



Title	Drug-resistant Mycobacterium tuberculosis and its genotypes isolated from an outbreak in western Thailand
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Citation	Transactions of the Royal Society of Tropical Medicine and Hygiene, 115(8), 886-895 https://doi.org/10.1093/trstmh/traa148
Issue Date	2021-08-01
Doc URL	https://hdl.handle.net/2115/86428
Rights	This is a pre-copyedited, author-produced version of an article accepted for publication in Transactions of the Royal Society of Tropical Medicine and Hygiene following peer review. The version of record is available online at: http://doi.org/10.1093/trstmh/traa148 .
Type	journal article
File Information	Transactions of the Royal Society of Tropical115 (8)_886-895.pdf



Drug-resistant *Mycobacterium tuberculosis* and its genotypes isolated from an outbreak in western Thailand

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Running title: Drug-resistant tuberculosis and its genotypes in western Thailand

Keyword: Tuberculosis, Spoligotyping, MIRU-VNTR, Mutation, Outbreak, Thailand,

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28

29 **Abstract**

30 **Background**

31 Multidrug-resistant tuberculosis outbreaks have occurred in the Thamaka district, Kanchanaburi
32 province in Thailand. Here, we aimed to characterise this pathogen and its genotypes.

33 **Methods**

34 Seventy-two *Mycobacterium tuberculosis* isolates were collected and genotyped by spoligotyping
35 15-locus mycobacterial interspersed repetitive unit-variable-number tandem repeat (MIRU-VNTR)
36 and single nucleotide polymorphism genotyping, and their drug resistance was analysed.

37 **Results**

38 The spoligotyping results showed that Beijing SIT1 was the predominant sub-lineage (n=38,
39 52.8%) whilst the remaining were non-Beijing sub-lineages (n=34). The MIRU-VNTR analysis
40 showed that the most Beijing isolates (n=37) belonged to the modern type, forming 5 clusters and
41 13 individual patterns. In *katG*, only mutation Ser315Thr was identified. In *rpoB*, Ser531Leu was
42 the predominant except His526Arg and Leu533Pro found in two isolates. A cluster of 14 Beijing
43 strains contained these common mutations and shared the MIRU-VNTR genotype with isolates
44 spreading previously in the Thamaka district. Two U SIT523 isolates contained mutations A1400G
45 in *rrs* and Asp94Gly in *gyrA* genes, indicating a spread of extensively drug-resistant tuberculosis
46 (XDR-TB).

47 **Conclusions**

48 Most mutations were associated with drug resistance, and the specific MDR Beijing and XDR-TB
49 in U SIT523 isolates are remaining. This genotyping was likely a key tool for tracking TB
50 transmission in the Thamaka district, Thailand.

51

52 **Keyword:** MIRU-VNTR, Mutation, Outbreak, Spoligotyping, Thailand, Tuberculosis

53

54 **Introduction**

55 Tuberculosis (TB) caused by the *Mycobacterium tuberculosis* complex is a serious health-
56 deteriorating disease and one of the leading causes of deaths in the world. Thailand has a high TB
57 burden, with approximately 120 000 new cases in 2018, and ranked amongst the top 30 TB-
58 burdened countries. TB infection in Thailand, with a population of about 69 million people, was
59 reported to have an incidence rate of 153 new cases per 100 000 inhabitants. Multidrug-resistant
60 tuberculosis (MDR-TB) caused by *Mycobacterium tuberculosis* (MTB) resistant to at least isoniazid
61 (INH) and rifampicin (RIF), was detected at about 2.3% and 24% of both new cases and retreated
62 patients, respectively.¹ The Kanchanaburi province, located in western Thailand, is one of the areas
63 that has a high incidence of MDR-TB. In particular, the Thamaka district in Kanchanaburi had been
64 documented as a hotspot, with multiple community outbreaks of MDR-TB from 2002 to 2010.² An
65 epidemiological investigation, proper strategies, and rapid responses, including effective treatments,
66 are deemed necessary to control TB transmission in the epidemic area.

67 Occurrence of drug-resistant TB can be categorised into primary and secondary drug
68 resistance. The primary drug resistance is caused by an infection with drug-resistant MTB, whilst
69 secondary drug resistance is an acquired resistance developed at a later stage by drug-susceptible
70 strains.³ Major mechanisms of drug resistance that primarily involve genetic mutations have been
71 reported in a number of studies.⁴⁻⁵ For example, mutations in the *katG* gene account for 30-75 % of
72 INH resistance and are associated with a high-level resistance to INH. The most frequent mutation
73 in the *katG* gene is Ser315Thr. In addition, a point mutation in the regulatory region of *inhA*
74 promoter, mainly at the positions -15 and -8, accounts for 6-30% of INH resistance, resulting in a
75 low-level resistance to INH.⁶⁻⁷ Meanwhile, 90-96% of rifampicin resistance is caused by mutations
76 in the *rpoB* gene in which the 81 bp of the rifampicin resistance determining region (RRDR)
77 between codons 507–533 is considered a mutation hotspot region.⁷ The increasing incidence of
78 MDR and extensive drug-resistant tuberculosis (XDR-TB; MDR plus resistance to fluoroquinolone
79 and aminoglycoside) have become major hurdles to treat TB effectively.⁸ To date, molecular

80 epidemiological data from many countries have demonstrated that MDR and XDR-TB have been
81 detected around the world, and hence become a serious threat to proper TB control. To overcome
82 this epidemiological problem, efficient epidemiological strategies are needed.^{9,10}

83 For the purpose of molecular epidemiology, a number of genotyping methods have been
84 reported and used to differentiate MTB strains. For example, spoligotyping¹¹, analysis of MIRU-
85 VNTR¹², single nucleotide polymorphism (SNPs)¹³, region of differences (RDs), large sequence
86 polymorphisms (LSP)¹³ and restriction fragment length polymorphism (RFLP)¹³ can be listed
87 amongst the methods extensively used for typing MTB strains. Recently, whole genome sequencing
88 (WGS) has been used to genetically characterise MTB.¹⁴ This method can provide comprehensive
89 genetic information and help deepen our understanding of the factors affecting TB transmission.
90 The genotyping of MTB is a molecular tool that can be used to investigate possible epidemiological
91 links between TB patients, detect suspected outbreaks and distinguish exogenous re-infection from
92 endogenous reactivation in relapse cases.¹² For example, spoligotyping is a simple and rapid
93 genotyping method that focuses on the detection of polymorphisms based on the presence or
94 absence of 43 unique spacer sequences in the direct repeat (DR) regions.¹¹ Spoligotyping has been
95 used to categorise MTB strains into several families, such as Beijing, East-African Indian (EAI),
96 Central Asian (CAS), Latin American Mediterranean (LAM), Unclassified (U) and T families.¹⁵
97 The analysis of variable-number tandem repeat of mycobacterial interspersed repetitive units
98 (MIRU-VNTR) is another genotyping method that has been widely used for monitoring the global
99 spread of TB.¹⁶ The combination of spoligotyping and MIRU-VNTR typing methods has become a
100 traditional and practical tool providing a resolution high enough for the clear identification of MTB
101 strains.¹⁷ Generally, MIRU-VNTR has been used after spoligotyping to further subtype major MTB
102 groups including the Beijing family, which commonly causes several major outbreaks worldwide, is
103 over-represented in drug-resistant isolates and seems to have unique virulence properties.¹³

