

# 1 Drug resistance prediction for *Mycobacterium* 2 *tuberculosis* with reference graphs

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## 16 1.4 Keywords

17 genome graphs, reference graphs, drug resistance prediction, *Mycobacterium tuberculosis*,  
18 benchmark, software

## 19 2. Abstract

20 The dominant paradigm for analysing genetic variation relies on a central idea: all genomes  
21 in a species can be described as minor differences from a single reference genome. However,  
22 this approach can be problematic or inadequate for bacteria, where there can be significant  
23 sequence divergence within a species.

24 Reference graphs are an emerging solution to the reference bias issues implicit in the “single-  
25 reference” model. Such a graph represents variation at multiple scales within a population –  
26 e.g., nucleotide- and locus-level.

27 The genetic causes of drug resistance in bacteria have proven comparatively easy to decode  
28 compared with studies of human diseases. For example, it is possible to predict resistance to  
29 numerous anti-tuberculosis drugs by simply testing for the presence of a list of single  
30 nucleotide polymorphisms and insertion/deletions, commonly referred to as a catalogue.

31 We developed DrPRG (Drug resistance Prediction with Reference Graphs) using the bacterial  
32 reference graph method Pandora. First, we outline the construction of a *Mycobacterium*  
33 *tuberculosis* drug resistance reference graph, a process that can be replicated for other  
34 species. The graph is built from a global dataset of isolates with varying drug susceptibility  
35 profiles, thus capturing common and rare resistance- and susceptible-associated haplotypes.

36 We benchmark DrPRG against the existing graph-based tool Mykrobe and the pileup-based  
37 approach of TBProfiler using 44,709 and 138 publicly available Illumina and Nanopore  
38 datasets with associated phenotypes. We find DrPRG has significantly improved sensitivity  
39 and specificity for some drugs compared to these tools, with no significant decreases. It uses  
40 significantly less computational memory than both tools, and provides significantly faster  
41 runtimes, except when runtime is compared to Mykrobe on Illumina data.

42 We discover and discuss novel insights into resistance-conferring variation for *M.*  
43 *tuberculosis* - including deletion of genes *katG* and *pncA* – and suggest mutations that may  
44 warrant reclassification as associated with resistance.  
45

### 46 **3. Impact statement**

47 *Mycobacterium tuberculosis* is the bacterium responsible for tuberculosis (TB). TB is one of  
48 the leading causes of death worldwide; before the coronavirus pandemic it was the leading  
49 cause of death from a single pathogen. Drug-resistant TB incidence has recently increased,  
50 making the detection of resistance even more vital. In this study, we develop a new software  
51 tool to predict drug resistance from whole-genome sequence data of the pathogen using new  
52 reference graph models to represent a reference genome. We evaluate it on *M. tuberculosis*  
53 against existing tools for resistance prediction and show improved performance. Using our  
54 method, we discover new resistance-associated variations and discuss reclassification of a  
55 selection of existing mutations. As such, this work contributes to TB drug resistance  
56 diagnostic efforts. In addition, the method could be applied to any bacterial species, so is of  
57 interest to anyone working on antimicrobial resistance.

### 58 **4. Data summary**

59 **The authors confirm all supporting data, code and protocols have been provided within**  
60 **the article or through supplementary data files.**

61 The software method presented in this work, DrPRG, is freely available from GitHub under  
62 an MIT license at <https://github.com/mbhall88/drprg>. We used commit [9492f25](#) for all results  
63 via a Singularity[1] container from the URI

64 `docker://quay.io/mbhall88/drprg:9492f25`.

65 All code used to generate results for this study are available on GitHub at

66 <https://github.com/mbhall88/drprg-paper>. All data used in this work are freely available from  
67 the SRA/ENA/DRA and a copy of the datasheet with all associated phenotype information  
68 can be downloaded from the archived repository at <https://doi.org/10.5281/zenodo.7819984>  
69 or found in the previously mentioned GitHub repository.

70 The *Mycobacterium tuberculosis* index used in this work is available to download through  
71 DrPRG via the command `drprg index --download mtb@20230308` or from  
72 GitHub at <https://github.com/mbhall88/drprg-index>.

### 73 **5. Introduction**

74 Human industrialisation of antibiotic production and use over the last 100 years has led to a  
75 global rise in prevalence of antibiotic resistant bacterial strains. The phenomenon  
76 was even observed within patients in the first clinical trial of streptomycin as a drug for  
77 tuberculosis (TB) in 1948[2], and indeed as every new drug class has been introduced, so has  
78 resistance followed. Resistance mechanisms are varied, and can be caused by point mutations  
79 at key loci (e.g., binding sites of drugs[3,4]), frame-shifts rendering a gene non-functional[5],  
80 horizontal acquisition of new functionality via a new gene[6], or by up-regulation of efflux  
81 pumps to reduce the drug concentration within the cell[7].  
82

83 Phenotypic and genotypic methods for detecting reduced susceptibility to drugs play  
84 complementary roles in clinical microbiology. Carefully defined phenotypic assays are used  
85 to give (semi)quantitative or binary measures of drug susceptibility; these have the benefit of

86 being experimental, quantitative measurements, and are able to detect resistance caused by  
87 hitherto unknown mechanisms. Prediction of drug resistance from genomic data has different  
88 advantages. Detection of a single nucleotide polymorphism (SNP) is arguably more  
89 consistent than a phenotypic assay, as it is not affected by whether the resistance it causes is  
90 near some threshold defining a resistant/susceptible boundary. Additionally, combining  
91 sequence datasets from different labs is more reliable than combining different phenotypic  
92 datasets, and using sequence data allows one to detect informative genetic changes (e.g., a  
93 tandem expansion of a single gene to form an array, thus increasing dosage). More subtly,  
94 defining the cut-off to separate resistant from susceptible is only simple when the minimum  
95 inhibitory concentration distribution is a simple bimodal distribution; in reality it is  
96 sometimes a convolution of multiple distributions caused by different mutations, and genetic  
97 data is sometimes needed to deconvolve the data and choose a threshold[8,9].

