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2

3 Title: Whole genome sequencing *Mycobacterium tuberculosis* directly from sputum

4 identifies more genetic diversity than sequencing from culture

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13

Abstract

Background

Repeated culture reduces within-sample *Mycobacterium tuberculosis* genetic diversity due to selection of clones suited to growth in culture and/or random loss of lineages, but it is not known to what extent omitting the culture step altogether alters genetic diversity. We compared *M. tuberculosis* whole genome sequences generated from 39 paired clinical samples. In one sample DNA was extracted directly from sputum then enriched with custom-designed SureSelect (Agilent) oligonucleotide baits and in the other it was extracted from mycobacterial growth indicator tube culture.

Results

DNA directly sequenced from sputum showed significantly more within-sample diversity than that from mycobacterial growth incubator tube culture. This was demonstrated by more variants present as heterozygous alleles (HAs) where both a variant and wild type allele were present within a given sample ($p<0.001$) and greater within-sample Shannon diversity ($p<0.001$). Seven genes with high within-sample diversity have previously been identified as targets for positive selection, highlighting their potential role in adaptation to survival within the host and under drug pressure. Resistance associated variants present as HAs occurred in six patients, and in four cases may have provided a genotypic explanation for phenotypic resistance.

Conclusions

37 Culture-free *M. tuberculosis* whole genome sequencing detects more within-sample

38 diversity and may allow detection of mycobacteria that are not actively replicating.

39

40 Key words: Mycobacterium tuberculosis; drug-resistant tuberculosis; whole genome

41 sequencing; sputum; within-patient diversity; heteroresistance

Background

International efforts to reduce tuberculosis (TB) infections and mortality over the last two decades have only been partially successful. In 2017, 10 million people developed TB and it has overtaken HIV as the infectious disease responsible for the most deaths worldwide(1, 2). Drug resistance is a major concern with a steady rise in the number of reported cases globally and rapid increases in some areas(1). Patients with *Mycobacterium tuberculosis* resistant to the first line drugs rifampicin and isoniazid are classed as having multidrug-resistant (MDR) TB and usually treated with a standardised second-line drug regimen for at least nine months, which is also used for rifampicin monoresistance(3, 4). With the emergence of resistance to fluoroquinolones and aminoglycosides (extensively drug-resistant [XDR] TB) there is an increasing need for individualised therapy based on drug susceptibility testing (DST). Individualised therapy ensures patients are treated with sufficient active drugs which can prevent selection of additional resistance, improve treatment outcomes and reduce duration of infectiousness(5-8).

Traditionally, phenotypic culture-based DST was used to identify drug resistance but this is being replaced by rapid genetic tests that detect specific drug resistance conferring mutations. Next generation whole genome sequencing (WGS) of *M. tuberculosis* is being increasingly used in research and clinical settings to comprehensively identify all drug resistance associated mutations(9). *M. tuberculosis* has a conserved genome with little genetic diversity between strains(10), but more detailed analysis of individual patient samples with WGS has identified genetically separate bacterial subpopulations in sequential sputum samples(11-15) and across different anatomical sites(16). This within-patient

66 diversity can occur as a result of mixed infection with genetically distinct strains or within-
67 host evolution of a single infecting strain(17).
68
69 Bacterial subpopulations can be detected in clinical samples after sequencing reads are
70 mapped to a reference genome where multiple base calls are detected at a single genomic
71 site. These heterozygous alleles (HAs) at sites associated with drug resistance (resistance
72 associated variants, RAVs) may reflect heteroresistance, where a fraction of the total
73 bacterial population is drug susceptible while the remainder is resistant(18). Identification
74 of genetic diversity within clinical samples is important as it may improve detection of RAVs
75 over currently available genetic tests and consensus-level WGS(18). Identifying RAVs could
76 improve individualised therapy, prevent acquired resistance(12), and give insight into
77 bacterial adaptation to the host.
78
79 *M. tuberculosis* WGS is usually performed on cultured isolates to obtain sufficient purified
80 mycobacterial DNA. However, the culture process can change the population structure from
81 that of the original sample due to genetic drift (random loss of lineages) and/or the
82 selection of subpopulations more suited to growth in culture(19-21), and repeated
83 subculture leads to loss of genetic diversity and heteroresistance(22). Additionally, in the
84 normal course of *M. tuberculosis* infection, some bacteria exist as viable non-culturable
85 persister organisms that are hypothesised to cause the high relapse rate seen following
86 treatment of insufficient duration(23). These organisms are likely to be missed by any
87 sequencing method reliant on culture.
88

89 WGS directly from sputum without enrichment is challenging(24). It has recently been
90 improved by depleting human DNA during DNA extraction(25). We have previously reported
91 the use of oligonucleotide enrichment technology SureSelect (Agilent, CA, USA) to sequence
92 *M. tuberculosis* DNA directly from sputum(26) and demonstrated its utility in determining a
93 rapid genetic drug resistance profile(27, 28).
94
95 It remains unclear to what extent WGS of cultured *M. tuberculosis* samples underestimates
96 the genetic diversity of the population in sputum samples. One previous study of 16 patients
97 did not identify increased genetic diversity in *M. tuberculosis* DNA sequenced directly from
98 sputum compared to DNA from culture(25), whereas another study of mostly drug
99 susceptible patients showed sequencing directly from sputum identified a slight excess of
100 HAs relative to culture(27). Here we reanalyse heterozygous alleles (HAs) present in that
101 study(27) in addition to newly collected samples from patients with MDR-TB, use a more
102 sensitive analysis to measure overall within-sample genetic diversity and further explore the
103 genomic location of the additional diversity identified.
104