104 Outbreaks of TB along the western border of Thailand with Myanmar are still a problem as
105 genotyping is not routinely conducted during these events. The present study was conducted to gain

106 an insight into the spread of TB in the Thamaka district of Kanchanaburi province, Thailand and
107 neighbouring regions. Genotyping was conducted by spoligotyping, MIRU-VNTR, and SNP
108 analysis. Mutation characteristics in targeted genes associated with INH, RIF, fluoroquinolone and
109 aminoglycoside resistance in MTB isolates were also analysed.

110 **Materials and Methods**

111 **Processing of MTB strains and samples**

112 A total of 72 MTB isolates from Thai pulmonary-TB patients (registered for TB treatment
113 during 2013-2014 MDR-TB outbreak in Thamaka District, Kanchanaburi Province) and strains
114 isolated from surrounding areas in western Thailand, Samut Sakhon and Prachuap Khiri Khan
115 Provinces, were analysed. The locations of the sample collection sites are showed in Figure 1.
116 These isolates were obtained from decontaminated sputum samples that were inoculated into
117 BACTEC MGIT™ liquid media (Becton Dickinson®,USA). Drug susceptibility testing (DST) for
118 first-line drugs including INH, RIF, ethambutol (EMB), and streptomycin (STM) was conducted
119 using BACTEC MGIT™ 960 SIRE Kit, as per the manufacturer's instructions. A second-line drug
120 susceptibility test was carried out only on some isolates (data not shown).

121 DNA was extracted from 500 µl of MTB cell culture suspension recovered from the
122 BACTEC 960 MGIT media using magnetic bead-based nucleic acid extraction (SEEPREP 12™,
123 Seegene, Inc., Korea) as per the manufacturer's protocol. The extracted DNA with a final volume of
124 50 µl was kept at -20⁰ C until further molecular analyses.

125 **Genotyping**

126 MTB isolates were genotyped by spoligotyping as previously described.¹¹ Briefly, the direct
127 repeat (DR) region in chromosomal DNA of MTB was amplified by PCR. The hybridisation of
128 PCR products to specific DNA probes was then screened for spoligo-patterns. The obtained
129 spoligo-patterns were converted to an octal code and compared with those in the international
130 spoligotyping database. SpolDB4 was used to identify spoligo-international types (SITs).¹⁵

131 To subtype isolates, the selected 15 MIRU-VNTR (424, 580, 802, 960, 1644, 1955, 2163b,
132 2165, 2401, 2996, 3192, 3690, 4052, 4156, 4348) were analysed according to a method previously
133 described.^{17,18} After amplification, the copy numbers of tandem repeats were determined from the
134 sizes of amplicons by agarose gel electrophoresis using 50 bp DNA ladders (New England,
135 Biolabs® Inc.) as markers.¹² A numerical value profile was assigned to each strain according to the
136 number of variable repeats in each VNTR allele.

137 The allelic diversity amongst the MIRU-VNTR isolates was determined. The Hunter-Gaston
138 Discriminatory Indexes (HGDI) were calculated by the following equation: $HGDI = 1 - (1/N(N-1))$
139 $(\sum n_j(n_j-1))$, where N is the total number of isolates in the sample population, S is the total number
140 of allelic, and n_j is the frequency of the allele in the locus. The discriminatory power was considered
141 high if the HGDI value was >0.6, moderate if HGDI was between 0.3 to 0.6 and low if HGDI was
142 <0.3, according to the definition by Sola *et al.*, 2003.¹⁹

143 **Analysis of clustering**

144 Spoligotyping patterns and 15-locus MIRU-VNTR typing data were analysed by MIRU-
145 VNTRplus (<http://www.miru-vntrplus.org/MIRU/index.faces>) to establish the lineages and sub-
146 lineages.²⁰ Dendrograms by the unweighted pair group method with arithmetic means mode
147 (UPGMA) were generated. In the present study, the genotypes of Beijing isolates were compared
148 with those of a previously published study.¹⁰ A mini spanning tree (MST) was constructed based on
149 the data of 15- locus MIRU-VNTR analysis using BioNumerics software version 6.6 (Applied
150 Maths, Kortrijk, Belgium). Clusters were defined as two or more isolates sharing an identical 15-
151 locus MIRU-VNTR pattern. The clustering rate was calculated using the following formula:
152 number of clustered isolates/ total number of isolates.

153 **SNP analysis and gene mutation detection by sequencing**

154 Single nucleotide polymorphism (SNP) at the 1477596 locus was examined to subdivide
155 modern and ancestral Beijing genotypes.¹³ DNA samples of Beijing isolates were amplified by PCR.
156 The PCR mixture contained 1x GoTaq buffer (Promega Co., Madison, WI), 0.26 mM of each dNTP,

157 0.3 µM of each primer, 0.5 M Betaine, 0.5 units of GoTaq DNA polymerase and DNA of MTB.
158 DNA sequences were aligned through pairwise alignment of the *M. tuberculosis* H37Rv using Bio-
159 Edit software version 7.09.²¹

160 DNA samples from drug-resistant strains were subjected to an analysis of mutations in
161 genes associated with drug resistance, targeting the following: the *katG* coding sequence and *inhA*
162 promoter region for INH; rifampicin resistance determining region (RRDR) in *rpoB* for RIF;
163 quinolone resistance determining region (QRDR) in *gyrA* for fluoroquinolone; and 16S ribosomal
164 RNA gene in *rrs* for kanamycin or aminoglycosides.^{8,22} The PCR components used were the same
165 as those described above. The amplified DNA fragments were subjected to sequence analysis using
166 BigDye terminator V3.1 (Life Technologies Co., CA) reagents and a 3130 genetic analyser (Life
167 Technologies Co., CA), as per the manufacturer's protocol. Sequences were compared with those of
168 wild-type reference H37Rv and analysed using Bio-Edit software.

