

1 **Comparative genomics of *Mycobacterium africanum* Lineage 5 and Lineage 6 from Ghana** 2 **suggests different ecological niches.**

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37 **Abstract:**

38 *Mycobacterium africanum* (*Maf*) causes up to half of human tuberculosis in West Africa, but little is
 39 known on this pathogen. We compared the genomes of 253 *Maf* clinical isolates from Ghana,
 40 including both L5 and L6. We found that the genomic diversity of L6 was higher than in L5, and
 41 the selection pressures differed between both groups. Regulatory proteins appeared to evolve
 42 neutrally in L5 but under purifying selection in L6. Conversely, human T cell epitopes were under
 43 purifying selection in L5, but under positive selection in L6. Although only 10% of the T cell
 44 epitopes were variable, mutations were mostly lineage-specific. Our findings indicate that *Maf* L5
 45 and L6 are genomically distinct, possibly reflecting different ecological niches.

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47

48 **Introduction:**

49 The global phylogeography of the human-adapted *Mycobacterium tuberculosis* complex (MTBC)
 50 demonstrates highest diversity in West Africa, with six out of the seven known lineages
 51 represented^{1,2}. Two of these lineages, Lineage 5 (L5) and Lineage 6 (L6), together originally known
 52 as *Mycobacterium africanum* (*Maf*), are restricted to West Africa for unknown reasons. By contrast,
 53 MTBC lineages belonging to *Mycobacterium tuberculosis* sensu stricto (*Mtbss*), in particular
 54 Lineage 4 (L4), are more geographically widespread¹. *M. africanum* has remained an important
 55 pathogen in West Africa since its first description in 1968³, and is responsible for up to half of
 56 human tuberculosis (TB) in some regions⁴.

57 The MTBC is thought to have originally emerged in Africa and subsequently spread to other parts
 58 of the world following waves of human migrations, trade and conquests⁵⁻⁸. Yet the reason(s) why
 59 *Maf* is limited to West Africa despite, for example, centuries of the trans-Atlantic slave trade
 60 remains unknown. Some comparative studies have identified phenotypic differences between the
 61 two *Maf* lineages^{9,10}, suggesting they might be fundamentally distinct and occupy different
 62 ecological niches.

63 Three hypotheses have been put forward to explain the restriction of *Maf* to West Africa. The first
 64 hypothesis proposes that *Maf* might have emigrated outside of Africa but was later outcompeted by
 65 *Mtbss*, which has been shown to be more virulent than *Maf* in animal models¹¹. The second
 66 hypothesis states that the restriction of *Maf* to West Africa is due to its adaptation to West African
 67 human populations^{9,12}. Finally, according to the third hypothesis, *Maf* might be zoonotic with an
 68 animal reservoir restricted to West Africa.

69 Some evidence in support of the first hypothesis is the reported association of *Maf* (L6) with HIV
 70 co-infection, attenuated ESAT-6 responses and delayed progression to active disease relative to
 71 *Mtbss*^{9,13-16}. In addition, both *Maf* lineages as well as *Mtbss* L1, together described as “ancestral”
 72 MTBC lineages, have been shown to elicit a stronger early production of pro-inflammatory
 73 cytokines compared to the “modern” MTB L2, L3 and L4¹⁷. The delayed pro-inflammatory

74 immune response in the “modern” MTBC lineages might allow for more rapid disease progression
 75 and transmission¹⁷. The second hypothesis is supported by the statistical association of L5 with the
 76 native West African ethnic group known as “Ewe” reported by two independent studies in
 77 Ghana^{9,12}. The third hypothesis is mainly supported by the phylogenetic placement of *Maf* (L6)
 78 amidst the cluster of the animal-adapted members of the MTBC in the various phylogenies of the
 79 MTBC^{5,7,18}.

80 If the first hypothesis is true, the proportion of *Maf* associated TB in West Africa is expected to
 81 decline over time. However, there are conflicting reports of the proportion of *Maf* associated TB in
 82 West Africa. Even though the report of a steady decline of *Maf* associated TB in some settings
 83 seems to support the first hypothesis^{19–21}, other studies indicate that *Maf* remains an important cause
 84 of TB in West-Africa^{22–24}. In Ghana for instance, a recent study showed that the proportion of TB
 85 due to *Maf* remained constant over the 8 year study period²⁵. Even though, the reported statistical
 86 association of L5 with ethnicity in Ghana suggests a possible co-evolutionary scenario in favour of
 87 the second hypothesis, genetic evidence of co-evolution/co-adaptation remains to be demonstrated.
 88 In the case of the third hypothesis, the environmental or zoonotic reservoir(s) need to be identified.

89 In this study, we used whole genome sequencing of Ghanaian *Maf* clinical strains to explore
 90 genomic differences between the two *Maf* lineages that might support one or more of these
 91 hypotheses.

92

93

94 Results

95 Whole genome SNP distance, average nucleotide diversity and phylogeny of *Maf* in Ghana

96 Our data set comprised *Maf* isolates obtained from TB patients reporting to various hospitals in
 97 Ghana. After excluding genomes that did not meet the criteria for mapping (Supplementary figure
 98 S1), 253 *Maf* genomes (175 L5 and 78 L6) were used for the analysis. Patients' residential regions
 99 are provided (Supplementary figure S2). The upper right pie chart indicates those 97 patients (55
 100 infected with L5 and 42 with L6) with no information on region of residence. We found the number
 101 of fixed SNPs (SNPs found in more than 95% of genomes) in a genome compared to the MTBC
 102 ancestor²⁶ to be significantly higher in L6 (1,037) compared to L5 (928) (Wilcoxon rank-sum test, p
 103 < 0.0001) (Fig 1A). Moreover, despite the larger number of L5 genomes (more than twice the
 104 number of L6 genomes) analyzed, the mean pairwise SNP distance between any two strains was
 105 significantly higher in L6 (360) compared to L5 (223) (Wilcoxon rank-sum test, $p < 0.0001$; Fig
 106 1B). Finally, the whole genome average nucleotide diversity (π) for L6 (0.000110) was significantly
 107 higher compared to L5 (0.00007) (Fig 1C, non-overlapping 95% confidence interval (CI)). Taken
 108 together, these findings show that L6 in Ghana is significantly more genetically diverse than L5
 109 irrespective of sample size. The whole genome-based phylogenetic tree of the Ghanaian *Maf* strains
 110 generated from 11,027 total polymorphic positions between the *Maf* strains and the MTBC ancestor
 111 reference excluding repetitive and mobile genetic element rooted on *M. canettii* is shown in Figure
 112 2. The *Maf* lineages were resolved as two distinct branches of the genome-based tree with possible
 113 sub-groups (Fig 2).

114

115 Genetic diversity of L6 is significantly higher than L5 among T cell epitopes and genes of 116 other functional categories.

117 We found that the higher diversity of L6 compared to L5 was reflected across all the 8 functional
 118 categories of genes analyzed (Fig. 3). Whereas pairwise nucleotide diversity (π) for L5 was below
 119 0.0001 across all functional categories, the estimates for L6 were all above 0.0001. The most
 120 prominent difference between L6 and L5 was within 1,226 experimentally confirmed human T cell

121 epitopes of MTBC which we downloaded from the Immune Epitope Database (IEDB)²⁷, for which
122 the mean π for L5 was 0.000063 compared to the 0.000149 estimated for L6, reflecting more than a
123 two-fold difference in diversity (non-overlapping 95% CI).

124 Within L5, there was no difference between the estimated π for the T cell epitopes and any of the
125 other functionally categorized genes. However, within L6, genes encoding regulatory proteins and
126 those involved with virulence, detoxification and adaptation were more diverse compared to those
127 for lipid metabolism as well as intermediate metabolism and respiration (non-overlapping 95% CI).
128 In addition, genes encoding regulatory proteins were more diverse compared to those involved with
129 lipid metabolism (non-overlapping 95% CI).