105 Results

106

107 Patient Characteristics and Drug Susceptibility Testing

108
109 Whole genome sequences were obtained for 39 patients from both mycobacterial growth
110 indicator tube (MGIT) culture and direct sputum sequencing. The patients were
111 predominantly of black African ethnicity (80%) and 50% were HIV positive (Table 1). First-
112 line phenotypic drug susceptibility testing (DST) results identified 22 patients with MDR-TB

113 and two with rifampicin monoresistance. In addition there were three isoniazid
114 monoresistant patients and ethambutol resistance was detected in 8 patients. Second-line
115 phenotypic DST was performed for patients with rifampicin-resistant or MDR-TB and
116 identified one case of kanamycin resistance (Table 2).
117
118 We observed greater median coverage depth in sputum-derived sequences than MGIT
119 sequences (164.3 vs 136.6, p=0.068). A genotypic susceptibility profile was determined by
120 evaluating MGIT WGS for consensus-level RAVs using a modified version of a publicly
121 available list(29). Genotypic RAVs predicted all rifampicin phenotypic resistance and >90% of
122 isoniazid phenotypic resistance. Ethambutol genotypic RAVs were poorly predictive of
123 phenotypic resistance in line with findings from other studies(30) (Table 2). The patient with
124 kanamycin phenotypic resistance was correctly identified by an *rrs* a1401g RAV. No full
125 phenotypic fluoroquinolone phenotypic resistance was identified, but several colonies from
126 patient F1013 did grow in the presence of ofloxacin (although not enough to be classified as
127 resistant). The consensus sequences from this patient harboured a *gyrB* E501D mutation
128 which is believed to confer resistance to moxifloxacin but not other fluoroquinolones, which
129 may explain the borderline phenotypic DST result(31).
130
131 **Genetic Diversity**
132
133 To compare consensus sequences from sputum and MGIT, a WGS consensus sequence-level
134 maximum likelihood phylogenetic tree was constructed (Supplementary Material: Figure 1).
135 Four previously sequenced strains from KwaZulu-Natal were included(32). As expected, all
136 paired sequences were closely related, with a mean difference of 1.30 (range 0-9) single

137 nucleotide polymorphisms (SNPs). Samples from patients F1066 and F1067 were closely
138 related with only one consensus-level SNP separating all four consensus sequences. There
139 was no obvious epidemiological link between these patients (although this study was not
140 designed to collect comprehensive epidemiological information) and they lived 20km apart
141 in Durban. However, both patients were admitted contemporaneously to an MDR
142 treatment facility and sampled on the same day. DNA extraction and sequencing occurred
143 on different runs so the close genetic linkage may represent direct transmission within a
144 hospital setting, a community transmission chain or an unlikely cross-contamination during
145 sample collection.
146
147 Having established congruence between sputum and MGIT sequences at the consensus
148 level we then compared genetic diversity by DNA source. We first defined a threshold for
149 calling variants present as heterozygous alleles (HAs) in our entire dataset by using a range
150 of minimum read count frequencies as described in the methods (Figure 1). Below a
151 minimum of five supporting reads there was an exponential increase in the number of HAs
152 identified, which may be indicative of the inclusion of sequencing errors. To reduce this risk,
153 we used a threshold of a minimum of five supporting reads.
154
155 Genetic diversity may occur because of within-host evolution or mixed infection. To identify
156 mixed infection we used a molecular barcode(33) to scan all HAs for a panel of 413
157 phylogenetic SNPs that can resolve *M. tuberculosis* into one of seven lineages and 55 sub-
158 lineages. We found three phylogenetic SNPs among the HAs. In all cases the heterozygous
159 phylogenetic SNP originated from the same sublineage as other SNPs present at 100%
160 frequency, and there were no cases of HAs indicating the presence of more than one lineage

161 or sublineage. This suggests that the genetic diversity identified is mostly or exclusively due
162 to within-host evolution, although there remains a small possibility that mixed infections
163 with two strains from the same sub-lineage could have occurred.
164
165 As a first step to comparing diversity between sputum and MGIT sequenced samples we
166 looked at the location of genetic diversity within the *M. tuberculosis* genome. Variants were
167 called in the MGIT and sputum sequences for each patient and classified as present in MGIT
168 only, sputum only or shared (present in both). HAs were widely dispersed across the
169 genome at similar sites in both sputum and MGIT samples but some genes had multiple HAs
170 (Table 3). The highest genetic diversity was found in the ribosomal RNA (rRNA) genes (*rrs*
171 and *rrl*) with 358 HAs, of which 98.6% were only found in sputum-derived sequences.
172
173 As rRNA contains regions that are highly conserved across bacteria, it was considered a
174 possibility that SureSelect baits targeting rRNA genes were capturing both *M. tuberculosis*
175 and other bacterial species. To evaluate this, metagenomic assignment was performed on
176 all reads. Sampling reads not assigned to *M. tuberculosis* (i.e. presumed contaminants from
177 other bacteria) and performing a BLAST search against *M. tuberculosis* 16S and 23S rRNA
178 genes indicated that a sizeable proportion of these reads from directly sequenced sputum
179 had a BLAST hit of at least 30 bases (median 11% v 0% of equivalent reads from MGIT
180 sequencing, $p<0.001$, Supplementary Material: Figure 2). The taxonomic assignment of
181 these reads were indeed typical of genera composing the oral flora, with a high
182 representation of *Actinomyces, Fusobacterium, Prevotella,* and *Streptococcus*
183 (Supplementary Material: Figure 3).
184