169 **Results**

170 **MTB phenotyping by drug susceptibility testing**

171 Based on the drug susceptibility testing, phenotypes resistant to four first-line anti-TB drugs
172 were identified in all 72 MTB isolates. Of them, 62.5% (n=45) were susceptible to INH and RIF,
173 whilst the rest (n=27) were drug-resistant MTB, including RIF mono-, INH mono-resistant, and
174 MDR at 5.6% (n=4), 1.4 % (n=1) and 30.6% (n=22), respectively (Table 1).

175 **Spoligotyping**

176 The spoligotyping results showed clustering amongst 59 isolates and 13 different non-
177 clustered spoligotypes (Table 2). Overall, there were 20 spoligotype patterns and the clustering rate
178 was 82%. The largest cluster belonged to the Beijing SIT1 family (n=38, 53%). Non-Beijing (n=34,
179 47.2%) included EAI2_NTB (n=6, 8.3%), EAI5 (n=8, 11.2%), EAI6_BGD1 (n=4, 5.6%), H3 (n=1,
180 1.4%), T1 (n=2, 2.8%) U (n=6, 8.4%) and new spoligotypes (n=7, 9.8%) (Table 2). There were 6
181 small clusters of T1 SIT53 (n=2), EAI2_NTB SIT89 (n=6), EAI5 SIT236 (n=3), EAI5 SIT256
182 (n=4), U SIT523 (n=4), and EAI6_BGD1 SIT591 (n=2) amongst 21 non-Beijing, as shown in Table

183 2. The rest of the non-Beijing group (n=13, 18%) was non-clustered (Table 2). Of 38 Beijing (SIT1)
184 genotypes, 19 (50%) were drug-resistant and 19 (50%) susceptible to both INH and RIF.

185 **MIRU-VNTR analysis**

186 According to the 15-locus MIRU-VNTR analysis, 5 different subtypes were generated
187 amongst 25 Beijing isolates consisting of Bj-I (n=16), Bj-II (n=2), Bj-III (n=2), Bj-IV (n=2) and Bj-
188 V (n=3). The remaining of the 13 Beijing isolates were single or non-clustered Beijing subtypes
189 (Figure 2A). The largest cluster was a group of Bj-I subtypes that contained 16 isolates (42%, 16 of
190 38 Beijing), including 14 MDR-TB isolates, 1 mono RIF- resistant and 1 susceptible isolate. All
191 members of Bj-I cluster were from the Kanchanaburi Province. In addition, Beijing drug-
192 susceptible strains were found in clusters of Bj-II, Bj-III, and Bj-IV. The other three Beijing isolates
193 were categorised into the Bj-V cluster. These three isolates were from the Samut Sakorn Province.
194 Two of these isolates were MDR and one was INH mono-resistant. The remaining Beijing isolate
195 was from the Prachuap Khiri Khan Province and non-clustered (Figure 2A). In non-Beijing groups,
196 the U SIT523 was found to have an identical MIRU-VNTR pattern in 2 of 4 isolates (Figure 2B).
197 The MIRU-VNTR pattern in the matching U strains was 443824652641113. Overall, 59 clustered
198 isolates that shared spoligotypes were subtyped by the MIRU-VNTR analysis, generating 38
199 different MIRU-VNTR patterns (Figure 2). Of these, 6 patterns formed major clusters containing 27
200 isolates, while 32 unique individual MIRU-VNTR patterns were identified. The dendrogram
201 resulting from clustering analysis is shown in Figure 2.

202 **Analysis of discriminatory power and diversity**

203 A discriminatory power of the selected 15-locus MIRU-VNTR for differentiating the Beijing
204 genotype was observed (Table 3). The comparison results showed that the HGDI value of 19
205 Beijing drug-resistant isolates (MDR, INH mono-resistant and RIF mono-resistant) was 0.38 lower
206 than that of drug-susceptible Beijing isolates, with a value of 0.98. In the drug-susceptible Beijing
207 group, allelic diversity was highly discriminated in locus 2163b and moderate in loci 0424, 0802,
208 1955, and 2996. Thus, 2 clusters consisting of 4 isolates and 15 single isolates were discriminated

209 from drug-susceptible Beijing strains. Nonetheless, although this selected 15-locus MIRU-VNTR
210 had a high power to distinguish Beijing isolates susceptible to INH and RIF, it could not
211 differentiate the MDR Beijing family that included 14 of 16 isolates in the Bj I cluster and 2 of 3
212 isolates in the Bj-V cluster.

213 **SNP for identification of modern and ancestral Beijing**

214 SNP data from locus 1477596 indicated that 37 of 38 Beijing isolates were either a SNP-T
215 type or a modern Beijing sub-lineage (97%). Only one isolate was observed to have a SNP-C type
216 (ancestral Beijing subtype) (Figure 2A).

217 **Identical MTB genotypes**

218 MIRU-VNTR data from Beijing isolates that were collected from past TB outbreaks
219 occurring during the periods 2006-2012 and 2013-2014 were retrieved from a previous report¹⁰ and
220 compared with those from the present study (Figure 3). The results showed a major clonal
221 expansion of 62 MDR-MTB isolates originated from previous outbreaks in 2006 (n=3), 2007 (n=1),
222 2008 (n=5, including 1 XDR-TB isolate), 2009 (n=8), 2010 (n=5), 2011 (n=1), and 2012 (n=1), and
223 isolates in the Bj- I cluster (Figure 3) from the present study (n=38).

224 **Mutations associated with drug resistance**

225 The sequencing results showed a single type of mutation in the *katG* gene at a codon Ser315Thr
226 in all INH-resistant isolates. Meanwhile, a substitution of Ser531Leu in *rpoB* was found in almost
227 all RIF-resistant strains except for two isolates carrying His526Arg and Ser533Pro. Of those 22
228 MDR, 20 isolates, which included 16 Beijing, 1 EAI6_BGD1 (SIT591) and 3 U (SIT523), had a
229 common mutation of Ser315Thr in the *katG* gene and Ser531Leu in the *rpoB* gene for typical INH
230 and RIF resistances, respectively (Figure 2). Only one Beijing MDR strain was found to carry
231 Ser315Thr in the *katG* gene but had a different mutation of His526Arg in the *rpoB* gene. This
232 isolate was from the Samut Sakorn Province. Another one had no data of DNA sequences in any of
233 the targeted genes. As for MDR, a common mutation of Ser531Leu in the *rpoB* gene was found in 3
234 of 4 mono-RIF-resistant isolates that were Beijing, EAI5 SIT526, and new spoligotypes, while

235 Leu533Pro was carried by EAI6_EGD1 SIT591. Most of the MDR isolates had no mutations in the
236 *rrs* and the *gyrA* genes, which conferred resistance to kanamycin and fluoroquinolone, respectively.
237 The exceptions were two isolates of the U SIT523 strain, which were observed to have the
238 additional mutations A1400G and Asp94Gly in the *rrs* and the *gyrA* genes, respectively (Figure 2B).
239 Therefore, these two identical U SIT523 isolates were considered to be strains that cause a
240 transmission of XDR-TB.

