completed L5 genomes from
134 clinical isolates, all sequenced with the PacBio SMRT technology, were analyzed. One genome
135 was from a Benin isolate (PcbL5Ben; sequenced in this study), one from an isolate from The

136 Gambia (PcbL5Gam, WBB1453_11-00429-1) (Phelan et al. 2018), and one from Nigeria
137 (PcbL5Nig, WBB1454_IB091-1) (Phelan et al. 2018). These genomes are also from disparate
138 parts of the L5 diversity, representing a broad range of L5 sub-lineages, as were recently
139 defined (Coscolla et al. 2020). The previously published reference/complete genomes H37Rv
140 (L4) (NC_000962.3)(Cole et al. 1998; Camus et al. 2019; Lew et al. 2011), L6 (GM041182,
141 Genbank accession n°: FR878060.1, GCF_0001593225.1_ASM159322v1) (Bentley et al. 2012)
142 and *M. bovis* (AF2122/97, accession: LT708304.1) (Malone et al. 2017) were also included.

143 **Whole genomic DNA extraction**. Genomic DNA extraction was performed on growth from
144 fresh Löwenstein-Jensen slants using the semi-automated Maxwell 16 Cell DNA purification
145 kit in the Maxwell 16 machine, or from the late exponential phase of growth in 7H9 medium,
146 using the CTAB method (Belisle and Sonnenberg 1998; Coscolla et al. 2020)

147 **Assembly and annotation of the completed PacBio genomes.** The Benin Pacbio genome was
148 assembled using HGAP (Chin et al. 2013) and Quiver (Chin et al. 2013) and checked for
149 sufficient quality and coverage (a 10,000 bp sliding window coverage was always above 65x
150 and average coverage across entire genome is 107x). The genome sequences of all three
151 completed PacBio genomes were annotated using Prokka-1.12 (Seemann 2014) based on the
152 reference genome H37Rv annotation.

153 **Gene presence-absence analysis**

154 Gene content differences were assessed with an all-vs-all BLASTn approach, using BLAST+
155 version+2.8.1 (Altschul et al. 1990; Camacho et al. 2009) (Fig.1). For a specific genome-
156 genome comparison, the following procedure was used: the gene sequences of genome 1 (ffn
157 file; coding regions only) were compared to those of genome 2 using the default cut-offs to
158 look for any homology for each gene. Those genes found in genome 1 and not in genome 2

159 were then compared to the completed PacBio genome (fna file; coding and non-coding
160 regions) of genome 2 to look for pseudogenes (herein qualified as ''suspected pseudogenes'')
161 using BLASTn. These ''suspected pseudogenes'' were then confirmed using tBLASTn of the
162 genome 1 protein sequences (faa file) compared to the completed PacBio genome (fna file)
163 of genome 2. This procedure was used to compare all three L5 completed PacBio genomes to
164 each other as well as each to H37Rv. Those genes present or absent in H37Rv (or pseudogenes
165 in either) were compared in a similar manner to the L6 and *M. bovis* reference strains to
166 determine if these genes/pseudogenes are L5-specific (i.e. present in L5, but not in H37Rv, L6
167 and *M. bovis*).

168 The ffn files of H37Rv, the three L5 completed PacBio genomes and the L6 complete genomes,
169 were also aligned using progressive Mauve (ver 20150226)(Darling et al. 2004) to identify
170 rearrangements and examine synteny.

171 **L5 Illumina (short reads) genomes.** In total 355 L5 isolates genomes sequenced on the
172 Illumina platform from various countries were included in the study. These genomes were
173 derived from a larger study on genetic diversity of L5 and L6 (Coscolla et al. 2020). After
174 reducing isolate redundancy (i.e. one representative retained for those that were extremely
175 closely related), 205 L5 isolates genomes formed a non-redundant dataset. These 205 isolates
176 (genomes) originated from West, South, East and Central Africa (Table S8), but primarily
177 (n=155) from two regions within West Africa: the Western part of West Africa (including The
178 Gambia, Sierra Leone, Ivory Coast, Liberia, Guinea and Mali) and the Eastern part of West
179 Africa (including Ghana, Benin, and Nigeria) (Coscolla et al. 2020). In general, L5 and L6 are
180 geographically restricted to West and Central-Africa, making this L5 genome selection
181 representative of all the geographical regions affected.

182 **Mapping of L5 Illumina reads to H37Rv and L5 completed PacBio genomes.**

183 Raw reads (fastq files) of the 205 non-redundant Illumina genomes were mapped respectively
184 to H37Rv and each of the 3 L5 completed PacBio genomes, using the MTBseq pipeline (Kohl
185 et al. 2018). The depth mapping coverage of the samples in the clinical WGS data Illumina
186 genomes against the reference genomes (percent read mapping, unambiguous coverage
187 mean) was compared between the three L5 completed PacBio genomes and between each
188 L5 completed PacBio genome and H37Rv. Mapping statistics parameters such as percent
189 unambiguous total base, uncovered bases, SNPs, deletions, insertions, substitutions, percent
190 genes mapped to reference, were also compared using each L5 completed PacBio genome or
191 H37Rv as a reference.

192 **Checking unique versus missing genes in Illumina L5 genomes, and SNPs in those confirmed**
193 **as L5 specific.** The genes found present or absent in L5 based on genome comparisons of the
194 three L5 completed PacBio genomes with H37Rv were checked for their expected presence
195 or absence in the L5 Illumina genomes. Using the Position Tables produced by the MTBseq
196 pipeline, a gene was considered absent if 95% of its position in the genome had less than 8
197 reads covering them. From this data, a gene presence/absence matrix was generated for L5
198 Illumina genomes mapped to H37Rv and each of the three L5 completed PacBio genomes.
199 Genes found to be L5-specific (present in completed PacBio and Illumina genomes) were also
200 checked for SNPs in these genes using each of the L5 completed PacBio genomes as a
201 reference. The script used for undertaking this analysis can be found at
202 https://github.com/conmeehan/pathophy.

203 **Calculating the effect of reference genome selection on pairwise SNP distances**

204 Transmission analysis of MTBC isolates often involves clustering isolates together based on
205 specific SNP cut-offs (Meehan et al. 2019, 2018; Walker et al. 2013). We assessed whether
206 the selection of reference genome (H37Rv, PcbL5Ben, PcbL5Gam or PcbL5Nig) changed the
207 clustering rate of L5 isolates. For this we used the non-redundant set of L5 genomes from a
208 previous study of L5 diversity (Coscolla et al. 2020), which included the 205 detailed above
209 and a further 145 isolates that were closely related to at least one of these 205 isolates.
210 Additionally, the TB-Profiler online SRA search tool (https://tbdr.lshtm.ac.uk/sra) was used to
211 identify a further five isolates that were not in this dataset, resulting in 355 isolates included
212 in this clustering study.

213 SNP alignments of the Illumina-sequenced genomes were created by first mapping to each of
214 the four reference genomes (H37Rv and three PcbL5). The Amend function of MTBseq does
215 this automatically for H37Rv, including masking of repetitive regions, accounting for 10% of
216 the genome (Cole et al. 1998), and exclusion of columns with 95% ambiguous calls. To
217 undertake this for the three L5 reference genomes, repetitive regions were first determined
218 from the annotations of the genes. All genes whose description contained one of the
219 following words was excluded: integrase, PE family, PE-PGRS, phage, transposase. SNP
220 alignments were then created using MTBseq as was done for H37Rv. Pairwise distance
221 matrices, one for each alignment based on each reference genome, were created using a
222 python script which can be found at https://github.com/conmeehan/pathophy. Loose
223 transmission clusters were created from these matrices at a cut-off of one, five and twelve
224 SNPs, as previously described (Meehan et al. 2018). Clustering rates and the presence of a
225 strain in a transmission cluster was then compared across the four reference genome
226 mapping approaches.

