bioRxiv preprint doi: https://doi.org/10.1101/173229; this version posted January 8, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

1 **Title:** Signatures of selection at drug resistance loci in *Mycobacterium tuberculosis*

2 **Authors:** Tatum D. Mortimer[1], Alexandra M. Weber[1], Caitlin S. Pepperell[1*]

3 [1] Department of Medicine, Division of Infectious Diseases and Department of Medical
4 Microbiology and Immunology, University of Wisconsin-Madison

5 *Address correspondence to Caitlin S. Pepperell, cspepper@medicine.wisc.edu

6 Running title: Selection at drug resistance loci in *M. tb*

7

8 Word counts:

9 Abstract- 250

10 Importance- 134

11 Main Text- 5,560

12

bioRxiv preprint doi: https://doi.org/10.1101/173229; this version posted January 8, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

**Abstract:**

Tuberculosis (TB) is the leading cause of death by an infectious disease, and global TB control efforts are increasingly threatened by drug resistance in *Mycobacterium tuberculosis* (*M. tb*). Unlike most bacteria, where lateral gene transfer is an important mechanism of resistance acquisition, resistant *M. tb* arises solely by *de novo* chromosomal mutation. Using whole genome sequencing data from two natural populations of *M. tb*, we characterized the population genetics of known drug resistance loci using measures of diversity, population differentiation, and convergent evolution. We found resistant sub-populations to be less diverse than susceptible sub-populations, consistent with ongoing transmission of resistant *M. tb*. A subset of resistance genes ("sloppy targets") were characterized by high diversity and multiple rare variants; we posit that a large genetic target for resistance and relaxation of purifying selection contribute to high diversity at these loci. For "tight targets" of selection, the path to resistance appeared narrower, evidenced by single favored mutations that arose numerous times on the phylogeny and segregated at markedly different frequencies in resistant and susceptible sub-populations. These results suggest that diverse genetic architectures underlie drug resistance in *M. tb*, and combined approaches are needed to identify causal mutations. Extrapolating from patterns observed in well-characterized genes, we identified novel candidate variants involved in resistance. The approach outlined here can be extended to identify resistance variants for new drugs, to investigate the genetic architecture of resistance, and, when phenotypic data are available, to find candidate genetic loci underlying other positively selected traits in clonal bacteria.

**Importance:**

*Mycobacterium tuberculosis* (*M. tb*), the causative agent of tuberculosis (TB), is a significant burden on global health. Antibiotic treatment imposes strong selective pressure on *M. tb* populations. Identifying the mutations that cause drug resistance in *M. tb* is important for guiding TB treatment and halting the spread of drug resistance. Whole genome sequencing (WGS) of *M. tb* isolates can be used to identify novel mutations mediating drug resistance and to predict resistance patterns faster than

bioRxiv preprint doi: https://doi.org/10.1101/173229; this version posted January 8, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

43 traditional methods of drug susceptibility testing. We have used WGS from natural
44 populations of drug resistant *M. tb* to characterize effects of selection for advantageous
45 mutations on patterns of diversity at genes involved in drug resistance. The methods
46 developed here can be used to identify novel advantageous mutations, including new
47 resistance loci, in *M. tb* and other clonal pathogens.

48

bioRxiv preprint doi: https://doi.org/10.1101/173229; this version posted January 8, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

## Introduction:

*Mycobacterium tuberculosis* (*M. tb*), the causative agent of tuberculosis (TB), is estimated to have caused 1.4 million deaths in 2015, making it the leading cause of death due to an infectious disease. The proportion of TB due to MDR (multi-drug resistant) *M. tb* resistant to first line anti-tuberculosis drugs isoniazid (INH) and rifampin (RIF)) is increasing (1), which poses a major threat to global public health. Unlike most bacteria, *M. tb* evolves clonally, so resistance cannot be transferred among strains or acquired from other species of bacteria: drug resistance in *M. tb* results from *de novo* mutation within patients and transmission of drug resistant clones (2–4). The relative contributions of *de novo* emergence and transmitted drug resistance varies across sampling locations (5–9). Another potential variable in the emergence of drug resistant TB is *M.tb*'s lineage structure: seven distinct lineages have been identified among globally extant populations of *M. tb*. Among these, lineage 2 (L2) has been associated with relatively high rates of drug resistance, and it has been postulated that the acquisition of resistance is a result of higher rates of mutation in this lineage (10). Studies of *M.tb* evolution within hosts with TB have shown that emergence of drug resistance is associated with clonal replacements that lead to reductions in genetic diversity of the bacterial population (11, 12).

Many of the methods developed to identify advantageous mutations, such as those conferring antibiotic resistance, depend on recombination to differentiate target loci from neutral variants (13). However, in clonal organisms like *M. tb*, neutral and deleterious mutations that are linked to advantageous variants will evolve in tandem with them. This linkage among sites can also cause competition between genetic backgrounds with beneficial mutations, decreasing the rate of fixation for beneficial alleles, while deleterious alleles are purged less efficiently (14–16).

While the majority of the *M. tb* genome is subject to purifying selection (i.e. selection against deleterious mutations) (3), antibiotic pressure exerts strong selection for advantageous variants that confer resistance. *M. tb* drug resistance has been the focus of extensive investigation, and a variety of resistance mutations have been characterized for commonly used anti-tuberculosis drugs (17). Drug resistance

bioRxiv preprint doi: https://doi.org/10.1101/173229; this version posted January 8, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

mutations can be associated with fitness costs (18–20), and compensatory mutations that ameliorate these fitness costs have been identified in the context of rifampicin resistance (21, 22). Resistance mutations found to have lower fitness costs *in vitro* - as measured by competition assays - are found at higher frequencies among *M. tb* clinical isolates and appear to be transmitted at higher rates relative to mutations with high *in vitro* fitness costs (18, 23). Candidate loci involved in resistance and compensation for its fitness effects have been identified previously by screening for homoplastic variants (i.e. mutations that emerge more than once on the phylogeny) that are significantly associated with drug resistant phenotypes (24) and genes with higher *dN*/*dS* (ratio of non-synonymous versus synonymous mutations) in resistant compared to sensitive isolates (25). Application of these methods to whole genome sequence data from *M. tb* clinical isolates has recovered known drug resistance loci, as well as loci associated with cell surface lipids and biosynthesis, DNA replication, and metabolism.

The goal of the present study was to use patterns of genetic diversity at known drug resistance loci to identify the population genomic signatures of positive selection in natural populations of *M. tb*. Using whole genome sequence data from two populations for which phenotypic resistance data were available, we have identified several distinct signatures associated with these loci under selection. Based on these results, we propose methods of identifying loci under positive selection, including novel drug resistance loci, in clonal bacteria such as *M. tb*.

**Results:**

We inferred the phylogeny of 1161 *M. tb* isolates from Russia and South Africa (see Methods, Supplementary Table 1) using the approximate maximum likelihood method implemented in FastTree2 (Figure 1). The majority of the isolates belong to L2 (*n* = 667) and L4 (*n* = 481). *M. tb* nucleotide diversity was similar to previous estimates from a globally distributed sample (26). We identified lineage-specific patterns in overall diversity, with L4 having higher diversity than L2 ($\pi_{L2}$: $3.6 \times 10^{-5}$, $\pi_{L4}$: $1.5 \times 10^{-4}$). Previously published analyses of whole genome sequence data from L2 indicate that the majority of L2 isolates worldwide belong to a sub-lineage that has undergone relatively recent expansion (27, 28). In this sample from Russia and South Africa, the

bioRxiv preprint doi: https://doi.org/10.1101/173229; this version posted January 8, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

majority of L2 isolates belong to this sub-lineage, while the L4 isolates are associated with deeper branching sub-lineages. This likely contributes to the observed differences in diversity.

Overall diversity of L2 was lower than L4 in our sample (Figure 2, $p < 2.2 \times 10^{-16}$). Seven hundred and sixty two of the isolates in our sample (66%) are resistant to one or more anti-tuberculosis drugs (Table 1).

Drug resistant TB can be acquired as a result of *de novo* mutations within a patient or by infection with a resistant strain. When resistance is primarily mediated by *de novo* mutations, diversity should be similar in susceptible and resistant populations as resistance will arise on multiple genetic backgrounds. By contrast, if resistance develops primarily *via* transmission of resistant strains, the resistant sub-population should be less diverse than the susceptible sub-population. We compared the nucleotide diversity of susceptible and resistant sub-populations and found genome wide estimates of nucleotide diversity to be higher in isolates susceptible to a range of drugs for which phenotyping data were available (paired t-test, $p = 0.029$). In comparisons of gene-wise diversity in susceptible and resistant populations, we found that resistant isolates had a greater number of genes with no diversity, but levels of diversity within genes in which it was measurable were similar between resistant and susceptible populations (Figure 3).

Of the 3,162 genes included in our analyses, 109 (3%) were invariant across all isolates in our sample. This is likely due to strong purifying selection on these genes. An additional 136 genes harbored variation in the drug susceptible populations but were invariant across all of the drug resistant populations. We did not observe the converse, i.e. genes that were invariant in susceptible isolates specifically, which supports the conclusion from genome wide diversity estimates that resistant isolates represent a subset of the diversity found in susceptible populations and suggests that there may be purifying selection that is specific to the setting of drug resistance. In order to evaluate whether the observed pattern was likely to arise by chance, we performed weighted random sampling of genes. The weighting was based on diversity in susceptible populations, assuming that genes with low diversity in susceptible populations are more

bioRxiv preprint doi: https://doi.org/10.1101/173229; this version posted January 8, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

likely to be invariant in resistant populations. After randomly sampling genes in each drug resistant population 1000 times, we found that no samples contained shared genes amongst all resistant populations (first and second line drugs). This suggests that specific genes tend to lose diversity in the setting of drug resistance, which could result from purifying selection specific to this setting. A potentially important caveat is that in our data set, drug resistant populations are not independent and the same isolates are often resistant to multiple drugs. Since resistance to second line drugs frequently arises on genetic backgrounds already resistant to one or more first line drugs, we repeated the sampling with only first line drugs and found that the maximum number of sampled genes shared across all populations was 2 (median 0). Overall, these results suggest that certain genes are more likely to lose diversity as drug resistance evolves, but we cannot completely rule out the possibility that the pattern arose as a result of overlapping membership in resistant populations.

We compared diversity of drug resistance associated genes (Table 2) with the rest of the genome using two measures of diversity: average pairwise differences ($\pi$) and number of segregating sites ($\theta$). We found the resistance genes *gid*, *rpsL,* and *pncA* to be in the top 5$^{th}$ percentile of gene-wise $\pi$ and/or $\theta$ values. *rrs* and *ethA* are in the top 5$^{th}$ percentile of $\theta$, but not $\pi$. Surprisingly, despite being a target of multiple drug resistance mutations (Table 2), we did not identify extreme levels of diversity in *katG* (80$^{th}$ and 82$^{nd}$ percentile of $\pi$ and $\theta$, respectively).