98  
99 The key requirement for a genomic predictor is to have an encodable understanding of the  
100 genotype-to-phenotype map. Research has focussed on clinically important pathogens,  
101 primarily *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella enterica*, *Pseudomonas*  
102 *aeruginosa* and *Mycobacterium tuberculosis* (MTB). The challenges differ across species;  
103 almost all bacterial species are extremely diverse, with non-trivial pan-genomes and  
104 considerable horizontal gene transfer causing transmission of resistance genes[10]. In these  
105 cases, species are so diverse that detection of chromosomal SNPs is affected heavily by  
106 reference bias[11]. Furthermore, there is an appreciable proportion of resistance which is not  
107 currently explainable through known SNPs or genes [12–14]. At the other extreme, MTB has  
108 almost no accessory genome, and no recombination or plasmids[15]. Resistance appears to be  
109 caused entirely by mutations, indels, and rare structural variants, and simple sets of rules ("if  
110 any of these mutations are present, or any of these genes inactivated, the sample is resistant")  
111 work well for most drugs[16]. MTB has an exceptionally slow growth rate, meaning culture-  
112 based drug susceptibility testing (DST) is slow (2-4 weeks depending on media), and  
113 therefore sequencing is faster[17]. As part of the end TB strategy, the WHO strives towards  
114 universal access to DST[18], defining Target Product Profiles for molecular  
115 diagnostics[19,20] and publishing a catalogue of high-confidence resistance mutations  
116 intended to provide a basis for commercial diagnostics and future research[16]. There was a  
117 strong community-wide desire to integrate this catalogue into software for genotypic  
118 resistance prediction, although independent benchmarking confirmed there was still need for  
119 improvement[12]. Hence, there is a continuing need to improve the understanding of the  
120 genetic basis of resistance and integrate it into software for genotypic DST.

121  
122 In this paper we develop and evaluate a new software tool for genotypic DST for MTB, built  
123 on a generic framework that can be used for any bacteria. Several tools have been developed  
124 previously[21–25]. Of these, only Mykrobe and TBProfiler work on Illumina and Nanopore  
125 data, and both have been heavily evaluated previously[22,23,26,27] - so we benchmark  
126 against these. Mykrobe uses de Bruijn graphs to encode known resistance alleles and thereby  
127 achieves high accuracy even on indel calls with Nanopore data[27]. However it is unable to  
128 detect novel alleles in known resistance genes, nor to detect gene truncation or deletion,  
129 which would be desirable. TBProfiler is based on mapping and variant calling (by default  
130 using Freebayes[28]), and detects gene deletions using Delly[29].

131  
132 Our new software, called DrPRG (Drug resistance Prediction with Reference Graphs), builds  
133 on newer pan-genome technology than Mykrobe[11] using an independent graph for each  
134 gene in the catalogue, which makes it easier to go back-and-forth between VCF and the

135 graph. To build an index, it takes as input a catalogue of resistant variants (a simple 4-column  
136 TSV file), a file specifying expert rules (e.g. any missense variant between codons X and Y in  
137 gene Z causes resistance to drug W), and a VCF of population variation in the genes of  
138 interest. This allows it to easily incorporate the current WHO-endorsed catalogue[16], which  
139 is conservative, and for the user to update the catalogue or rules with minimal effort. Finally,  
140 to provide resistance predictions, it takes a prebuilt index (an MTB one is currently provided)  
141 and sequencing reads (FASTQ).

142

143 We describe the DrPRG method, and to evaluate it, gather the largest MTB dataset of  
144 sequencing data with associated phenotype information and reveal novel insights into  
145 resistance-determining mutations for this species.

## 146 **6. Methods**

147 DrPRG is a command-line software tool implemented in the Rust programming language.  
148 There are two main subcommands: `build` for building a reference graph and associated  
149 index files, and `predict` for producing genotypic resistance predictions from sequencing  
150 reads and an index (from `build`).

### 151 **6.1 Constructing a resistance-specific reference graph and index**

152 The `build` subcommand of DrPRG requires a Variant Call Format (VCF) file of variants  
153 from which to build a reference graph, a catalogue of mutations that confer resistance or  
154 susceptibility for one or more drugs, and an annotation (GFF) and FASTA file of the  
155 reference genome.

156 For this work, we used the reference and annotation for the MTB strain H37Rv (accession  
157 NC\_000962.3) and the default mutation catalogue from Mykrobe (v0.12.1)[12,26].

158 To ensure the reference graph is not biased towards a particular lineage or susceptibility  
159 profile, we selected samples from a VCF of 15,211 global MTB samples[30]. We randomly  
160 chose 20 samples from each lineage 1 through 4, as well as 20 samples from all other  
161 lineages combined. In addition, we included 17 clinical samples representing MTB global  
162 diversity (lineages 1-6)[31,32] to give a total of 117 samples. In the development phase of  
163 DrPRG we also found it necessary to add some common mutations not present in these 177  
164 samples; as such, we added 48 mutations to the global VCF (these mutations are listed in  
165 archived repository – see Data summary). We did not add all catalogue mutations as there is a  
166 saturation point for mutation addition to a reference graph, and beyond this point,  
167 performance begins to decay[33].

168 The `build` subcommand turns this VCF into a reference graph by extracting a consensus  
169 sequence for each gene and sample. We use just those genes that occur in the mutation  
170 catalogue and include 100 bases flanking the gene. A multiple sequence alignment is  
171 constructed for each gene from these consensus sequences with MAFFT (v7.505)[34,35] and  
172 then a reference graph is constructed from these alignments with `make_prg` (v0.4.0)[11].  
173 The final reference graph is then indexed with `pandora`[11].

### 174 **6.2 Genotypic resistance prediction**

175 Genotypic resistance prediction of a sample is performed by the `predict` subcommand of  
176 DrPRG. It takes an index produced by the `build` command (see [Constructing a resistance-  
177 specific reference graph and index](#)) and sequencing reads – Illumina or Nanopore are  
178 accepted. To generate predictions, DrPRG discovers novel variants (`pandora`), adds these to  
179 the reference graph (`make_prg` and MAFFT), and then genotypes the sample with respect

180 to this updated graph (*pandora*). The genotyped VCF is filtered such that we ignore any  
181 variant with less than 3 reads supporting it and require a minimum of 1% read depth on each  
182 strand. Next, each variant is compared to the catalogue. If an alternate allele has been called  
183 that corresponds with a catalogue variant, resistance ('R') is noted for the drug(s) associated  
184 with that mutation. If a variant in the VCF matches a catalogue mutation, but the genotype is  
185 null ('.'), we mark that mutation, and its associated drug(s), as failed ('F'). Where an alternate  
186 allele call does not match a mutation in the catalogue, we produce an unknown ('U')  
187 prediction for the drug(s) that have a known resistance-conferring mutation in the relevant  
188 gene.

189 DrPRG also has the capacity to detect minor alleles and call minor resistance ('r') or minor  
190 unknown ('u') in such cases. Minor alleles are called when a variant (that has passed the  
191 above filtering) is genotyped as being the susceptible (reference) allele, but there is also read  
192 depth on the resistant (alternate) allele above a given minor allele frequency parameter (`--`  
193 `maf`; default is 0.1 for Illumina data). Minor allele calling is turned off by default for  
194 Nanopore data as we found it led to a drastic increase in the number of false positive calls  
195 (this is also the case for Mykrobe and TBProfiler).