227 **Determination of putative function of genes differentially present or absent in the L5**
228 **completed PacBio genomes**

229 To find the (putative) function of genes present or absent only in L5 strains, and genes present
230 in L5 strains but pseudogenes in H37Rv/L6/*M. bovis* or vice-versa, the fasta sequence of the
231 genes were searched against the NR database of NCBI using BLASTX-2.8.1+ (Altschul et al.
232 1997) as well as against the Tuberculist database (http://tuberculist.epfl.ch), Mycobrowser
233 (https://mycobrowser.epfl.ch/) (Kapopoulou, Lew, and Cole 2011) and literature.
234 Furthermore, the gene function group/class was found using the COG database (Tatusov et
235 al. 2000) and Mycobrowser.

236 **Determination of the sublineage of the L5 strains**

237 The sublineage of the strains was determined by looking for sublineage defining SNP in the L5
238 genomes as previously described (Coscolla et al. 2020; Ates et al. 2018). The sublineage
239 defining SNP were searched in the SNP data generated using the MTBseq pipeline with H37Rv
240 as the reference genome.

241

242 ## Results

243 **Comparative analysis of the completed L5 PacBio genomes and other lineages reveal**
244 **differences in gene content**

245 The number of genes, including paralogs, in the completed L5 PacBio genomes was: 4189 in
246 the PcbL5Ben genome, 4162 in PcbL5Gam, and 4134 in PcbL5Nig versus 4126 in H37Rv, 4126
247 in the reference L6 genome and 4059 in the reference *M. bovis* genome. Hence, the maximum
248 gene count difference was 55 genes among the three L5 isolates from this study, 63 genes

249 among the human-adapted lineages (H37Rv, L5, L6), and 130 genes among the human- and
250 animal adapted lineage (H37Rv, L5, L6 and *M. bovis*).

251 The visualization of the structure of the genomes showed that all three L5 genomes had a
252 region (herein called region A) that was absent in H37Rv (gap between 1683331-1716665 on
253 Fig.2). Furthermore, the Nigerian L5 completed PacBio genome (PcbL5Nig) was missing an
254 additional region (herein called region B, between 1966666-2000000 in Fig.2) present in the
255 Benin and Gambian L5 genomes (Fig.2) and also present in H37Rv.

256 The Benin genome contained 5 genes (a block of 3 genes (1148 bp, 1104 bp, 2840 bp) and the
257 other 2 (152 bp, 368 bp) randomly spread in the genome) that were neither present in the
258 Nigerian or Gambian genomes, while the others contained respectively 1 (150 bp) and 2 (152
259 bp, 366 bp) unique genes randomly spread in the genome. These increased the pangenome
260 of L5 by at least 6,280 bp. The Benin and Gambian strains each contained 33 genes that were
261 not present in the Nigerian strain (Fig.3, Table 1, Table S1). Interestingly, 32 of these genes
262 were also present in H37Rv (Fig.4, Table 1, Table S1). These 32 genes were thus missing in the
263 Nigerian L5 strain exclusively, meaning that the default/ancestral state have the genes and
264 then is loosing them. Those 32 genes- absent in the Nigerian L5 strain only- were sequentially
265 contiguous and formed three blocks of 18, 12 and 2 genes (Table S1). We found that 30 of
266 those genes, the blocks of 18 and 12 genes were separated by one gene and represented the
267 region B (mentioned in the section above, Fig 2) found missing in the Nigerian L5 genome
268 during another analysis (comparison of the structure of the 3 L5 completed PacBio genomes
269 using Mauve pipeline). Ten genes (region A mentioned above) that were shared by the three
270 L5 genomes were absent in H37Rv, while 9 genes (spread across the genome) present in
271 H37Rv were not present in any of the three L5 genomes. Two genes (*Rv2073c*, *Rv2074*) of

272 those 9 genes were only present in H37Rv but completely absent in the L5, L6 and *M. bovis*
273 complete genomes.

274 Six of the genes shared by the three L5 completed PacBio genomes were confirmed
275 pseudogenes in H37Rv (Table S2). Three genes present in H37Rv were confirmed to be
276 pseudogenes in the three L5 completed PacBio genomes (Table S3).

277 **Gene presence/absence and related SNPs in lineage specific genes in a wider set of clinical**
278 **isolates**

279 The mapping estimates of the Illumina genomes are presented in Table 2. Mapping quality
280 and coverage against a completed L5 reference was superior to the H37Rv reference
281 approach (Table 2, Fig.5), as expected. Using the completed PacBio genomes from Benin and
282 The Gambia had similar mapping estimates that were better than those of the Nigerian L5
283 genome, likely due to the large deletion in this genome. The genes specific to PcbL5Ben (5),
284 PcbL5Gam (2), PcbL5Nig (1) were each found at various rates among the 205 L5 Illumina
285 genomes (77.6%-97.1%, 159-199/205, Table 3).

286 Interestingly, 2.9% of the Illumina L5 genomes (6/205) had similar patterns of large gene loss
287 as the Nigerian completed PacBio genome as they missed 30 of the 33 genes present in the
288 Benin and Gambian genomes and H37Rv. These six PcbL5Nig-like Illumina L5 genomes formed
289 a monophyletic group, within the L5 clade (Fig. 6), suggesting a single loss of these gene
290 clusters, although those six L5 genomes originated from several different countries including
291 Benin, Ghana and Nigeria. The two blocks of 18 (14835 bp, *Rv1493* through *Rv1509*) and 12
292 genes (10209 bp, *Rv1511* through *Rv1521*), separated by one gene (3441 bp, *Rv1510*)
293 amounted in total to 25044 bp which is around 0.6% of the length (around 4 million bp) of a

294 typical MTBC (H37Rv) genome. The two blocks contain genes whose annotations include
295 *mutB*, *mazE4*, *mazF4* and others with various putative functions (Table S4).

296 Four of the ten genes present in the three completed L5 PacBio genomes but absent in H37Rv
297 were found in all the 205 L5 Illumina genomes while the other were found in variable amounts
298 81-98% (165-201) (Table S5). These four gene amounted to a total of 2382 bp (Table S5),
299 representing around 0.05% of the length of a typical MTBC genome. These four genes include:
300 *Mb2048c*, PE35, a hypothetical protein- possibly an IS*256* transposase, and a hypothetical
301 protein possibly related to the CAAX conserved domain. Two of these four genes were present
302 in L5 genomes only (PE35 and hypothetical protein possibly CAAX conserved domain) while
303 the remaining two (*Mb2048c* and hypothetical protein possibly IS256 transposase) were
304 found in L6 and *M. bovis* as well (Table S5). Importantly for phylogenetic purposes, SNPs were
305 detected in all four genes in 1.5-3.4% of the 205 L5 Illumina genomes (Table S5). Predicted
306 functions for all L5-specific genes are listed in table S5.

307 Five (*Rv1977*, *Rv1979c*, *Rv1993c*, *Rv1995*, *Rv2073c*) of the 9 genes present in H37Rv and
308 absent in the three completed L5 PacBio genomes were absent in all the 205 L5 Illumina
309 genomes. These five genes amounted to a total of 4,245 bp (Table S6), representing around
310 0.1% of the length of a typical MTBC genome. Four of these genes (*Rv1977*, *Rv1979c*, *Rv1993c*,
311 *Rv1995*) were absent in L5 strains only (i.e. present in L6 and *M. bovis* reference genomes)
312 while the fifth (*Rv2073c*) was absent in L6 and *M. bovis* as well (Table S6). The four other
313 genes absent from the completed PacBio genomes were present in a minority of the L5
314 Illumina genomes (Table S6, Fig. 5). Predicted functions for all nine genes are listed in table
315 S6.

316 **Sublineage of the strains (PacBio completed and Illumina genomes)**

317 The determination of the strains sublineage revealed that the PcbL5Ben is an L5.3 strain that
318 contains the 30 genes (two blocks of 18 and 12 genes: *Rv1493* through *Rv1509* and *Rv1511*
319 through *Rv1521*) whereas PcbL5Nig is also a L5.3 strain, but situated in a different clade (Fig.
320 6). Based on this region (a new RD), we classified PcbL5Ben as an L5.3.1 strain and the
321 PcbL5Nig as a L5.3.2 strain; the PcbL5Gam isolate is an L5.1.5 strain. The Illumina genomes
322 were distributed as follows (Table S7): 90 L5.1.1; 17 L5.1.2; 12 L5.1.3; 21 L5.1.4; 9 L5.1.5
323 amounting to a total of 149 L5.1 strains (72.7%); 13 (6.3%) L5.2 strains; 8 L5.3.1; 6 L5.3.2
324 amounting to a total of 14 L5.3 strains (6.8%) and 29 (14.2%) strains of unknown sublineages
325 (potentially new sublineages). All L5 strains including L5.1, L5.2, L5.3.1, unknown sublineages,
326 except L5.3.2 strains have that 30 genes region.