We also examined gene-wise diversity values within each lineage to look for lineage specific high diversity genes. In both L2 and L4, *gid, rpsL, pncA, ethA,* and *thyA* were in the top 5$^{th}$ percentile of diversity ($\pi$ and/or $\theta$). In L2, *rpoB, embB, Rv1772*, and *folC* were additionally in the top 5$^{th}$ percentile of gene-wise $\pi$ and/or $\theta$ values. In L4, *Rv0340* was in the top 5$^{th}$ percentile of gene-wise $\pi$ and/or $\theta$. While *rpoB* and *embB* were not in the top 5$^{th}$ percentile of gene-wise $\theta$ in L4, they still had high diversity (91$^{st}$ and 82$^{nd}$ percentile, respectively). The lineage specific differences in diversity of *Rv1772*, *folC*, and *Rv0340* suggest that there are interactions between these loci and loci that differentiate L2 and L4.

bioRxiv preprint doi: https://doi.org/10.1101/173229; this version posted January 8, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

We used gene-wise estimates of Tajima's D to investigate gene specific skews in the site frequency spectrum that could result from selection, where negative values indicate an excess of rare variants and positive values indicate an excess of intermediate frequency variants. We previously identified a relationship between gene length and gene-wise estimates of Tajima's D for *M. tb* (26), and this finding was corroborated here ($R^2$ = 0.3 after $\log_2$ transformation). In order to identify genes with extreme values of Tajima's D - out of proportion with their length - we performed linear regression on $\log_2$ transformed gene lengths and Tajima's D values and identified genes with the largest residuals (Figure 4). *pncA, ethA*, and *embC* all had Tajima's D values lower than expected based on their length ($5^{th}$ percentile of residual values). This indicates that these genes contain an excess of rare variants compared to other genes in the genome. Excess rare variants can result from a population expansion, a selective sweep, or purifying selection.

We calculated the ratio of π and θ of resistance associated genes in isolates susceptible and resistant to first line drugs and identified genes with markedly different diversities in resistant and susceptible sub-populations (Figure 5A). Among resistance genes in the top $5^{th}$ percentile of gene-wise π and θ overall, diversity of *pncA* and *ethA* is relatively high among resistant isolates, whereas diversity of *gid* is similar in resistant and susceptible populations. We also examined differences in this ratio between isolates in L2 and L4 (Figure 5B). *Rv1772* and *embR* were more diverse in resistant isolates in L2, and *kasA* and *tlyA* were more diverse in resistant isolates in L4.

We used $F_{ST}$ outlier analysis to identify single nucleotide polymorphisms (SNPs) and indels that exhibited extreme differences in frequency between susceptible and resistant populations. Our *a priori* expectation was that variants mediating resistance would be at markedly higher frequency in the drug resistant sub-population and that drug targets would be enriched among genes harboring variants with high $F_{ST.}$ After removing SNPs in regions corresponding to indels and variants at sites missing data for greater than 5% of isolates, the highest $F_{ST}$ SNPs in comparisons of resistant and susceptible sub-populations to first line drugs are in *katG* (2155168, $F_{ST}$ = 0.89, INH), *rpoB* (761155, $F_{ST}$ = 0.72, RIF), and *rpsL* (781687, $F_{ST}$ = 0.37, streptomycin (STR)). These SNPs were also

bioRxiv preprint doi: https://doi.org/10.1101/173229; this version posted January 8, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

198 $F_{ST}$ outliers in the lineage specific analyses. We used a randomization procedure to
199 assess the significance of observed $F_{ST}$ values and found the maximum $F_{ST}$ values after
200 randomly assigning resistant and susceptible designations to be 0.023 for INH, 0.019
201 for RIF, and 0.018 for STR. In addition to SNPs within known drug resistance
202 associated genes, we identified $F_{ST}$ outliers in genes that may be novel targets for drug
203 resistance (Table 3).

204 Homoplastic SNPs – i.e. SNPs that evolve more than once on a phylogeny – are
205 candidate loci under positive selection and have previously been used to identify
206 resistance associated mutations in *M. tb* (24). Of the 235 genes with homoplastic SNPs
207 that we identified in our sample, 13 are known to be associated with drug resistance
208 (Figure 6), and resistance genes were significantly enriched among genes with
209 homoplastic SNPs (Fisher's Exact Test, $p = 1.2 \times 10^{-4}$). *pncA* had the largest number of
210 homoplastic SNPs of any gene in the genome ($n = 27$ distinct SNPs that appear > 1 on
211 the phylogeny). The SNPs identified in $F_{ST}$ analysis were also identified as homoplastic
212 (Figure 6). Our results suggest that complementary approaches based on homoplasy
213 and $F_{ST}$ outlier analysis can be used to identify SNPs associated with a trait of interest
214 (in this case drug resistance). In addition to genic SNPs, we observed homoplastic
215 SNPs that are also $F_{ST}$ outliers in intergenic regions upstream of drug resistance
216 associated genes (Table 3). These are candidate resistance and compensatory
217 mutations with a regulatory mechanism of action.

218 In our analyses of indels, we controlled for the possibility that indels affecting the same
219 gene may not be called in exactly the same position by considering indels within the
220 same gene as identical. We identified four drug resistance associated genes with
221 homoplastic indels: *gid, ethA*, *rpoB,* and *pncA*. $F_{ST}$ values for the deletion in *gid* were in
222 the top 5$^{th}$ percentile for capreomycin (CAP), ethambutol (EMB), ethionamide (Et),
223 kanamycin (K), ofloxacin (OFL), and pyrazinamide (PZA) resistant populations, but,
224 interestingly, the deletion was not associated with STR resistance ($F_{ST} = 0.04$). Unlike
225 homoplastic SNPs, homoplastic indels were not significantly enriched for drug
226 resistance associated loci ($p = 1$).

bioRxiv preprint doi: https://doi.org/10.1101/173229; this version posted January 8, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

We recovered 20 out of 40 known drug targets by identifying genes with extreme values of diversity, homoplastic SNPs, or SNPs that are $F_{ST}$ outliers in comparisons of resistant and susceptible subpopulations. All genes with both extremely high diversity (top 5$^{th}$ percentile) and homoplastic mutations were drug resistance associated (i.e. *gid, ethA, pncA,* and *rpsL*). We identified 67 genes with high diversity and Tajima's D values more negative than expected based on gene length; only two of these were associated with drug resistance (i.e. *ethA* and *pncA*). Twenty out of 51 homoplastic SNPs that are also $F_{ST}$ outliers fall within or upstream of known drug resistance associated genes. The remaining SNPs may be false positives or novel drug resistance mutations.

**Discussion:**

Highly virulent bacterial pathogens such as *M. tb, Yersinia pestis* (29), *Francisella tularensis* (30), and *Mycobacterium ulcerans* (31) appear to evolve clonally, i.e. with little to no evidence of lateral gene transfer. It is important to identify advantageous mutations in these and other organisms, as they are likely to be associated with phenotypes such as drug resistance, heightened transmissibility, or host adaptation. However, few methods are available for identifying loci under positive selection in the setting of clonal evolution. We adopted an empirical approach to this problem and used natural population data to characterize patterns of diversity at loci known to be under positive selection in *M. tb*.

In this analysis of clinical isolates from settings with endemic drug resistance, we found genome-wide diversity to be higher in susceptible *M. tb* sub-populations than in those resistant to first- and second- line drugs (with the exception of protionamide (PRO) and moxifloxacin (MOX) resistant populations). The observation of higher diversity in drug susceptible populations is consistent with a significant role for transmitted resistance in the propagation of drug resistant *M. tb*. A recent study of extensively drug resistant (XDR) *M. tb* infection in South Africa concluded that XDR cases result primarily from transmission of resistance, rather than *de novo* evolution of resistance mutations during infection (9). The primary studies for the sequence data analyzed here also identified clusters of drug resistant isolates (5, 6), suggesting that resistant isolates were being transmitted. Our results, along with these previously published observations, suggest

bioRxiv preprint doi: https://doi.org/10.1101/173229; this version posted January 8, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

that the fitness of drug resistant isolates can be high enough to allow them to circulate in endemic regions. As discussed below, the fitness effects of *M. tb* drug resistance mutations appear to vary substantially; the finding of transmitted resistance in this and other studies suggests that the fitness of isolates harboring low-cost mutations is comparable to that of susceptible *M. tb*. The populations in our study have a high burden of drug resistant TB, and the role of transmitted drug resistance may differ in other settings.

An alternative – but not mutually exclusive – explanation for the observation of higher diversity in susceptible populations is that drug resistant *M. tb* is under distinct evolutionary constraints that reduce average genome-wide levels of diversity. In support of this hypothesis, we identified a specific subset of genes that were invariant across drug resistant populations. Interestingly, while average diversity was lower for resistant sub-populations, the gene-wise diversity distributions had heavier tails, indicating there were more genes with extreme levels of diversity.

We found the genetic architecture of resistance to vary among targets, and resistance-associated genes tended to fall within categories that we term “sloppy”, “tight”, and “hybrid” targets of selection (the latter has a combination of tight and sloppy features and applies to *rpsL, embB,* and *rpoB*). “Sloppy” resistance genes are characterized by high levels of diversity. Genes associated with PZA, EMB, Et, and STR resistance (i.e. *pncA*, *gid, rpsL, rrs, ethA*) have high levels of diversity; some also had an excess of rare variants (*pncA*, *ethA*, *embC*). The finding that these genes accumulate multiple, individually rare mutations implies that there is a large target for resistance and/or compensatory mutations within the gene: that is, resistance can result from multiple different variants acting individually or in concert. In addition to its numerous rare mutations, *pncA* also contains the highest number of homoplastic SNPs (27 SNPs emerged more than once on the phylogeny) of any gene in the data set. Among the 62 non-synonymous *pncA* mutations in our dataset, 55 have been previously reported in association with drug resistance (TB Drug Resistance Mutation Database (32)). The newly described SNPs may mediate drug resistance or compensation for the fitness

bioRxiv preprint doi: https://doi.org/10.1101/173229; this version posted January 8, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

effects of other variants. Relaxed purifying selection is likely to play a role in concert with selection for diverse advantageous resistance mutations in the accumulation of diversity in *pncA* and other sloppy targets. The fact that numerous mutations are segregating in a natural population suggests that alterations to these genes are generally associated with negligible fitness costs. An *M. tb* strain harboring a deletion in *pncA* conferring resistance to PZA was estimated to be endemic in Quebec by 1800, long before the use of PZA for the treatment of TB (33–35). This supports the idea that purifying selection on *pncA* is relatively weak, which would contribute to its exceedingly high diversity and broaden the adaptive paths to resistance.

In contrast to *pncA*, *gid,* which is associated with low level STR resistance (36), does not appear to have the signatures of a “sloppy” target for resistance despite its high diversity. We identified just three homoplastic SNPs within *gid*, and previous studies have found that STR resistant isolates do not encode the same *gid* mutations (37). This could indicate that a multitude of mutations within *gid* confer resistance, but levels of diversity in the gene were similar in resistant and susceptible isolates. Previous studies of sequence polymorphism in *gid* have identified high diversity in this gene in both resistant and susceptible isolates (37–39): *gid* appears to be subject to relaxed purifying selection in the presence and absence of antibiotic pressure. Since *gid* mutations confer low level resistance, it’s also possible that mis-classification of resistance phenotypes contributed to the lack of differentiation we and others have observed between putatively STR resistant and susceptible sub-populations. In addition, mutations in *rpsL*, which cause high level resistance, could mask the contribution of *gid* to STR resistance.

We found some drug targets to be highly diverse in resistant sub-populations of either L2 or L4 (but not both), suggesting that resistance mutations in these genes interact with the genetic background; the fitness effects of mutations in these genes could, for example, vary on different genetic backgrounds. Lineage-specific $F_{ST}$ outliers are another category of candidate locus with lineage dependent roles in drug resistance (Table 3). Epistatic interactions between drug resistance mutations and *M. tb* lineage have been reported previously: for example, specific mutations in the *inhA* promoter have been associated with the L1 and *M. africanum* genetic backgrounds (40, 41).

bioRxiv preprint doi: https://doi.org/10.1101/173229; this version posted January 8, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

317 In contrast to “sloppy” targets, we discovered individual homoplastic SNPs associated
318 with drug resistant sub-populations (i.e. with high $F_{ST}$) representing “tight” targets of
319 selection in genes conferring resistance to INH, RIF, and STR. Numerous resistance
320 mutations have been described in *katG*, *rpoB, rpsL, embB,* and *gyrA,* but we find drug
321 resistant sub-populations to be defined by a specific subset of mutations in these genes.
322 This suggests that certain mutations are strongly favored relative to others conferring
323 resistance to the same drugs when *M. tb* is in its natural environment. Antibiotic
324 resistance can impose fitness costs on *M. tb* during *in vitro* growth, with the range of
325 fitness costs varying among mutations, even within the same gene (18). Mutations can
326 also have different fitness effects depending on the genetic background, but the most fit
327 mutants were the same across *M. tb* lineages in a study of RIF resistance (18).