130

131 **Different selection pressures within L5 and L6 in human T cell epitopes and regulatory** 132 **proteins**

133 The average pairwise dN/dS of the concatenates of T cell epitopes as well as genes of the seven
134 other functional categories were calculated for all genomes and compared between L5 and L6.
135 Apart from sequences encoding human T cell epitopes and regulatory proteins that had median
136 average pairwise dN/dS ratios greater than 1.0 in L5 and L6, respectively (Fig 4, panel a and b), all
137 the remaining functional categories showed a dN/dS ratio of less than 1.0 in both lineages
138 (Supplementary figure S3). Human T cell epitopes of *Maf* L5 (median pairwise dN/dS = 0.64) were
139 significantly more conserved compared to L6, which exhibited higher diversity (median pairwise
140 dN/dS = 1.53) (Wilcoxon rank-sum test, $W = 2265$, $p < 0.0001$). Conversely, genes encoding
141 regulatory proteins were more diverse among L5 genomes (with median pairwise dN/dS = 1.03)
142 (Wilcoxon rank-sum test, $W = 6303$, $p = 0.0010$) compared to L6 (with median pairwise dN/dS =
143 0.85). To account for the different sample sizes; 147 L5 compared to 67 L6 genomes after
144 excluding 43 genomes differing from others with less than 10 SNPs difference (see Methods and
145 supplementary figure S1), we repeated the analysis using mean values of 10 randomly sampled sets
146 of L5 genomes with sample size 67 among human T cell epitopes (Fig 4, panel c) and regulatory
147 proteins (Fig 4 panel d) and got similar results (Wilcoxon rank-sum test, $W = 1300$, $p < 0.0001$, W

148 = 3466, $p < 0.0001$ for T cell epitopes and regulatory proteins, respectively).

149
150 **Lineage-specific accumulation of mutations within human T cell epitopes.**

151 When we compared the number of epitopes with amino acid mutations between lineages, we found
152 more epitopes mutated in L6 (57) compared to L5 (45), but no statistically significant difference
153 (Fig 5). In addition, we compared the number of nonsynonymous polymorphic sites between the
154 two *Maf* lineages within the human T cell epitopes (Supplementary Fig S4), and found that these
155 were more frequent in L6 (38) compared to L5 (28) but with no statistically significant difference
156 between L5 and L6. We compared the identity of the mutant human T cell epitopes between the two
157 *Maf* lineages (Fig 6A) and found 72 epitopes that were uniquely mutated in L5 (among 174
158 genomes) compared to 54 epitopes in L6 (among 67 genomes). Only two epitopes (IEDB IDs
159 178644 and 178609) were mutated in both lineages. However, the mutations were at different loci
160 with different amino acid substitutions (A183G and G278D in L5 compared to A177V and D277N
161 in L6). In terms of T cell antigens, there were 28 uniquely mutated in L5 compared to 19 in L6 and
162 12 mutated in both lineages involving different epitopes within the respective antigens (Fig 6B).
163 The 12 T cell antigens mutated in both lineages are summarized in Table 1. All T cell epitopes and
164 antigens with mutations among the two *Maf* lineages are listed in Supplementary table S5.

165
166 **Conservation of human T cell epitopes of L5 is not affected by patient ethnicity.**

167 We previously reported an association between L5 and Ewe patient ethnicity^{9,12}. Hence to test if
168 conservation of T cell epitopes and/or the diversity of regulatory proteins in L5 was influenced by
169 patient ethnicity, we estimated pairwise dN/dS for sequences encoding T cell epitopes and
170 regulatory proteins of L5 genomes stratified by patient ethnicity (Fig 7). The median dN/dS of T
171 cell epitopes were all below 1.0 irrespective of patient ethnicity (Fig 7A). However, the median
172 dN/dS of regulatory proteins were marginally above 1.0 among L5 from Ewe TB patients and
173 below 1.0 among L5 from non-Ewe TB patients (Fig 7B). There was no statistically significant

174 difference between the estimated dN/dS of either the sequences encoding T cell epitopes (Fig 7A)
 175 or regulatory proteins (Fig 7B) between L5 from TB patients of Ewe and non-Ewe ethnicities. In
 176 addition, there was no difference in either the number of T cell epitopes with amino acid
 177 substitutions (Supplementary figure S6A) or the number of non-redundant SNPs (Supplementary
 178 figure S6B) between L5 strains from patients of the Ewe ethnicity and those of other ethnicities.
 179
 180

181 Discussion

182 In this study, we compared the largest collection of *Maf* genomes including both L5 and L6
183 reported so far. We found that, 1) at the whole genome level, L6 had significantly more pairwise
184 nucleotide diversity, higher number of fixed SNPs as well as lower average pairwise SNPs relative
185 to L5, 2) L5 had overall more conserved human T cell epitopes compared to L6, 3) conservation of
186 T cell epitopes in L5 was not influenced by patient ethnicity, and 4) genes encoding regulatory
187 proteins of L5 had lower pairwise nucleotide diversity but a higher ratio of non-synonymous to
188 synonymous substitution rate than L6.

189
190 Our finding that *Maf* L6 has a higher number of fixed SNPs and higher average pairwise SNPs
191 relative to L5 suggests that L6 has diversified more compared to L5 since the emergence of the two
192 lineages^{5,28,29}. This observation is corroborated by the higher genome-wide nucleotide diversity of
193 L6 compared to L5. The higher diversity of L6 might be linked to an earlier emergence. However,
194 recent whole genome-based phylogenies rooted on *Mycobacterium canettii* show that following the
195 branch leading to *Maf* and all animal-adapted members of the MTBC defined by the characteristic
196 deletion in RD9^{30,31}, L5 branches off much earlier than L6³². Hence, L5 is ancestral to L6, a notion
197 which is also supported by genomic deletion analyses which show that in addition to RD9, L6 and
198 all the animal-adapted members of the MTBC harbor the deletions of RD7, RD8 and RD10³⁰.
199 Hence other factors are likely to account for the higher diversity of L6 compared to L5 in Ghana.

200
201 *Maf* is highly restricted to West Africa, and thus could be seen as an ecological specialist compared
202 to the other MTBC lineages. Specialists are expected to harbour less diversity across strains
203 compared to generalists⁸. The observed lower genome-wide nucleotide diversity of L5 hence
204 supports the hypothesis that L5 might be a specialist maintained in West Africa by adaptation to
205 specific human genotypes. In contrast, the higher genome-wide diversity of L6 indicates a
206 generalist pathogen, and hence would have been expected to be globally distributed instead of

displaying restriction to West Africa^{4,8}. The observed diversity of L6 therefore may indicate a pathogen with a wider host range, supporting the hypothesis of maintenance in West Africa by possible environmental or zoonotic reservoir(s). Alternatively, the higher diversity of L6 coupled with the higher number of fixed SNPs could mean that it has a higher intrinsic mutation rate compared to L5.

Even though 90% of T cell epitopes were highly conserved in both L5 and L6 (Fig 5), which is in line with previous reports for the whole MTBC^{26,8,33}, we found T cell epitopes in L5 to exhibit less nucleotide diversity and to be under purifying selection compared to L6. The purifying selection of mutations within L5 is comparable to that reported for the specialist sub-lineages of L4⁸. Interestingly, dN/dS within essential genes for survival in macrophages did not differ between L5 and L6 (Supplementary Figure S3) supporting the notion that the genes in this category perform key functions in both L5 and L6. Since T cell response might partially drive the pathogenesis of TB³⁴, the relative conservation of T cell epitopes in L5 indicate that it might elicit a more efficient T cell response compared to L6 in its particular host population. This therefore suggests L5 may be a more human-specific pathogen and L6, with significantly more diverse T cell epitopes, a potential opportunistic environmental or zoonotic pathogen. Even though the conserved T cell epitopes of L5 could account for geographical restriction to West Africa and the association with the Ewe ethnicity^{1,4,9,12}, we found no difference between the diversity of L5 isolated from TB patients of Ewe and those of non-Ewe ethnic backgrounds. The limited number of L5 genomes from Ewe TB patients could possibly account for the lack of observed difference in diversity of T cell epitopes of L5 from TB patients of Ewe and non-Ewe ethnicities, and hence larger sample sizes are required to explore this further. L5 isolated from TB patients of the Ewe ethnicity were shown to be distributed all across the L5 clade of the *Maf* phylogeny instead of clustering in a particular sub-clade (Supplementary figure S7). This suggests that, if L5 is indeed maintained in West Africa by its co-evolution/adaptation with the Ewe ethnic group of West Africa (Cote d'Ivoire, Ghana, Nigeria,

233 Togo and Benin)^{9,12}, there is no specific sub-group of L5 that is responsible for this association but
234 rather the whole of L5.