327 **Changes to transmission clustering rates based on selection of reference genome**

328 Transmission analysis was undertaken on an expanded set of 355 L5 isolates using each of the
329 four reference genomes (H37Rv, PcbL5Ben, PcbL5Gam and PcbL5Nig) for the SNP mapping.
330 The current gold standard is to use H37Rv for calling SNPs and then creating transmission
331 clusters at a specific SNP cut-off. For this dataset, using a 12 SNP cut-off, it was found that
332 40.6% (n=144) isolates were within a transmission cluster with at least one other isolate (Table
333 4), for a otal of 55 clusters. Substituting PcbL5Ben instead of H37Rv as the reference genome
334 reduced the number of clusters to 54, removing 3 isolates from transmission clusters, resulting
335 in a clustering rate of 39.7% at a 12 SNP cut-off (Table 4). A similar reduction in transmission
336 clustering was observed when using the PcbL5Nig genome as a reference but not when using
337 the PcbL5Gam. When a more conservative SNP cut-off was used, such as 1 SNP or 5 SNPs,
338 the reduction in transmission clustering rates was more pronounced and was observed for all
339 the PcbL5 genomes (Table 4). For example, at a 1 SNP cut-off, H37Rv-based mapping resulted
340 in 100 isolates (28.2%) being placed inside transmission clusters, but this reduced to 94 or 95

341 isolates (26.5-26.8%) when using a L5 genome as the reference. This demonstrates that even
342 on this small dataset, transmission cluster estimations are affected by the selection of reference
343 genome, with potential over-estimation of recent transmission when using H37Rv as the
344 reference genome.

345 **Discussion**

346 Comparison of completed PacBio and Illumina short-reads genomes from clinical isolates
347 revealed gene content diversity within L5 isolates as well as between L5 and other lineages.

348 We found that four genes, present in all the L5 isolates of our multi-country collection, were
349 absent in the H37Rv genome. Those genes represent 2,382 bp which is around 0.05% of the
350 total length (base-pair) of a typical MTBC genome. Furthermore, those genes contain SNPs
351 that can be used to identify additional diversity between L5 strains. This has implications
352 when using precise SNP cut-offs to define transmission clusters (Walker et al. 2013; Meehan
353 et al. 2018) as SNPs in these genes would be missed with H37Rv mapping, resulting in
354 underestimation of genome diversity of L5 strains. Our findings suggest, that using an L5
355 genome as a reference would likely increase the resolution of L5 genome analyses over the
356 H37Rv approach and can potentially increase the discrimination power, leading to a reduction
357 of the amount of recent L5 transmission inferred.

358 Our findings are in line with other reports indicating that some genes present in genomes of
359 clinical MTBC strains were absent in the H37Rv genome (Periwal et al. 2015; O'Toole and
360 Gautam 2017), and with other recommendations to use additional reference genomes
361 different to H37Rv (Norman et al. 2019; O'Toole and Gautam 2017; Lee et al. 2020). Although
362 Lee *et al*. (Lee and Behr 2016) - after comparing various lineages as reference genome for L4
363 genome analysis - concluded that there is no need to use a lineage-specific reference genome,

364 their observation was only based on the analysis of L4 clinical isolates and focused on SNPs
365 and short indels, not larger gene deletions or SNPs within these regions. In contrast, our
366 findings indicate that mapping of NGS data from L5 strains to L4 reference will have an impact,
367 in terms of both reference genome coverage and coverage of lineage-specific genes.

368 Although the use of an L5 genome as reference would have many benefits over H37Rv for
369 particular study questions, several gene content differences were still observed within the L5
370 lineage. Each of the three L5 PacBio genomes had genes unshared with the two others that
371 were all found in L5 Illumina genomes (thus excluding exogenous contamination). The
372 absence of 30 genes in the Nigerian L5 completed PacBio genome (but shared by the Benin
373 and Gambian long read genomes), also found in six L5 genomes from different countries,
374 confirmed the large deletions in the PcbL5Nig genome and showed that it is representative
375 of some L5 strains in some countries. Deletions in the genomes of strains of MTBC lineages
376 were previously reported (Tsolaki et al. 2005; Gagneux et al. 2006; Kato-Maeda et al. 2001).
377 The fact that, on the phylogenetic tree the six isolates formed a monophyletic group of the
378 (SNP defined) L5.3 sub-lineage (Coscolla et al. 2020) suggests that the loss of these 30 genes
379 (*Rv1493* through *Rv1509*, and *Rv1511* through *Rv1521*) is a marker of the L5.3.2 sub-lineage,
380 compared to the sublineage L5.3.1 strains that have these genes intact. Thus, we find that
381 regions of difference (RDs) are present both between lineages and between sub-lineages.

382 We found that the genes *Rv2073c* and *Rv2074* that were absent in L5, L6 and *M. bovis*. It was
383 previously reported that L5, L6 and *M. bovis* all lack the RD9 region (Brosch et al. 2002;
384 Mostowy et al. 2002; Gagneux et al. 2006), which contains *Rv2073c*, *Rv2074* and partly
385 *Rv2075* (Gordon et al. 1999). The lack of RD9 by L5, L6 and *M. bovis* is thus confirmed by our
386 data, however one (1/205) of the L5 genomes had *Rv2074* present. Of the five genes (*Rv1977*,

387 *Rv1979c*, *Rv1993c*, *Rv1995*, *Rv2073c*) absent in all L5 strains, besides *Rv2073c* included in RD9,
388 one (*Rv1977*) was part of the RD7 region (Gordon et al. 1999), another (*Rv1979c*) was part of
389 the RD2 region (Gordon et al. 1999), and *Rv1993c* and *Rv1995* potentially included in other
390 RD regions. These findings show that gene presence/absence approach sheds more light on
391 the differences between lineages than the RD approach alone would do.

392 The classification of the L5-specific genes into functional groups based on Mycobrowser and
393 Tuberculist databases (Table 4) revealed a variety of functions including: a PE/PPE protein
394 family (possibly PE35) which may be linked to antigenic variation (NCBI annotation) and
395 virulence (STRING database annotation (Szklarczyk et al. 2017)); two ABC-transporters; an
396 IS*256*-like transposase, whose homology (IS256) is a marker of specific virulent lineages of
397 staphylococci (Murugesan et al. 2018; Gu et al. 2005); and a gene with a CAAX conserved
398 domain, implicated in post-translation modification or signal transduction (Gao, Liao, and
399 Yang 2009a, 2009b). Only one (*Rv1979c*) of the four genes absent in only L5 strains had a
400 predicted function, labelled as "cell wall and cell processes" (Table S6). While *Rv1979c* is
401 associated with clofazimine and bedaquiline resistance (Zhang et al. 2015; Ghodousi et al.
402 2019), two of the drugs used for the treatment of RIF-resistant/MDR-TB (WHO 2016, 2019),
403 minimal inhibitory concentration testing of clofazimine in five L5 strains did not find the
404 deletion conferred resistance (Merker et al. 2020). Further studies and protein function
405 discovery is needed to investigate the consequences of the absence of those genes in L5.

406 The genes *Rv1978* and *Rv1994c* present in H37Rv and the completed reference genomes of
407 L6 and *M. bovis*, were absent in the 3 completed Pacbio genomes of L5 strains, yet present in
408 few L5 isolates Illumina genomes (3/205, 1.5% for *Rv1978*; 1/205, 0.5% for *Rv1994c*). *Rv1978*
409 is required for bacterial survival in macrophages, and non-essential for *in vitro* growth of

410 H37Rv (Rengarajan, Bloom, and Rubin 2005). *Rv1994c* is involved in the regulation and
411 transport (efflux) of toxic metals, especially copper, which is toxic in excess (Samanovic et al.
412 2012) and may hamper *in vitro* growth, and survival during the chronic phase of infection,
413 similar to the effect of disruption of *csoR* (Marcus et al. 2016; Rowland and Niederweis 2012;
414 Ward, Hoye, and Talaat 2008; Cavet et al. 2003) (Table S6).