196 When building the index for DrPRG and making predictions, we also accept a file of "expert  
197 rules" for calling variants of a certain class. A rule is associated with a gene, an optional  
198 position range, a variant type, and the drug(s) that rule confers resistance to. Currently  
199 supported variant types are missense, nonsense, frameshift, and gene absence.

200 The output of running `predict` is a VCF file of all variants in the graph and a JSON file of  
201 resistance predictions for each drug in the index, along with the mutation(s) supporting that  
202 prediction and a unique identifier to find that variant in the VCF file (see Supplementary  
203 Section S1 for an example). The reference graph gene presence/absence (as determined by  
204 *pandora*) is also listed in the JSON file.

### 205 **6.3 Benchmark**

206 We compare the performance of DrPRG against Mykrobe (v0.12.1)[26] and TBProfiler  
207 (v4.3.0)[22] for MTB drug resistance prediction. Mykrobe is effectively a predecessor of  
208 DrPRG; it uses genome graphs, in the form of de Bruijn graphs, to construct a graph of all  
209 mutations in a catalogue and then genotypes the reads against this graph. TBProfiler is a more  
210 traditional approach which aligns reads to a single reference genome and calls variants from  
211 that alignment via a pileup.

212 A key part of such a benchmark is the catalogue of mutations, as this generally accounts for  
213 the majority of differences between tools[26]. As such, we use the same catalogue for all  
214 tools to ensure any differences are method-related - not catalogue disparities. The catalogue  
215 we chose is the default one provided in Mykrobe[12]. It is a combination of the catalogue  
216 described in Hunt *et al.* [26] and the category 1 and 2 mutation and expert rules from the  
217 2021 WHO catalogue[16]. This catalogue contains mutations for 14 drugs: isoniazid,  
218 rifampicin, ethambutol, pyrazinamide, levofloxacin, moxifloxacin, ofloxacin, amikacin,  
219 capreomycin, kanamycin, streptomycin, ethionamide, linezolid, and delamanid.

220 We used Mykrobe and TBProfiler with default parameters, except for a parameter in each  
221 indicating the sequencing technology of the data as Illumina or Nanopore and the TBProfiler  
222 option to not trim data (as we do this in [Quality control](#)).

223 We compare the prediction performance of each program using sensitivity and specificity. To  
224 calculate these metrics, we consider a true positive (TP) and true negative (TN) as a case  
225 where a program calls resistance and susceptible, respectively, and the phenotype agrees; a  
226 false positive (FP) as a resistant call by a program but a susceptible phenotype, with false

227 negatives (FN) being the inverse of FP. We only present results for drugs in the catalogue and  
228 where at least 10 samples had phenotypic data available.  
229 To benchmark the runtime and memory usage of each tool, we used the Snakemake  
230 benchmark feature within our analysis pipeline[36].

#### 231 **6.4 Datasets**

232 We gathered various MTB datasets where whole-genome sequencing data (Nanopore or  
233 Illumina) were available from public repositories (ENA/SRA/DRA) and associated  
234 phenotypes were accessible for at least one drug present in our catalogue[16,27,37–49].  
235 All data was downloaded with `fastq-dl` (v1.1.1; <https://github.com/rpetit3/fastq-dl>).

#### 236 **6.5 Quality control**

237 All downloaded Nanopore fastq files had adapters trimmed with `porechop` (v0.2.4;  
238 <https://github.com/rrwick/Porechop>), with the option to discard any reads with an adapter in  
239 the middle, and any reads with an average quality score below 7 were removed with `nanoq`  
240 (v0.9.0)[50]. Illumina reads were pre-processed with `fastp` (v0.23.2)[51] to remove adapter  
241 sequences, trim low quality bases from the ends of the reads, and remove duplicate reads and  
242 reads shorter than 30bp.

243 Sequencing reads were decontaminated as described in Hall *et al.*[27] and Walker *et al.*[16].  
244 Briefly, sequenced reads were mapped to a database of common sputum contaminants and the  
245 MTB reference genome (H37Rv; accession NC\_000962.3)[52] keeping only those reads  
246 where the best mapping was to H37Rv.

247 After quality control, we removed any sample with average read depth less than 15, or where  
248 more than 5% of the reads mapped to contaminants.