235

236 Members of the MTBC survive in the host mostly by modulation of the host immune response via
237 the action of secretory proteins which form part of regulons controlled by specific regulatory
238 proteins^{35,36}. In addition, some regulatory proteins are involved in the regulation of transcription and
239 translation of these secretory effectors as well as gene expression of other proteins involved with
240 diverse functions^{36,37}. Regulatory proteins in the MTBC hence play an important role in the survival
241 and propagation of the bacteria. Therefore, our finding that regulatory proteins in L5 are under
242 neutral selection (pairwise dN/dS = 1.03) compared to L6 in which they appear under purifying
243 selection indicates that the mutations within regulatory proteins might be lineage-specific. This
244 result is comparable to an earlier report comparing mutations within regulatory proteins between
245 *Mtbss* and *M. bovis*, which found most of the *M. bovis* to harbor majority of the mutations³⁶. As
246 mutations within some regulatory proteins have been associated with attenuated virulence³⁸⁻⁴⁰, our
247 observation could account for the reported attenuated virulence of *Maf* relative to *Mtbss*^{14,15,17,41}.
248 This calls for further comparative studies of regulatory proteins between L5, L6 and other MTBC
249 lineages to ascertain the role of regulatory proteins.

250

251 Our data is limited by the fact that, the number of L5 genomes was almost 3 times the number of L6
252 genomes; however, we used 1,000x bootstrap sampling with replacement of both L5 and L6 of
253 equal sample size to limit any possible bias when comparing both lineages due to differences in
254 sample size. In addition, a number of the L5 genomes did not have data on ethnicity and hence
255 affected the number of L5 isolated from patients of the Ewe ethnicity for which we used average
256 estimates of 10 random samples of L5 isolated from patients of non-Ewe origin in comparisons to
257 account for the different sample sizes.

258

259 In conclusion, our findings indicate that the two *Maf* lineages L5 and L6 are distinct in terms of
 260 genomic diversity, and selection pressure on T cell epitopes and regulatory proteins, possibly
 261 reflecting different ecological niches. Whereas L5 may be maintained in West Africa by its co-
 262 evolution or adaptation with native West Africans, L6 may be maintained by an environmental
 263 reservoir, possibly a zoonotic source. This genomic analysis of *Maf* from Ghana gives a glimpse of
 264 the often neglected diversity within *Maf* and the MTBC overall. More studies are needed from
 265 representative genomes of *Maf* from across West Africa to understand the full diversity of these
 266 members of the MTBC. Improved knowledge of *Maf* will have implications for our understanding
 267 of human TB and the development of better control tools.

268

269

270 Tables

271 **Table 1: Functions of the 12 T cell antigens mutated in both L5 and L6**

T cell antigen	Function
Rv0288	encodes low molecular weight antigen 7 EsxH involved with cell wall and cell processes
Rv0934	encodes periplasmic phosphate-binding lipoprotein PstS1 involved with cell wall and cell processes
Rv2029c	encodes 6-phosphofructokinase PfkB involved with intermediate metabolism and respiration
Rv2627c	encoding a conserved hypothetical protein
Rv3003c	Encodes the large subunit of acetolactate synthase involved with valine and isoleucine biosynthesis
Rv3024c	encodes a probable tRNA involved with information pathways
Rv3763	encodes a 19 kDa lipoprotein antigen precursor LpqH involved with cell wall and cell processes
Rv3804c	encodes the secreted antigen 85-a FbpA involved with lipid metabolism
Rv3823c	encodes conserved integral membrane transport protein MmpL8 involved with cell wall and cell processes
Rv3825c	encodes polyketide synthase Pks2 involved with lipid metabolism
Rv3879c	encodes ESX-1 secretion-associated protein EspK involved with cell wall and cell processes
Rv3883c	encodes membrane-anchored myosin MycP1 involved with intermediate metabolism and respiration

272

273

274 **Figure legends**

275 Figure 1: Number of SNPs per *Maf* Lineage (175 L5 and 78 L6 genomes). *a*: Number of SNPs
276 between *Maf* genomes and the hypothetical MTBC ancestor (the median fixed SNPs of L5 (934) is
277 lower ($W = 417$, $p\text{-value} < 2.2e-16$) compared to L6 (1,039). *b*: Pairwise SNPs between genomes
278 within each lineage (the median of the pairwise SNPs is lower ($W = 234$, $p\text{-value} < 2.2e-16$) in L5
279 (212) compared to L6 (334). *c*: Whole genome average nucleotide diversity (π) between L5 and L6
280 (the mean diversity of L5 (0.000076) is significantly (non-overlapping 95% confidence intervals)
281 lower than L6 (0.000110). Error bars indicate 95% confidence intervals.

282

283 Figure 2: Phylogeny of Ghanaian *Maf* strains. (The maximum likelihood phylogenetic tree of 253
284 Ghanaian *Maf* isolates is based on 11,027 variable positions. The tree was rooted on *M. canettii* and
285 the confidence of nodes was assessed by bootstrapping 1000 pseudo replicates. Each lineage clade
286 is colored according to the conventional MTBC lineage color codes¹.

287

288 Figure 3: Averaged nucleotide diversity (π) of *Maf* within genes of eight functional categories. *epit*
289 – genes encoding human T cell epitopes, *esmac* – genes essential for growth in macrophages,
290 *intmedres* – genes involved with intermediate metabolism and respiration, *lipmet* – genes involved
291 with lipid metabolism, *virdetad* – genes involved with virulence, detoxification and adaptation,
292 *cwallproc* – genes involved with cell wall and cell processes, *regprot* – genes encoding regulatory
293 proteins and *infopath* – genes involved with information pathways. Error bars are indications of
294 95% confidence intervals.

295

296 Figure 4: Pairwise dN/dS of genes encoding human T cell epitopes and regulatory proteins in L5
297 and L6. Estimation of pairwise dN/dS of epitopes (*a*) and regulatory proteins (*b*) using the entire
298 147 L5 against the 67 L6 genomes. Estimation of pairwise dN/dS of epitopes (*c*) and regulatory

299 proteins (*d*) using the mean dN/dS values of 10 random samples (size =67, with replacement) of L5
300 against the 67 L6 genomes.

301

302 Figure 5: Number of human T cell epitopes with nonsynonymous SNPs (nsSNPs) stratified by *Maf*
303 lineage. No significant difference (X-squared = 1.487, df = 1, p-value = 0.22) between the number
304 of epitopes with nsSNPs among the 67 L6 genomes and L5 (mean values of 10 random samples of
305 size=67 with replacement).

306

307 Figure 6: Number of human T cell epitopes (*a*) and human T cell antigens (*b*) with amino acid
308 substitutions stratified by *Maf* lineage. Green represents L6-specific mutant antigens or epitopes.
309 Brown represents L5-specific mutant antigens or epitopes. Yellow represents antigens or epitopes
310 mutated in both L5 and L6 but at different loci with different amino acid substitutions.

311

312 Figure 7: Pairwise dN/dS of sequences encoding human T cell epitopes (*a*) and genes encoding
313 regulatory proteins (*b*) of L5 by patient ethnicity. L5 genomes from strains isolated from patients of
314 the Ewe ethnicity (15 genomes) against, average values of 10 random samples of size 15 of L5
315 genomes of isolates from Non-Ewe patients.

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 453

454 **Acknowledgements**

455 Bacterial Isolation and DNA preparations were done in the Biosafety level 3 facility at the Noguchi
456 Memorial Institute for Medical Research, University of Ghana. Bioinformatics analyses were
457 performed using the scientific computing core (sciCORE) at the University of Basel and the
458 computing facility of the Wellcome Trust Sanger Institute, Genome Campus, Cambridge
459 University. This work was supported by the Wellcome Trust Intermediate Fellowship awarded to
460 DYM (Grant Number 097134/Z/11/Z) and by the Swiss National Science Foundation (grants
461 310030_166687, IZRJZ3_164171 and IZLSZ3_170834), the European Research Council (309540-
462 EVODRTB) and SystemsX.ch.

463

464

465 **Author contributions**

466 Conceived the idea: DYM, SG

467 Designed experiments: DYM, SG, IDO, MC, SRH, JP

468 Contributed reagents and performed experiments: IDO, MC, AAP, LSB, MC, SOW, AF, CL, GAA,

469 AIY, AB, SYA, PA, CL, DB, SB, FG, PB, TK, SN, MA, SN, CB, BCDJ, JP and SRH

470 Analysed Data: IDO, MDC, SRH, LSB, SG, DYM

471 Wrote manuscript: IDO, MC, SG and DYM

472 All authors critically reviewed the manuscript

473

474 **Competing financial interests**

475 None declared.

476

477 **Data availability.**

478 All the analyzed and/or generated data in this study are included in this article and its

479 supplementary information files. Whole genome sequence reads have been submitted to the EMBL-
480 EBI European Nucleotide Archive (ENA) Sequence Read Archive (SRA) with accession numbers
481 provided in the supplementary document attached (Supplementary data S8).