241 **Discussion**

242 A number of MDR cases has been detected in the Thamaka district, in the Kanchanaburi
243 province, Thailand, and epidemic outbreaks of MDR-TB have since been continuously reported in
244 these areas. Previously, the potential of a specific MDR-TB strain was reported to cause a
245 continuous outbreak of drug-resistant TB, and showed the genetic diversity of MTB strains in these
246 region.^{2,3} Currently, molecular detection is being conducted for rapid detection of drug-resistant
247 cases, but no genotyping is routinely carried out. Therefore, the genetic information of clinical
248 isolates currently available is still limited. A previous study using retrospective isolates from the
249 year 2006 showed that most of the MDR strains belonged to the Beijing genotype and that U
250 SIT523 was the XDR-TB strain.¹⁰ The findings of the present work confirmed that primary
251 transmission of drug-resistant TB was the major factor driving the increase of TB drug resistance in
252 local outbreaks in Thailand and that a drug resistance mechanism related to INH and RIF was
253 caused by common mutations in genes *katG* and *rpoB*.

254 Molecular epidemiology has helped deepen the understanding of the TB epidemic in the
255 studied Thai regions. Furthermore, our spoligotyping results indicated that more than 50% of MTB
256 strains were clustered and belonged to the Beijing family. Based on the DST results and
257 spoligotyping, we showed that the Beijing family was prevalent and the majority of the strains were
258 drug-resistant. In addition, almost all Beijing members in the present study belonged to the modern
259 type, which was determined by SNP at the position of 1477596.^{13,24,25} The modern Beijing strain
260 has become a great concern in epidemiology because it is spreading worldwide and causing

261 outbreaks in many countries.^{26,27} In the present work, only one isolate was the ancestral Beijing type,
262 which is usually associated with low virulence of tuberculosis.^{13,24} This finding was in agreement
263 with previous reports of TB incidence in many countries where the Beijing type was
264 predominant.^{26,27,28} A recently study reported that about 66% of Beijing strains were drug-resistant
265 and that were prevalent in Thai tuberculosis patients.²⁹ Beijing strains have become a global health
266 threat because of their frequent association with a high mutation rate, hypervirulence, immune
267 evasion, treatment failure, and drug resistance.³⁰

268 In the present study, the analysis of molecular data based on 15- locus MIRU VNTR
269 showed diverse genetic backgrounds amongst drug-susceptible Beijing strains. In contrast, MDR
270 Beijing strains had restricted genetic backgrounds. As a result, we proposed that the method
271 provided a discrimination power high enough to differentiate MTB strains in the epidemic Thai
272 areas. We found that the largest cluster of Beijing isolates circulating in these areas was the Bj-I
273 subtype. Compared with a previous study (Figure 2), the specific Bj-I genotype of MDR-MTB was
274 also found to be dominant, forming the largest cluster among Beijing strains. As shown in Figure
275 2A, the successful expansion of a specific clone of the Bj-I sub-lineage resulted in restricted genetic
276 backgrounds. From this evidence, we proposed that the specific clone of Beijing sub-lineage with
277 multidrug resistance was highly transmissible and may be the major cause of MDR-TB outbreaks in
278 the Thamaka district. Our mini span diagram clearly showed that predominant cluster-sharing
279 genotypes, identical to past strains, have been continuously spreading to date. Rapid identification
280 of TB cases, patient isolation, and appropriate treatments are required for a future, more effective
281 outbreak control.

282 Molecular drug resistance analysis by DNA sequencing showed that genetic alterations were
283 highly associated with drug-resistant phenotypes and identified common mutations in the *katG* and
284 the *rpoB* genes. Additional mutations conferring resistance to a fluoroquinolone and injectable
285 aminoglycosides were further identified in two of three U SIT523 isolates, suggesting the
286 emergence of XDR-TB. The absence of second-line drug resistance amongst a large number of

MDR-TB genotypes suggested that under a MDR treatment, acquisition of additional resistance to second-line drugs was limited. MDR was also found in non-Beijing strains, but in a low percentage, accounting for merely 12.5 % (4 of 22) (Figure 1). Occurrence of MDR with individual unique genotypes in Beijing and non-Beijing groups could be explained by the fact that MTB developed a spontaneous mutation or acquired drug resistance resulting in the development of new drug-resistant strains.

It should be mentioned that the present study had several limitations. For example, the sample size of the MTB population was limited by the short time of the sample collection. In addition, the demographic and clinical data obtained for each patient was limited. Nonetheless, even though the number of samples were small, we were able to find clusters of MTB amongst the isolates from the Kanchanaburi Province. It is also worth noting that in neighbouring provinces, no identical genotypes to those identified in Kanchanaburi were identified. These results may imply that there was no TB spread across the provinces, but the evidence remains inconclusive due to the sample sizes from other adjunct provinces were small.

Conclusions

Our data suggested that the specific Beijing genotype strain with MDR was the most prevalent MTB strain causing a continuous TB transmission in the Thai areas studied. Our results also showed that this specific MTB strain, which was dominant, virulent, and resistant to isoniazid and rifampicin, has not been eliminated from the region. In addition, it was demonstrated that XDR-TB concurrently emerged and caused transmission. Finally, in the present work, we showed that genotyping based on spoligotyping and 15-MIRU-VNTR analysis was a useful tool for successfully discriminating MTB strains.

Author Contributions

311 SB, KS, NT, YF conducted the analysis and interpretation of data. JR and BP designed the
312 study, the analysis and the interpretation of data, and drafted the manuscript. CN and YS carried out
313 the analysis and interpretation of data and edited and approved the final version.

314

315 **Acknowledgment**

316 The authors are grateful for the support from the Research Center for Zoonosis Control,
317 Hokkaido University. We thank the staff of the mycobacteria group at the Sasakawa Research
318 Building, the National Institute of Health, in Thailand for their assistance.