415 The functional annotation for the H37Rv-specific genes *Rv2073c* (short chain dehydrogenase)
416 and *Rv2074* (pyridoxamine-5-phosphate oxydase: vitamin B6 (pyridoxine)) absent in L5, L6
417 and *M. bovis* strains is "intermediary metabolism and respiration". Those two genes are part
418 of the RD9 region, which confirmed their absence in L5, L6 and *M. bovis* that have that region
419 deleted (Gordon et al. 1999). *Rv2073c* is an NAD(P)-dependent oxidoreductase (NCBI) that
420 catalyzes a wide range of reactions and is involved in redox sensor mechanisms (Kavanagh et
421 al. 2008; Sellés Vidal et al. 2018). *Rv2074* was previously thought to be a pyridoxine (vitamin
422 B6) oxidase, but is now known to be a F420 dependent biliverdin reductase, a cofactor of
423 vitamin B6 synthesis (UniProt) (Ahmed et al. 2016; Selengut and Haft 2010). Vitamin B6 is
424 essential for survival and virulence of *M. tuberculosis* (Dick et al. 2010) and its cofactor (F420
425 dependent biliverdin reductase) is implicated in immune-evasive mechanisms to allow
426 bacterial persistence (Ahmed et al. 2016; Selengut and Haft 2010)(Table S6). Recently, it has
427 been reported that the synthesis of F420 might be stimulated by phosphoenolpyruvate
428 (Grinter et al. 2020), which is the precursor to pyruvate, a supplement often added to culture
429 medium to improve the *in vitro* growth of L5, L6 and *M. bovis*. Furthermore, there is a
430 suggestion that F420 is needed for the activation of the antituberculosis drugs pretomanid
431 and delamanid (Grinter et al. 2020).

432 The absence of these genes in most L5 isolates suggests that L5 strains would be less likely to
433 survive in macrophages (*Rv1978*), have reduced growth *in vitro* (*Rv1994c* , as previously found
434 by Sanoussi et al (Sanoussi et al. 2017)), be less immune-evasive, less persistent and less
435 virulent (*Rv2074*) than L4 strains (at least H37Rv; as previously suggested for L5 and L6 strains
436 (Coscolla and Gagneux 2014; Reiling et al. 2013)). In addition, the absence of *Rv2074* in most
437 L5 (and in the complete genomes of L6 and *M. bovis* as well) suggests that L6 and *M. bovis*
438 strains would also be less likely to be immune-evasive, to be persistent and to survive than
439 their L4 counterparts. Despite the presence of vitamin B6 in boiled egg (thus Lowenstein-
440 Jensen medium), its quantity in the L.J medium is probably insufficient to allow a high yield of
441 L5, L6 and *M. bovis* growth in culture (survival). Further studies, including vitamin B6
442 supplementation, could investigate the consequences of the absence of those genes in most
443 L5 strains (and L6 and *M. bovis* for *Rv2074*).

444 Transmission clustering analyses revealed differences in clustering rates and inclusion of
445 isolates in clusters based on the selection of the reference genome (Table 4). Overall, H37Rv-
446 based mapping was found to place a small percentage more isolates in transmission clusters
447 than any L5-based mapping approach. This is due to the additional genes found in these
448 genomes also being present in the clinical isolates and these genes containing differentiating
449 SNPs. Thus, with an increased discovery of SNPs in non-H37Rv genes, some isolates that
450 previously satisfied the SNP distance criterion for clustering were now excluded as they are
451 less similar than they appear to be when using H37Rv as a reference. This was most
452 pronounced at the lower SNP cut-offs (Table 4). This has strong implications for molecular
453 epidemiology in West Africa, where most L5 strains are found. Additionally, if similar
454 scenarios exist for other lineages, this may result in changes to all non-L4 transmission
455 analyses.

456 The within-lineage differences in both gene content and potential functionality indicates a
457 need for more closed genomes of MTBC sub-lineages and that perhaps a sub-lineage-specific
458 reference genome approach would be appropriate for MTBC WGS and molecular
459 epidemiology. However, although an *ad hoc* (e.g. outbreak-specific) reference genome
460 provides a more accurate and precise comparison of strains involved in a specific situation,
461 that reference is only specific to that particular situation/outbreak/population/lineage and
462 cannot be used in another context, making comparisons between lineages and settings
463 difficult.

464 As an alternative to using a single MTBC-wide, lineage or sub-lineage specific reference
465 genome approach, the variable presence of genes within one lineage suggests that an L5
466 pangenome-based reference genome capturing all the known diversity may be required
467 instead. Such a genome would be a composite of all the genes found in all lineages and sub-
468 lineages of the MTBC (i.e. both the core and accessory genome) (McInerney et al. 2020) and
469 is usually represented as a graph instead of a single sequence (Rakocevic et al. 2019; Paten
470 et al. 2017; Martiniano et al. 2019; Marschall et al. 2018; Church et al. 2015). Alternatively, it
471 can be a selection of reference genomes, with mapping to all or a subset undertaken, as has
472 been done with *M. chimaera* and other pathogens (van Ingen et al. 2017; Garimella et al.
473 2019). Other authors have also reported that because of the genetic variability between
474 strains, using a single strain genome as reference genome lacks accuracy (Ballouz, Dobin, and
475 Gillis 2019; Sherman et al. 2019; Li et al. 2010). This MTBC-wide pangenome approach has
476 been suggested before, including for other organisms (Periwal et al. 2015; Meehan et al.
477 2019; Jandrasits et al. 2019; Ballouz, Dobin, and Gillis 2019; Medini et al. 2005; Tettelin et al.
478 2005; Sherman et al. 2019; Li et al. 2010). However, such an approach also has its own
479 drawbacks, including difficulty in mapping reads that bridge the boundaries between

480 accessory genes and the rest of the genome, comparing strains of different lineages including
481 phylogenetic analyses and retention of gene names and codes in clinical use, where H37Rv is
482 deeply embedded (Marschall et al. 2018). The specific sequence of each gene would also need
483 to be chosen for such a reference, with the inferred ancestral genome representative of MTBC
484 lineages approach being the most likely method (Goig et al. 2020, 2018; Comas et al. 2013)

485 Another alternative approach is a *de novo* assembly reference free approach (Maretty et al.
486 2017; Iqbal et al. 2012; Meehan et al. 2019; Li et al. 2010) where strains are either assembled
487 into contigs without the use of a reference genome, or compared to each other without first
488 calling SNPs. This would allow for clustering of samples regardless of lineage, e.g. for
489 genotyping or transmission analyses using Mash (software for fast/meta -genome distance
490 estimation technique) (Ondov et al. 2016) but would require new cut-offs for defining clusters
491 and many additional steps for further gene annotation and between sample comparisons,
492 making its clinical use potentially confusing.

493 A limitation of this study is that the completed (PacBio) and short-read (Illumina) genomes
494 were derived from positive cultures, excluding possible minority L5 strain diversity as L5
495 strains are overrepresented in negative cultures (Sanoussi et al. 2017). WGS applied directly
496 on sputum is more and more needed, especially for ancestral lineages (including L5 and L6)
497 where growth in culture is problematic (negative culture or dysgonic isolates that are a
498 challenge to grow for DNA extraction (Sanoussi et al. 2018, 2017)). Also, a limitation regarding
499 the comparison of gene presence/absence in L5 versus L4 (including H37Rv), L6, *M. bovis*
500 complete genomes, is that our study included only a single complete genome of L4, L6 and
501 *M. bovis*, while these lineages may also display variability similar to the intra-L5 variability we
502 observed in our study.