482 **Supplementary Information**

483

484 Supplementary figure S1: Flow chart of Ghanaian *Maf* genomes used for the study. Sites of DNA
485 sequencing as well as number of genomes used for each specific analysis are indicated.

486

487 Supplementary figure S2: Map of Ghana showing the regional distribution of *Maf* isolates. The
488 sizes of pie charts correspond to number of isolates from the respective regions.

489

490 Supplementary figure S3: Pairwise dN/dS of genes of six functional categories using all the 147 L5
491 against the 67 L6 genomes. *esmac* – genes essential for growth in macrophages, *intmedres* – genes
492 involved with intermediate metabolism and respiration, *lipmet* – genes involved with lipid
493 metabolism, *viridetad* – genes involved with virulence, detoxification and adaptation, *cwallproc* -
494 genes involved with cell wall and cell processes and *infopath* – genes involved with information
495 pathways

496

497 Supplementary figure S4: Number of non-redundant SNPs within human T cell epitopes stratified
498 by *Maf* lineage. No difference (X-squared = 0.0055391, df = 1, p-value = 0.9407) between the
499 proportion of nsSNPs between L6 (67 genomes) and L5 (mean values of 10 random samples of size
500 = 67 with replacement)

501

502 Supplementary table S5: Mutated Human T cell antigens and epitopes of *Maf* with mutations
503 stratified by lineage

504

505 Supplementary figure S6: Number of epitopes with and without nsSNPs stratified by Lineage 5
 506 with patient ethnicity (a). Number of non-redundant SNPs in epitopes stratified by ethnicity of L5
 507 infected patients (b). (The values for the non-Ewe associated L5 are mean estimates of 10 random
 508 samples of size 15 with replacement)

509

510 Supplementary figure S7: Uniform distribution of L5 isolated from ethnic Ewe TB patients among
 511 the L5 clade.

512

513 Supplementary data S8: Genomes and accession numbers of *Maf* from Ghana.

514

515 **Methods**

516 **Ethical Statement and Participant Enrollment**

517 The study and its protocols were reviewed by the Scientific and Technical Committee and
518 approved by the Institutional Review Board (IRB) of the Noguchi Memorial Institute for Medical
519 Research, Legon-Ghana with Federal Wide Assurance number FWA00001824.

520

521 ***Mycobacterium africanum* Strains**

522 Isolates used for this study were cultivated from July 2007 to November 2014 in Ghana^{9,12}, West
523 Africa, involving consecutive sputum smear positive pulmonary TB cases recruited from two
524 different studies with isolates spanning four regions of Ghana (three in the south and one from the
525 North) (supplementary figure S2).

526

527 **Mycobacterial Sub-Culturing and Chromosomal DNA Extraction**

528 *Mycobacterium africanum* strains were revived by sub-culturing on Lowenstein Jensen (LJ) slants;
529 one supplemented with 0.4% sodium pyruvate the other with glycerol to enhance the growth of
530 Lineage 5 and Lineage 6 strains of respectively. The cultures were incubated at 37 °C and
531 monitored regularly until growth was observed. When confluence was achieved, five loops full of
532 colonies were fetched into 2 mL cryo-vials containing 1 mL of sterile nuclease-free water, heat-
533 inactivated at 98 °C for 60 minutes for DNA extraction using a hybrid DNA extraction protocol⁴².
534 The isolates were confirmed MTBC by PCR amplification of IS6110, genotyped as *Maf* by large
535 sequence polymorphism (LSPs) detecting region of difference (RD) 9 and 12⁴³. Lineage
536 identification was achieved by spoligotyping as previously described⁴⁴. Strains confirmed as
537 belonging either L5 or L6 were sequenced by the illumina platform at the Wellcome Trust Sanger
538 Institute, United Kingdom.

539

540 **DNA Sequencing, Mapping of Sequence Reads, Variance Calling and Generation of Whole**

541 **Genome Fasta files**

542 Samples were sequenced as multiplexed libraries on the Illumina HiSeq platform to produce paired
543 end reads of 125 nt in length. Genomes provided by the Research Center Borstel was obtained by
544 sequencing DNA libraries prepared with the Nextera XT kit and run on Illumina MiSeq (250 and
545 300 bp, paired end) and NextSeq (150 bp, paired end) according to the manufacturer's instruction
546 (Illumina, San Diego, USA). The FastQ files containing the raw paired-end reads were processed
547 using a python pipeline developed in house as follows. The reads were first adapter- and quality-
548 trimmed with Trimmomatic v0.33⁴⁵. Reads lower than 20 bp were not kept for the downstream
549 analysis. Overlapping paired-end reads were then merged with SeqPrep
550 (<https://github.com/jstjohn/SeqPrep>). The resulting filtered reads were mapped to a hypothetical
551 reconstructed MTBC ancestor²⁶ with BWA v0.7.12⁴⁶. Duplicated reads were marked by the
552 MarkDuplicates module of Picard v 2.1.1 (<https://github.com/broadinstitute/picard>). The
553 RealignerTargetCreator and IndelRealigner modules of GATK v.3.4.0
554 (<https://software.broadinstitute.org/gatk/download/archive>) were used to perform local realignment
555 of reads around indels. SNPs were called with Samtools v1.2
556 (<https://sourceforge.net/projects/samtools/files/samtools/1.2/>) and VarScan v2.4.1⁴⁷ using the
557 following thresholds: minimum mapping quality of 20, minimum base quality at a position of 20
558 and minimum read depth at a position of 7X. SNPs were considered fixed at a frequency of $\geq 90\%$
559 and alleles were considered ancestral when the SNP frequency was $\leq 10\%$. Furthermore, SNPs were
560 called only if the alternative basecall was supported by at least five reads and without strand bias.
561 All variants were annotated using snpEff v4.11⁴⁸, in accordance with the *M. tuberculosis* H37Rv
562 reference annotation (AL123456.3). SNPs falling in regions with at least 50 bp identity to other
563 regions in the genome were excluded from the analysis.

564

565 **Generation of Variable Positions and Phylogenetic Analysis**

566 The variable SNPs alignment was obtained by concatenating the SNP calls present in the variant

calling file of each genome, using the IUPAC nucleotide ambiguity codes for heterozygous calls. A position was considered variable if at least one genome had a SNP at that position. Called deletions and positions not called according to the minimum threshold of 7 were encoded as gaps. Positions for which the proportion of gaps exceeded 50% were excluded from the alignment. Maximum likelihood phylogeny of the variable positions with 1000 bootstraps was then generated using RAxML version 8.2.3⁴⁹ with GTR substitution matrix and other default settings with the final tree evaluated and optimized under GAMMA with accuracy of 0.1 Log likelihood units. The best tree was then, rooted on *M. canettii* and annotated using figtree (<http://www.webcitation.org/getfile?fileid=27177ee8dd2f34cfd254b9c5e6c6fdf4b65329f6>).

Comparative genomics analysis of isolates using genes encoding proteins of 8 functional categories.

Experimentally confirmed human MTBC T cell epitope (1,226 epitopes) sequences (spanning 304 antigens with some overlapping sequences) retrieved from the Immune Epitope Database (IEDB), tested in human T cell assays, with no major histocompatibility complex (MHC) restrictions and have genomic coordinates in the H37Rv reference strain^{32,8} were *in silico* extracted from the fasta whole genome files and concatenated excluding sequence redundancy using customized bash algorithms. Complementary sequences of epitopes encoded by the reversed strand were first transcribed before the concatenation to have all the sequences in the same direction. In addition, MTBC genes of other seven functional categories namely those encoding regulatory proteins (regprot; 196), genes involved with lipid metabolism (limpet; 267), genes involved with intermediate metabolism (intmedres; 917), genes involved with virulence, detoxification and adaptation (virdetad; 216), genes involved with information pathways (infopath; 234), genes involved with cell wall and cell processes (cwallproc; 768) and genes essential for growth in macrophages (esmac; 125) according to the tuberculist database⁵⁰ were also retrieved and concatenated as described above excluding genes involved with drug resistance.

594 ***Estimation of Pairwise Nucleotide Diversity***

595 Pairwise SNP distances of the whole genome excluding sites associated with drug resistance,
 596 concatenates of T cell epitopes and the genes of other seven functional categories were calculated
 597 with the *dna.dist* function of *ape* package⁵¹ of R version 3.2.3⁵² as previously described⁸. Average
 598 pairwise nucleotide diversity per site (π) and confidence intervals for the π was calculated as
 599 previously described⁸ and plotted with *ggplot2* package implemented in R. The upper and lower
 600 levels of confidence were attained by estimating the 97.5th and 2.5th quantiles of the π distribution
 601 obtained by bootstrapping (1000 replicates) as previously described⁸. Non-overlapping confidence
 602 intervals of π were taken as evidence of statistically significant differences^{53,54}. Details of the
 603 algorithm for this analysis are available upon request.