328 In our analyses, we found the dominant INH resistance mutation in *katG* to affect the
329 serine at position 315. This change reduces affinity to INH but preserves catalase
330 activity (42) and is associated with lower fitness costs than other *katG* mutants, both *in*
331 *vitro* and in a mouse model (43, 44). This mutation was recently shown to precede
332 mutations conferring resistance to other drugs during accumulation of resistance in
333 evolution of multi-drug resistant *M. tb* (45). The dominant mutations we identified in
334 *rpoB* (codon 450) and *rpsL* (codon 43) have also been found to have lower fitness costs
335 *in vitro* compared to other mutations conferring resistance to RIF and STR in these
336 genes (18, 44, 46). These results suggest that many of the findings regarding the
337 relative fitness costs of *M. tb* resistance mutations *in vitro* and in animal models are
338 relevant to the pathogen’s natural environment.

339 The fitness effects of mutations in *gyrA* (codon 94) and *embB* (codon 306) have not
340 been measured; based on our homoplasy and $F_{ST}$ results, we propose that they have
341 lower fitness costs than other mutations in these genes and that they represent “tight”
342 targets of selection. Mutations at *gyrA* codon 94 were previously found to be the most
343 prevalent in a survey of *gyrA* and *gyrB* mutations in fluoroquinolone resistant *M. tb*
344 clinical isolates (47). Interestingly, the mutation in *embB* codon 306 has been previously
345 associated with acquisition of multiple resistances (48), and we find that this position is
346 an $F_{ST}$ outlier for all first line drugs in L4. This mutation is not an $F_{ST}$ outlier in L2 (i.e top

bioRxiv preprint doi: https://doi.org/10.1101/173229; this version posted January 8, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

347 5$^{th}$ percentile), with percentiles for $F_{ST}$ values ranging from 0.07-0.68 for first line drugs
348 in this lineage. These observations suggest that the genetic background affects
349 interactions among resistance mutations, and that *embB* 306 is important for acquisition
350 of multidrug resistance in L4 but not L2.

351 We searched for indels with the signature of a "tight" target, i.e. homoplastic mutations
352 segregating at markedly different frequencies in drug susceptible and resistant sub-
353 populations. Unlike the pattern observed with SNPs, genes associated with drug
354 resistance were not significantly enriched among those harboring homoplastic indels.
355 We identified one homoplastic indel that was also an $F_{ST}$ outlier - a deletion in *gid* that
356 causes a frameshift. Patterns of variation in *gid* are complex and suggest a role for
357 relaxation of purifying selection (i.e. in the accumulation of excess SNPs in both
358 resistant and susceptible isolates) and perhaps a tight target associated with multi-
359 resistance (i.e. this homoplastic/$F_{ST}$ outlier deletion that was associated with resistance
360 to CAP, EMB, Et, K, OFL, and PZA).

361 Our finding that, save for the frameshift mutation in *gid*, indels in resistance genes do
362 not have the signature of "tight" targets suggests that they are generally associated with
363 higher fitness costs than SNPs. Fifteen drug targets have been found in transposon
364 mutagenesis experiments to be essential for *M. tb* growth *in vitro*, including *rpoB* and
365 *rpsL*; deletions in these genes are likely to interrupt important functions (49). Deletions
366 in non-essential genes could also have fitness costs. Deletions in *katG,* which is non-
367 essential, can result in INH resistance but they are not observed as frequently in clinical
368 isolates as the KatG S315 SNP, particularly among transmitted INH-resistant strains
369 (23).

370 There are several limitations to our study. Resistance to multiple drugs was common in
371 our sample, and in some cases it was difficult to identify patterns of diversity and
372 population differentiation that were specific to individual drugs. Our results are also
373 limited by the accuracy with which drug resistance phenotypes were determined and a
374 lack of phenotypic data for some drugs (particularly second line drugs). Our sample
375 was heavily skewed to lineages 2 and 4, and the results are not necessarily applicable
376 to other *M. tb* lineages. Finally, the data analyzed here were generated with short

bioRxiv preprint doi: https://doi.org/10.1101/173229; this version posted January 8, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

sequencing read technologies, and we were thus limited to characterizing diversity in regions of the *M. tb* genome that can be resolved with these methods: regions that were masked from analysis (e.g. due to sequence repeats) may include unknown resistance targets. We also used an L4 genome (H37Rv) as a reference, and gene content specific to L2 may not have been identified.

We were not able to recover all drug resistance associated genes using the analyses performed here. This is likely a result of limited phenotypic data for some drugs and their associated targets (e.g. *thyA* and *folC*, which are associated with aminosalicylic acid (PAS) resistance). Our list of drug targets was dominated by genes associated with INH resistance, and signatures in genes that harbor rare resistance associated alleles may be subtle compared to the KatG S315 mutation found at high frequency in drug resistant populations.

We identified 31 SNPs that do not fall within the list of known drug resistance genes, which both emerged more than once on the phylogeny (homoplasies) and were segregating at markedly different frequencies in resistant and susceptible sub-populations ($F_{ST}$ outliers). These SNPs may be novel resistance determinants; notably, all non-synonymous SNPs within this group are in genes linked with drug resistance in other studies (i.e. they are in genes encoding efflux pumps, genes differentially regulated in resistant isolates or in response to the presence of drug, potential drug targets, or genes in the same pathways as drug targets or resistance determinants) (50–54). In addition to a direct, previously unrecognized role in resistance, these SNPs could compensate for fitness costs of drug resistance. For example, we identified a homoplastic $F_{ST}$ outlier in *rpoC*, and mutations in *rpoC* have been shown to compensate for RIF resistance in experimental evolution studies (22).

Intriguingly, we found lipid metabolism genes to be enriched in the list of genes harboring homoplastic SNPs ($p = 0.013$). We've previously shown that these genes have extreme values of diversity in a global sample of *M. tb* isolates and within individual hosts (26), suggesting that lipid metabolism genes may also be under positive selection in *M. tb* populations. The results presented here could be extended by

bioRxiv preprint doi: https://doi.org/10.1101/173229; this version posted January 8, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

406 phenotypic characterization of lipid profiles and identification of homoplastic variants
407 that are at markedly different frequencies in isolates with distinct lipid profiles.

408

409 Here we have used drug resistance loci in *M. tb* to identify the signatures of positive
410 selection in a clonal bacterium. We found these loci to be associated with distinct
411 patterns of diversity that likely reflect differing genetic architectures underlying the traits
412 under selection. The evolutionary path to resistance is broad for some drugs with
413 "sloppy targets", whereas for drugs with "tight targets" the means of acquiring resistance
414 appear more limited. This is likely due to fitness effects of resistance mutations in *M.*
415 *tb*'s natural environment, as numerous resistance mutations have been identified in tight
416 target genes. We also found evidence suggesting that there are important interactions
417 among loci during the evolution of resistance. Our results suggest that purifying
418 selection on a subset of genes intensifies in the setting of resistance, which could reflect
419 epistatic interactions and/or a response to the metabolic milieu imposed by
420 antimycobacterial agents. The results presented here can be used to create more
421 realistic models of resistance evolution in *M. tb* and to develop novel strategies of
422 preventing or mitigating the acquisition of resistance. For example, the narrow path to
423 resistance for drugs with tight targets reveals potentially exploitable vulnerabilities, as
424 does the finding of interdependencies among specific loci and the genetic background
425 in the evolution of resistance and multi-resistance. As new TB drugs become available
426 for clinical use, the approach outlined here can be extended to investigate their
427 architectures of resistance.

428 Efforts are underway to sequence and perform drug susceptibility testing on thousands
429 of *M. tb* isolates with the goal of creating an exhaustive catalogue of drug resistance
430 mutations and eventually using WGS to diagnose drug resistance in clinical settings
431 (CRyPTIC project, http://modmedmicro.nsms.ox.ac.uk/cryptic/, last accessed: May 24,
432 2017). We found that loci under positive selection can be identified using relatively
433 simple methods: "tight" targets are highly differentiated in their allele frequencies across
434 phenotypic groups (i.e. $F_{ST}$ outliers) and appear as homoplasies on the phylogeny;
435 "sloppy" targets are characterized by high diversity and/or low Tajima's D, as well as

bioRxiv preprint doi: https://doi.org/10.1101/173229; this version posted January 8, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

436 homoplasies. Extrapolating from patterns observed among known resistance variants,
437 we have discovered new candidate regulatory and genic resistance variants. The
438 methods used in this study are widely available and should scale to analysis of the large
439 collections of genomic and phenotypic data that are currently being generated. This
440 approach can be extended to identify novel resistance loci in bacteria for which drug
441 susceptibility phenotypes are defined, as well as other positively selected loci in clonal
442 bacterial populations.

443 **Methods:**

444 Reference guided assembly

445 We downloaded sequencing read data from two large surveys of drug resistant *M. tb* in
446 Russia (5) and South Africa (6). We used FastQC (55) and TrimGalore (56) for quality
447 assessment and adaptor trimming of the reads. Trimmed reads were mapped to *M. tb*
448 H37Rv (NC_000962.3) using BWA-MEM v 0.7.12 (57). We used Samtools v 1.2 (58)
449 and Picard Tools (https://broadinstitute.github.io/picard/) for sorting, format conversion,
450 and addition of read group information. Variants were identified using Pilon v 1.16 (59).
451 A detailed description of the reference guided assembly pipeline is available at
452 https://github.com/pepperell-lab/RGAPepPipe. We removed isolates with mean
453 coverage less than 20X, isolates with percentage of the genome covered at 10X less
454 than 90%, isolates where a majority of reads did not map to H37Rv, and isolates where
455 greater than 10% of sites were unknown after mapping. The final data set contains 1161
456 *M. tb* isolates (Supplementary Table 1). The alignment was masked to remove repetitive
457 regions including PE/PPE genes.

458 Phylogenetic analysis

459 We estimated the approximately maximum likelihood phylogeny using the masked
460 alignment from reference guided assembly with FastTree-2.1.9 (60). We compiled
461 FastTree using the double precision option to accurately estimate branch lengths of
462 closely related isolates. We used FigTree (http://tree.bio.ed.ac.uk/software/figtree/) for
463 tree visualization.

bioRxiv preprint doi: https://doi.org/10.1101/173229; this version posted January 8, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

## SNP annotation

A VCF of single nucleotide variants was created from the masked alignment using SNP-sites v 2.3.2 (61). SNPs were annotated using SnpEff v 4.1j (62) to identify synonymous, non-synonymous, and intergenic SNPs based on the annotation of *M. tb* H37Rv.

## Indel identification

Insertions and deletions were identified during variant calling with Pilon. We used Emu (63) to normalize indels across multiple isolates. We used a presence/absence matrix for the normalized indels for further analyses of indel diversity.

## Population genetics statistics

Whole genome and gene-wise diversity ($\pi$ and $\theta$) and neutrality (Tajima's D) statistics were calculated using Egglib v 2.1.10 (64) for whole genome alignments and gene-wise alignments. Isolates were further divided by lineage and drug resistance phenotype. Sites with missing data due to indels or low quality base calls more than 5% of isolates in the alignment were not included in calculation of statistics. Values of Tajima's D showed a correlation with gene length in our sample. To find genes with extreme values of Tajima's D, we performed linear regression in R (65) on log transformed Tajima's D values and gene length and identified genes with large residual values. To identify alleles with marked differences in frequency in resistant and susceptible isolates, Weir and Cockerham's $F_{ST}$ (66) was calculated using populations of resistant and susceptible isolates for each drug using vcflib v1.0.0-rc0-262-g50a3 (https://github.com/vcflib/vcflib). For non-biallelic SNPs, we calculated $F_{ST}$ for the two most common variants.