319

320 **Funding**

321 This work was supported in part by the Japan Society for the Promotion of Science
322 RONPAKU (Dissertation Ph.D.) Program (Grant No. NRCT—11141) to JR, in part by a grant from
323 National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Thailand
324 to BP, in part by a grant from the Ministry of Education, Culture, Sports, Science and Technology
325 (MEXT), Japan, for the Joint Research Program of the Hokkaido University Research Center for
326 Zoonosis Control to YS, and in part by Japan Agency for Medical Research and Development
327 (AMED) under Grant Number JP20jk0210005, JP20jm0110021 and JP20wm0125008 to YS.

328

329 **Competing interests:** None declared.

330

331 **Ethical approval:** Not required.

332

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1 **Legend**

2 **Figure 1.** Map of sample collection sites and the number (*n*) of isolates from the Thamaka District,
3 Kanchanaburi Province and surrounding areas in western Thailand, and Samut Sakhon and
4 PrachuapKhiri Khan Provinces, Thailand.

5 **Figure 2.** Clustering analysis; A, Dendrogram of genetic relationships between 38 Beijing isolates
6 from Kanchanaburi; B, Dendrogram of genetic relationships between 21 non-Beijing isolates based
7 on the 15 loci-MIRU-VNTR analysis. Spoligotypes, phenotypic and genotypic characteristics of