185 This supported the hypothesis that the baits may enrich rRNA from other organisms so rRNA
186 genes were excluded from further analysis. The difference in diversity between sputum and
187 MGIT sequences can be explained by the selective nature of MGIT media which will enrich
188 *M. tuberculosis* sequences. Importantly the frequency of HAs in other highly diverse genes
189 between sequencing strategies was more balanced (Table 3). Pertinently seven of these
190 genes (*Rv1319c(34)*, *lppB(35)*, *Rv2082(35)*, *ppsA(34, 36)*, *ponA1(36)*, *lppA(37)*, and *pks12(35,*
191 *36)*) with high numbers of HAs have been previously identified as highly diverse in
192 comparative genomic studies suggesting the detected HAs are not artefactual. The
193 frequencies at which HAs in these genes were present in MGIT and sputum is shown in
194 Supplementary Material Figure 4.
195
196 After confirming the absence of mixed infections and removing rRNA gene sequences we
197 compared the frequency of HAs in sputum and MGIT. There were 2048 variants across the
198 dataset that were present as a HA in either MGIT, sputum or both sequences (Table 4).
199 Variants present in both MGIT and sputum derived sequences were more likely to be
200 present as a HA in the sputum-derived sequence (3.2% v 1.9%, $p<0.0001$). Of the other
201 variants present as HAs, 821 were unique to direct sputum sequencing and 153 were unique
202 to MGIT sequencing (Table 4). Variants found only in sputum were more likely to be
203 heterozygous than those found only in MGIT or in both ($p<0.0001$). The distribution of HAs
204 by patient is shown in Figure 2A. HAs found only by one modality were more likely to be
205 SNPs than shared HAs, where the majority were insertions or deletions. The ratio of non-
206 synonymous to synonymous HAs was similar for those that were shared or MGIT only, but
207 was lower for sputum only HAs. Frameshift mutations were most prevalent among shared
208 HAs (Table 4).

209

210 To confirm our findings of increased diversity in sputum we calculated mean within-sample

211 diversity (*H*), excluding rRNA genes and repeat regions (see methods). The mean diversity

212 was significantly greater in sputum than MGIT-derived sequences (Figure 2B: 0.116±0.078 v

213 0.054±0.026 , mean H_{sputum}/H_{MGIT} = 2.66, p=3.0 x 10^{-5}).

214

215 **Genetic diversity in drug resistance genes**

216

217 HAs in drug resistance-associated regions, including promoters and intergenic regions, were

218 individually assessed. Five of the 39 patients had RAVs present as HAs in at least one gene,

219 which are shown in Table 5. F1002 had three compensatory mutations in *rpoC* present at

220 HAs in both sequences. F1007 had high-level phenotypic isoniazid resistance despite wild

221 type *katG* and *inhA* genes, but did have two *ahpC* promoter variants present as HAs. Neither

222 of these variants are reported frequently but both have been previously associated with

223 resistance in limited numbers of samples(38). As described above F1066 and F1067 were

224 highly related with only one consensus SNP difference between all four sequences. Both had

225 phenotypic high level isoniazid resistance with no consensus-level *katG* or *inhA* mutation,

226 but had frameshift *katG* mutations present as HAs which have the potential to cause

227 resistance(39). F1066 and RF021 had *Rv1979c* and *pncA* mutations respectively at low

228 frequency in sputum only which have the potential to confer phenotypic resistance to

229 clofazimine (*Rv1979c*) and pyrazinamide (*pncA*), although no phenotypic testing was

230 performed for these drugs.

231

Discussion

In this study we whole genome sequenced DNA from sputum and MGIT culture in paired samples from 39 patients and compared within-patient genetic diversity of the bacterial genome identified from each source. All paired sequences were closely related at the consensus level, and WGS predicted phenotypic drug susceptibility with over 90% sensitivity and specificity for rifampicin and isoniazid in line with published data(40).

The understanding of within-patient *M. tuberculosis* genetic diversity is becoming increasingly important as the detection of rare variants has been shown to improve the correlation between phenotypic and genotypic drug resistance profiles(18) and can identify emerging drug resistance(11, 12). Here we have demonstrated that significantly more genetic diversity is identified by WGS performed directly from enriched sputum than MGIT culture. Not including a culture step avoids the introduction of bias towards culture-adapted subpopulations and the impact of random chance and is also likely to incorporate DNA from viable non-culturable mycobacteria. A reduction in genetic diversity has previously been shown with sequential *M. tuberculosis* subculture(19, 22), but was not confirmed by a study performing WGS directly from sputum(25). However, the 16 paired sputum and MGIT samples compared by Votintseva(25) had a minimum of 5x coverage compared to a minimum 40x coverage in this study, and were likely to contain less genetic material as they were surplus clinical rather than dedicated research samples.

We found that the rRNA genes have high levels of diversity in sputum samples, but believe this is due to non-mycobacterial DNA hybridising to the capture baits — a conclusion borne