## Homoplasy

We used TreeTime (67) to perform ancestral reconstruction and place SNPs and indels on the phylogeny. We identified homoplastic SNPs and indels as those arising multiple times on the phylogeny.

## Data availability

bioRxiv preprint doi: https://doi.org/10.1101/173229; this version posted January 8, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

Unless otherwise noted, all data and scripts associated with this study are available at https://github.com/pepperell-lab/mtbDrugResistance.

**Funding information:**

This material is based upon work supported by the National Science Foundation Graduate Research Fellowship Program under Grant No. DGE-1256259 to TDM. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the National Science Foundation. TDM is also supported by National Institutes of Health National Research Service Award (T32 GM07215). CSP is supported by National Institutes of Health (R01AI113287). Funding for this project was provided by the University of Wisconsin Madison School of Medicine and Public Health from the Wisconsin Partnership Program.

**Acknowledgments:**

We thank members of the Pepperell Lab for their input on analyses and data visualization.

bioRxiv preprint doi: https://doi.org/10.1101/173229; this version posted January 8, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

## References:

1. World Health Organization. 2016. Global tuberculosis report 2016.

2. Supply P, Warren RM, Bañuls A-L, Lesjean S, Van Der Spuy GD, Lewis L-A, Tibayrenc M, Van Helden PD, Locht C. 2003. Linkage disequilibrium between minisatellite loci supports clonal evolution of Mycobacterium tuberculosis in a high tuberculosis incidence area. Mol Microbiol 47:529–538.

3. Pepperell CS, Casto AM, Kitchen A, Granka JM, Cornejo OE, Holmes EC, Birren B, Galagan J, Feldman MW. 2013. The Role of Selection in Shaping Diversity of Natural M. tuberculosis Populations. PLoS Pathog 9:e1003543.

4. Eldholm V, Balloux F. 2016. Antimicrobial Resistance in Mycobacterium tuberculosis: The Odd One Out. Trends Microbiol 24:637–648.

5. Casali N, Nikolayevskyy V, Balabanova Y, Harris SR, Ignatyeva O, Kontsevaya I, Corander J, Bryant J, Parkhill J, Nejentsev S, Horstmann RD, Brown T, Drobniewski F. 2014. Evolution and transmission of drug-resistant tuberculosis in a Russian population. Nat Genet 46:279–286.

6. Cohen KA, Abeel T, Manson McGuire A, Desjardins CA, Munsamy V, Shea TP, Walker BJ, Bantubani N, Almeida DV, Alvarado L, Chapman SB, Mvelase NR, Duffy EY, Fitzgerald MG, Govender P, Gujja S, Hamilton S, Howarth C, Larimer JD, Maharaj K, Pearson MD, Priest ME, Zeng Q, Padayatchi N, Grosset J, Young SK, Wortman J, Mlisana KP, O'Donnell MR, Birren BW, Bishai WR, Pym AS, Earl AM. 2015. Evolution of Extensively Drug-Resistant Tuberculosis over Four Decades: Whole Genome Sequencing and Dating Analysis of Mycobacterium tuberculosis Isolates from KwaZulu-Natal. PLoS Med 12:e1001880.

7. Coscolla M, Barry PM, Oeltmann JE, Koshinsky H, Shaw T, Cilnis M, Posey J, Rose J, Weber T, Fofanov VY, Gagneux S, Kato-Maeda M, Metcalfe JZ. 2015. Genomic Epidemiology of Multidrug-resistant Mycobacterium tuberculosis During Transcontinental Spread. J Infect Dis jiv025.

8. Guerra-Assunção JA, Crampin AC, Houben R, Mzembe T, Mallard K, Coll F, Khan P, Banda L, Chiwaya A, Pereira RPA, McNerney R, Fine PEM, Parkhill J, Clark TG, Glynn JR. 2015. Large-scale whole genome sequencing of M. tuberculosis provides insights into transmission in a high prevalence area. eLife 4:e05166.

9. Shah NS, Auld SC, Brust JCM, Mathema B, Ismail N, Moodley P, Mlisana K, Allana S, Campbell A, Mthiyane T, Morris N, Mpangase P, van der Meulen H, Omar SV, Brown TS, Narechania A, Shaskina E, Kapwata T, Kreiswirth B, Gandhi NR. 2017. Transmission of Extensively Drug-Resistant Tuberculosis in South Africa. N Engl J Med 376:243–253.

10. Ford CB, Shah RR, Maeda MK, Gagneux S, Murray MB, Cohen T, Johnston JC, Gardy J, Lipsitch M, Fortune SM. 2013. Mycobacterium tuberculosis mutation rate estimates from different lineages predict substantial differences in the emergence of drug-resistant tuberculosis. Nat Genet 45:784–790.

bioRxiv preprint doi: https://doi.org/10.1101/173229; this version posted January 8, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

11. Eldholm V, Norheim G, von der Lippe B, Kinander W, Dahle UR, Caugant DA, Mannsåker T, Mengshoel AT, Dyrhol-Riise AM, Balloux F. 2014. Evolution of extensively drug-resistant Mycobacterium tuberculosisfrom a susceptible ancestor in a single patient. Genome Biol 15:490.

12. Black PA, Vos M de, Louw GE, Merwe R van der, Dippenaar A, Streicher EM, Abdallah AM, Sampson SL, Victor TC, Dolby T, Simpson JA, Helden P van, Warren RM, Pain A. 2015. Whole genome sequencing reveals genomic heterogeneity and antibiotic purification in Mycobacterium tuberculosis isolates. BMC Genomics 16:857.

13. Vitti JJ, Grossman SR, Sabeti PC. 2013. Detecting Natural Selection in Genomic Data. Annu Rev Genet 47:97–120.

14. Hill WG, Robertson A. 1966. The effect of linkage on limits to artificial selection. Genet Res.

15. Felsenstein J. 1974. The Evolutionary Advantage of Recombination. Genetics 78:737–756.

16. Birky CW, Walsh JB. 1988. Effects of linkage on rates of molecular evolution. Proc Natl Acad Sci 85:6414–6418.

17. Silva PEAD, Palomino JC. 2011. Molecular basis and mechanisms of drug resistance in Mycobacterium tuberculosis: classical and new drugs. J Antimicrob Chemother 66:1417–1430.

18. Gagneux S, Long CD, Small PM, Van T, Schoolnik GK, Bohannan BJM. 2006. The Competitive Cost of Antibiotic Resistance in Mycobacterium tuberculosis. Science 312:1944–1946.

19. Böttger EC, Springer B. 2008. Tuberculosis: drug resistance, fitness, and strategies for global control. Eur J Pediatr 167:141–148.

20. Strauss OJ, Warren RM, Jordaan A, Streicher EM, Hanekom M, Falmer AA, Albert H, Trollip A, Hoosain E, Helden PD van, Victor TC. 2008. Spread of a Low-Fitness Drug-Resistant Mycobacterium tuberculosis Strain in a Setting of High Human Immunodeficiency Virus Prevalence. J Clin Microbiol 46:1514–1516.

21. Brandis G, Wrande M, Liljas L, Hughes D. 2012. Fitness-compensatory mutations in rifampicin-resistant RNA polymerase. Mol Microbiol 85:142–151.

22. Comas I, Borrell S, Roetzer A, Rose G, Malla B, Kato-Maeda M, Galagan J, Niemann S, Gagneux S. 2012. Whole-genome sequencing of rifampicin-resistant Mycobacterium tuberculosis strains identifies compensatory mutations in RNA polymerase genes. Nat Genet 44:106–110.

23. Gagneux S, Burgos MV, DeRiemer K, Enciso A, Muñoz S, Hopewell PC, Small PM, Pym AS. 2006. Impact of Bacterial Genetics on the Transmission of Isoniazid-Resistant Mycobacterium tuberculosis. PLOS Pathog 2:e61.

24. Farhat MR, Shapiro BJ, Kieser KJ, Sultana R, Jacobson KR, Victor TC, Warren RM, Streicher EM, Calver A, Sloutsky A, Kaur D, Posey JE, Plikaytis B, Oggioni MR, Gardy JL,

bioRxiv preprint doi: https://doi.org/10.1101/173229; this version posted January 8, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

Johnston JC, Rodrigues M, Tang PKC, Kato-Maeda M, Borowsky ML, Muddukrishna B, Kreiswirth BN, Kurepina N, Galagan J, Gagneux S, Birren B, Rubin EJ, Lander ES, Sabeti PC, Murray M. 2013. Genomic analysis identifies targets of convergent positive selection in drug-resistant Mycobacterium tuberculosis. Nat Genet advance online publication.

25. Zhang H, Li D, Zhao L, Fleming J, Lin N, Wang T, Liu Z, Li C, Galwey N, Deng J, Zhou Y, Zhu Y, Gao Y, Wang T, Wang S, Huang Y, Wang M, Zhong Q, Zhou L, Chen T, Zhou J, Yang R, Zhu G, Hang H, Zhang J, Li F, Wan K, Wang J, Zhang X-E, Bi L. 2013. Genome sequencing of 161 Mycobacterium tuberculosis isolates from China identifies genes and intergenic regions associated with drug resistance. Nat Genet advance online publication.

26. O'Neill MB, Mortimer TD, Pepperell CS. 2015. Diversity of Mycobacterium tuberculosis across Evolutionary Scales. PLoS Pathog 11:e1005257.

27. Luo T, Comas I, Luo D, Lu B, Wu J, Wei L, Yang C, Liu Q, Gan M, Sun G, Shen X, Liu F, Gagneux S, Mei J, Lan R, Wan K, Gao Q. 2015. Southern East Asian origin and coexpansion of Mycobacterium tuberculosis Beijing family with Han Chinese. Proc Natl Acad Sci 112:8136–8141.

28. O'Neill MB, Kitchen A, Zarley A, Aylward W, Eldholm V, Pepperell CS. 2017. Lineage specific histories of Mycobacterium tuberculosis dispersal in Africa and Eurasia. bioRxiv 210161.

29. Achtman M, Zurth K, Morelli G, Torrea G, Guiyoule A, Carniel E. 1999. Yersinia pestis, the cause of plague, is a recently emerged clone of Yersinia pseudotuberculosis. Proc Natl Acad Sci 96:14043–14048.

30. Larsson P, Elfsmark D, Svensson K, Wikström P, Forsman M, Brettin T, Keim P, Johansson A. 2009. Molecular Evolutionary Consequences of Niche Restriction in Francisella tularensis, a Facultative Intracellular Pathogen. PLoS Pathog 5:e1000472.

31. Vandelannoote K, Meehan CJ, Eddyani M, Affolabi D, Phanzu DM, Eyangoh S, Jordaens K, Portaels F, Mangas K, Seemann T, Marsollier L, Marion E, Chauty A, Landier J, Fontanet A, Leirs H, Stinear TP, Jong D, C B. 2017. Multiple Introductions and Recent Spread of the Emerging Human Pathogen Mycobacterium ulcerans across Africa. Genome Biol Evol 9:414–426.

32. Sandgren A, Strong M, Muthukrishnan P, Weiner BK, Church GM, Murray MB. 2009. Tuberculosis Drug Resistance Mutation Database. PLOS Med 6:e1000002.

33. Nguyen D, Brassard P, Westley J, Thibert L, Proulx M, Henry K, Schwartzman K, Menzies D, Behr MA. 2003. Widespread Pyrazinamide-Resistant Mycobacterium tuberculosis Family in a Low-Incidence Setting. J Clin Microbiol 41:2878–2883.