8 drug resistance were incorporated. **Abbreviations:** ID, identification; SIT, spoligo-international
9 types; DST, drug susceptibility testing; MIRU-VNTR, mycobacterial interspersed repetitive unit-
10 variable number tandem repeat; SNP, single nucleotide polymorphism; INH, Isoniazid; RIF,
11 Rifampicin; Kan, Kanamycin; S, Susceptible; R, Resistance; WT, wildtype; Ser, Serine; Thr,
12 Threonine; His, Histidine; Arg, Arginine; Leu, Leucine; Ser, Serine; Thr, Threonine; His, Histidine;
13 Arg, Arginine; Leu, Leucine; Asp, Asparagine; Gly, Glycine; A, Adenine; G, Guanine; NA, Not
14 applicable.

15 **Figure 3.** Comparison of genotypes among Beijing isolates in Thai outbreak areas in previous
16 (during the year 2006-2012) and recent (during the year 2013-2014) outbreaks, based on MIRU-
17 VNTR; A minimum spanning tree was constructed based on 15 loci of MIRU-VNTR genotyping of
18 the Beijing strains (*n*=24) isolated during the period 2006-2013, as reported in a previous study and
19 the present work (*n*=38). Circles denote different types discriminated by 15 locus-MIRU-VNTR
20 genotypes. The origins of each isolate are represented by different colours.

21

22

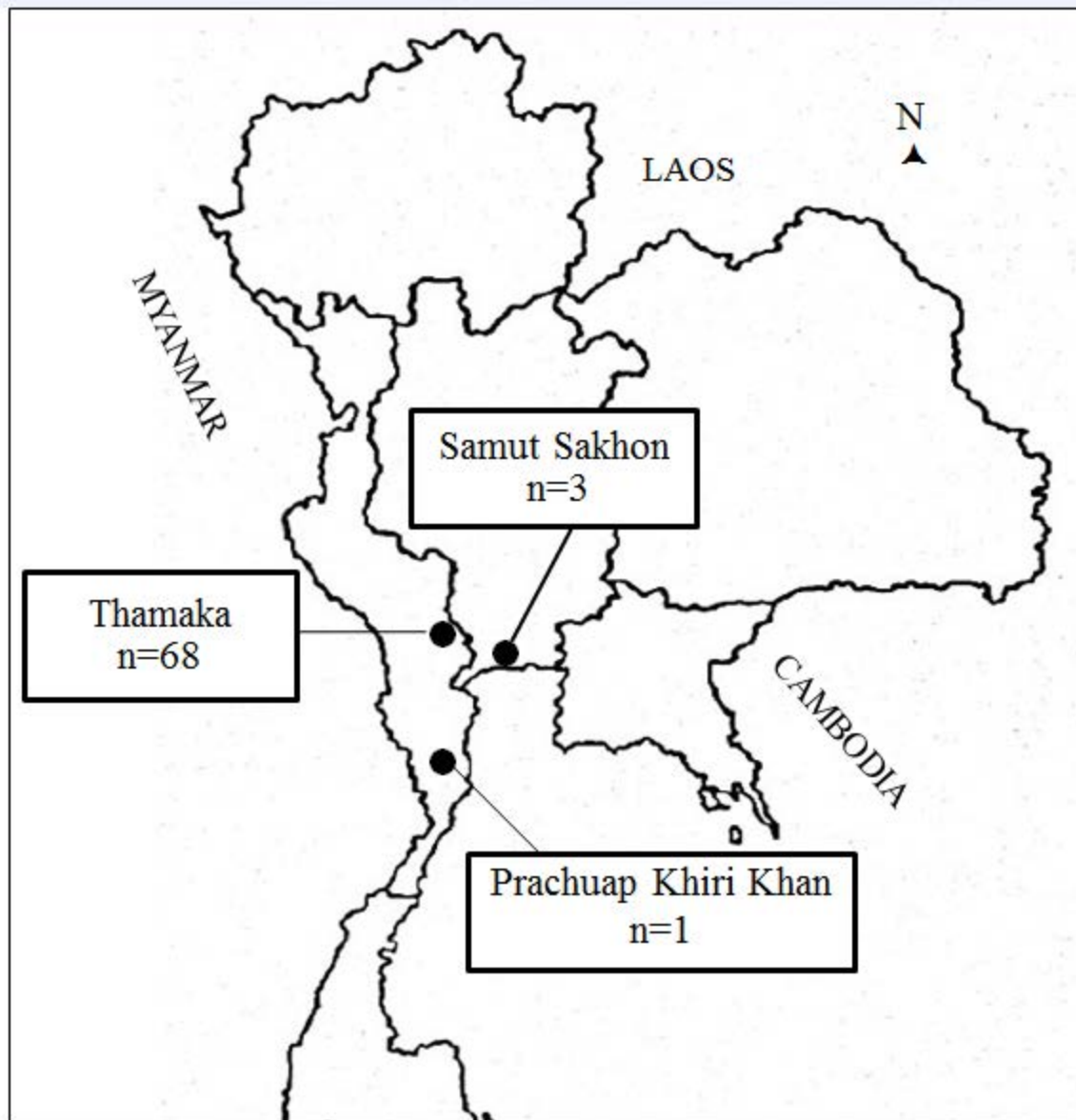
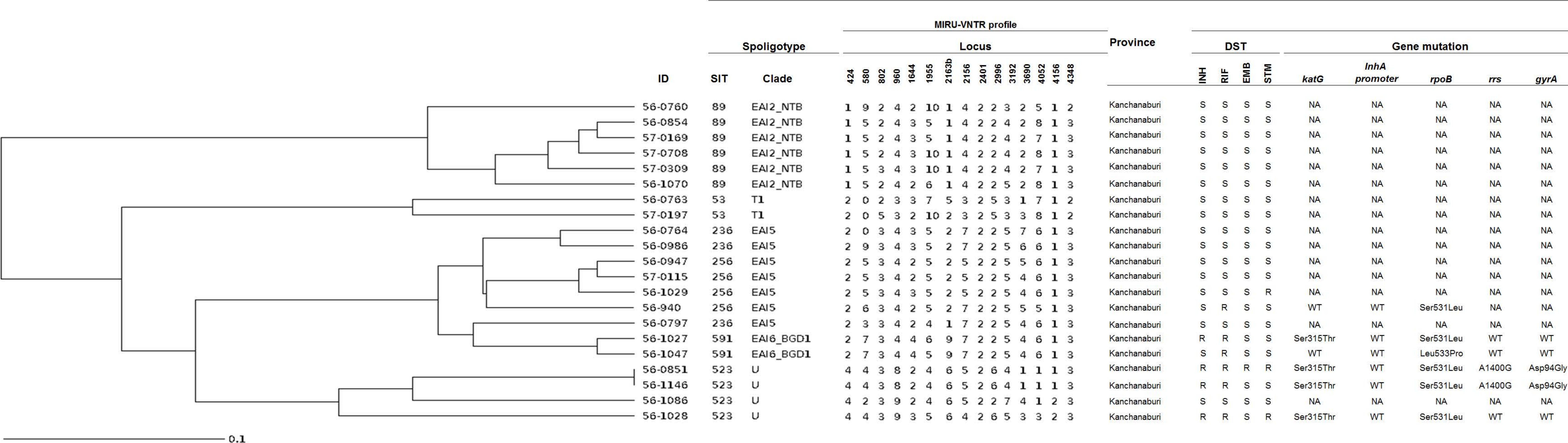