249 Lineage information was extracted from the TBProfiler results (see [Benchmark](#)).

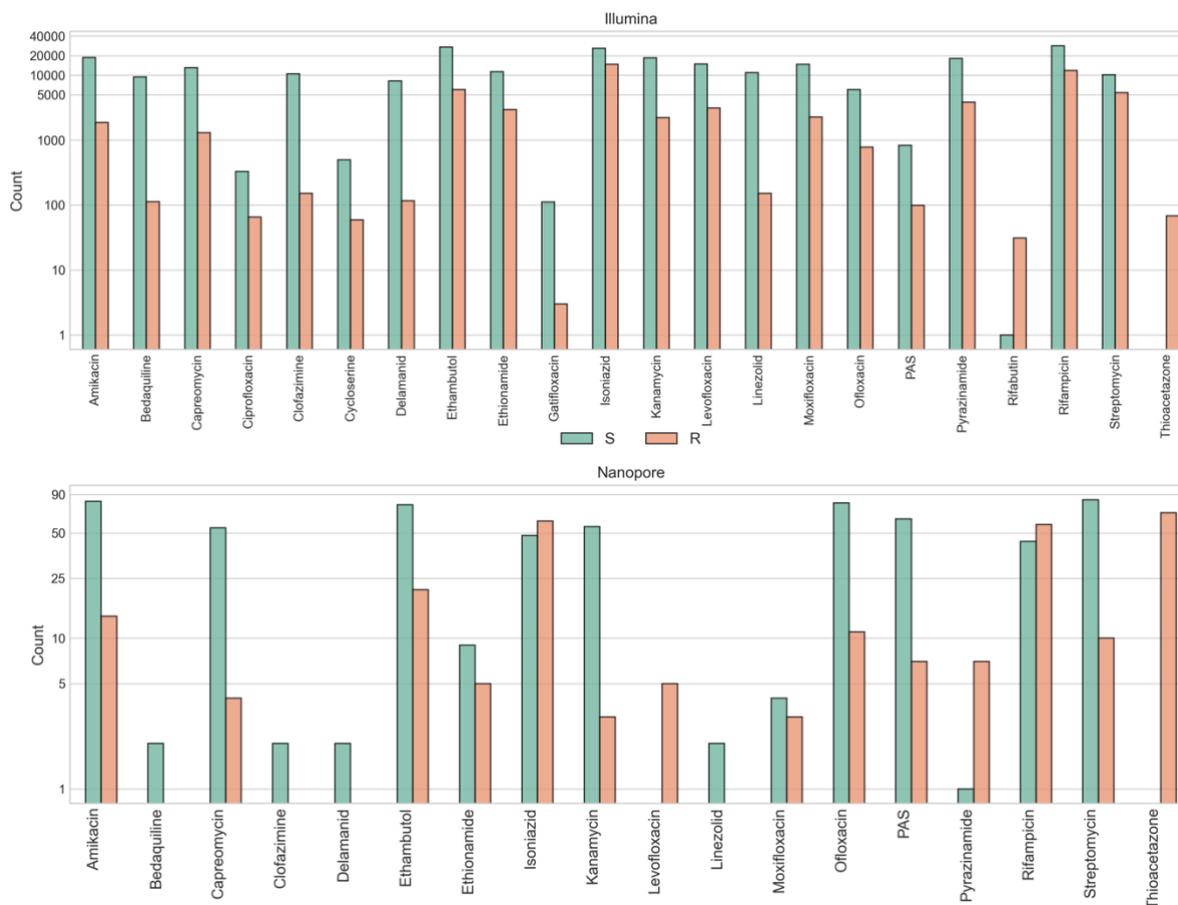
#### 250 **6.6 Statistical Analysis**

251 We used a Wilcoxon rank-sum paired data test from the Python library SciPy[53] to test for  
252 significant differences in runtime and memory usage between the three prediction tools.

253 The sensitivity and specificity confidence intervals were calculated with a Wilson's score  
254 interval with a coverage probability of 95%.

### 255 **7. Results**

256 To benchmark DrPRG, Mykrobe, and TBProfiler, we gathered an Illumina dataset of 45,702  
257 MTB samples with a phenotype for at least one drug. After quality control (see [Quality  
258 control](#)), this number reduced to 44,709. In addition, we gathered 142 Nanopore samples, of  
259 which 138 passed quality control. In Figure 1 we show all available drug phenotypes for  
260 those interested in the dataset, yet our catalogue does not offer predictions for all drugs listed  
261 (see [Benchmark](#)). Lineage counts for all samples that passed quality control and have a  
262 single, major lineage call can be found in Table 1.



263  
264 **Figure 1: Drug phenotype counts for Illumina (upper) and Nanopore (lower) datasets. Bars are stratified and**  
265 **coloured by whether the phenotype is resistant (R; orange) or susceptible (S; green). Note, the y-axis is log-scaled.**  
266 **PAS=para-aminosalicylic acid**

267 **Table 1: Lineage counts from the Illumina and Nanopore datasets, covering main lineages 1-9 (L1-L9) and the three**  
268 **livestock-associated lineages (La1-La3) as defined in [54]**

Lineage	Illumina	Nanopore
La1	239	0
La2	7	0
La3	71	0
L1	3907	32
L2	12870	38
L3	5803	9
L4	20731	59
L5	63	0
L6	78	0
L7	3	0
L9	1	0

269

## 270 7.1 Sensitivity and specificity performance

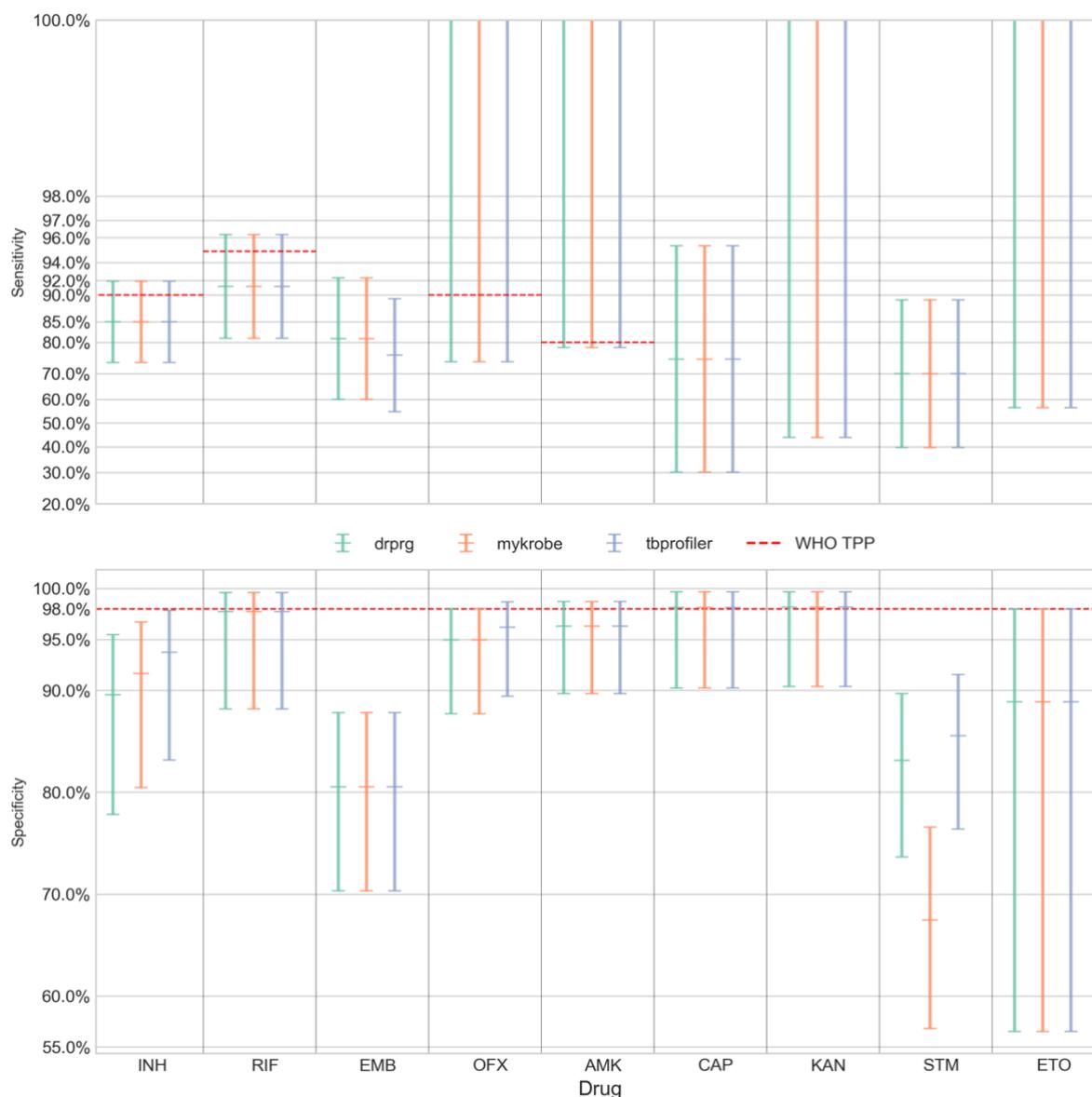
271 We present the sensitivity and specificity results for Illumina data in Figure 2 and Suppl.  
272 Table S1 and the Nanopore data in Figure 3 and Suppl. Table S2.