34. Nguyen D, Brassard P, Menzies D, Thibert L, Warren R, Mostowy S, Behr M. 2004. Genomic Characterization of an Endemic Mycobacterium tuberculosis Strain: Evolutionary and Epidemiologic Implications. J Clin Microbiol 42:2573–2580.

35. Brassard P, Henry KA, Schwartzman K, Jomphe M, Olson SH. 2008. Geography and genealogy of the human host harbouring a distinctive drug-resistant strain of tuberculosis. Infect Genet Evol 8:247–257.

bioRxiv preprint doi: https://doi.org/10.1101/173229; this version posted January 8, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

625 36. Okamoto S, Tamaru A, Nakajima C, Nishimura K, Tanaka Y, Tokuyama S, Suzuki Y, Ochi
626 K. 2007. Loss of a conserved 7-methylguanosine modification in 16S rRNA confers low-
627 level streptomycin resistance in bacteria. Mol Microbiol 63:1096–1106.

628 37. Spies FS, Ribeiro AW, Ramos DF, Ribeiro MO, Martin A, Palomino JC, Rossetti MLR,
629 Silva PEA da, Zaha A. 2011. Streptomycin Resistance and Lineage-Specific
630 Polymorphisms in Mycobacterium tuberculosis gidB Gene. J Clin Microbiol 49:2625–2630.

631 38. Feuerriegel S, Oberhauser B, George AG, Dafae F, Richter E, Rüsch-Gerdes S, Niemann
632 S. 2012. Sequence analysis for detection of first-line drug resistance in Mycobacterium
633 tuberculosis strains from a high-incidence setting. BMC Microbiol 12:90.

634 39. Jagielski T, Ignatowska H, Bakuła Z, Dziewit Ł, Napiórkowska A, Augustynowicz-Kopeć E,
635 Zwolska Z, Bielecki J. 2014. Screening for Streptomycin Resistance-Conferring Mutations
636 in Mycobacterium tuberculosis Clinical Isolates from Poland. PLOS ONE 9:e100078.

637 40. Homolka S, Meyer CG, Hillemann D, Owusu-Dabo E, Adjei O, Horstmann RD, Browne
638 ENL, Chinbuah A, Osei I, Gyapong J, Kubica T, Ruesch-Gerdes S, Niemann S. 2010.
639 Unequal distribution of resistance-conferring mutations among Mycobacterium tuberculosis
640 and Mycobacterium africanum strains from Ghana. Int J Med Microbiol 300:489–495.

641 41. Fenner L, Egger M, Bodmer T, Altpeter E, Zwahlen M, Jaton K, Pfyffer GE, Borrell S,
642 Dubuis O, Bruderer T, Siegrist HH, Furrer H, Calmy A, Fehr J, Stalder JM, Ninet B, Böttger
643 EC, Gagneux S, Group for the SHCS and the SME of TS. 2012. Effect of Mutation and
644 Genetic Background on Drug Resistance in Mycobacterium tuberculosis. Antimicrob
645 Agents Chemother 56:3047–3053.

646 42. Wengenack NL, Uhl JR, Amand S, L A, Tomlinson AJ, Benson LM, Naylor S, Kline BC,
647 Cockerill FR, Rusnak F. 1997. Recombinant Mycobacterium tuberculosis KatG(S315T) Is a
648 Competent Catalase-Peroxidase with Reduced Activity toward Isoniazid. J Infect Dis
649 176:722–727.

650 43. Pym AS, Saint-Joanis B, Cole ST. 2002. Effect of katG Mutations on the Virulence of
651 Mycobacterium tuberculosis and the Implication for Transmission in Humans. Infect Immun
652 70:4955–4960.

653 44. Spies FS, von Groll A, Ribeiro AW, Ramos DF, Ribeiro MO, Dalla Costa ER, Martin A,
654 Palomino JC, Rossetti ML, Zaha A, da Silva PEA. 2013. Biological cost in Mycobacterium
655 tuberculosis with mutations in the rpsL, rrs, rpoB, and katG genes. Tuberculosis 93:150–
656 154.

657 45. Manson AL, Cohen KA, Abeel T, Desjardins CA, Armstrong DT, Barry Iii CE, Brand J,
658 TBResist Global Genome Consortium, Chapman SB, Cho S-N, Gabrielian A, Gomez J,
659 Jodals AM, Joloba M, Jureen P, Lee JS, Malinga L, Maiga M, Nordenberg D, Noroc E,
660 Romancenco E, Salazar A, Ssengooba W, Velayati AA, Winglee K, Zalutskaya A, Via LE,
661 Cassell GH, Dorman SE, Ellner J, Farnia P, Galagan JE, Rosenthal A, Crudu V,
662 Homorodean D, Hsueh P-R, Narayanan S, Pym AS, Skrahina A, Swaminathan S, Van der
663 Walt M, Alland D, Bishai WR, Cohen T, Hoffner S, Birren BW, Earl AM. 2017. Genomic
664 analysis of globally diverse Mycobacterium tuberculosis strains provides insights into the
665 emergence and spread of multidrug resistance. Nat Genet advance online publication.

bioRxiv preprint doi: https://doi.org/10.1101/173229; this version posted January 8, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

666 46. Billington OJ, McHugh TD, Gillespie SH. 1999. Physiological Cost of Rifampin Resistance
667 Induced In Vitro in Mycobacterium tuberculosis. Antimicrob Agents Chemother 43:1866–
668 1869.

669 47. Eilertson B, Maruri F, Blackman A, Herrera M, Samuels DC, Sterling TR. 2014. High
670 Proportion of Heteroresistance in gyrA and gyrB in Fluoroquinolone-Resistant
671 Mycobacterium tuberculosis Clinical Isolates. Antimicrob Agents Chemother 58:3270–
672 3275.

673 48. Hazbón MH, Valle MB del, Guerrero MI, Varma-Basil M, Filliol I, Cavatore M, Colangeli R,
674 Safi H, Billman-Jacobe H, Lavender C, Fyfe J, García-García L, Davidow A, Brimacombe
675 M, León CI, Porras T, Bose M, Chaves F, Eisenach KD, Sifuentes-Osornio J, León AP de,
676 Cave MD, Alland D. 2005. Role of embB Codon 306 Mutations in Mycobacterium
677 tuberculosis Revisited: a Novel Association with Broad Drug Resistance and IS6110
678 Clustering Rather than Ethambutol Resistance. Antimicrob Agents Chemother 49:3794–
679 3802.

680 49. DeJesus MA, Gerrick ER, Xu W, Park SW, Long JE, Boutte CC, Rubin EJ, Schnappinger
681 D, Ehrt S, Fortune SM, Sassetti CM, Ioerger TR. 2017. Comprehensive Essentiality
682 Analysis of the Mycobacterium tuberculosis Genome via Saturating Transposon
683 Mutagenesis. mBio 8:e02133-16.

684 50. Shekar S, Yeo ZX, Wong JCL, Chan MKL, Ong DCT, Tongyoo P, Wong S-Y, Lee ASG.
685 2014. Detecting Novel Genetic Variants Associated with Isoniazid-Resistant
686 Mycobacterium tuberculosis. PLOS ONE 9:e102383.

687 51. Danilchanka O, Mailaender C, Niederweis M. 2008. Identification of a Novel Multidrug
688 Efflux Pump of Mycobacterium tuberculosis. Antimicrob Agents Chemother 52:2503–2511.

689 52. Fu LM, Shinnick TM. 2007. Genome-wide exploration of the drug action of capreomycin on
690 Mycobacterium tuberculosis using Affymetrix oligonucleotide GeneChips. J Infect 54:277–
691 284.

692 53. Phong TQ, Ha DTT, Volker U, Hammer E. 2015. Using a Label Free Quantitative
693 Proteomics Approach to Identify Changes in Protein Abundance in Multidrug-Resistant
694 Mycobacterium tuberculosis. Indian J Microbiol 55:219–230.

695 54. Phelan J, Coll F, McNerney R, Ascher DB, Pires DEV, Furnham N, Coeck N, Hill-
696 Cawthorne GA, Nair MB, Mallard K, Ramsay A, Campino S, Hibberd ML, Pain A, Rigouts
697 L, Clark TG. 2016. Mycobacterium tuberculosis whole genome sequencing and protein
698 structure modelling provides insights into anti-tuberculosis drug resistance. BMC Med
699 14:31.

700 55. Andrews S. 2012. FastQC.

701 56. Kreuger F. 2013. TrimGalore!

702 57. Li H. 2013. Aligning sequence reads, clone sequences and assembly contigs with BWA-
703 MEM. ArXiv13033997 Q-Bio.

bioRxiv preprint doi: https://doi.org/10.1101/173229; this version posted January 8, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

58. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R. 2009. The Sequence Alignment/Map format and SAMtools. Bioinformatics 25:2078–2079.

59. Walker BJ, Abeel T, Shea T, Priest M, Abouelliel A, Sakthikumar S, Cuomo CA, Zeng Q, Wortman J, Young SK, Earl AM. 2014. Pilon: An Integrated Tool for Comprehensive Microbial Variant Detection and Genome Assembly Improvement. PLOS ONE 9:e112963.

60. Price MN, Dehal PS, Arkin AP. 2010. FastTree 2 – Approximately Maximum-Likelihood Trees for Large Alignments. PLOS ONE 5:e9490.

61. Page AJ, Taylor B, Delaney AJ, Soares J, Seemann T, Keane JA, Harris SR. 2016. SNP-sites: rapid efficient extraction of SNPs from multi-FASTA alignments. biorxiv;038190v1.

62. Cingolani P, Platts A, Wang LL, Coon M, Nguyen T, Wang L, Land SJ, Lu X, Ruden DM. 2012. A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff. Fly (Austin) 6:80–92.

63. Salazar A, Earl A, Desjardins C, Abeel T. 2015. Normalizing alternate representations of large sequence variants across multiple bacterial genomes. BMC Bioinformatics 16:A8.

64. De Mita S, Siol M. 2012. EggLib: processing, analysis and simulation tools for population genetics and genomics. BMC Genet 13:27.

65. R Core Team. 2015. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

66. Weir BS, Cockerham CC. 1984. Estimating F-Statistics for the Analysis of Population Structure. Evolution 38:1358–1370.