503 In conclusion, the use of a (sub-)lineage reference genome can increase the resolution for
504 strains comparison in comparison to a H37Rv based mapping approach for L5 genome
505 analyses for epidemiology (transmission), phylogeny and sub-lineage determination. Still, the
506 use of a (sub-)lineage reference genome may miss some within-lineage gene differences. For
507 drug-resistance detection in clinical L5 strains or strains of other lineages, H37Rv could still be
508 used as reference genome as resistance-related mutations are usually among core genes
509 (shared across all lineages). The high within-lineage gene content variability suggests the
510 pangenome of MTBC strains may be larger (at least by 6,280 bp) than previously thought,
511 implying a reference-free genome assembly (*de novo assembly*) approach may be needed.

512

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515 curation, writing of the original draft, review/editing the manuscript and visualization.

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Conflict of interest

The authors declare no conflicts of interest

Funding information

This work was supported by funds from the Directorate General for Development (DGD), Belgium (FA4 to C.N.S., B.C.D.J., D.A., and L.R.); the European Research Council-INTERRUPTB starting grant (number 311725 to B.C.D.J., C.J.M., and L.R.)

M.C. is supported by ESCMID, Ministerio de Ciencia (RYC-2015-18213 and RTI2018-094399-A-I00) and Generalitat Valenciana (SEJI/2019/011). S.G. is supported by the Swiss National Science Foundation (grants 310030_188888, IZRJZ3_164171, IZLSZ3_170834 and CRSII5_177163) and the European Research Council (883582-ECOEVODRTB).

The funders had no role in study design, data collection and interpretation or the decision to submit the work for publication.

Ethical approval

The PacBio sequenced L5 strain from Benin was isolated during the multicentric OFLOTUB study approved by the National Ethics Committee in Cotonou, Benin and each of the participating countries (Merle et al. 2012).

Acknowledgements

We would like to acknowledge input from Pim de Rijk Willem and Patrick Beckert for their technical inputs.

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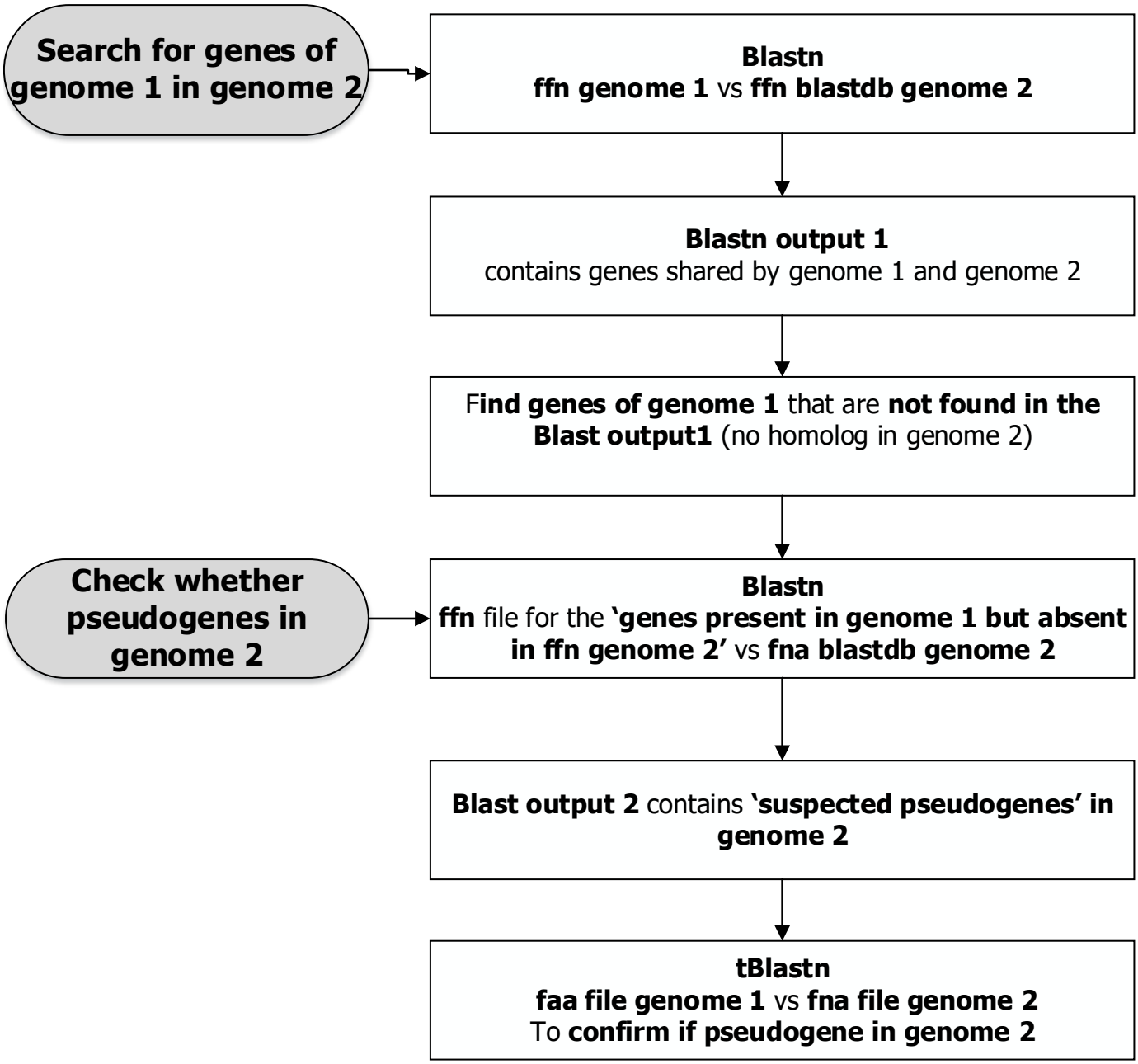