604

605 ***Estimation of Pairwise dN/dS***

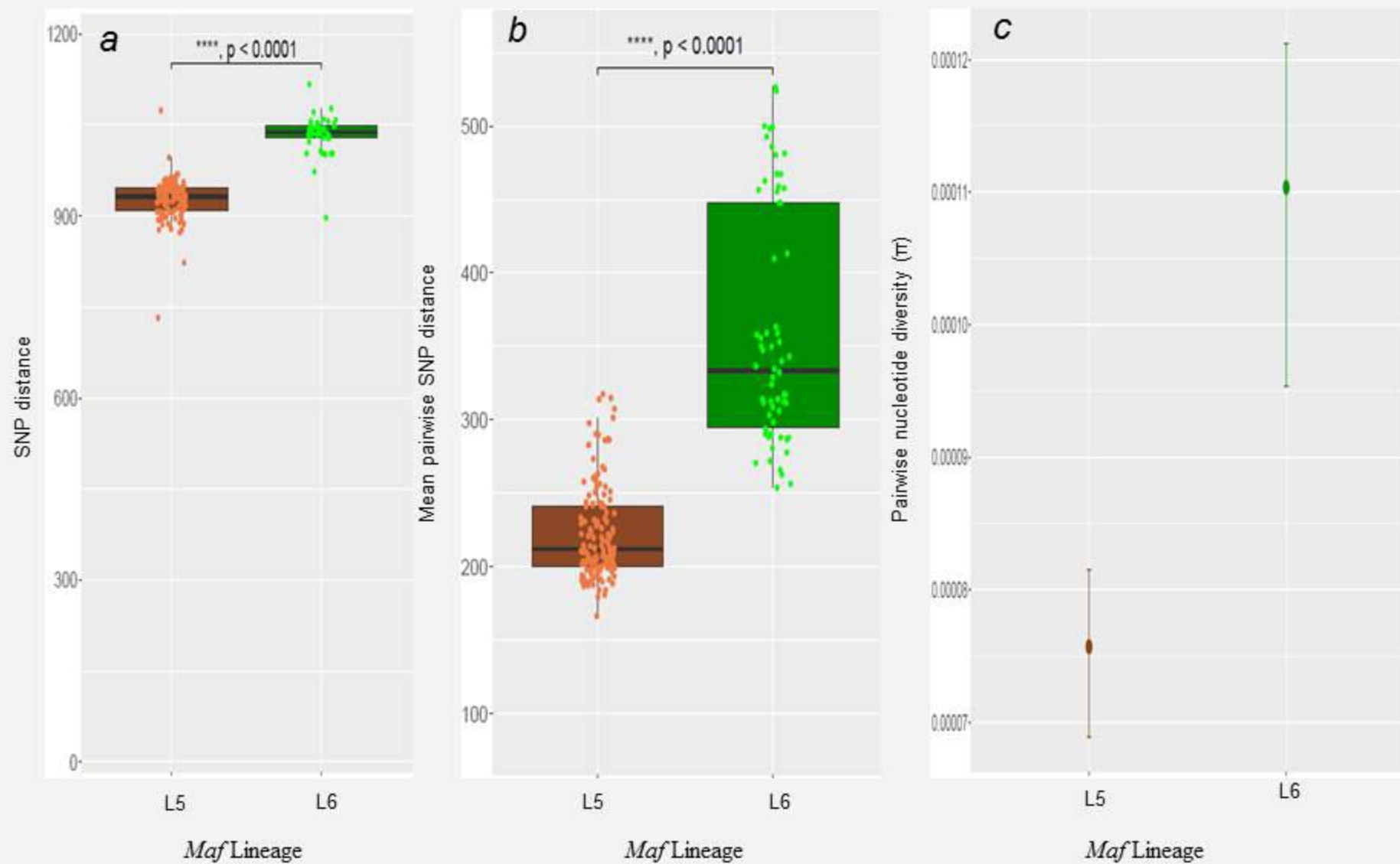
606 The concatenates of the human T cell epitopes and the other genes of seven functional categories
 607 were also used for estimation of dN/dS ratios stratified by lineage. As a follow up, dN/dS of T cell
 608 epitopes and regulatory proteins were also estimated for 15 L5 genomes from Ewe TB patients and
 609 77 from non-Ewe TB patients. The dN/dS estimates were calculated with all polymorphic sites
 610 within each lineage using the *kaks* function of the *seqinr* package⁵⁵ as previously described⁸ and
 611 box plotted using *ggplot2* package in R version 3.2.3. Statistical difference of the estimates
 612 between the *Maf* lineages was accessed using the non-parametric Wilcoxon rank-sum tests with
 613 continuity correction in R version 3.4.0.