67. Sagulenko P, Puller V, Neher R. 2017. TreeTime: maximum likelihood phylodynamic analysis. bioRxiv 153494.

bioRxiv preprint doi: https://doi.org/10.1101/173229; this version posted January 8, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

729 **Table 1. Frequency of resistance in data set.** AMI- amikacin, CAP- capreomycin,
730 EMB- ethambutol, Et- ethionamide, INH- isoniazid, K- kanamycin, MOX- moxifloxacin,
731 OFL- ofloxacin, PRO- protionamide, PZA- pyrazinamide, RIF- rifampin, STR-
732 streptomycin.

| Drug | Resistant | Susceptible | Unknown |
|---|---|---|---|
| **INH** | 0.59 | 0.33 | 0.08 |
| **STR** | 0.53 | 0.39 | 0.07 |
| **RIF** | 0.50 | 0.43 | 0.07 |
| **EMB** | 0.30 | 0.59 | 0.11 |
| **PZA** | 0.21 | 0.67 | 0.12 |
| **OFL** | 0.16 | 0.39 | 0.45 |
| **PRO** | 0.15 | 0.22 | 0.62 |
| **CAP** | 0.10 | 0.41 | 0.49 |
| **MOX** | 0.09 | 0.41 | 0.49 |
| **Et** | 0.06 | 0.07 | 0.88 |
| **AMI** | 0.05 | 0.34 | 0.61 |
| **K** | 0.05 | 0.12 | 0.83 |

733

734

bioRxiv preprint doi: https://doi.org/10.1101/173229; this version posted January 8, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

735 **Table 2. Signatures of selection in known drug resistance genes.** The number of
736 distinct entries in the TB Drug Resistance Mutation Database for each gene is reported
737 in TB Dream column. π and θ are the percentiles for each diversity value, respectively.
738 TD is the percentile of the residual after linear regression of Tajima's D with gene
739 length. Genes with homoplastic SNPs are indicated with 'Y' in the Homoplasy column. If
740 a homoplastic SNP was also an $F_{ST}$ outlier, it is indicated with a 'Y' in the $F_{ST}$ column.
741 Genes are classified as tight, sloppy, or hybrid targets of selection based on diversity,
742 homoplasy, and $F_{ST}$ results. (IG) indicates an intergenic SNP.

| Gene | Rv Number | Drug | TB Dream | π | θ | TD | Homoplasy | $F_{ST}$ | Type |
|---|---|---|---|---|---|---|---|---|---|
| *katG* | *Rv1908c* | INH | 226 | 0.80 | 0.82 | 0.34 | Y | Y | tight |
| *pncA* | *Rv2043c* | PZA | 195 | 0.97 | 1.00 | 0.00 | Y | N | sloppy |
| *embB* | *Rv3795* | EMB | 117 | 0.77 | 0.89 | 0.08 | Y | Y | hybrid |
| *ahpC* | *Rv2428* | INH | 31 | 0.20 | 0.21 | 0.61 | Y | N | - |
| *tlyA* | *Rv1694* | CAP | 28 | 0.37 | 0.89 | 0.06 | N | N | - |
| *embC* | *Rv3793* | EMB | 28 | 0.59 | 0.74 | 0.01 | N | N | - |
| *embR* | *Rv1267c* | EMB | 25 | 0.46 | 0.49 | 0.28 | N | N | - |
| *rrs* | *Rvnr01* | STR, K, CAP | 24 | 0.89 | 1.00 | 0.08 | N | N | - |
| *ethA* | *Rv3854c* | Et | 23 | 0.72 | 1.00 | 0.00 | Y | Y (IG) | sloppy, tight (IG) |
| *gid* | *Rv3919c* | STR | 22 | 1.00 | 1.00 | 0.07 | Y | N | sloppy |
| *gyrB* | *Rv0005* | MOX, OFL | 15 | 0.58 | 0.91 | 0.00 | Y | N | - |
| *fabG1* | *Rv1483* | INH, Et | 13 | 0.60 | 0.66 | 0.30 | Y | Y (IG) | tight |
| *inhA* | *Rv1484* | INH, Et | 13 | 0.56 | 0.59 | 0.32 | Y | N | - |
| *rpsL* | *Rv0682* | STR | 13 | 0.99 | 0.95 | 0.80 | Y | Y | hybrid |
| *gyrA* | *Rv0006* | MOX, OFL | 12 | 0.81 | 0.94 | 0.10 | Y | Y | tight |
| *embA* | *Rv3794* | EMB | 11 | 0.77 | 0.38 | 0.79 | N | N | - |
| *kasA* | *Rv2245* | INH | 7 | 0.73 | 0.18 | 0.86 | N | N | - |
| *ndh* | *Rv1854c* | INH | 5 | 0.57 | 0.52 | 0.28 | N | N | - |
| *iniA* | *Rv0342* | EMB, INH | 4 | 0.64 | 0.33 | 0.56 | N | N | - |
| *Rv0340* | *Rv0340* | INH | 3 | 0.89 | 0.88 | 0.57 | N | N | - |
| *iniB* | *Rv0341* | EMB, INH | 3 | 0.07 | 0.07 | 0.79 | N | N | - |
| *fbpC* | *Rv0129c* | INH | 3 | 0.78 | 0.19 | 0.89 | N | N | - |
| *rmlD* | *Rv3266c* | EMB | 2 | 0.75 | 0.36 | 0.75 | N | N | - |
| *iniC* | *Rv0343* | EMB, INH | 2 | 0.49 | 0.67 | 0.08 | N | N | - |
| *thyA* | *Rv2764c* | PAS | 2 | 0.84 | 0.94 | 0.28 | N | N | - |
| *nat* | *Rv3566c* | INH | 2 | 0.76 | 0.55 | 0.63 | N | N | - |
| *accD6* | *Rv2247* | INH | 1 | 0.90 | 0.63 | 0.90 | N | N | - |
| *furA* | *Rv1909c* | INH | 1 | 0.80 | 0.63 | 0.62 | N | N | - |
| *Rv1772* | *Rv1772* | INH | 1 | 0.50 | 0.35 | 0.54 | N | N | - |
| *fabD* | *Rv2243* | INH | 1 | 0.26 | 0.28 | 0.54 | N | N | - |
| *fadE24* | *Rv3139* | INH | 1 | 0.36 | 0.58 | 0.12 | N | N | - |
| *rpoB* | *Rv0667* | RIF | 1 | 0.82 | 0.92 | 0.18 | Y | Y | hybrid |
| *efpA* | *Rv2846c* | INH | 1 | 0.10 | 0.11 | 0.65 | N | N | - |

bioRxiv preprint doi: https://doi.org/10.1101/173229; this version posted January 8, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *ethR* | *Rv3855* | Et | - | 0.58 | 0.77 | 0.22 | N | N | - |
| *Rv0678* | *Rv0678* | BDQ | - | 0.37 | 0.72 | 0.25 | N | N | - |
| *eis* | *Rv2416c* | K | - | 0.51 | 0.28 | 0.54 | N | N | - |
| *mshA* | *Rv0486* | Et | - | 0.86 | 0.48 | 0.87 | N | N | - |
| *rpsA* | *Rv1630* | PZA | - | 0.88 | 0.62 | 0.84 | N | N | - |
| *folC* | *Rv2447c* | PAS | - | 0.66 | 0.78 | 0.11 | Y | N | - |
| *rplC* | *Rv0701* | LZD | - | 0.57 | 0.77 | 0.21 | N | N | - |

743

744 **Table 3. Homoplastic $F_{ST}$ outliers.** Weir and Cockerham's $F_{ST}$ (wcFst) values in the top 1% of values genome wide are
745 reported for each drug. For intergenic SNPs, the closest gene is listed. We identified mutations in genes previously
746 associated with drug resistance (Known = Y) and novel putative resistance or compensatory mutations (Known = N).

| Location | Gene | Type | AMI | CAP | EMB | Et | INH | K | MOX | OFL | PRO | PZA | RIF | STR | Known | Lineage |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1821 | *dnaN* | intergenic | - | - | - | 0.43 | - | 0.57 | - | 0.10 | - | - | - | - | N | all |
| 7570 | *gyrA* | missense | - | 0.33 | 0.11 | 0.46 | - | 0.66 | - | 0.29 | - | 0.18 | - | - | Y | all |
| 7572 | *gyrA* | missense | - | - | - | - | - | - | 0.06 | - | - | - | - | - | Y | all |
| 7581 | *gyrA* | missense | - | - | - | - | - | - | 0.07 | - | - | - | - | - | Y | all |
| 7582 | *gyrA* | missense | - | - | - | - | - | - | 0.35 | 0.22 | - | - | - | - | Y | all |
| 75233 | *icd2* | intergenic | - | - | - | - | - | - | 0.05 | - | - | - | - | - | N | all |
| 94388 | *hycQ* | synonymous | - | - | 0.07 | - | 0.12 | - | - | - | - | - | 0.12 | 0.13 | N | all |
| 230170 | *Rv0194* | missense | - | - | - | - | 0.12 | - | 0.05 | - | - | - | 0.12 | 0.13 | N | all |
| 332916 | *vapC25* | missense | - | - | 0.10 | - | - | - | - | 0.09 | - | 0.20 | - | - | N | all |
| 761155 | *rpoB* | missense | - | - | 0.31 | - | 0.58 | - | - | - | - | 0.10 | 0.72 | 0.41 | Y | all |
| 761161 | *rpoB* | missense | - | 0.33 | 0.09 | 0.51 | - | 0.71 | - | 0.13 | - | 0.16 | - | - | Y | all |
| 764817 | *rpoC* | missense | 0.19 | - | - | - | - | - | - | - | - | - | - | - | N | all |
| 781687 | *rpsL* | missense | - | - | 0.10 | - | 0.32 | - | - | - | - | 0.15 | - | 0.37 | Y | all |
| 922004 | *Rv0830* | missense | - | 0.30 | 0.12 | 0.43 | - | - | - | 0.10 | - | 0.21 | - | - | N | all |
| 1076880 | *Rv0965c* | synonymous | - | - | - | - | 0.12 | - | - | - | - | - | 0.12 | 0.13 | N | all |
| 1673425 | *fabG1* | intergenic | - | - | - | - | - | - | - | - | 0.11 | - | - | - | Y | all |
| 1673432 | *fabG1* | intergenic | - | - | - | 0.52 | - | 0.65 | - | - | - | - | - | - | Y | all |
| 1722228 | *pks5* | missense | - | - | 0.08 | - | 0.28 | - | - | 0.07 | - | 0.17 | - | 0.26 | N | all |
| 2122395 | *lldD2* | synonymous | - | - | - | - | - | - | 0.06 | - | - | - | - | - | N | all |
| 2155168 | *katG* | missense | - | - | 0.36 | - | 0.89 | - | - | 0.13 | - | 0.32 | 0.60 | 0.66 | Y | all |
| 2174216 | *Rv1922* | synonymous | - | - | - | - | - | - | - | - | - | 0.08 | - | - | N | all |
| 2207525 | *Rv1958c* | intergenic | - | - | - | - | - | - | - | - | - | 0.09 | - | - | N | all |
| 2422824 | *Rv2161c* | missense | - | 0.30 | - | 0.43 | - | 0.57 | - | 0.10 | - | - | - | - | N | all |
| 2660319 | *mbtF* | missense | - | - | 0.06 | - | - | - | - | - | - | - | - | - | N | all |
| 2715369 | *Rv3413c* | intergenic | 0.17 | - | 0.09 | - | 0.28 | - | - | - | - | - | 0.30 | 0.13 | N | all |
| 2866647 | *lppA* | synonymous | - | - | - | - | 0.12 | - | 0.07 | - | - | - | - | - | N | all |

bioRxiv preprint doi: https://doi.org/10.1101/173229; this version posted January 8, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