Figure 1. Methodology for finding gene presence-absence between two genomes

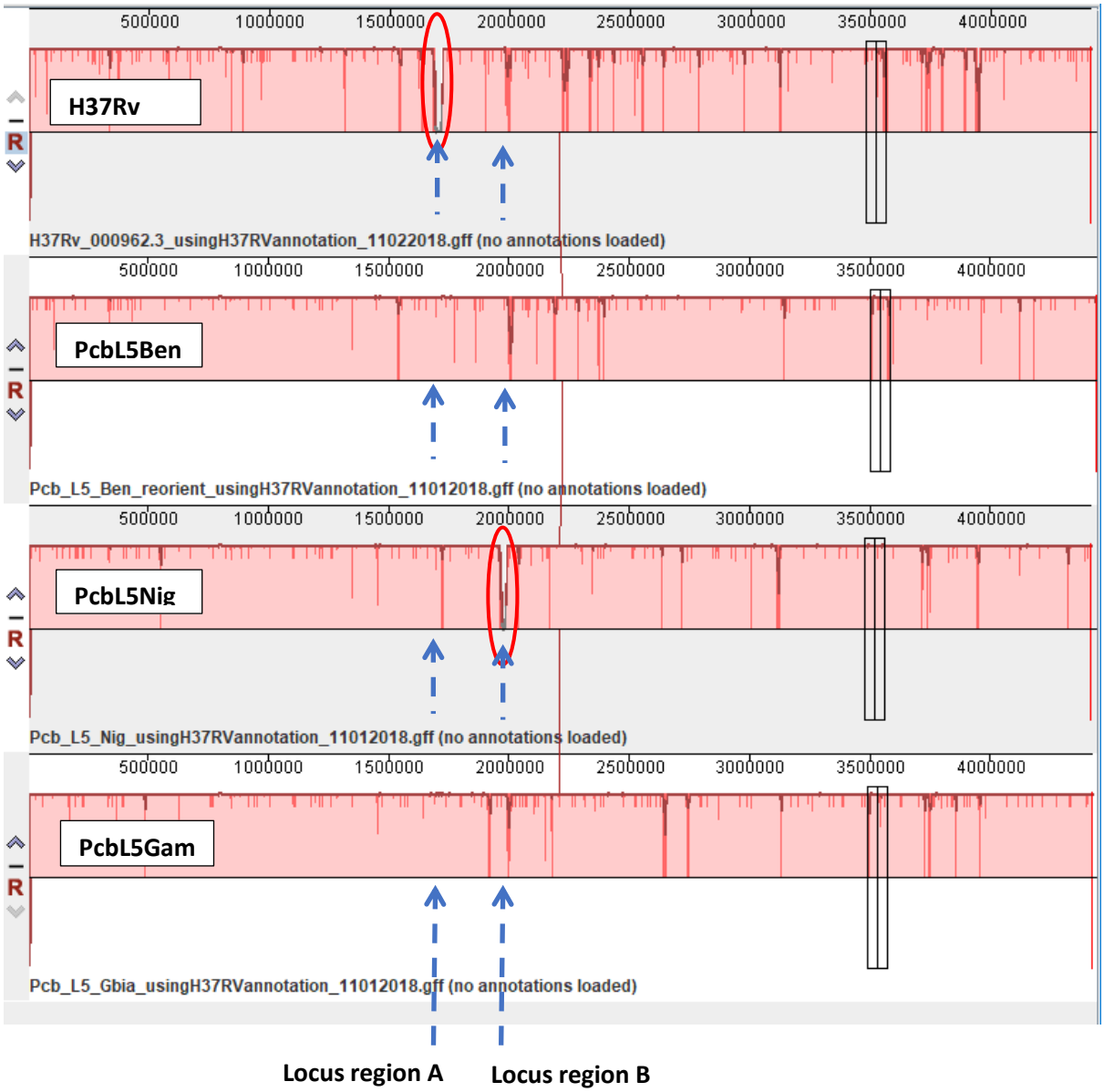


Figure 2. Visualization of possible rearrangements using Mauve pipeline: H37Rv (L4) genome and three completed PacBio genomes of *M. africanum* L5 isolates from Benin, The Gambia and Nigeria.

The region circled in red (one on the H37Rv genome (missing region A), and one on the Nigerian completed PacBio genome (missing region B)) represents the regions absent in that strain genome but present in the other three strains complete genomes.

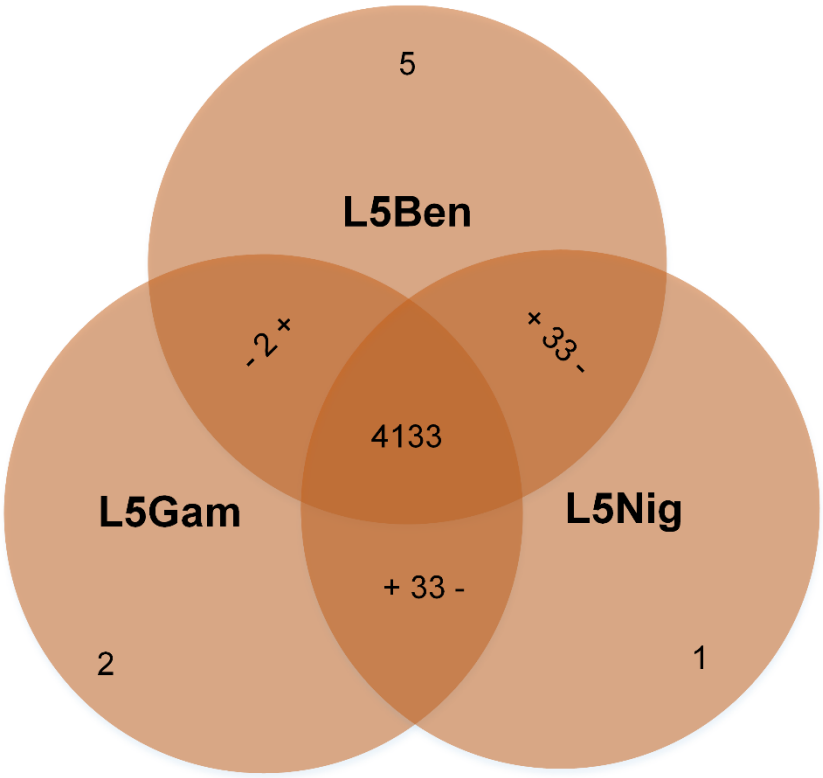


Figure 3. Gene count difference between three completed PacBio genomes of *M. africanum* L5 from Benin, The Gambia and Nigeria: L5Ben, L5Gam and L5Nig. The numbers are the count/number of gene difference (present or absent) between genomes. The sign (+) indicates the presence of genes while (-) indicates the absence of genes. For example the "- 2 +" gene difference between L5Gam and L5Ben, indicates that 2 genes are present in the L5Ben genome but absent in the L5Gam genome. 5, 2, 1 are the number of genes only found in respectively L5Ben, L5Gam, L5Nig.

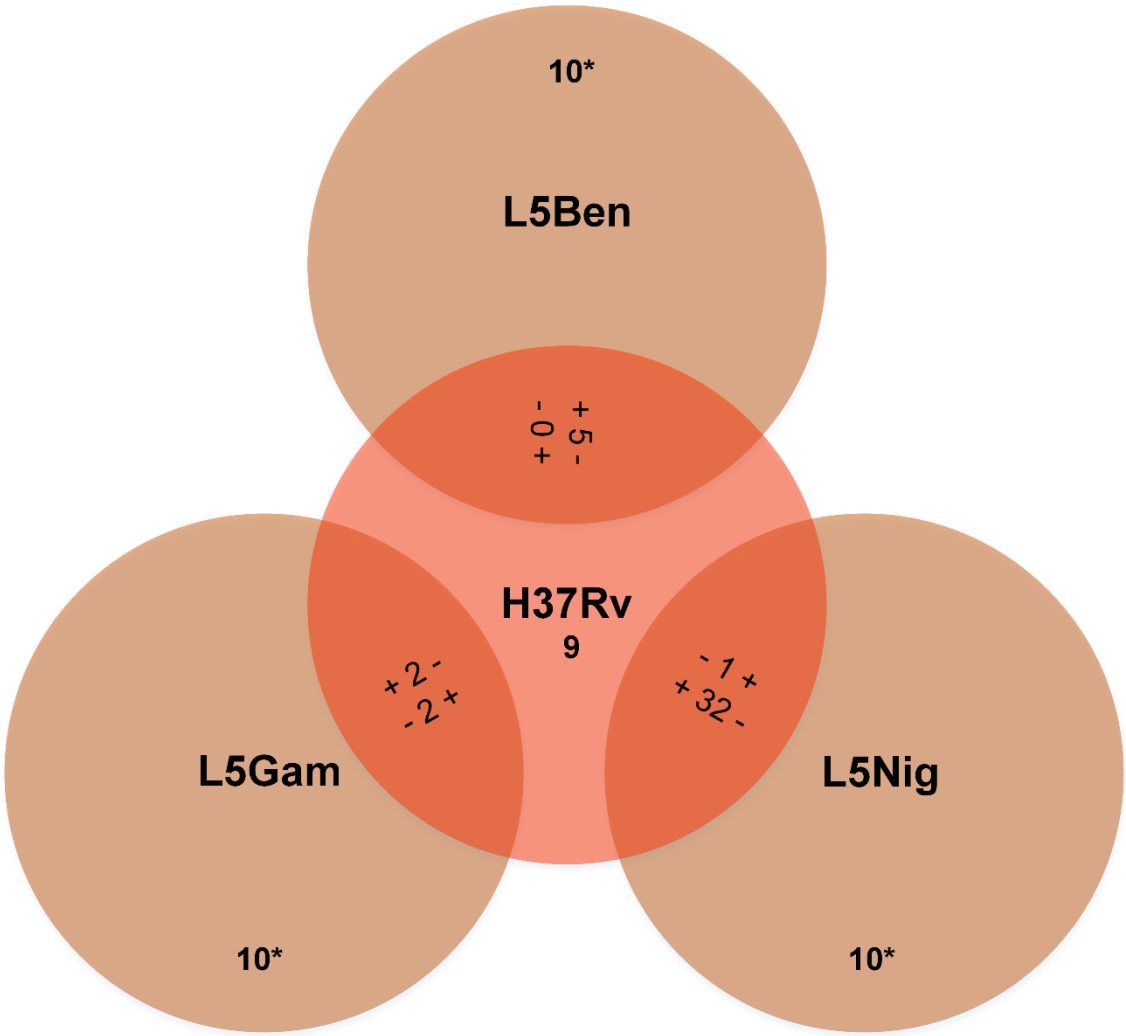
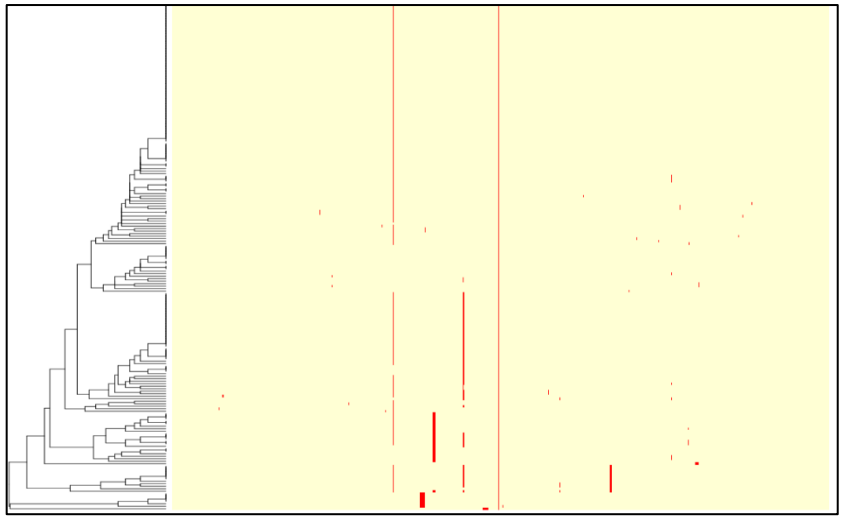
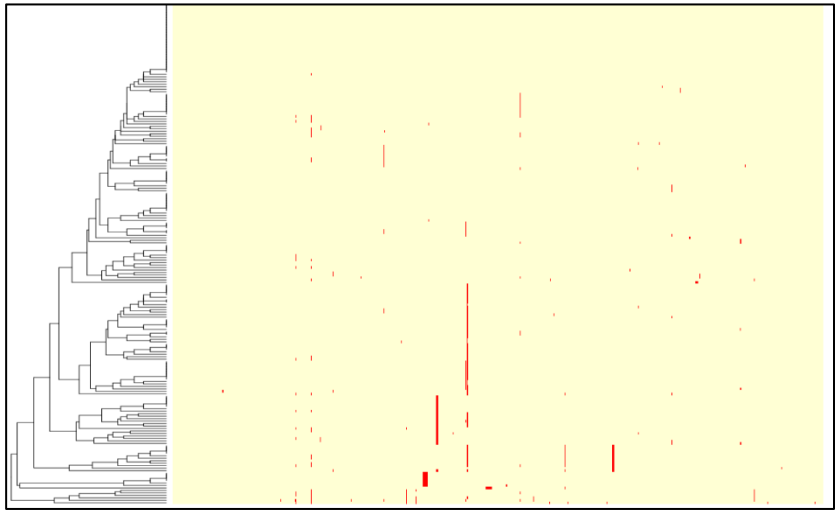