273 When comparing DrPRG's performance to that of Mykrobe and TBProfiler, we look for  
 274 instances where the confidence intervals do not overlap; indicating a significant difference.  
 275 With Illumina data (Figure 2 and Suppl. Table S1), DrPRG achieves significantly greater  
 276 sensitivity than Mykrobe for rifampicin (96.4% [96.0-96.7] vs. 95.6% [95.2-95.9]),  
 277 streptomycin (85.3% [84.4-86.3] vs. 83.1% [82.1-84.1]), amikacin (85.6% [83.9-87.1] vs.  
 278 80.8% [78.9-82.5]), capreomycin (77.5% [75.2-79.7] vs. 71.8% [69.3-74.1]), kanamycin  
 279 (83.7% [82.1-85.2] vs. 79.9% [78.2-81.5]), and ethionamide (75.2% [73.7-76.8] vs. 71.4%  
 280 [69.7-73.0]), with no significant difference for all other drugs. In terms of sensitivity, there  
 281 was no significant difference between DrPRG and TBProfiler except for ethionamide, where  
 282 DrPRG was significantly more sensitive (75.2% [73.7-76.8] vs. 71.5% [69.8-73.1]). For  
 283 specificity, there was no significant difference between the tools except that DrPRG and  
 284 Mykrobe were significantly better than TBProfiler for rifampicin (97.8% [97.6-98.0] vs.  
 285 97.2% [97.0-97.4]). There was no significant difference in sensitivity or specificity for any  
 286 drug with Nanopore data.



287  
 288 **Figure 2: Sensitivity (upper panel; y-axis) and specificity (lower panel; y-axis) of resistance predictions for different**  
 289 **drugs (x-axis) from Illumina data. Error bars are coloured by prediction tool. The central horizontal line in each**  
 290 **error bar is the sensitivity/specificity and the error bars represent the 95% confidence interval. Note, the sensitivity**  
 291 **panel's y-axis is logit-scaled. This scale is similar to a log scale close to zero and to one (100%), and almost linear**

292 around 0.5 (50%). The red dashed line in each panel represents the minimal standard WHO target product profile  
 293 (TPP; where available) for next-generation drug susceptibility testing for sensitivity and specificity. INH=isoniazid,  
 294 RIF=rifampicin, EMB=ethambutol, PZA=pyrazinamide, LFX=levofloxacin, MFX=moxifloxacin, OFX=ofloxacin,  
 295 AMK=amikacin, CAP=capreomycin, KAN=kanamycin, STM=streptomycin, ETO=ethionamide, LZD=linezolid,  
 296 DLM=delamanid.



297 **Figure 3: Sensitivity (upper panel; y-axis) and specificity (lower panel; y-axis) of resistance predictions for different**  
 298 **drugs (x-axis) from Nanopore data. Error bars are coloured by prediction tool. The central horizontal line in each**  
 299 **error bar is the sensitivity/specificity and the error bars represent the 95% confidence interval. Note, the sensitivity**  
 300 **panel's y-axis is logit-scaled. This scale is similar to a log scale close to zero and to one (100%), and almost linear**  
 301 **around 0.5 (50%). The red dashed line in each panel represents the minimal standard WHO target product profile**  
 302 **(TPP; where available) for next-generation drug susceptibility testing for sensitivity and specificity. INH=isoniazid,**  
 303 **RIF=rifampicin, EMB=ethambutol, OFX=ofloxacin, AMK=amikacin, CAP=capreomycin, KAN=kanamycin,**  
 304 **STM=streptomycin, ETO=ethionamide.**  
 305

306 In both figures, we show the minimal requirements from the WHO target product profiles for  
 307 sensitivity and specificity of genotypic drug susceptibility testing[19] as red dashed lines.  
 308 Note, a sensitivity target is not specified by the WHO for ethambutol (EMB), capreomycin  
 309 (CAP), kanamycin (KAN), streptomycin (STM), or ethionamide (ETO). For Illumina data, all  
 310 tools' predictions for rifampicin, isoniazid, levofloxacin, moxifloxacin and amikacin are

311 above the sensitivity minimal requirement target. TBProfiler also exceeds the target for  
312 pyrazinamide, which DrPRG misses by 0.2%. No drug's sensitivity target was achieved with  
313 Nanopore data. For specificity, the tools are all very similar and either exceed or fall below  
314 the threshold together (see Figure 2). The target of >98% is met by all tools on Illumina data  
315 only for ofloxacin, amikacin, linezolid, and delamanid. Mykrobe also exceeds the target for  
316 capreomycin. As such, amikacin is the only drug where both sensitivity and specificity  
317 performance exceed the minimal requirement of the WHO target product profiles. Only  
318 capreomycin and kanamycin specificity targets are exceeded (by all tools) with Nanopore  
319 data.

320 However, for Illumina data, we did find that likely sample-swaps or phenotype instability[55]  
321 could lead to some drugs being on the threshold of the WHO target product profiles. If we  
322 excluded samples where all three tools make a FP call for the strong isoniazid and rifampicin  
323 resistance-conferring mutations *katG* S315T ( $n=152$ ) and *rpoB* S450L ( $n=119$ ) [16]  
324 respectively, all three tools would exceed the isoniazid specificity target of 98% - thus  
325 meeting both sensitivity and specificity targets for isoniazid. In addition, DrPRG and  
326 Mykrobe would meet the rifampicin specificity target of 98% – leading to both targets being  
327 met for rifampicin for these two tools. As previously reported [55,56], we also found a lot of  
328 instability in the ethambutol result caused by *embB* mutations M306I ( $n=827$ ) and M306V  
329 ( $n=519$ ) being called for phenotypically susceptible samples (FP) by all three tools. Other  
330 frequent consensus FP calls included: *fabG1* c-15t, which is associated with ethionamide  
331 ( $n=441$ ) and isoniazid ( $n=241$ ) resistance; *rrs* a1401g, which is associated with resistance to  
332 capreomycin ( $n=241$ ), amikacin ( $n=70$ ), and kanamycin ( $n=48$ ). In addition there were  
333 common false positives from *gyrA* mutations A90V and D94G, which are associated with  
334 resistance to the fluoroquinolones levofloxacin ( $n=108$  and  $n=70$ , respectively), moxifloxacin  
335 ( $n=419$  and  $n=349$ ) and ofloxacin ( $n=19$  and  $n=17$ ), and are known to cause heteroresistance  
336 and minimum inhibitory concentrations (MIC) close to the critical concentration  
337 threshold[57–59].