256 out by the taxonomic assignment of reads aligning to these genes in common oral bacteria.
257 We therefore exclude these from further analysis, and recommend others using enrichment
258 from sputum do similarly. We use two methods to evaluate within-sample *M. tuberculosis*
259 genetic diversity. First, we demonstrate increased diversity when sequencing directly from
260 sputum with significantly more unique heterozygous alleles (HAs) than sequencing from
261 MGIT culture. We also observed significantly higher genetic diversity in sputum-derived
262 sequences by comparing the Shannon diversity of variable sites across pairs of samples.
263
264 Many of the genes with high levels of within-sample diversity are also reported to be targets
265 for convergent evolution, independently accumulating similar mutations on a global scale.
266 This implies that diversity seen on a macroevolutionary scale has a basis in microevolution,
267 and reinforces the importance of accurately characterising the biological function of these
268 genes and their products to aid the identification of new therapeutic targets. Two-thirds of
269 the patients with MDR-TB had already been treated for drug-sensitive TB, and the diversity
270 identified in sputum samples may therefore represent early adaptation to drug pressure.
271 Importantly, as direct sputum sequencing does not rely on live mycobacteria, DNA from
272 recently killed *M. tuberculosis* is likely to also be sequenced, meaning that recent genomic
273 mutations are likely to be represented as HAs.
274
275 In four patients, RAVs present as HAs provided a likely genotypic basis for otherwise
276 unexplained phenotypic resistance. Given the small total number of resistance mutations in
277 this study, the excess of heterozygous known RAVs in directly sequenced sputum is not
278 statistically significant. However the presence of heterozygous RAVs in both MGIT and
279 sputum sequences reinforces the biological importance of these mutations.

A limitation of this study is that it can be difficult to distinguish low frequency variants from sequencing error. Ideally low frequency variants could be confirmed by resequencing the same DNA samples. To reduce the risk of sequencing errors yet still identify genetic diversity we used the lowest minimum read threshold at which the number of HAs remained stable. Also, it is reassuring that of all fixed and heterozygous variants called, more than 93% were identified from both DNA sources.

Conclusions

Directly sequencing *M. tuberculosis* from sputum is able to identify more genetic diversity than sequencing from culture. Understanding within-patient genetic diversity is important to understand bacterial adaptation to drug treatment and the acquisition of drug resistance. It also has potential to identify low frequency RAVs that may further enhance genotypic-phenotypic drug resistance correlation.

Methods

Patient enrolment

Adult patients presenting with a new diagnosis of sputum culture-positive TB were included in the study. Patients were recruited in London, UK (n=15) and Durban, South Africa (n=24). All patients recruited in Durban were Xpert MTB/RIF (Cepheid, CA, USA) positive for rifampicin resistance. Two sputum samples were collected prior to initiating treatment, with

303 one inoculated into mycobacterial growth indicator tube (MGIT) culture (BD, NJ, USA) and
304 the other used for direct DNA extraction.
305
306 **Ethics, Consent and Permissions**
307
308 All patients gave written informed consent to participate in the study. Ethical approval for
309 the London study was granted by NHS National Research Ethics Service East Midlands–
310 Nottingham 1 (reference 15/EM/0091). Ethical approval for the Durban study was granted
311 by University of KwaZulu-Natal Biomedical Research Ethics Committee (reference
312 BE022/13).
313
314 **Microbiology**
315 MGIT samples were incubated in a BACTEC MGIT 960 (BD, NJ, USA) until flagging positive.
316 Phenotypic DST data for London samples were those provided to treating hospitals by Public
317 Health England. Phenotypic DST for Durban samples was performed using the solid agar
318 proportion method (Supplementary Material: Methods).
319
320 **DNA extraction and sequencing**
321 Positive MGIT tubes were centrifuged at 16,000g for 15 minutes and the supernatant
322 removed. Cells were resuspended in phosphate-buffered saline before undergoing heat
323 killing at 95°C for 1 hour followed by centrifugation at 16,000g for 15 minutes. The
324 supernatant was removed and the sample resuspended in 1mL sterile saline (0.9% w/v). The
325 wash step was repeated. DNA was extracted with mechanical ribolysis before purification

326 with DiaSorin Liaison Ixt (DiaSorin, Italy) or CTAB(41). NEBNext Ultra II DNA (New England
327 Biolabs, MA, USA) was used for DNA library preparation.
328
329 Sputum samples for direct sequencing were similarly heat killed processed as for MGIT
330 samples. DNA extraction was performed with mechanical ribolysis followed by purification
331 using DiaSorin Liaison Ixt (DiaSorin, Italy) or DNeasy blood & tissue kit (Qiagen,
332 Germany)(41). Target enrichment was performed using SureSelect with a custom-designed
333 bait set providing coverage of the entire *M. tuberculosis* genome as described
334 previously(27). Batches of 48 multiplexed samples were sequenced on a NextSeq (Illumina,
335 CA, USA).
336
337 **Bioinformatic analysis**
338 Bioinformatic analysis was performed with CLC Genomics Workbench v11.0 (Qiagen,
339 Germany). DNA sequence reads were aligned to an H37Rv reference genome as detailed in
340 the Supplementary Material Methods section (GenBank accession NC_000962.3). All
341 samples had minimum 98% 1x reference genome coverage and mean coverage depth 40x
342 across the genome. Variants falling within or near hypervariable elements were excluded
343 (Supplementary Material: Table 1). A consensus sequence was extracted and used to
344 determine the genotypic drug susceptibility profile. To construct the maximum likelihood
345 phylogenetic tree, variants were called against the reference genome using VarScan v2.3.9
346 (Supplementary Material: Methods).
347
348 For the initial analysis of genetic diversity, variants were included if supported by ≥2 reads,
349 with ≥1 forward and reverse read. The minimum supporting read threshold was increased in