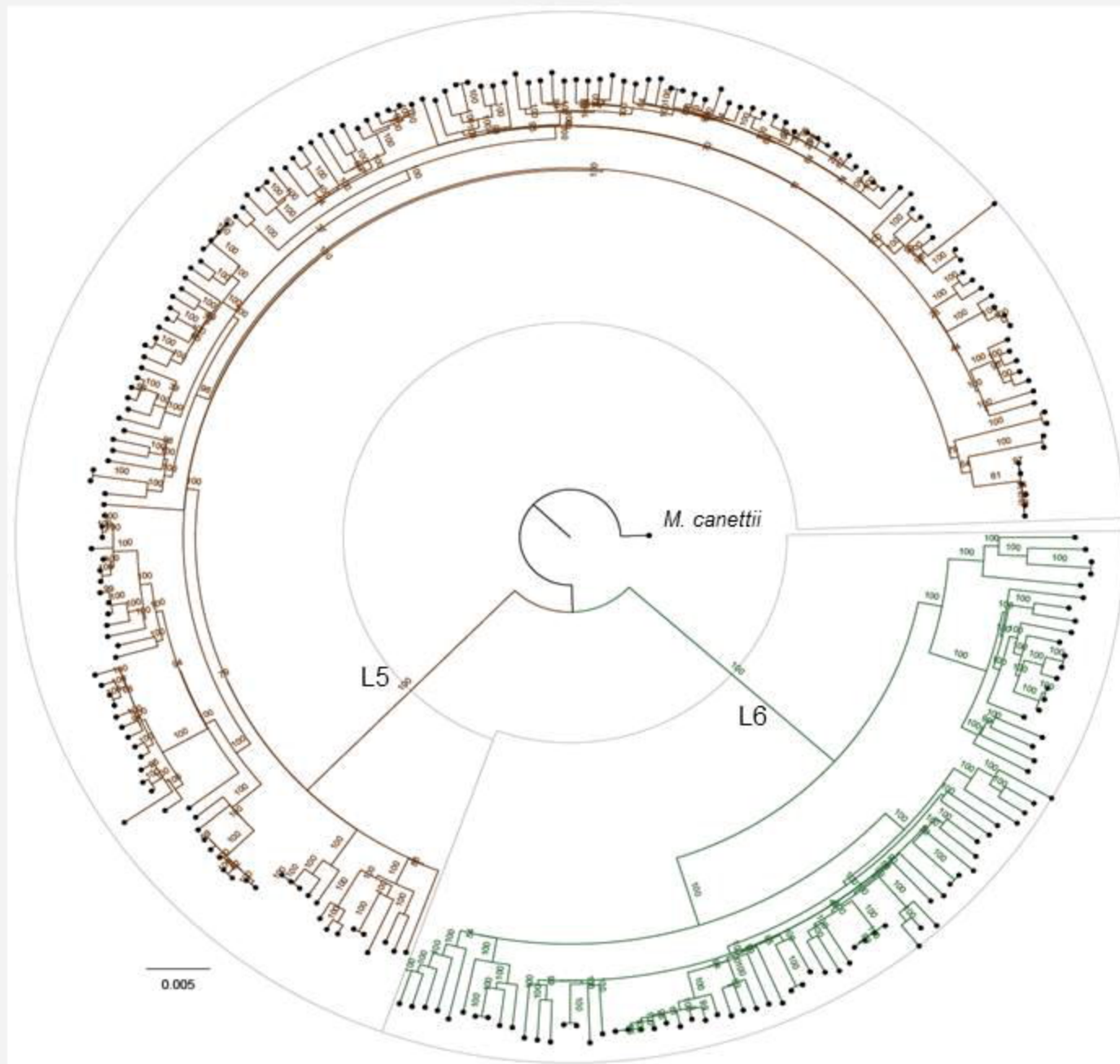
614

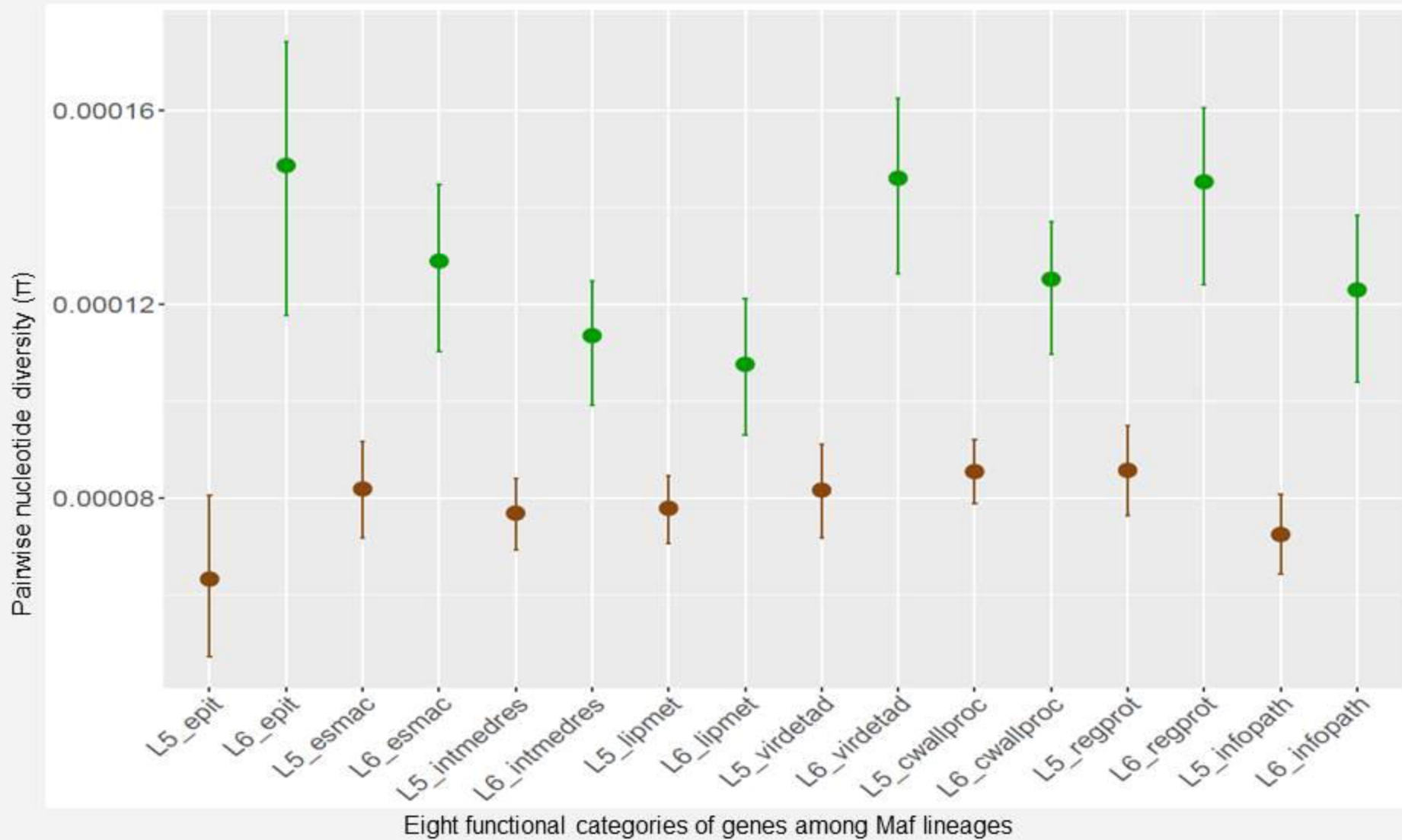
615 **Human T cell Epitopes with Non-Synonymous SNPs and Count of Non-Redundant SNPs**

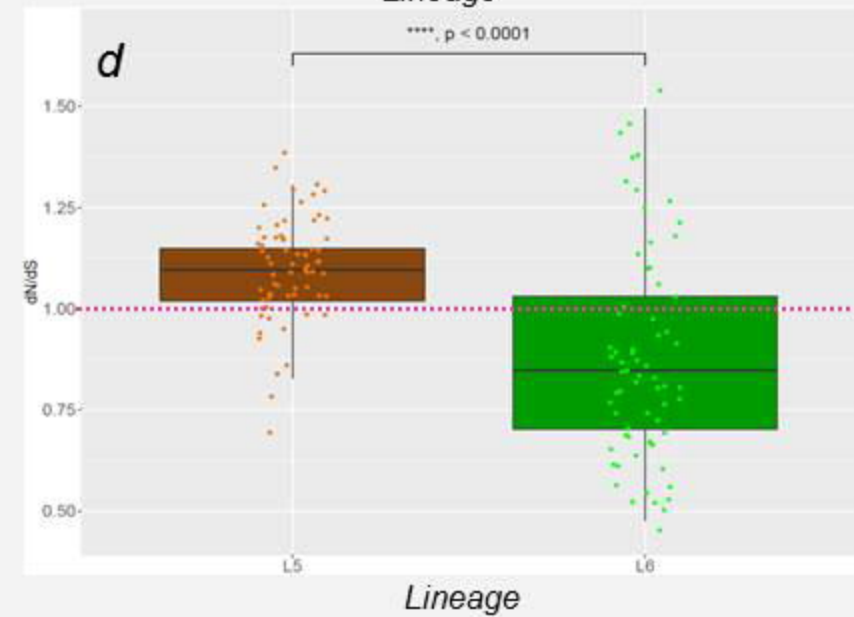
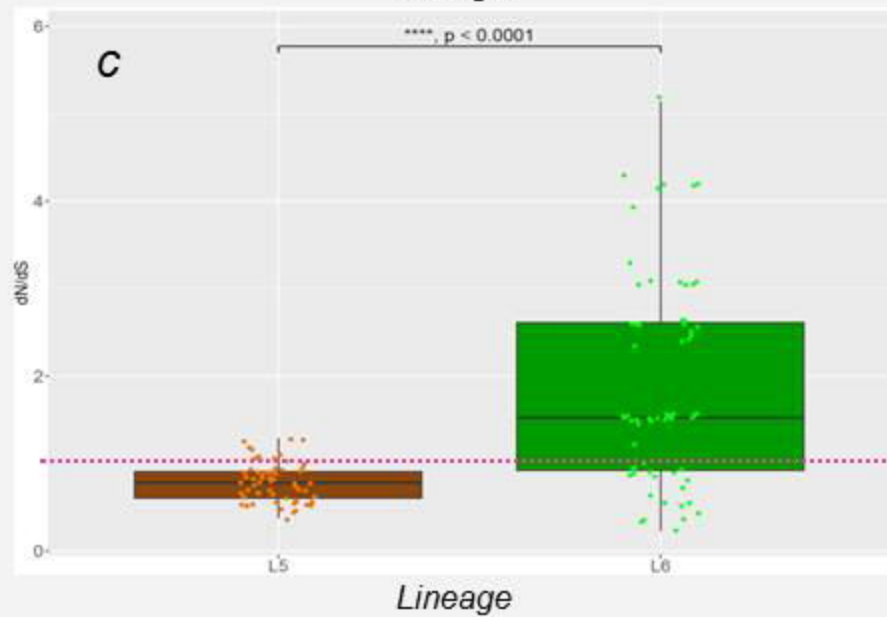
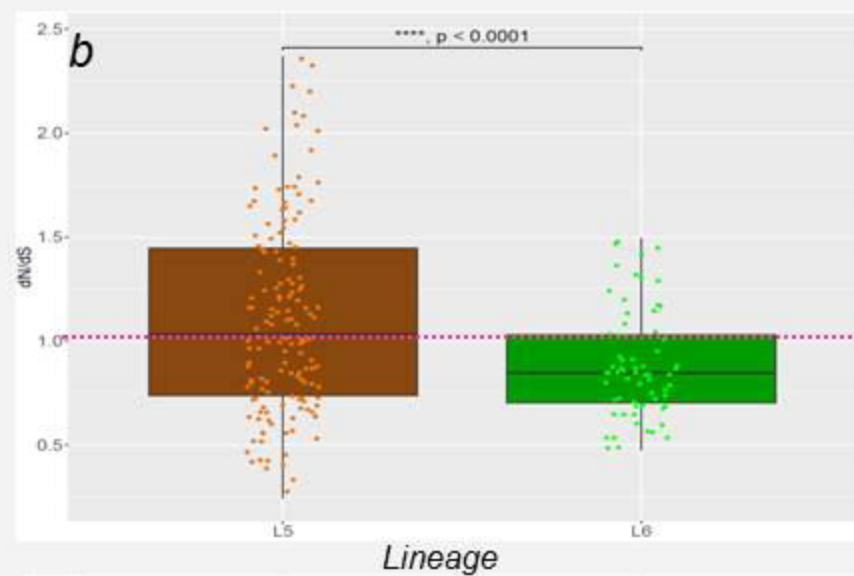
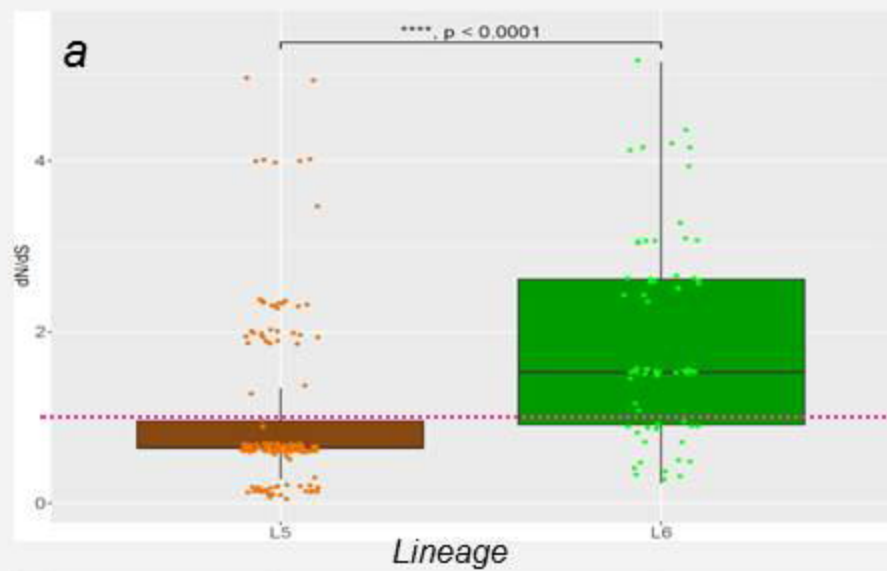
616 Synonymous and non-synonymous mutations within the coordinates of each epitope were extracted
 617 from the variant calling file (VCF) obtained for each genome. The specific human T cell epitopes
 618 with non-synonymous SNPs were compared between the *Maf* lineages for lineage-specific mutated
 619 epitopes and *Maf*-specific mutated epitopes.