## 338 **7.2 Evaluation of potential additions to the WHO catalogue**

339 False negatives are much harder to investigate as it is not known which mutation(s) were  
340 missed as they are presumably not in the catalogue if all tools failed to make a call. However,  
341 looking through those FNs where DrPRG makes an “unknown” resistance call, we note some  
342 potential mutations that may need reclassification or inclusion in the WHO catalogue. For  
343 delamanid FNs, we found five different nonsense mutations in the *ddn* gene in seven samples  
344 – W20\* ( $n=2$ ), W27\* ( $n=1$ ), Q58\* ( $n=1$ ), W88\* ( $n=2$ ), and W139\* ( $n=1$ ) – none of which  
345 occurred in susceptible samples. We also found 13 pyrazinamide FN cases with a nonstop  
346 (stop-loss) mutation in *pncA* – this mutation type was also seen in two susceptible samples.  
347 Another *pncA* mutation, T100P, was also observed in 10 pyrazinamide FN samples and no  
348 susceptible samples. T100P only appears once in the WHO catalogue data (“solo” in a  
349 resistant sample). As such, it was given a grading of uncertain significance. As our dataset  
350 includes those samples in the WHO catalogue dataset, this means an additional nine isolates  
351 have been found with this mutation - indicating this may warrant an upgrade to ‘associated  
352 with resistance’. We found an interesting case of allele combinations, where nine ethambutol  
353 FN samples have the same two *embA* mutation c-12a and c-11a and *embB* mutation P397T -  
354 this combination is only seen in two susceptible samples. Interestingly, *embB* P397T and  
355 *embA* c-12a don't appear in the WHO catalogue, but have been described as causing  
356 resistance previously[60]. Three *katG* mutations were also detected in isoniazid FN cases.  
357 First, G279D occurs in eight missed resistance samples and no susceptible cases. This  
358 mutation is graded as ‘uncertain significance’ in the WHO catalogue and was seen solo in

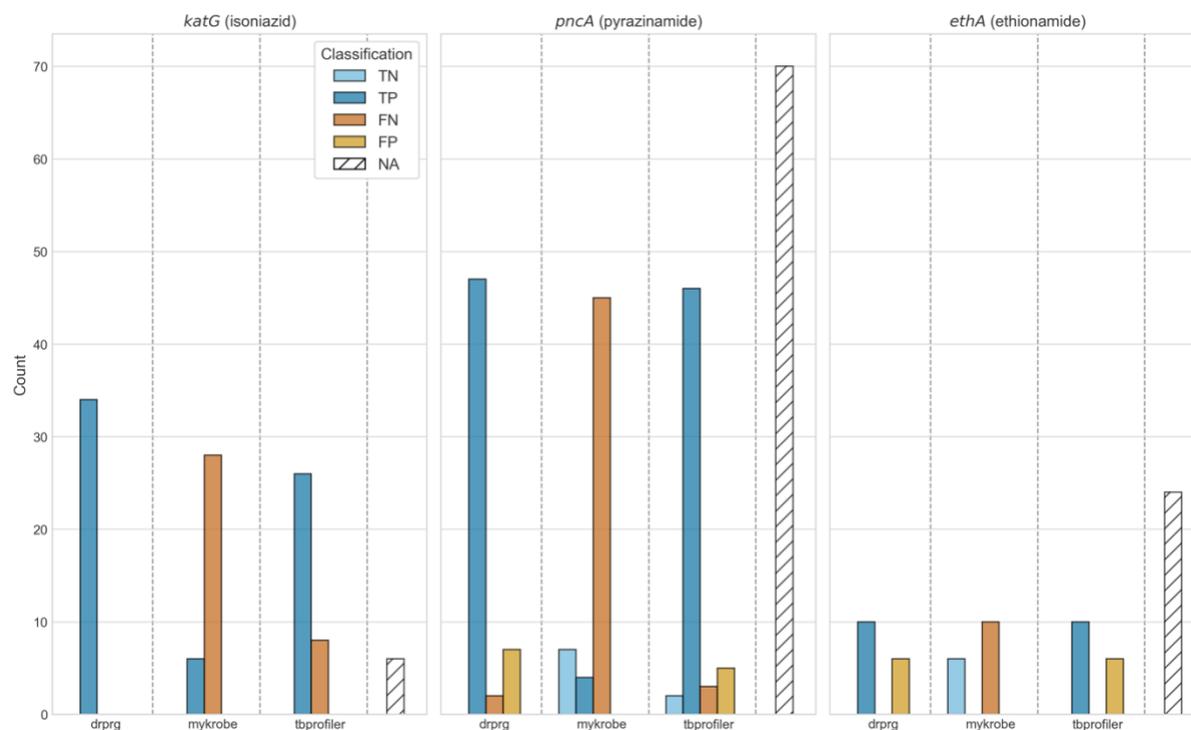
359 four resistant samples in that data. Singh *et al.* performed a protein structural analysis caused  
360 by this mutation and found it produced “an undesirable effect on the functionality of the  
361 protein”[61]. Second, G699E occurs in eight FN samples and no susceptible cases, but has a  
362 WHO grading of ‘uncertain significance’ based on six resistant isolates; thus, we add two  
363 extra samples to that count. And third, N138H occurs in 14 FN samples and one susceptible.  
364 In seven of these cases, it co-occurs with *ahpC* mutations t-75g ( $n=2$ ) and t-76a ( $n=5$ ). This  
365 mutation occurs in only three resistant isolates in the WHO catalogue dataset, giving it an  
366 uncertain significance, but we add a further 11 cases. This mutation has been found to cause a  
367 high isoniazid MIC and be associated with resistance[62,63].

### 368 **7.3 Detection of large deletions**

369 There are expert rules in the WHO catalogue which treat gene loss-of-function (any  
370 frameshift or nonsense mutation) in *katG*, *ethA*, *gid*, and *pncA* as causing resistance for  
371 isoniazid, ethionamide, streptomycin, and pyrazinamide, respectively[16]. Although  
372 examples of resistance caused by gene deletion are rare[64–68], with a dataset of this size  
373 ( $n=44,709$ ), we can both evaluate these rules, and compare the detection power of DrPRG  
374 and TBProfiler for identifying gene deletions (Mykrobe does not, although in principle it  
375 could). In total we found 206 samples where DrPRG and/or TBProfiler identified deletions of  
376 *ethA*, *katG*, or *pncA*. Although many of these isolates did not have phenotype information for  
377 the associated drug ( $n=100$ ), the results are nevertheless striking (Figure 4). Given the low  
378 false-positive rate of *pandora* for gene absence detection[11], these no-phenotype samples  
379 provide insight into how often gene deletions are occurring in clinical samples.