350 a stepwise fashion from 2 to 20. Further analyses were performed on variant tracks where
351 variants were supported ≥5 supporting reads including ≥1 forward and reverse read.
352
353 To compare diversity between paired samples, we first mapped reads to the reference
354 genome using bwa mem v0.7.12(42). After verifying all samples had adequate coverage with
355 qualimap(43) (mean ± standard deviation coverage at 10x: 98.0 ± 1.8%) and realigning
356 indels, variants were called with HaplotypeCaller in GATK v3.3.0(44) (Supplementary
357 Material: Methods). The gvcf files were combined for each pair of samples with
358 CombineGVCFs in GATK then screened to remove sites in variable regions and rRNA genes
359 with vcfintersect in vcflib, resulting in 39 paired gvcf files containing allele depths at variable
360 positions for diversity analysis.
361
362 **Metagenomic assignment**
363 Sequencing reads were classified using Kraken v0.10.6(45) against a custom Kraken
364 database previously constructed from all available RefSeq genomes for bacteria, archaea,
365 viruses, protozoa, and fungi, as well as all RefSeq plasmids (as of September 19th 2017) and
366 three human genome reference sequences(46). The size of the final database after shrinking
367 was 193 Gb, covering 38,190 distinct NCBI taxonomic IDs.
368
369 To assess the proportion of contaminating reads that could generate spurious diversity
370 when mapped to *M. tuberculosis* ribosomal genes, we randomly subsampled 100 reads
371 taxonomically assigned as non-*M. tuberculosis* and performed a BLAST search with blastn
372 v2.2.28(47) against rRNA genes from the H37Rv reference genome. We only analysed hits of
373 at least 30 bases.

Statistics

Statistical analyses were performed with Prism v7.0 (Graphpad, CA, USA). The number of HAs in paired samples were compared using a two-tailed Wilcoxon matched-pairs signed rank test. Numbers of HAs found between groups were compared with chi-squared.

Within-sample diversity (H) was calculated using Shannon diversity from the allele frequencies (p). The Shannon index (H_n) expresses the positional entropy at each position (n), with the mean positional entropy (H) indicating greater within-sample diversity:

$$H = \sum_{n} H_n = \sum_{n} \sum_{i \in \{A,C,G,T\}} p_{n,i} \log (p_{n,i})$$

To make a fair comparison for each pair of samples, after removing indels, sites were included if they contained a variant in at least one and had a depth coverage ≥30 in both. We found that the depth coverage cutoff had no qualitative effect on the conclusions. The difference in mean within-sample diversity depending on DNA source was compared with a two-tailed Wilcoxon matched-pairs signed rank test.

Abbreviations

DST	drug susceptibility testing
HA	heterozygous allele
MDR-TB	multidrug resistant-tuberculosis
MGIT	mycobacterial growth indicator tube

RAV	resistance-associated variant
rRNA	ribosomal RNA
SNP	single nucleotide polymorphism
TB	tuberculosis
WGS	whole genome sequencing

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392

393

Declarations

Ethics approval and consent to participate

All patients gave written informed consent to participate in the study. Ethical approval for the London study was granted by NHS National Research Ethics Service East Midlands–Nottingham 1 (reference 15/EM/0091). Ethical approval for the Durban study was granted by University of KwaZulu-Natal Biomedical Research Ethics Committee (reference BE022/13).

Consent for publication

Not applicable

Availability of data and materials

Original fastq files are available at NCBI Sequence Read Archive with BioProject reference PRJNA486713: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA486713/

Competing interests

The authors declare that they have no competing interests.

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419 analysis, data interpretation or manuscript writing.
420

421 **Authors' contributions**

422 Study conception: JB, ASP

423 Data collection: CB, KB

424 Analysis and interpretation: CN, LPS, RD, RW

425 Drafting of manuscript: CN, LPS

426 Revision of manuscript: FB, JB, ASP

427 Final approval of manuscript: CN, LPS, RD, RW, KB, CB, JB, FB, ASP

428

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Tables

	Count / mean	Range/percentage
Age	36.7	22 – 64
Male sex	23/37	59.0%
Ethnicity		
Asian	3/35	8.6%
Black African	28/35	80.0%
Caucasian	4/35	11.4%
HIV positive	19/38	50.0%
CD4 count (median)	296.5*	17 – 707
On antiretroviral therapy at time of diagnosis	8/19**	42.1%

Table 1. Baseline patient characteristics for 39 patients (or as otherwise specified where data were missing). *Data missing for 1 patient.

438

Drug	Resistance by phenotypic DST	Resistance by genotypic DST	Genotypic DST sensitivity	Genotypic DST specificity
First-line drugs				
Rifampicin	24/37 (64.9%)	24/39	24/24 (100%)*	24/24 (100%)
Isoniazid	25/37 (67.6%)	24/39	23/25 (92.0%)	23/24 (95.8%)
Ethambutol	8/37 (21.6%)	17/39	8/8 (100%)	8/17 (47.1%)
Second-line drugs				
Ofloxacin	0/24 (0.0%)	1/24	N/A	0/1 (0%)**
Kanamycin	1/24 (4.2%)	1/24	1/1 (100%)	1/1 (100%)

439

440 Table 2. Phenotypic and genotypic drug susceptibility testing (DST) results and sensitivity
441 and specificity of genotypic DST relative to phenotypic DST. Phenotypic DST available for
442 first-line drugs for 37 of the 39 patients, and for second-line drugs for 24 patients who
443 demonstrated rifampicin drug resistance. *In two directly-sequenced sputum samples
444 rifampicin RAVs were missed due to low coverage, although they were identified in the
445 corresponding MGIT sample. **This sample had <1% of colonies grow in the presence of
446 ofloxacin, so is categorised as sensitive but may have low-level or heteroresistance to
447 fluoroquinolones (see main text).