Figure 4. Gene count difference between *M. tuberculosis* H37Rv (L4) genome and three completed PacBio genomes of *M. africanum* L5 isolates from Benin, The Gambia and Nigeria. * These 10 genes are present in each of the three L5 completed PacBio genomes, and absent in H37RV.

H37Rv (L4) genes [A*]



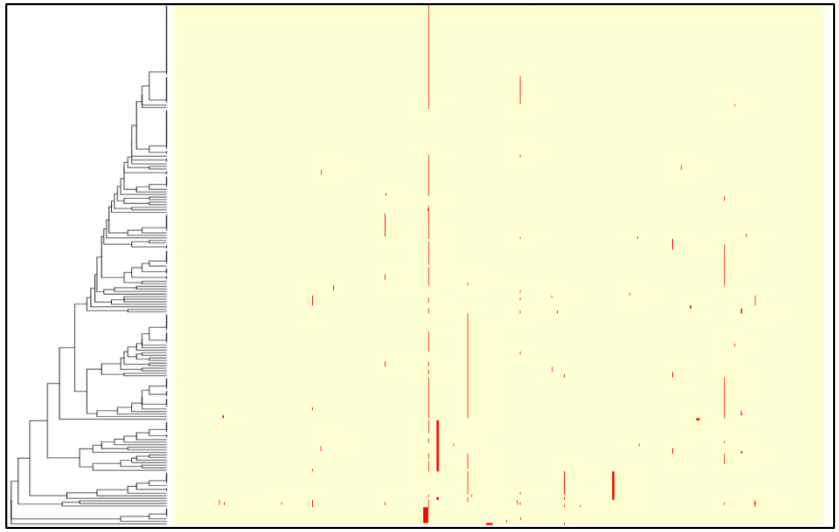
PcbL5Ben genes [B*]



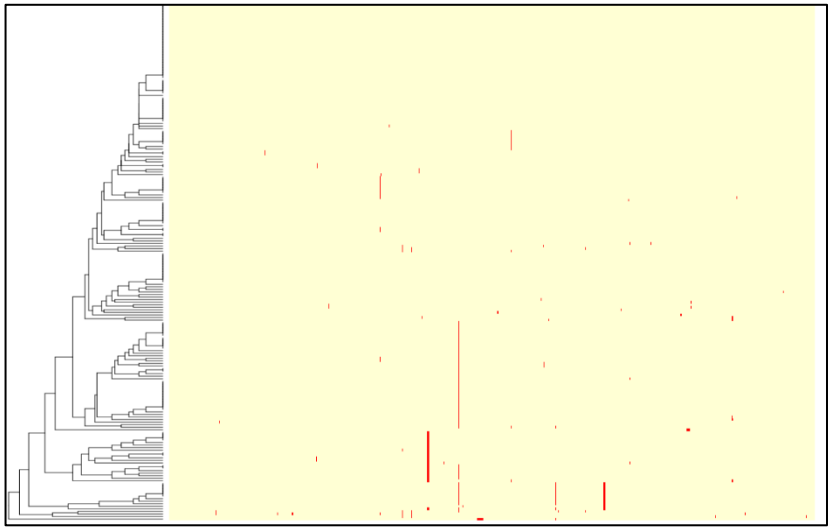
L5 Illumina genomes

1003

PcbL5Gam genes [C*]



PcbL5Nig genes [D]



L5 Illumina genomes

1013 **Figure 5. Mapping of 205 clinical (Illumina platform sequenced) genomes (L5_ig) of *M. africanum* L5 from various countries to *M. tuberculosis* H37Rv (L4)**
1014 **and three *M. africanum* L5 isolates (completed PacBio genomes) from Benin, The Gambia and Nigeria as reference (PcbL5Ben; PcbL5Gam; PcbL5Nig).**

1015 (A) **5**/9 genes present in H37Rv but absent in the three L5_cg were **absent in all 205 L5_ig.**
1016 (B) **5**/5 genes **present in PcbL5Ben but absent in the two other L5 completed PacBio genomes** were **present in 159-199/205 L5_ig.**
1017 (C) **2**/2 genes **present in PcbL5Gam but absent in the two other L5 completed PacBio genomes** were **present in 195 & 199/205 L5_ig.**
1018 (D) **1**/1 gene **present in PcbL5Nig but absent in the two other L5 completed PacBio genomes** was **present in 195/205 L5_ig.**

1019 * The 32-33 genes absent in PcbL5Nig but present in PcbL5Ben, PcbL5Gam and *H37Rv* were also absent in 6/205 L5_ig (Fig.9).