380 Of the 34 isolates where *katG* was identified as being absent, and an isoniazid phenotype was  
381 available, all 34 were phenotypically resistant. DrPRG detected all 34 (100% sensitivity) and  
382 TBProfiler identified 26 (76.5% sensitivity). Deletions of *pncA* were detected in 56 isolates,  
383 of which 49 were phenotypically resistant. DrPRG detected 47 (95.9% sensitivity) and  
384 TBProfiler detected 46 (93.9% sensitivity). Lastly, *ethA* was found to be missing in 16  
385 samples with an ethionamide phenotype, of which 10 were phenotypically resistant. Both  
386 DrPRG and TBProfiler correctly predicted all 10 (100% sensitivity). No *gid* deletions were  
387 discovered. We note that the TP calls made by Mykrobe were due to it detecting large  
388 deletions that are present in the catalogue, which is understandable given the whole gene is  
389 deleted.

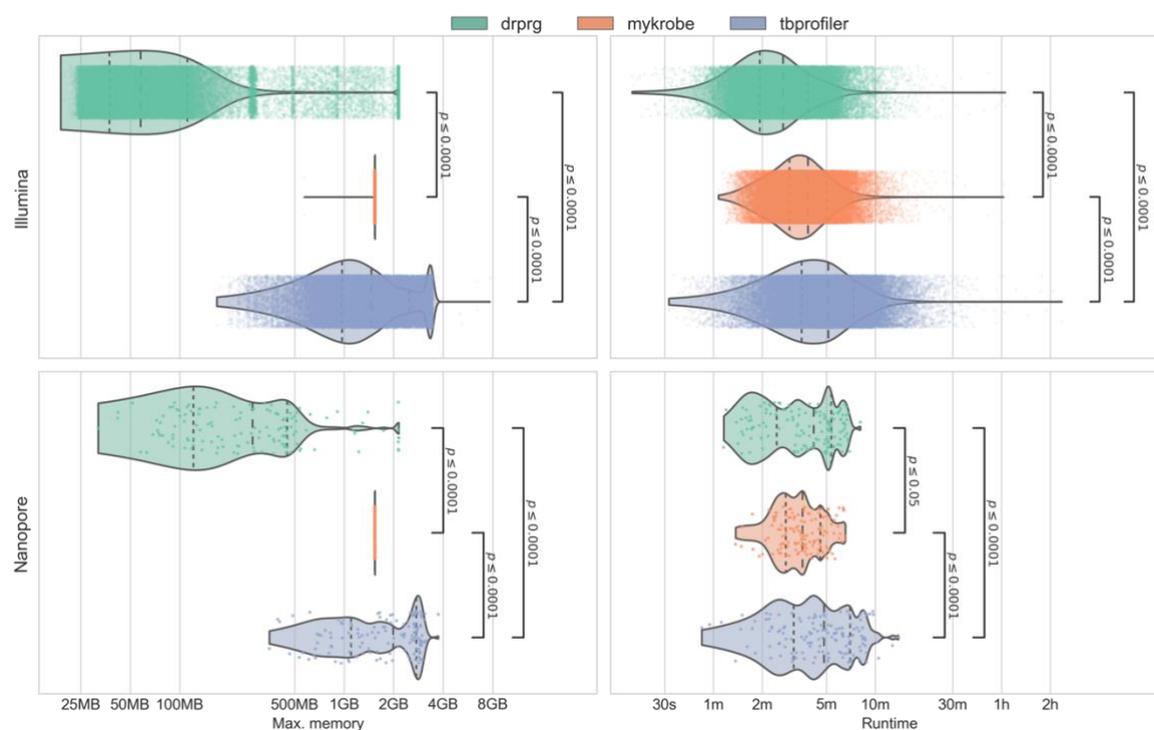
390 We conclude that DrPRG is slightly more sensitive at detecting large deletions than  
391 TBProfiler (and Mykrobe) for *katG*, and equivalent for *pncA* and *ethA*. However we note that  
392 the WHO expert rule which predicts resistance to isolates missing specific genes appears  
393 more accurate for *katG* (100% of isolates missing the gene are resistant) than for *pncA* (87%  
394 resistant) and *ethA* (62.5% resistant).



395  
 396 **Figure 4: Impact of gene deletion on resistance classification.** The title of each subplot indicates the gene and drug it  
 397 effects. Bars are coloured by their classification and stratified by tool. Count (y-axis) indicates the number of gene  
 398 deletions for that category. The NA bar (white with diagonal lines) indicates the number of samples with that gene  
 399 deleted but no phenotype information for the respective drug. TP=true positive; FN=false negative; TN=true negative;  
 400 FP=false positive; NA=no phenotype available.

#### 401 7.4 Runtime and memory usage benchmark

402 The runtime and peak memory usage of each program was recorded for each sample and is  
 403 presented in Figure 5. DrPRG (median 161 seconds) was significantly faster than both  
 404 TBProfiler (307 seconds;  $p \leq 0.0001$ ) and Mykrobe (230 seconds;  $p \leq 0.0001$ ) on Illumina data.  
 405 For Nanopore data, DrPRG (250 seconds) was significantly faster than TBProfiler (290  
 406 seconds;  $p \leq 0.0001$ ), but significantly slower than Mykrobe (213 seconds;  $p = 0.0347$ ). In  
 407 terms of peak memory usage, DrPRG (Illumina median peak memory 58MB; Nanopore  
 408 277MB) used significantly less memory than Mykrobe (1538MB; 1538MB) and TBProfiler  
 409 (1463MB; 1990MB) on both Illumina and Nanopore data ( $p \leq 0.0001$  for all comparisons).  
 410



411  
412 **Figure 5: Benchmark of the maximum memory usage (left panels) and runtime (right panels) from Illumina (upper**  
413 **row) and Nanopore (lower row) data. Each point and violin is coloured by the tool, with each point representing a**  
414 **single sample. Statistical annotations are the result of a Wilcoxon rank-sum paired data test on each pair of tools.**  
415 **Dashed lines inside the violins represent the quartiles of the distribution. Note, the x-axis is log-scaled.**

## 416 8. Discussion

417 In this work, we have presented a novel method for making drug resistance predictions with  
418 reference graphs. The method, DrPRG, requires only a reference genome and annotation, a  
419 catalogue of resistance-conferring mutations, a VCF of population variation from which to  
420 build a reference graph, and (optionally) a set of rules for types of variants in specific genes  
421 which cause resistance. We apply DrPRG to the pathogen *M. tuberculosis*, for which there is  
422 a great deal of information on the genotype/phenotype relationship, and a great need to  
423 provide good tools which implement and augment current and forthcoming versions of the  
424 WHO catalogue. We illustrate the performance of DrPRG against two existing methods for  
425 drug resistance prediction – Mykrobe and TBProfiler.