448

Gene	Heterozygous Allele Count				Gene length (base pairs)	Hypothesised gene function
	Shared	MGIT only	Sputum only	Total		
rrs	2	3	180	185	3138	23S rRNA
rrl	0	0	173	173	1537	16S rRNA
Rv1319c	70	1	24	95	1608	Metabolism and respiration
lppB	7	6	10	23	663	Surface lipoprotein
Rv2561	21	0	0	21	294	Unknown function
Rv3424c	1	1	19	21	363	Unknown function
Rv2082	16	1	2	19	2166	Unknown function
ppsA	7	0	11	18	1059	GGPP synthetase (lipid synthesis)
Rv1435c	3	9	6	18	609	Secreted protein
ponA1	5	3	9	17	2037	Cell wall biosynthesis
Rv2277c	2	0	15	17	906	Metabolism and respiration
vapC31	5	0	12	17	429	Possible toxin
Rv2823c	5	2	9	16	2430	Unknown function
lppA	1	3	11	15	660	Surface lipoprotein
pks12	5	5	4	14	12456	MPM synthesis (lipid metabolism)

Table 3. Genes with the most heterozygous alleles (HAs) identified across the entire dataset.

452

	Shared variants	MGIT only variants	Sputum only variants	Total
All variants vs H37Rv (fixed or heterozygous)				
Total variants	33 153	1162	1217	35532
Variants vs H37Rv present as heterozygous alleles (HAs) only				
Total variants present as HAs (% of total variants)	MGIT 645 (1.9%) Sputum 1074 (3.2%)	153 (13.2%)	821 (67.5%)	2048 (5.8%)
Median HAs per sample	21	3	15	40
Variant type (% all HAs)				
SNP	500 (46.6%)	127 (83.0%)	708 (86.2%)	1335 (65.2%)
MNP	12 (1.1%)	1 (0.7%)	24 (2.9%)	37 (1.8%)
Insertion	303 (28.2%)	8 (5.2%)	31 (3.8%)	342 (16.7%)
Deletion	259 (24.1%)	16 (10.5%)	57 (6.9%)	332 (16.2%)
Replacement	0 (0.0%)	1 (0.7%)	1 (0.1%)	2 (0.1%)
Coding change (% all HAs)				
Non-synonymous	395 (36.8%)	79 (51.6%)	318 (38.7%)	792 (38.7%)
Synonymous	159 (14.8%)	32 (20.9%)	171 (20.8%)	362 (17.7%)
Intergenic	520 (48.4%)	42 (27.5%)	332 (40.4%)	894 (43.7%)
Non-synon/synon ratio	2.48	2.47	1.86	2.19
Stop codon (% of all non-synonymous HAs)	4 (1.0%)	1 (1.3%)	9 (2.8%)	14 (1.8%)
Frameshift (% of all non-synonymous HAs)	185 (46.8%)	19 (24.1%)	47 (14.8%)	251 (31.7%)

453

454 Table 4. Variants identified in MGIT derived, sputum derived, or both sequences from paired

455 samples. Values given represent totals for the 39 paired samples. SNP = single nucleotide

456 polymorphism; MNP = multi-nucleotide polymorphism.

457

458

Patient ID	Phenotypic resistance	Mutation	Frequency (MGIT/sputum)	Description
F1002	Rifampicin	*rpoB* S450L	100%/100%	High confidence resistance mutation
F1002	Rifampicin	*rpoC* G332R(48)	82.6%/21.7%	Putative compensatory mutations
F1002	Rifampicin	*rpoC* L516P(48)	12.7%/7.7%	
F1002	Rifampicin	*rpoC* P1040S(49)	21.7%/12.3%	
F1007	Isoniazid (high)	*ahpC* c-52t(38)	60.0%/50.7%	Rare, have been associated with resistance
F1007	Isoniazid (high)	*ahpC* g-48a(38)	28.6%/30.3%	
F1061	Rifampicin	*rpoB* H445D	16.1%/0.0%*	High confidence resistance mutation
F1061	Rifampicin	*rpoB* S450W	84.4%/0.0%*	High confidence resistance mutation
F1066	Isoniazid (high)	*katG* N218fs	0.0%/6.9%	Possible resistance mutations, not previously described
F1066	Clofazimine – not tested	*Rv1979c* G376D	0.0%/0.5%	
F1067	Isoniazid (high)	*katG* N218fs	10.7%/7.6%	
RF021	Pyrazinamide – testing failed	*pncA* Q122H	0%/2.5%	

459

460 Table 5. Resistance-associated variants present as heterozygous alleles (HAs). *These

461 mutations were also present in sputum but due to low coverage of the area (3 and 4 reads

462 respectively) variant calling criteria were not met.

463

464

Figure legends

Figure 1. Variation in total number of heterozygous alleles (HAs) identified across all 39 patients in sequences generated from sputum and MGIT depending on minimum supporting read count threshold. Direct sputum samples indicated by red squares, MGIT samples blue circles.

Figure 2. (A) Number of heterozygous alleles (HAs) found in directly sequenced sputum only (sputum), MGIT (MGIT) only or in both samples (shared) by patient. (B) Mean Shannon diversity at variable positions across pairs of samples (*H*) as calculated for MGIT and sputum-derived sequences. Size of point indicates number of variable positions considered (see Methods).