1020 **.... or ....** gene absent

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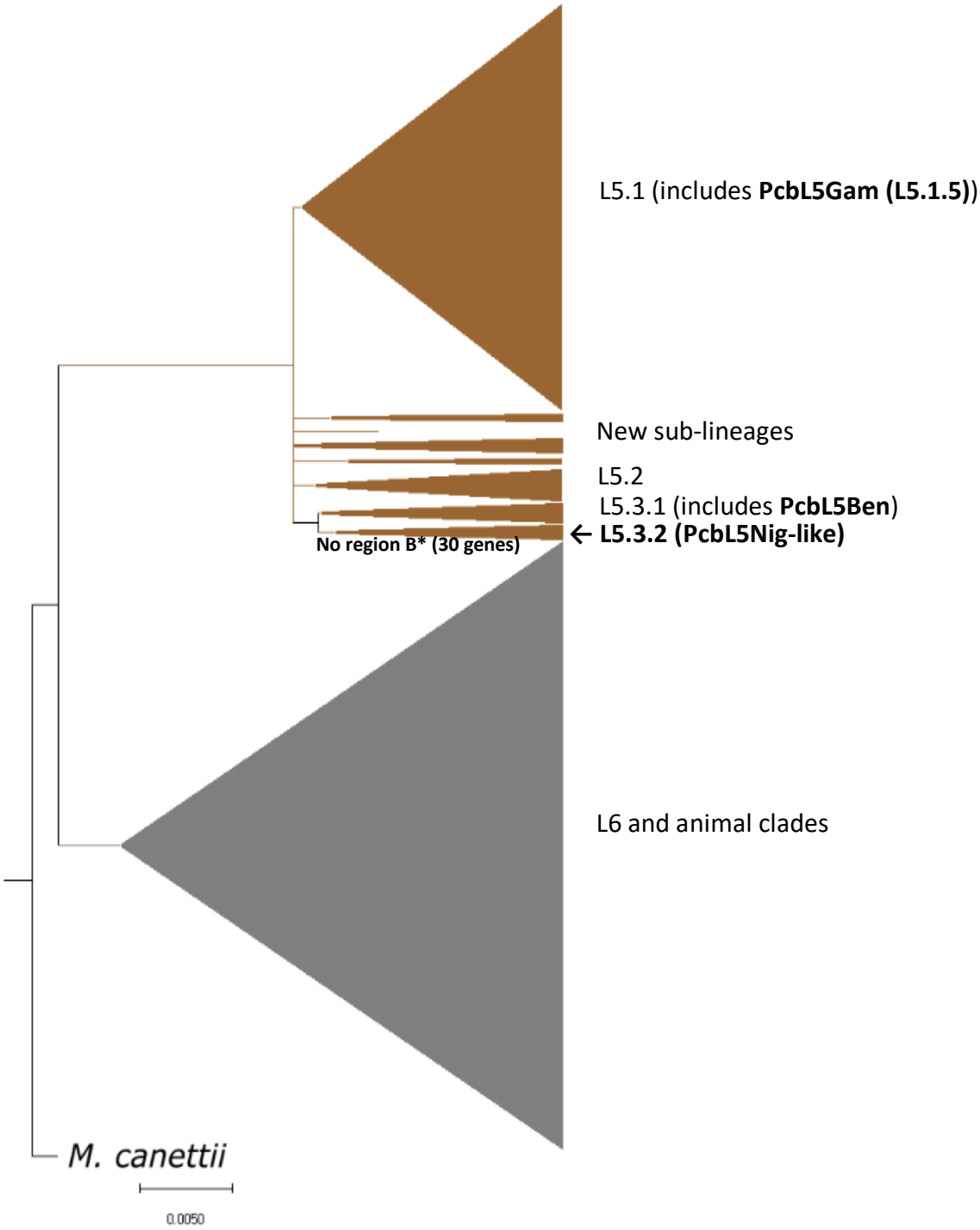

* Region B= region of 30 genes (two blocks of 18 and 12 genes: *Rv1493* through *Rv1509* and *Rv1511* through *Rv1521*) missing in L5.3.2 strains but present in all other L5 strains (L5.1, L5.2, L5.3.1 and new sublineages).

Figure 6. Phylogenetic tree showing the 6 L5 isolates Illumina genomes (L5.3.2) similar to the completed PacBio genome of the Nigerian L5 strain (PcbL5Nig) and the position of the other two L5 PacBio genomes (PcbL5Ben and PcbL5Gam)

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1064 **Table 1. Gene content difference between *M. tuberculosis* H37Rv (L4) genome and completed**
1065 **PacBio genomes of three L5 isolates from Benin, The Gambia and Nigeria.**

		Present in			
		PcbL5Ben	**PcbL5Gam**	**PcbL5Nig**	**H37Rv**
Absent in	**PcbL5Ben**		2[B]	1[C]	**9**[D]
	PcbL5Gam	2 + 5[A]		1[C]	2 + **9**[D]
	PcbL5Nig	33 + 5[A]	33 + 2[B]		32 + **9**[D]
	H37Rv	**10**[*] + 5[A]	**10**[*] + 2[B]	**10**[*] + 1[C]	
		4189	4162	4134	4126

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1067 **A:** include 5 only present in PcbL5Ben

1068 **B:** includes 2 only present in PcbL5Gam

1069 **C:** includes 1 only present in PcbL5Nig

1070 **D:** includes 9 only present in H37Rv

1071 *: includes 10 genes shared by the PcbL5 (Ben, Gam, Nig) and absent in H37Rv

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1089 **Table 2. Mapping of 205 L5 isolates Illumina genomes to the *M. tuberculosis* H37Rv (L4) genome**
1090 **and completed genomes of three L5 isolates from Benin, The Gambia and Nigeria (mapping**
1091 **statistics/estimates).** The best mapping results (numbers) are written in bold. When the best
1092 mapping result is obtained for PcbL5Nig as the reference, the next best result is also written in bold
1093 (as PcbL5Nig compared to the other two PcbL5 genomes missed a 30 genes region).

	H37Rv	**PcbL5Ben**	**PcbL5Gam**	**PcbL5Nig**
Reads				
Mean of Percent L5 reads mapped to	96.9	**97.5**	97.3	96.5
Mean of unambiguous coverage mean	121.9	122.7	**122.8**	122.5
Bases				
Mean of Percent unambiguous total bases	0.983	0.988	**0.989**	0.982
Mean uncovered	30444.8	18801.7	**14657.5**	35215.4
Mean SNP	2210	529.9	**513.2**	**503.7**
Mean deletions	373.3	95.9	**95.7**	97.2
Mean insertions	239.2	**77.3**	107.6	**60.8**
Mean substitutions (including stop codons)	1193	**0**	**0**	**0**
Genes				
Mean of Percent gene mapped (presence)	99.59	99.72	**99.79**	99.76
L5 illumina having all the ref. genome genes % (n=205)	0	2 (4)	7.3 (15)	0
Mean gene count difference between L5 Illumina genomes (gene count per Illumina-sequenced genome minus minimum gene count)	29.8	**38.3**	33.4	33

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1095 **Table 3. Presence in 205 L5 isolates Illumina genomes of genes detected in only one of the three L5**
1096 **completed genomes from Benin, Nigeria or The Gambia**

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	Gene	**Present in L5 Illumina genomes % (n=205)**
	PcbL5_01893	**77.6** (159)
	PcbL5_01894	**77.6** (159)
PcbL5Ben	PcbL5_01895	**77.6** (159)
	PcbL5_02043	**95.1** (195)
	PcbL5_03043	**97.1** (199)
PcbL5Gam	PcbL5_02028	**95.1** (195)
	PcbL5_03020	**97.1** (199)
PcbL5Nig	PcbL5_02001	**95.1** (195)

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1099 **Table 4. Comparison of transmission clustering rates based on choice of reference genome.** Short read data from 355 L5 isolates were mapped against
1100 each of the four reference genomes for SNP calling. Distance matrices between all isolates were constructed per reference approach and transmission
1101 clusters were defined based on specific SNP cut-offs

1 SNP

Reference used	In transmission cluster	Percentage of total dataset	Number of clusters
H37rv	100	28.17	44
PcbL5Ben	94	26.48	43
PcbL5Gam	95	26.76	43
PcbL5Nig	95	26.76	43

5 SNP

Reference used	In transmission cluster	Percentage of total dataset	Number of clusters
H37rv	129	36.34	53
PcbL5Ben	124	34.93	51
PcbL5Gam	124	34.93	51
PcbL5Nig	124	34.93	51

12 SNP

Reference used	In transmission cluster	Percentage of total dataset	Number of clusters
H37rv	144	40.56	55
PcbL5Ben	141	39.72	54
PcbL5Gam	144	40.56	55
PcbL5Nig	141	39.72	54

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