Figure 1. Map of sample collection sites and the number (*n*) of isolates from the Thamaka District, Kanchanaburi Province and surrounding areas in western Thailand, and Samut Sakhon and Prachuap Khiri Khan Provinces, Thailand

	ID	SIT	Clade	MIRU-VNTR														Drug resistance detection					Cluster	SNP 1477596						
				Locus	Province	DST	Gene mutation																							
							katG	inhA promoter	rpoB	gyrA	rrs																			
424	580	802	960	1644	1955	2163b	2156	2401	2996	3192	3690	4052	4156	4348	INH	RIF	EMB	STM												
	56-0754	1	BEIJING	4	2	3	3	3	5	4	4	4	7	5	3	8	2	3	Kanchanaburi	R	R	S	R	Ser315Thr	WT	Ser531Leu	WT	WT	Bj-I	T
	56-0755	1	BEIJING	4	2	3	3	3	5	4	4	4	7	5	3	8	2	3	Kanchanaburi	R	R	S	R	Ser315Thr	WT	Ser531Leu	WT	WT		T
	56-0781	1	BEIJING	4	2	3	3	3	5	4	4	4	7	5	3	8	2	3	Kanchanaburi	S	R	S	R	WT	WT	Ser531Leu	WT	WT		T
	56-0830	1	BEIJING	4	2	3	3	3	5	4	4	4	7	5	3	8	2	3	Kanchanaburi	R	R	S	R	Ser315Thr	WT	Ser531Leu	WT	WT		T
	56-0840	1	BEIJING	4	2	3	3	3	5	4	4	4	7	5	3	8	2	3	Kanchanaburi	R	R	S	R	Ser315Thr	WT	Ser531Leu	WT	WT		T
	56-0926	1	BEIJING	4	2	3	3	3	5	4	4	4	7	5	3	8	2	3	Kanchanaburi	R	R	R	R	Ser315Thr	WT	Ser531Leu	WT	WT		T
	56-0949	1	BEIJING	4	2	3	3	3	5	4	4	4	7	5	3	8	2	3	Kanchanaburi	R	R	S	R	Ser315Thr	WT	Ser531Leu	WT	WT		T
	56-1043	1	BEIJING	4	2	3	3	3	5	4	4	4	7	5	3	8	2	3	Kanchanaburi	R	R	S	R	Ser315Thr	WT	Ser531Leu	WT	WT		T
	56-1052	1	BEIJING	4	2	3	3	3	5	4	4	4	7	5	3	8	2	3	Kanchanaburi	R	R	S	R	Ser315Thr	WT	Ser531Leu	WT	WT		T
	56-1103	1	BEIJING	4	2	3	3	3	5	4	4	4	7	5	3	8	2	3	Kanchanaburi	R	R	R	R	Ser315Thr	WT	Ser531Leu	WT	WT		T
	57-0003	1	BEIJING	4	2	3	3	3	5	4	4	4	7	5	3	8	2	3	Kanchanaburi	R	R	R	R	Ser315Thr	WT	Ser531Leu	WT	WT		T
	57-0287	1	BEIJING	4	2	3	3	3	5	4	4	4	7	5	3	8	2	3	Kanchanaburi	R	R	R	R	Ser315Thr	WT	Ser531Leu	WT	WT		T
	57-0298	1	BEIJING	4	2	3	3	3	5	4	4	4	7	5	3	8	2	3	Kanchanaburi	R	R	S	R	Ser315Thr	WT	Ser531Leu	WT	WT		T
	57-0302	1	BEIJING	4	2	3	3	3	5	4	4	4	7	5	3	8	2	3	Kanchanaburi	R	R	R	R	Ser315Thr	WT	Ser531Leu	WT	WT		T
	57-0680	1	BEIJING	4	2	3	3	3	5	4	4	4	7	5	3	8	2	3	Kanchanaburi	S	S	S	S	NA	NA	NA	NA	NA	T	
	57-0754	1	BEIJING	4	2	3	3	3	5	4	4	4	7	5	3	8	2	3	Kanchanaburi	R	R	S	R	Ser315Thr	WT	Ser531Leu	WT	WT	T	
	56-0836	1	BEIJING	4	2	3	3	3	5	5	4	4	7	5	3	8	2	3	Kanchanaburi	R	R	S	R	Ser315Thr	WT	Ser531Leu	WT	WT	T	
	56-1149	1	BEIJING	4	2	3	3	3	5	5	4	4	7	5	3	8	2	3	Kanchanaburi	S	S	S	S	NA	NA	NA	NA	NA	Bj-II	T
	56-1023	1	BEIJING	4	2	3	3	3	5	4	4	4	5	5	3	8	2	3	Kanchanaburi	S	S	S	S	NA	NA	NA	NA	NA		T
	56-999	1	BEIJING	4	2	3	3	3	5	6	4	4	5	5	3	8	2	3	Kanchanaburi	S	S	S	S	NA	NA	NA	NA	NA		T
	57-0423	1	BEIJING	4	2	3	3	3	5	6	4	4	4	5	3	8	2	3	Kanchanaburi	S	S	S	S	NA	NA	NA	NA	NA		T
	56-0941	1	BEIJING	4	2	2	3	3	5	4	4	4	7	5	3	7	2	3	Kanchanaburi	S	S	S	S	NA	NA	NA	NA	NA	T	
	57-0375	1	BEIJING	4	2	2	3	3	5	4	4	4	7	5	3	8	2	3	Kanchanaburi	S	S	S	S	NA	NA	NA	NA	NA	T	
	57-0707	1	BEIJING	4	2	2	3	3	5	6	4	4	7	5	3	8	2	3	Kanchanaburi	S	S	S	S	NA	NA	NA	NA	NA	T	
	57-0043	1	BEIJING	4	2	3	3	3	5	6	4	5	7	5	3	8	2	3	Kanchanaburi	S	S	S	S	NA	NA	NA	NA	NA	Bj-III	T
	57-0114	1	BEIJING	4	2	3	3	3	5	6	4	5	7	5	3	8	2	3	Kanchanaburi	S	S	S	S	NA	NA	NA	NA	NA		T
	57-0198	1	BEIJING	4	2	3	3	3	5	6	4	4	8	5	3	8	2	3	Kanchanaburi	S	S	S	S	NA	NA	NA	NA	NA	T	
	57-0312	1	BEIJING	2	2	3	3	3	5	6	4	4	8	5	3	8	2	3	Kanchanaburi	S	S	S	S	NA	NA	NA	NA	NA	Bj-IV	T
	56-0769	1	BEIJING	3	2	3	3	3	7	6	4	4	7	5	3	8	2	3	Kanchanaburi	S	S	S	S	NA	NA	NA	NA	NA		T
	56-1010	1	BEIJING	3	2	3	3	3	7	6	4	4	7	5	3	8	2	3	Kanchanaburi	S	S	S	R	NA	NA	NA	NA	NA	T	
	56-799	1	BEIJING	3	2	3	3	3	2	5	4	4	7	5	3	8	2	3	Prachuap Khiri Khan	S	S	S	S	NA	NA	NA	NA	NA	T	
	56-0952	1	BEIJING	5	2	1	3	3	5	5	4	4	7	5	3	8	2	3	Kanchanaburi	S	S	S	R	NA	NA	NA	NA	NA	T	
	56-866	1	BEIJING	4	0	5	3	3	5	5	4	4	7	5	3	8	2	3	Samut Sakorn	R	R	S	R	Ser315Thr	WT	His526Arg	WT	WT	Bj-V	T
	56-998	1	BEIJING	4	0	5	3	3	5	5	4	4	7	5	3	8	2	3	Samut Sakorn	R	R	R	R	Ser315Thr	WT	Ser531Leu	WT	WT		T
	57-147	1	BEIJING	4	0	5	3	3	5	5	4	4	7	5	3	8	2	3	Samut Sakorn	R	S	S	R	Ser315Thr	WT	WT	NA	NA		T
	56-868	1	BEIJING	4	0	3	3	3	3	5	4	4	7	5	3	8	2	3	Kanchanaburi	S	S	S	S	NA	NA	NA	NA	NA		T
	57-0530	1	BEIJING	4	2	3	3	3	4	5	4	4	7	5	3	10	5	3	Kanchanaburi	S	S	S	S	NA	NA	NA	NA	NA	C	
	56-0828	1	BEIJING	3	2	3	2	3	5	2	4	4	7	5	4	8	2	4	Kanchanaburi	S	S	S	S	NA	NA	NA	NA	NA	T	