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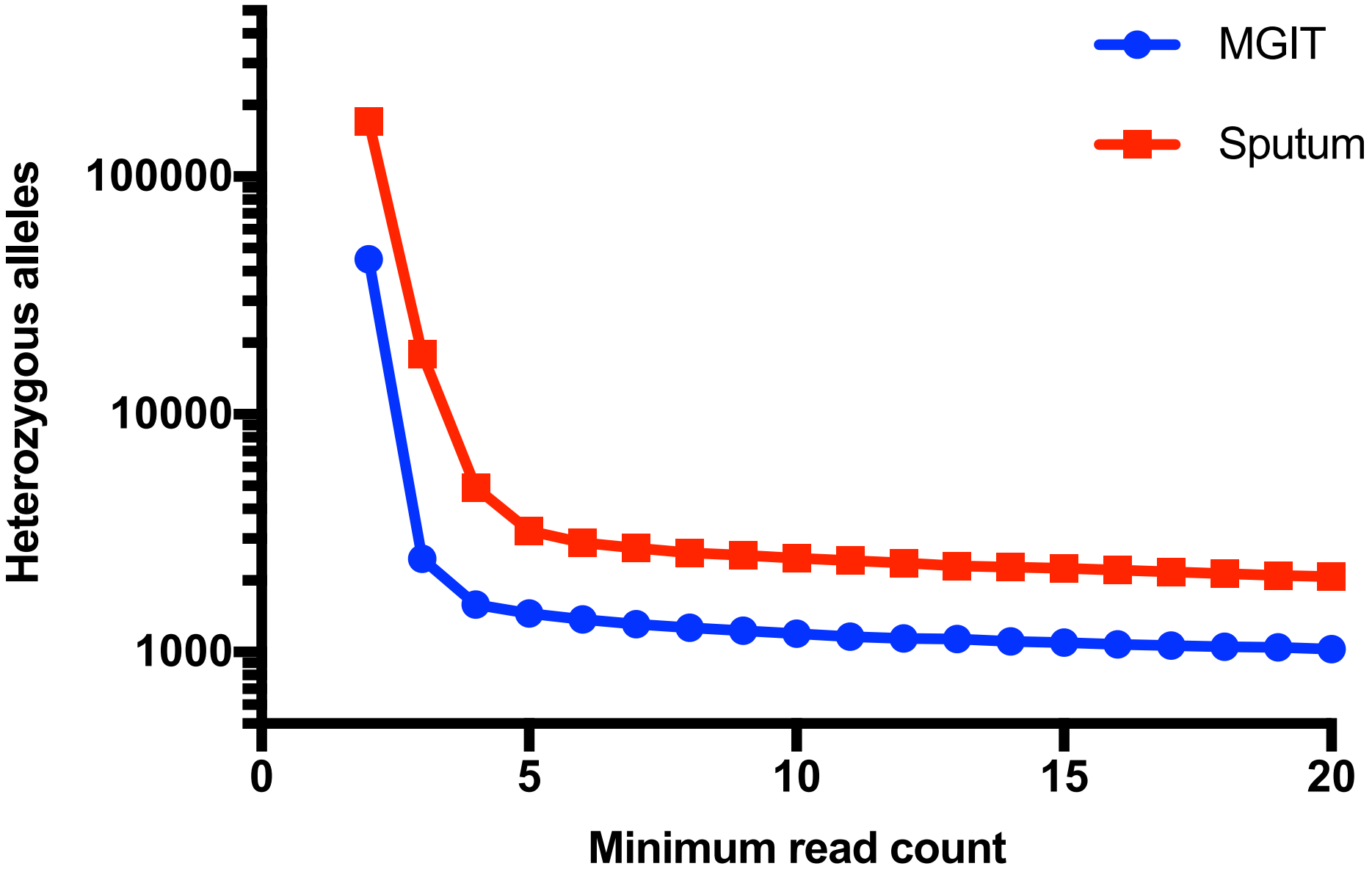
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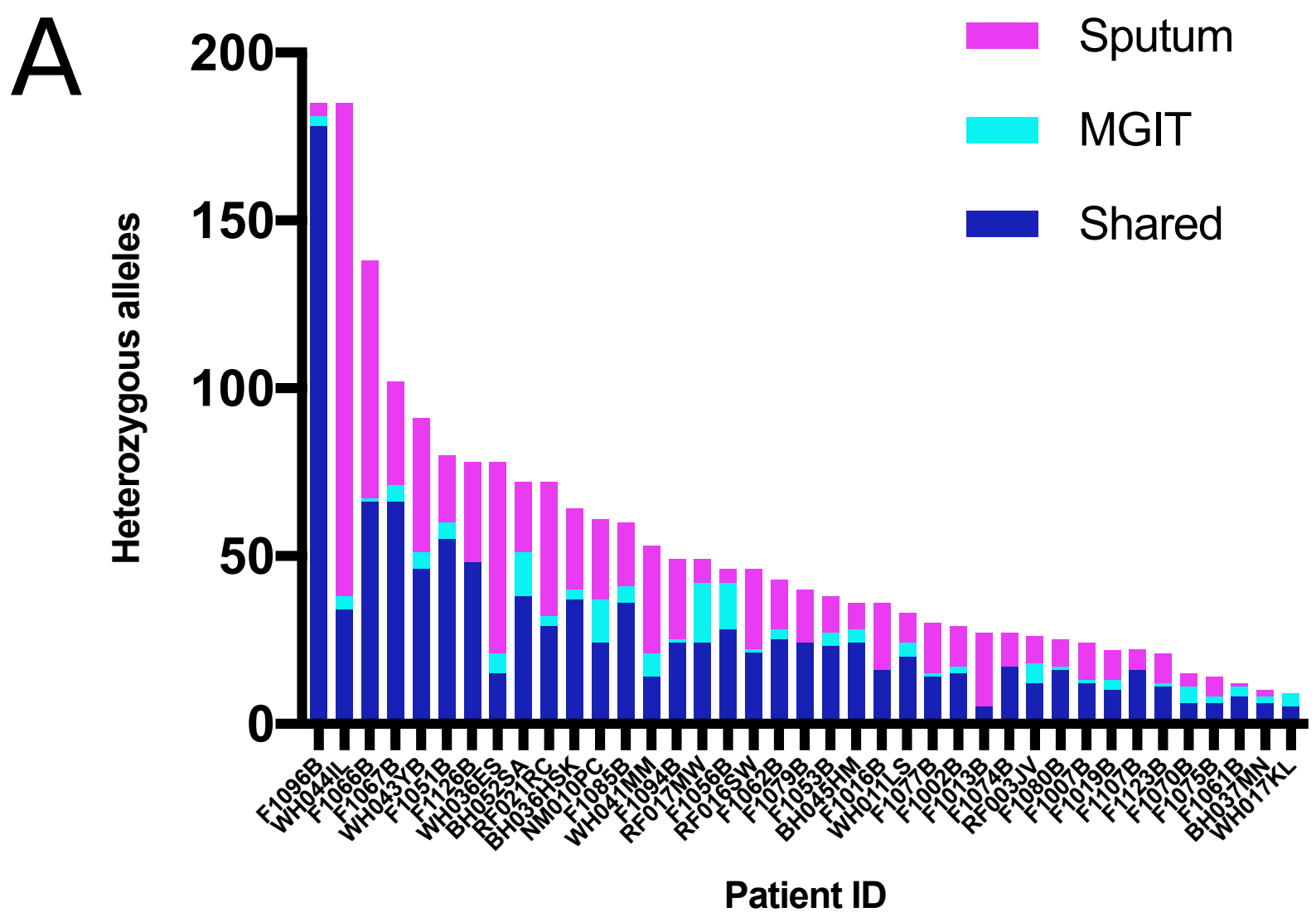
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