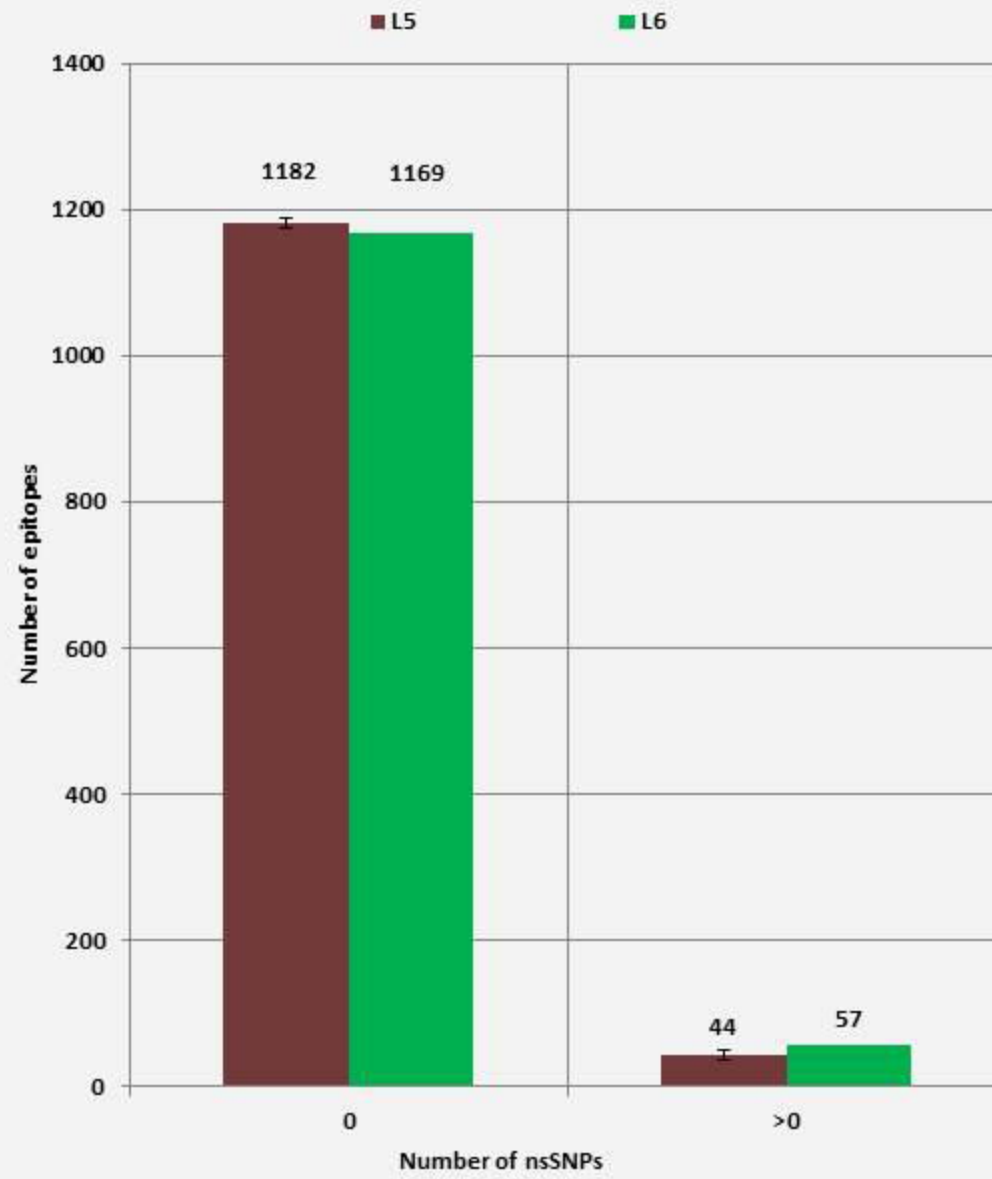
620 Furthermore, the number of pairwise non-redundant SNPs was estimated for the *Maf* lineages (67
621 L6 genomes and the 10 random samples of L5 of equal size as L6) as well as L5 genomes stratified
622 by patient ethnicity (15 L5 from Ewe patients and 10 random samples of L5 from non-Ewe TB
623 patients of size 15) using Mega6⁵⁶. The number of SNPs per each group was plotted and compared
624 between the groups using the fisher's exact test for statistical significance in R version 3.2.3.
625