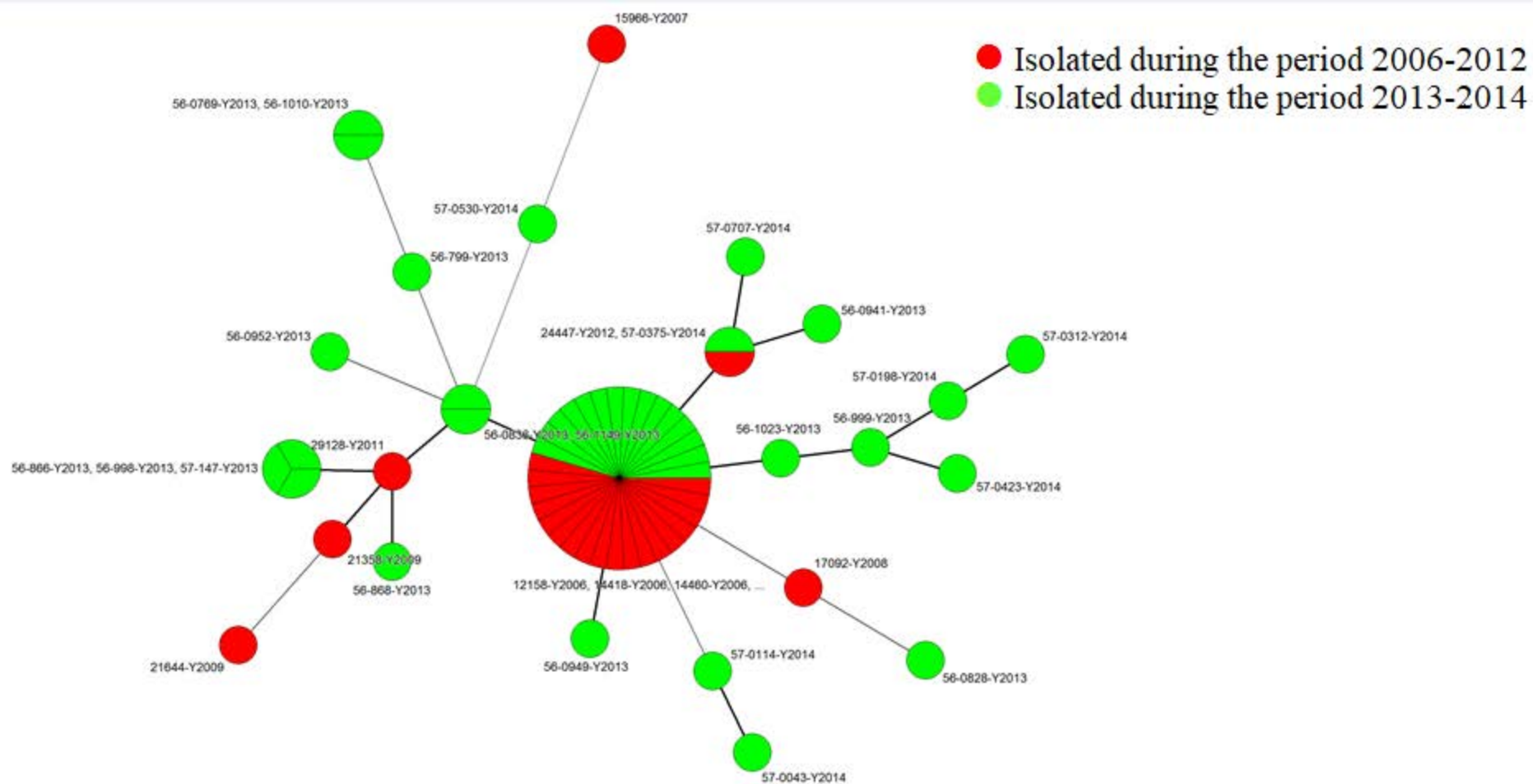


Table 1. Phenotypic characteristics of MTB isolates according to drug susceptibility tests with first-line anti-TB drugs

Strain	Drug resistant patterns				Frequency	Proportion		
	INH	RIF	EMB	STM		(%)		
Susceptible to INH and RIF					45	62.5		
	S	S	S	S	42	58.3		
	S	S	S	R	3	4.2		
Resistant to INH and RIF					27	37.5		
RIF mono-resistance	S	R	S	S	3	4	4.2	5.6
	S	R	S	R	1		1.4	
INH mono-resistance	R	S	S	R	1		1.4	
MDR and XDR	R	R	S	R	13	22	18.1	30.6
	R	R	S	S	2		2.8	
	R	R	R	R	7		9.7	
					72	100		

Abbreviations: MTB, *Mycobacterium tuberculosis*; INH, isoniazid; RIF, rifampicin; EMB, ethambutol; STM, streptomycin; S, susceptible; R, resistance; MDR, multi-drug resistance; XDR, extensively-drug resistance

Table 2. Classification of MTB strains based on spoligotyping

No.	Clade	SIT	Octal Code	Frequency	%	Clustering rate (%)
				59		82
1	BEIJING	1	000000000003771	38	52.8	53
2	EAI2_NTB	89	674000003413771	6	8.3	8
3	EAI5	236	777777777413771	3	4.2	4
4	EAI5	256	777777777413671	4	5.6	6
5	EAI5	1395	474377767413771	1	1.4	
6	EAI6_BGD1	292	777777757413371	1	1.4	
7		591	777777757413771	2	2.8	3
8	EAI6_BGD1	1414	777757757413371	1	1.4	
9	H3	50	777777777720771	1	1.4	
10	T1	53	777777777760771	2	2.8	3
11	U	523	777777777777771	4	5.6	6
12	U	1189	677777477403771	1	1.4	
13	U	1391	777777700003371	1	1.4	
14	NEW1	ND	737777747413771	1	1.4	
15	NEW2	ND	12004377763771	1	1.4	
16	NEW3	ND	674000003412771	1	1.4	
17	NEW4	ND	367777670020731	1	1.4	
18	NEW5	ND	774177774000071	1	1.4	
19	NEW6	ND	000000007777711	1	1.4	
20	NEW7	ND	674000003413731	1	1.4	
Total				72	100	

Abbreviations: SIT, spoligo-international type number; ND, no data

Table 3. Allelic distribution and discrimination power of MIRU-VNTR in Beijing genotypes resistant and susceptible to isoniazid and rifampicin

locus	DST result	N	MIRU-VNTR													Discreminatory power	
			Copy number														
			1	2	3	4	5	6	7	8	9	10	ND	HGDI	Level		
424	DR & MDR	19				19								0.00	Low		
	Susceptible to INH&RIF	19	1	4	13	1								0.51	Moderate		
580	DR & MDR	19		16									3	0.28	Low		
	Susceptible to INH&RIF	19		18									1	0.11	Low		
802	DR & MDR	19			16		3							0.28	Low		
	susceptible to INH&RIF	19	1	3	15									0.37	Moderate		
960	DR & MDR	19			19									0.00	Low		
	Susceptible to INH&RIF	19		1	18									0.11	Low		
1644	DR & MDR	19			19									0.00	Low		
	Susceptible to INH&RIF	19			19									0.00	Low		
1955	DR & MDR	19					19							0.00	Low		
	Susceptible to INH&RIF	19		1	1	1	14	2						0.46	Moderate		
2163b	DR & MDR	19				16	3							0.28	Low		
	Susceptible to INH&RIF	19		1		3	6	9						0.68	High		
2165	DR & MDR	19				19								0.00	Low		
	Susceptible to INH&RIF	19				19								0.00	Low		
2401	DR & MDR	19				19								0.00	Low		
	Susceptible to INH&RIF	19				17	2							0.20	Low		
2996	DR & MDR	19					1		18					0.11	Low		
	Susceptible to INH&RIF	19				1	1		15	2				0.38	Moderate		
3192	DR & MDR	19					19							0.00	Low		
	Susceptible to INH&RIF	19					19							0.00	Low		
3690	DR & MDR	19			19									0.00	Low		
	Susceptible to INH&RIF	19			18	1								0.11	Low		
4052	DR & MDR	19								19				0.00	Low		
	Susceptible to INH&RIF	19							1	17		1		0.20	Low		
4156	DR & MDR	19		19										0.00	Low		
	Susceptible to INH&RIF	19		18			1							0.11	Low		
4348	DR & MDR	19			19									0.00	Low		
	Susceptible to INH&RIF	19			18	1								0.11	Low		

Abbreviations: MIRU-VNTR, mycobacterial interspersed repetitive unit-variable-number tandem repeat;
DR, drug resistance; INH, isoniazid; RIF, rifampicin