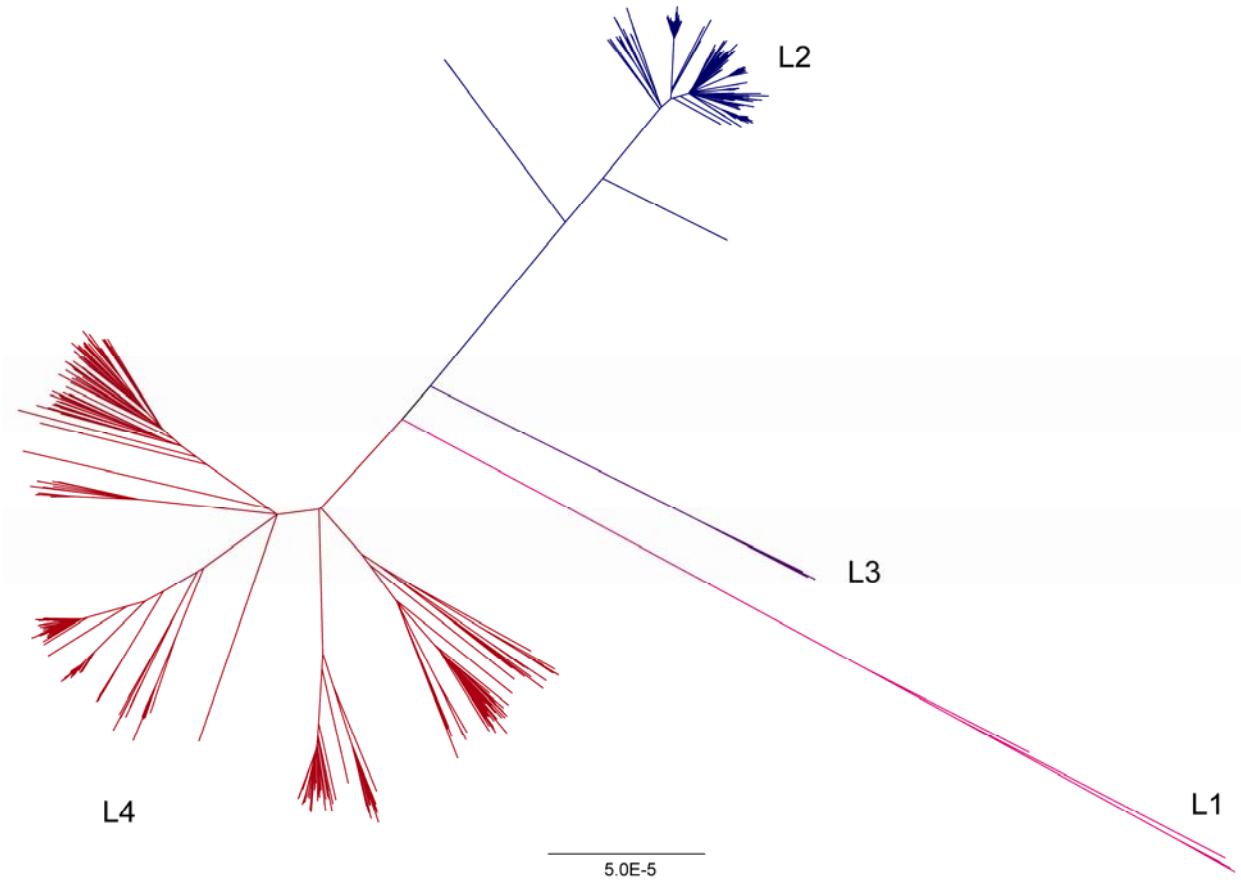
| | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2867298 | *lppB* | synonymous | - | - | - | - | 0.13 | - | - | - | - | - | - | - | N | all |
| 2867347 | *lppB* | synonymous | - | - | - | - | 0.13 | - | 0.06 | - | - | - | 0.12 | 0.14 | N | all |
| 2867756 | *lppB* | synonymous | - | - | - | - | 0.14 | - | - | - | - | - | - | - | N | all |
| 3500149 | *Rv3134c* | synonymous | - | - | - | - | - | - | - | - | - | - | 0.11 | - | N | all |
| 3550789 | *Rv3183* | synonymous | - | - | - | - | - | - | - | - | - | - | 0.12 | 0.13 | N | all |
| 3680932 | *lhr* | synonymous | - | - | - | - | 0.12 | - | - | - | - | - | 0.12 | 0.13 | N | all |
| 4001622 | *fadA6* | intergenic | - | - | - | - | - | - | - | - | - | - | 0.11 | - | N | all |
| 4247429 | *embB* | missense | - | - | 0.25 | 0.45 | 0.23 | - | 0.05 | 0.11 | - | 0.31 | 0.21 | 0.20 | Y | all |
| 4247574 | *embB* | synonymous | 0.19 | - | 0.07 | - | 0.27 | - | - | - | - | - | 0.30 | - | Y | all |
| 4327480 | *ethA* | intergenic | 0.20 | - | 0.07 | - | 0.27 | - | - | - | - | - | 0.30 | - | Y | all |
| 764948 | *rpoC* | missense | - | - | - | - | - | - | - | - | 0.06 | - | - | - | Y | L2 |
| 4248003 | *embB* | missense | - | 0.16 | - | - | - | - | - | - | - | - | - | - | Y | L2 |
| 698 | *dnaA* | missense | - | - | - | - | - | - | - | - | - | - | - | 0.10 | N | L4 |
| 60185 | *Rv0057* | missense | - | - | - | - | - | - | - | - | - | - | - | 0.06 | N | L4 |
| 761110 | *rpoB* | missense | 0.66 | - | - | - | - | - | - | - | - | - | - | - | Y | L4 |
| 764822 | *rpoC* | missense | - | - | - | - | - | - | - | - | - | - | - | 0.06 | Y | L4 |
| 781822 | *rpsL* | missense | - | - | - | - | 0.12 | - | - | - | - | - | 0.13 | 0.14 | Y | L4 |
| 2123145 | *lldD2* | missense | - | - | - | - | - | - | - | - | - | - | - | 0.06 | N | L4 |
| 2372550 | *dop* | missense | 0.64 | - | - | - | - | - | - | - | - | - | - | - | N | L4 |
| 2715344 | *Rv2413c* | intergenic | - | - | - | - | - | - | - | - | - | - | - | 0.06 | N | L4 |
| 2986827 | *Rv2670c* | missense | - | - | - | - | 0.16 | - | - | - | - | - | 0.17 | 0.15 | N | L4 |
| 4247431 | *embB* | missense | - | - | - | - | 0.11 | - | - | - | - | - | 0.11 | 0.07 | Y | L4 |
| 4248003 | *embB* | missense | - | - | - | - | - | - | - | - | - | - | - | 0.06 | Y | L4 |

747

748

bioRxiv preprint doi: https://doi.org/10.1101/173229; this version posted January 8, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

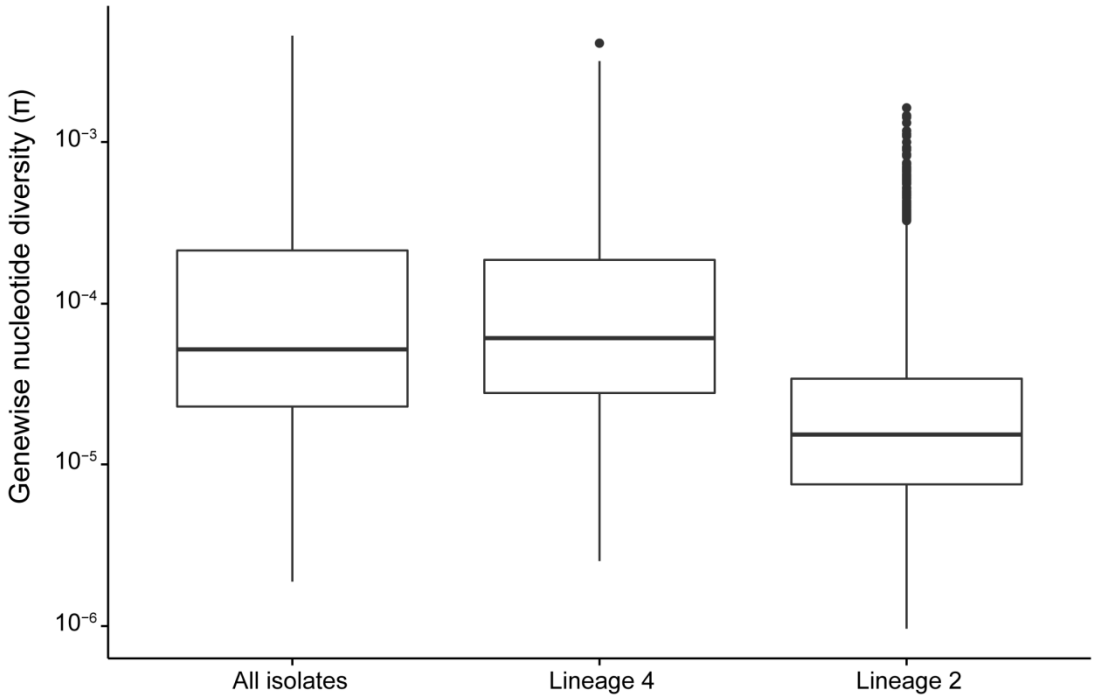
bioRxiv preprint doi: https://doi.org/10.1101/173229; this version posted January 8, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.




749

750 **Figure 1. Phylogeny of *Mycobacterium tuberculosis* sample.** The phylogeny was
751 inferred using FastTree (60). Lineages are colored as follows: lineage 1 (L1) - pink,
752 lineage 2 (L2) - blue, lineage 3 (L3) - purple, lineage 4 (L4) - red. Lineage 4 is
753 associated with deeper branching sub-lineages in comparison with lineage 2.

bioRxiv preprint doi: https://doi.org/10.1101/173229; this version posted January 8, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.




754

755 **Figure 2. Distributions of gene-wise nucleotide diversity for all isolates, as well as**
756 **lineages 4 and 2 considered separately.** Repetitive regions of the alignment were
757 masked. Sites were included in estimation of π if 95% of isolates in the alignment had a
758 valid nucleotide at the position. We used Egglib to calculate statistics (64). Nucleotide
759 diversity is lower in lineage 2 compared to lineage 4 (Welch Two Sample t-test, $p < 2.2 \times 10^{-16}$)

761

bioRxiv preprint doi: https://doi.org/10.1101/173229; this version posted January 8, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

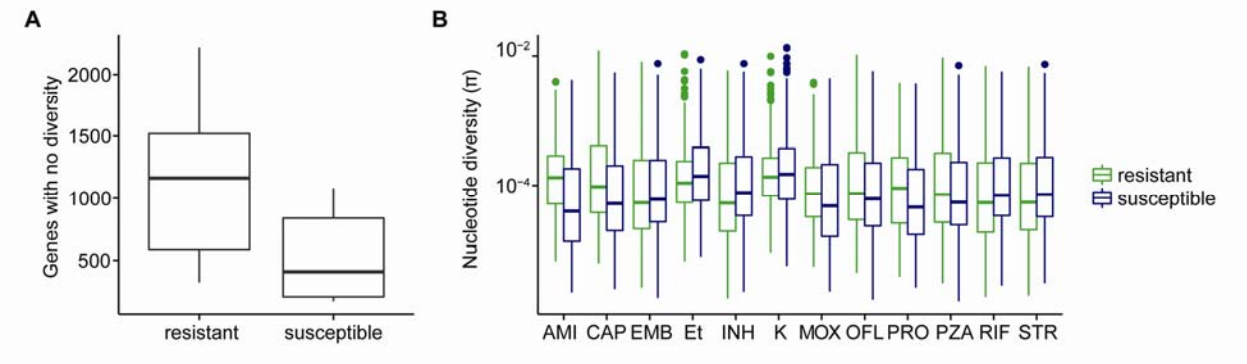



**Figure 3. Diversity of resistant and susceptible isolates.** A) Counts of genes with no nucleotide diversity in resistant and susceptible subpopulations. B) Genewise nucleotide diversity (excluding invariant genes) in susceptible and resistant isolates. Among genes in which it is measurable, nucleotide diversity is similar between resistant and susceptible isolates even when drug resistance associated genes and targets of independent mutation identified by Farhat et al. 2013 are removed ($p$ = 0.13).

bioRxiv preprint doi: https://doi.org/10.1101/173229; this version posted January 8, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