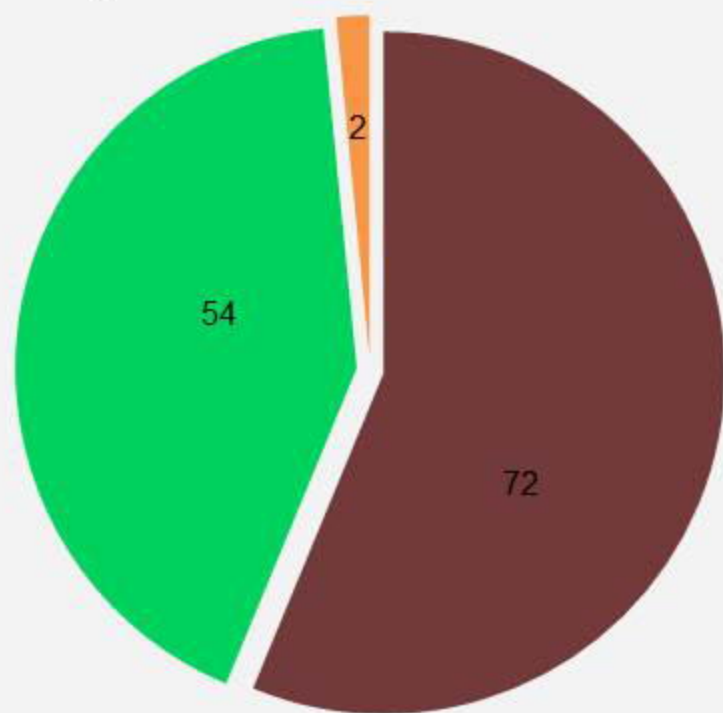






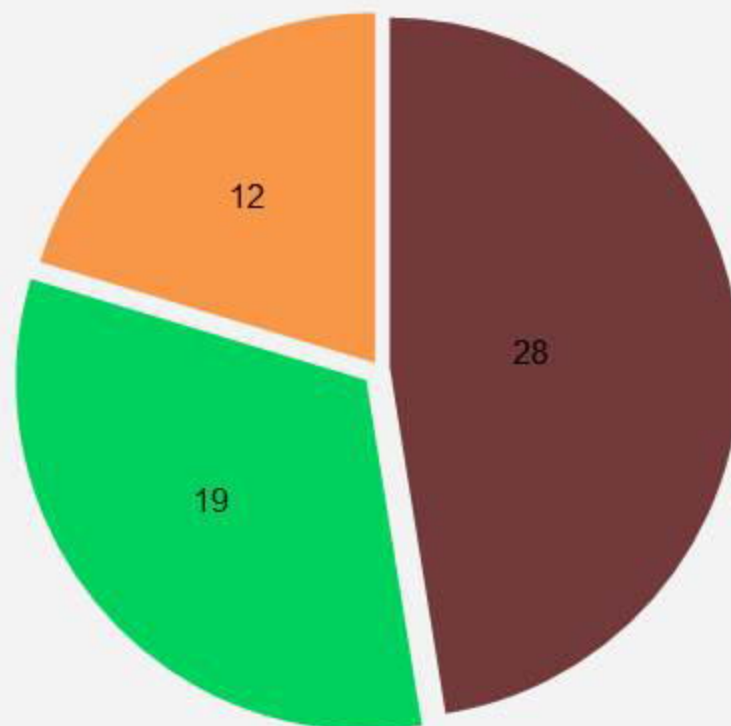


a

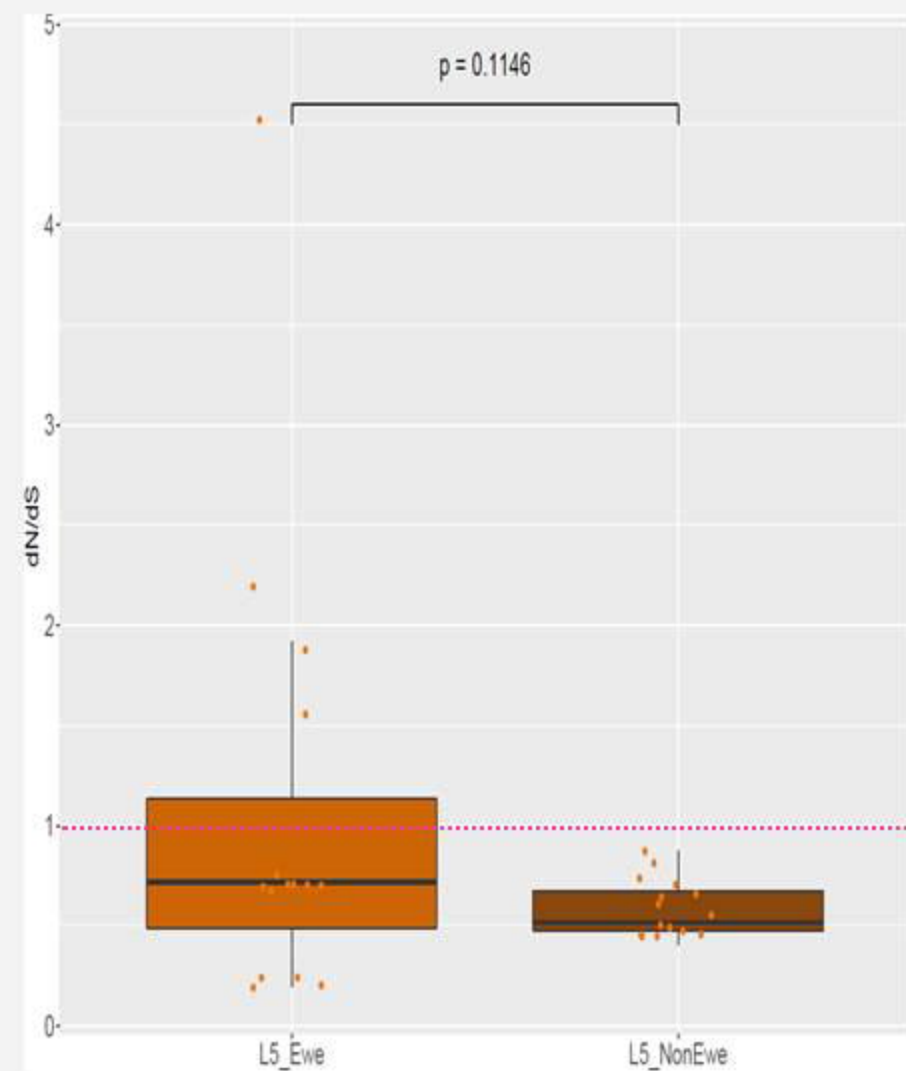
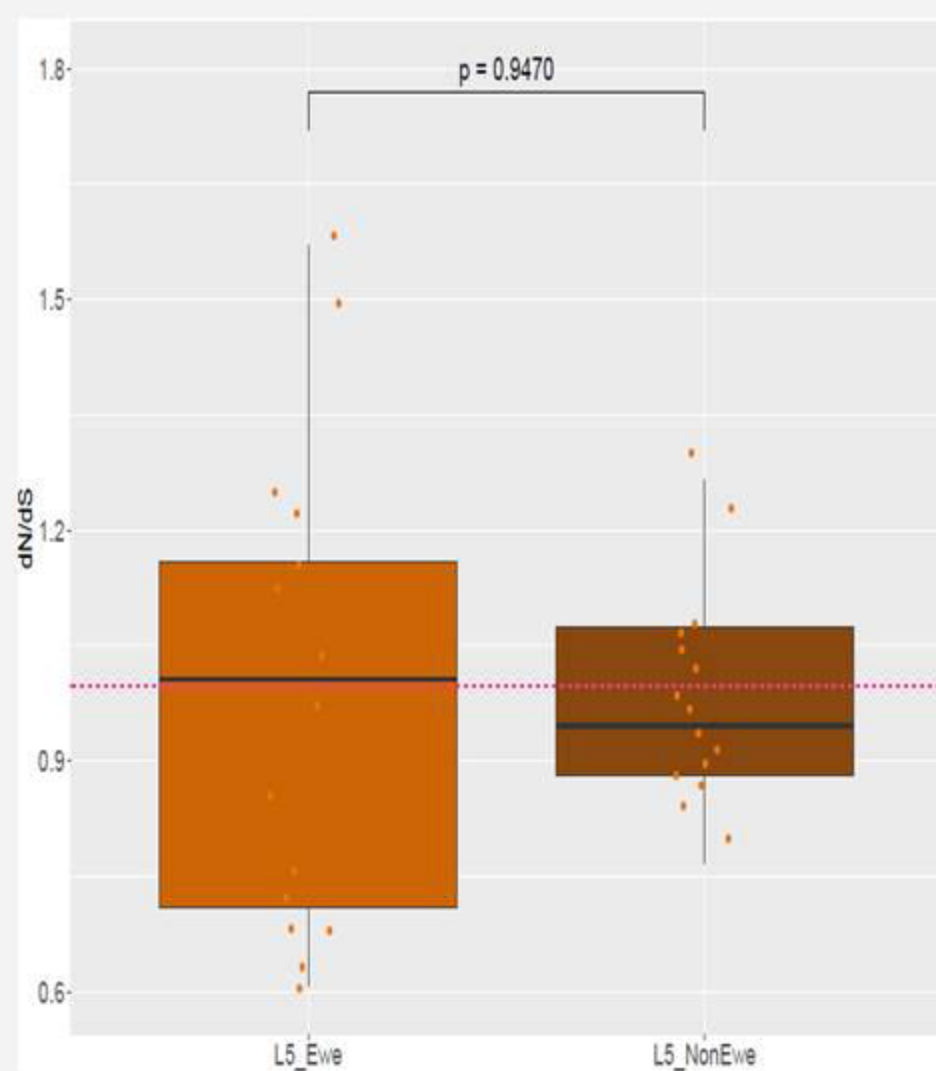


■ L5 ■ L6 ■ L5/L6

b



■ L5 ■ L6 ■ L5/L6

a*L5 with Ethnicity***b***L5 with Ethnicity*