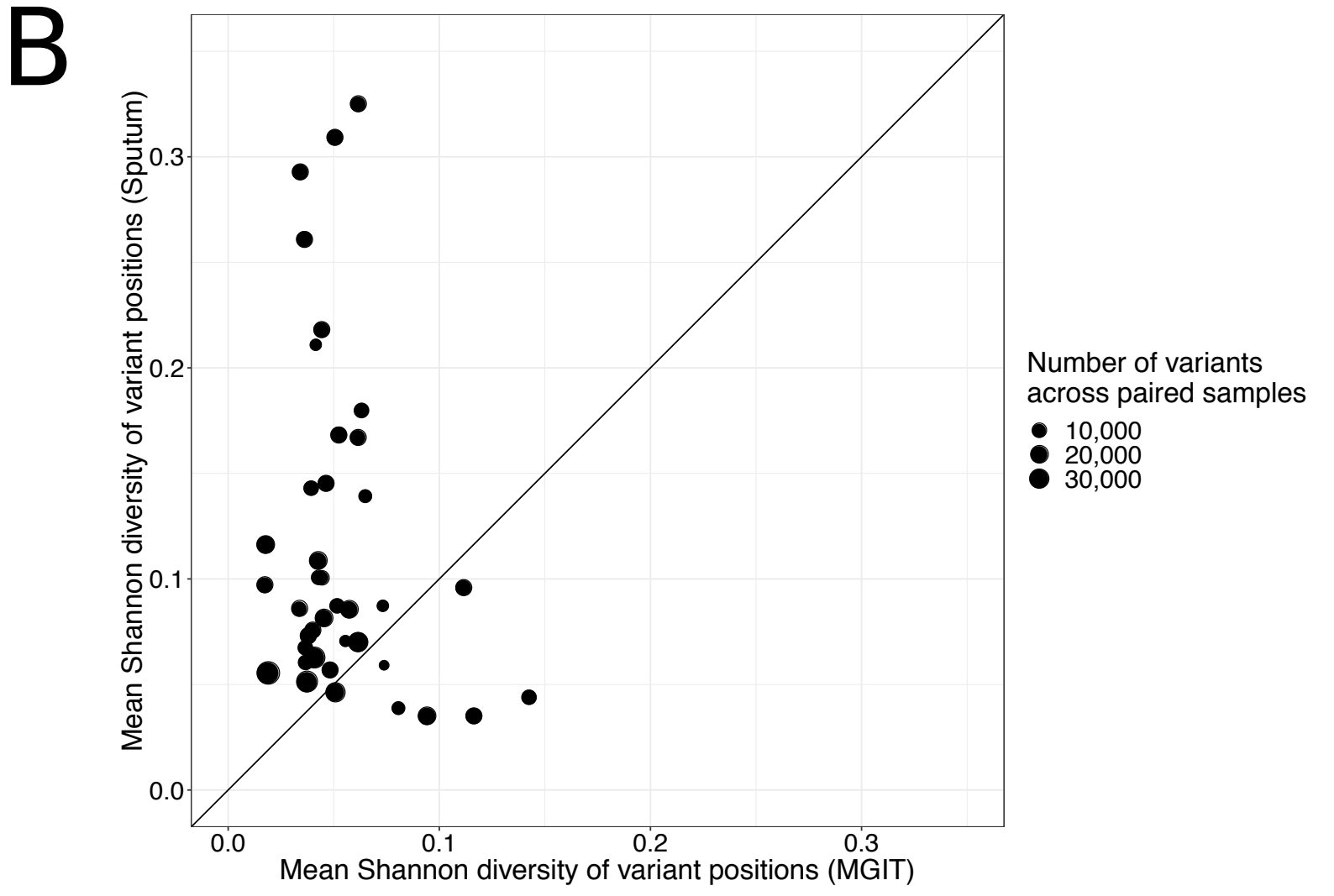
B
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0.1
0.2
0.3
Mean Shannon diversity of variant positions (MGIT)
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20,000
30,000