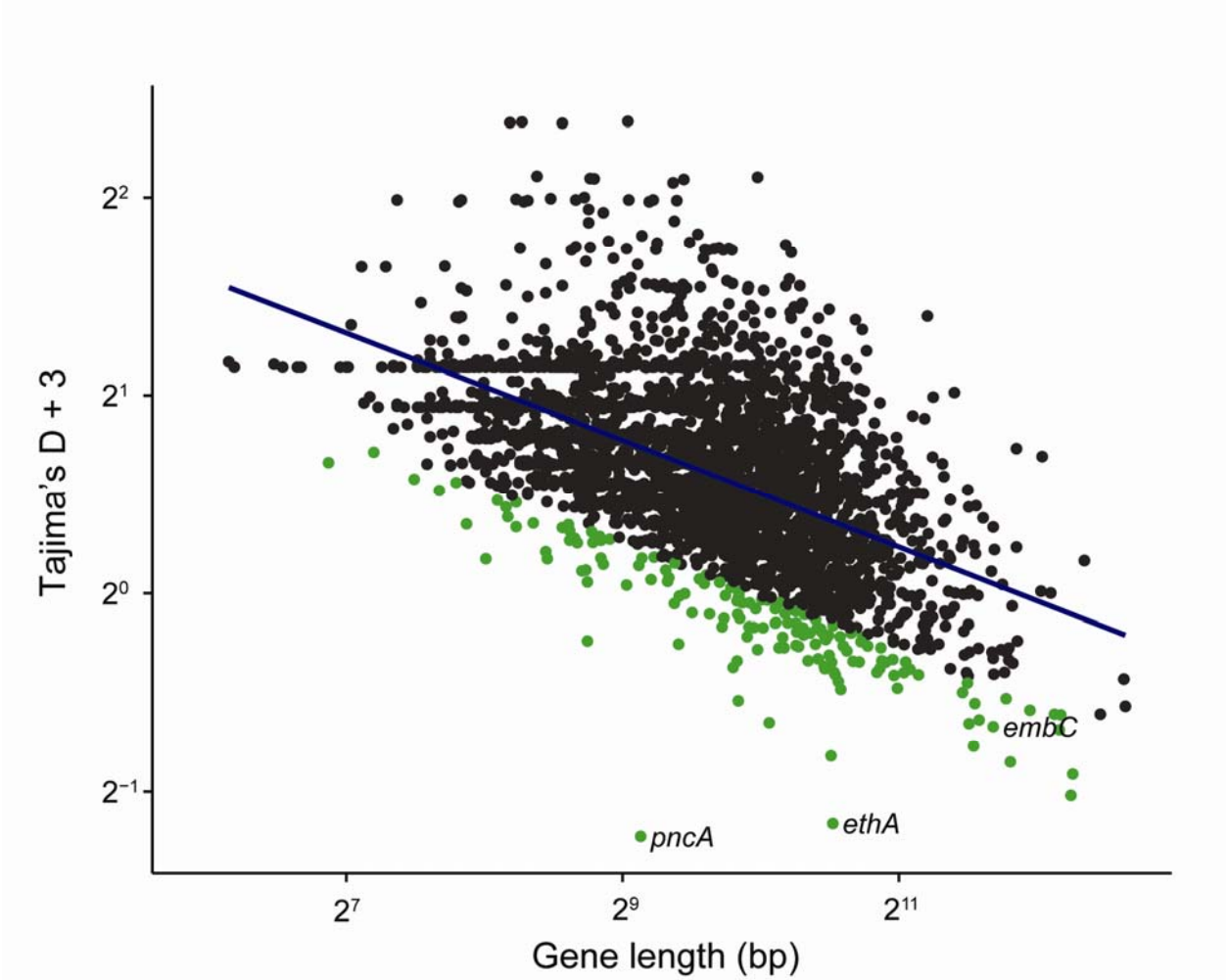
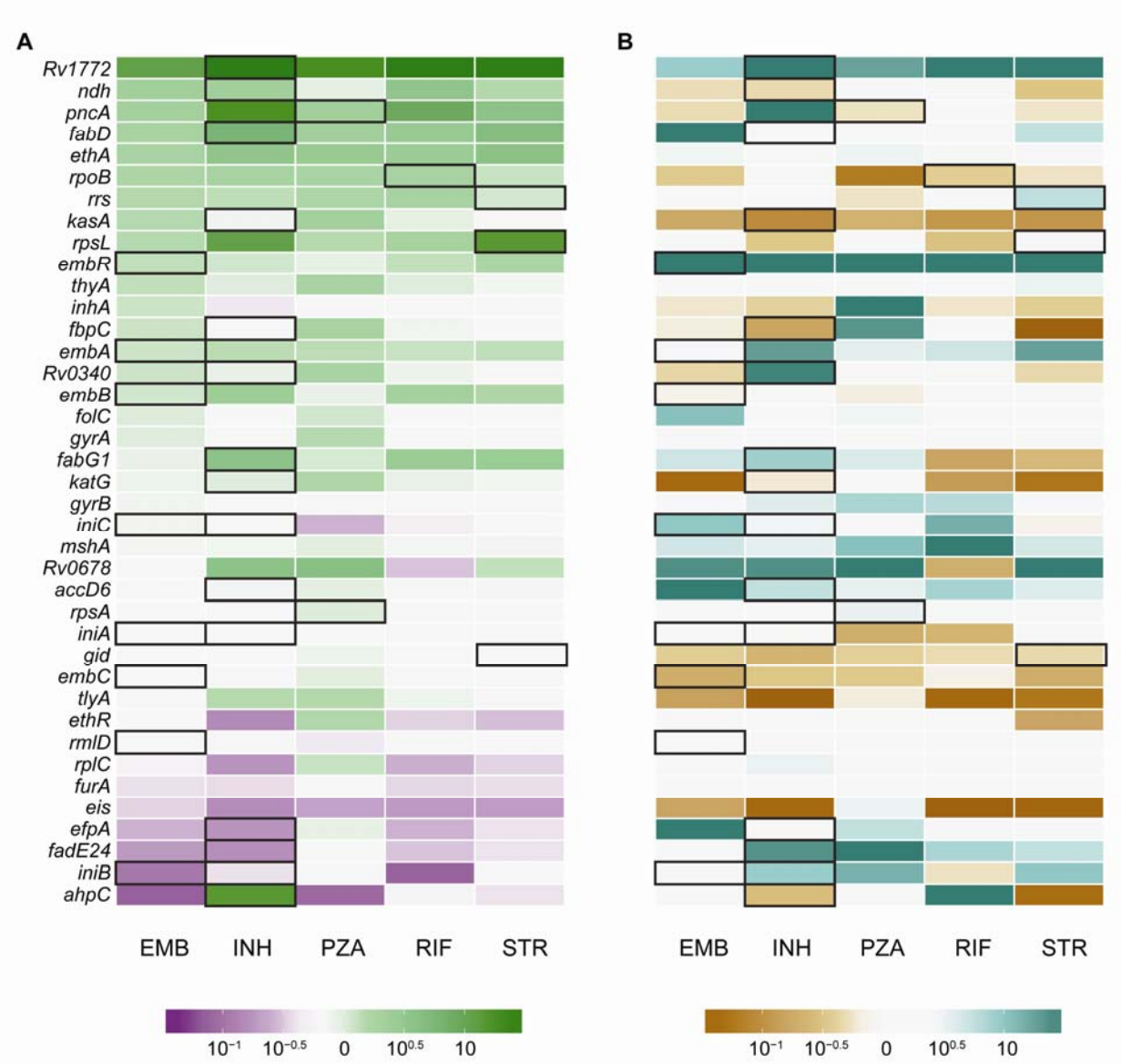



**Figure 4. Gene-wise Tajima's D and gene length.** Repetitive regions of the alignment were masked. Gene lengths have been log transformed (base 2). We added a constant value (3) to all Tajima's D values to make them positive and log transformed (base 2), as with the gene lengths. The linear regression line is plotted in blue. Genes with regression values in the lower 5% are highlighted in green. Drug resistance associated genes in this group are labelled. While negative Tajima's D is normally associated with purifying selection or a recent selective sweep, we find that drug resistance genes with negative Tajima's D also have high nucleotide diversity. We hypothesize that patterns of diversity at these genes have been affected by relaxation of purifying selection and positive selection in association with for drug resistance.

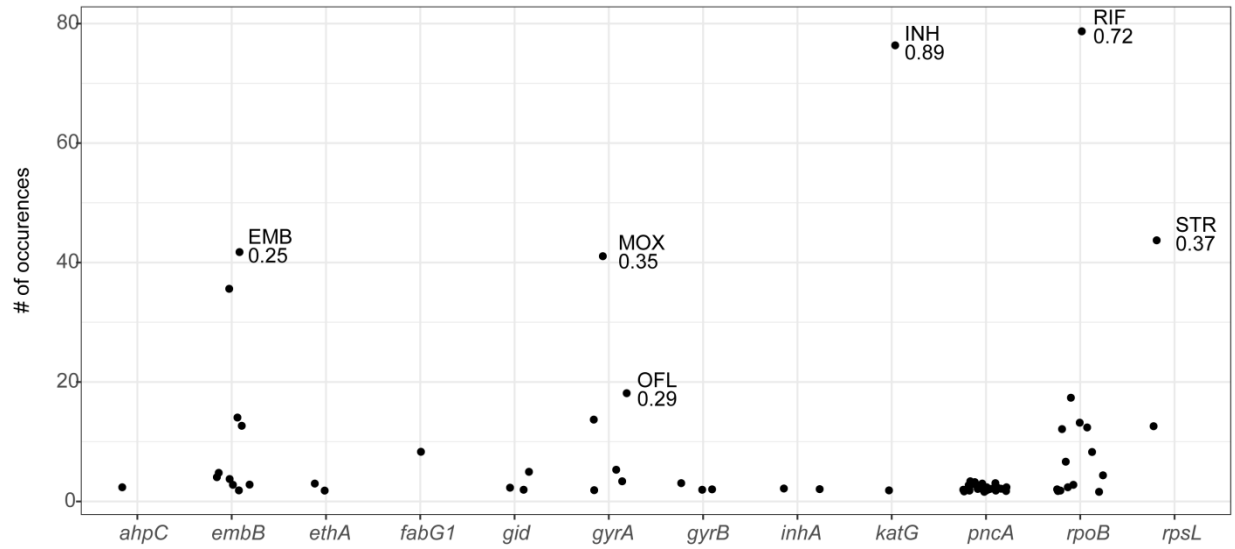
bioRxiv preprint doi: https://doi.org/10.1101/173229; this version posted January 8, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.




781

782 **Figure 5. Ratios of nucleotide diversity in resistance associated genes.** Genes with
783 zero diversity were transformed to 1 x $10^{-16}$ before calculating ratios. Genes with ratios
784 more extreme than $10^{-1.5}$ or $10^{1.5}$ are all filled with the deepest shade. Genes associated
785 with resistance to each drug are outlined in black. A) Ratio of nucleotide diversity in
786 resistant and susceptible isolates. Green genes are more diverse in resistant isolates,
787 which could be due to diversifying selection and/or relaxation of purifying selection.
788 Purple genes are more diverse in susceptible isolates, likely due to increased purifying
789 selection. White genes have similar diversity in resistant and susceptible isolates. B)
790 Comparison of ratios in lineage 2 and lineage 4. Teal genes are more diverse in lineage
791 2 resistant isolates, suggesting diversifying selection/relaxation of purifying selection
792 specific to this lineage. Brown genes are more diverse in lineage 4 resistant isolates.
793 White genes have similar diversity in lineages 2 and 4.

bioRxiv preprint doi: https://doi.org/10.1101/173229; this version posted January 8, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.




794
795 **Figure 6. Homoplastic SNPs in drug resistance associated genes.** SNPs with $F_{ST}$ in
796 the top 1% of genome-wide values are labeled with the population (associated drug
797 resistance) and the $F_{ST}$ value. *pncA* is remarkable for harboring diverse homoplastic
798 mutations, each of which occurs relatively infrequently ("sloppy target"). *embB*, *gyrA*,
799 *katG*, *rpoB* and *rpsL* harbor dominant mutations that occur frequently on the phylogeny
800 and are strongly associated with resistant populations ("tight targets").

801

bioRxiv preprint doi: https://doi.org/10.1101/173229; this version posted January 8, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

802 **Supplementary Table 1.** Accession numbers and lineage designation for sequence
803 data passing quality control filters.