426  
427 We benchmarked the methods on a high-quality Illumina sequencing dataset with associated  
428 phenotype profiles for 44,709 MTB genomes; the largest known dataset to-date[16]. All tools  
429 used the same catalogue and rules, and for most drugs, there was no significant difference  
430 between the tools. However, DrPRG did have a significantly higher specificity than  
431 TBProfiler for rifampicin predictions, and sensitivity for ethionamide predictions. DrPRG's  
432 sensitivity was also significantly greater than Mykrobe's for rifampicin, streptomycin,  
433 amikacin, capreomycin, kanamycin, and ethionamide. Evaluating detection of gene loss, we  
434 found DrPRG was more sensitive to *katG* deletions than TBProfiler.

435 We also benchmarked using 138 Nanopore-sequenced MTB samples with phenotype  
436 information, but found no significant difference between the tools. This Nanopore dataset  
437 was quite small and therefore the confidence intervals were large for all drugs. Increased  
438 Nanopore sequencing over time will provide better resolution of the overall sensitivity and  
439 specificity values and improve the methodological nuances of calling variants from this  
440 emerging, and continually changing, sequencing technology.

441 DrPRG also used significantly less memory than Mykrobe and TBProfiler on both Nanopore  
442 and Illumina data. In addition, the runtime of DrPRG was significant faster than both tools on  
443 Illumina data and faster than TBProfiler on Nanopore data. While the absolute values for  
444 memory and runtime for all tools mean they could all easily run on common computers found  
445 in the types of institutions likely to run them, the differences for the Nanopore data warrant  
446 noting. As Nanopore data can be generated “in the field”, computational resource usage is  
447 critical. For example, in a recent collaboration of ours with the National Tuberculosis  
448 program in Madagascar[27], Nanopore sequencing and analysis are regularly performed on a  
449 laptop, meaning memory usage is sometimes a limiting factor. DrPRG’s median peak  
450 memory was 277MB, meaning it can comfortably be run on any laptop and other mobile  
451 computing devices[69].

452 It is clear from the Illumina results that more work is needed to understand resistance-  
453 conferring mutations for delamanid and linezolid. However, we did find that nonsense  
454 mutations in the *ddn* gene appear likely to be resistance-conferring for delamanid – as has  
455 been noted previously[39,70–72]. We also found a novel (likely) mechanism of resistance to  
456 pyrazinamide - a nonstop mutation in *pncA*. Phenotype instability in *embB* at codon 306 was  
457 also found to be the main driver in poor ethambutol specificity, as has been noted  
458 elsewhere[55,56], indicating the need to further investigate cofactors that may influence the  
459 phenotype when mutations at this codon are present.

460 Gene absence/deletion detection allowed us to confirm that the absence of *katG* – a  
461 mechanism which is rare in clinical samples[64–67,73] - is highly likely to confer resistance  
462 to isoniazid. Additionally, we found that the absence of *pncA* is likely to cause resistance to  
463 pyrazinamide, as has been noted previously[68]. One finding that requires further  
464 investigation is the variability in ethionamide phenotype when *ethA* is absent. We found that  
465 only 63% of the samples with *ethA* missing, and an ethionamide phenotype, were resistant.  
466 An *et al.* have suggested that *ethA* deletion alone does not always cause resistance and there  
467 might be an alternate pathway via *mshA*[74].

468 Given the size of the Illumina dataset used in this work, the results provide a good marker of  
469 Illumina whole-genome sequencing’s ability to replace traditional phenotyping methods.  
470 With the catalogue used in this study, DrPRG meets the WHO’s target product profile for  
471 next-generation drug-susceptibility testing for both sensitivity and specificity for amikacin,  
472 and sensitivity only for rifampicin, isoniazid, levofloxacin, and moxifloxacin. However, if we  
473 exclude cases where all tools call *rpoB* S450L or *katG* S315T for phenotypically susceptible  
474 samples (these are strong markers of resistance[16] and therefore we suspect sample-swaps or  
475 phenotype error[75]), DrPRG also meets the specificity target product profile for rifampicin  
476 and isoniazid. For the other first-line drugs ethambutol and pyrazinamide, ethambutol does  
477 not have a WHO target and DrPRG’s sensitivity is 0.2% below the WHO target (although the  
478 confidence interval spans the target), while the specificity target is missed by 0.8%.

479 The primary limitation of the DrPRG method relates to minor allele calls. DrPRG uses  
480 *pandora* for novel variant discovery, which combines a graph of known population variants  
481 (which can be detected at low frequency) with *de novo* detection of other variants if present at  
482 above ~50% frequency. Thus, it can miss minor allele calls if the allele is absent from its  
483 reference graph. While this issue did not impact most drugs, it did account for the majority of  
484 cases where DrPRG missed pyrazinamide-resistant calls (in *pncA*), but the other tools  
485 correctly called resistance. Unlike most other genes, where there are a relatively small  
486 number of resistance-conferring mutations, or they’re localised to a specific region (e.g. the  
487 rifampicin-resistance determining region in *rpoB*), resistance-conferring mutations are  
488 numerous - with most being rare - and distributed throughout *pncA*[16,76,77]. Adding all of  
489 these mutations will, and does, lead to decreased performance of the reference graph[33], and

490 so improving minor allele calling for pyrazinamide remains a challenge we need to revisit in  
491 the future.

492 One final limitation is the small number of Nanopore-sequenced MTB isolates with  
493 phenotypic information. In order to get a clearer picture of the sensitivities and specificities  
494 this sequencing technology can provide, we need much larger and more diverse data.

495  
496 In conclusion, DrPRG is a fast, memory frugal software program that can be applied to any  
497 bacterial species. We showed that on MTB, it performs as well as, or better than two other  
498 commonly used tools for resistance prediction. We also collected and curated the largest  
499 dataset of MTB Illumina-sequenced genomes with phenotype information and hope this will  
500 benefit future work to improved genotypic drug susceptibility testing for this species. While  
501 we applied DrPRG to MTB in this study, it is a framework that is agnostic to the species.  
502 MTB is likely one of the bacterial species with the least to gain from reference graphs given  
503 its relatively conserved (closed) pan-genome compared to other common species[78]. As  
504 such, we expect the benefits and performance of DrPRG to improve as the openness of the  
505 species' pan-genome increases[11]; especially given its good performance on a reasonably  
506 closed pan-genome.

## 507 **9. Author statements**

### 508 **9.1 Author contributions**

509 M.B.H: conceptualisation, data curation, formal analysis, investigation, methodology,  
510 resources, software, visualisation, writing – original draft, writing – review & editing. L.L:  
511 resources, software, writing – review & editing. L.J.M.C: funding acquisition, methodology,  
512 supervision, writing – review & editing. Z.I: conceptualisation, funding acquisition,  
513 methodology, supervision, writing – original draft, writing – review & editing.

### 514 **9.2 Conflicts of interest**

515 The authors declare no conflicts of interest.

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