Insight into the Beijing genotype *Mycobacterium tuberculosis* clinical isolates in Myanmar

(ミャンマーにおける北京型結核菌臨床分離株に関する研究)

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Transboundary spread of high risk Beijing genotype *Mycobacterium tuberculosis* strains in Mawlamyine near Myanmar-Thailand border area

ABBREVIATIONS

CAS	Central Asian strain
CDS	Coding sequence
CRISPRs	Clustered regularly short palindromic repeats
RD	Regions of difference
EAI	East Asian Indian
LAM	Latin American Meditrranean
LSP	Large Sequence Polymorphism
MDR	Multidrug resistance
MIRU	Mycobacterial interspersed repetitive unit
MST	Minimum spanning tree
MTB	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* complex
NTF	Noise transfer region
NTM	Non-tuberculous *Mycobacterium*
PCR	Polymerase Chain Reaction
RFLP	Restriction fragment length polymorphism
SIT	Spoligo-international type
SNP	Single nucleotide polymorphism

sSNP	Synonymous single nucleotide polymorphism
TB	Tuberculosis
UPGMA	Unweighted pair group method with arithmetic averages
VNTR	Variable number of tandem repeat
XDR	Extensively drug resistance

PREFACE

Tuberculosis (TB) is one of the world leading cause of death by infectious disease with 10.4 million incidences. It is mainly caused by a bacterium called *Mycobacterium tuberculosis* (MTB). TB is threatening the world, especially in the region of socioecological disadvantages. The increasing number of multidrug resistance/rifampicin resistant (MDR/RR) as well as extensively drug resistant (XDR) TB causes obstacles for effective control. MDR/RR-TB was accounted for 4.1% of new and 19% of previously treated cases in the 2017 updated WHO report [1].

MTB is one of the members of the *M. tuberculosis* complex (MTBC), an acid-fast, intracellular, aerobic bacillus with a tough cell wall structure containing high content of mycolic acids, long-chain cross-linked fatty acids, and other cell-wall lipids. It is an intracellular pathogen and able to survive by slow growing in an adverse environment, such as inside of the macrophage.

MTB has a circular chromosome, which contains about 4,200,000 base pair consisting of 65% GC content. Several genotyping methods of MTB have been employed for the differentiation into lineages which dominate in certain regions of the world, for the estimation of transmission and for observation of evolution. Moreover, it is applicable for the determination of variable drug susceptibility pattern to help for anti-TB therapy. It is also useful for determination of relapse and recurrence of TB, and investigation of drug resistance development to evaluate anti-TB treatment strategy [2].

The most common genotyping methods for MTB are IS*6110* restriction fragment length polymorphism (RFLP) typing, the spacer oligonucleotide typing (spoligotyping), and Mycobacterial interspersed repetitive units-variable number of tandem repeats (MIRU-VNTR) typing. IS*6110* RFLP typing was formerly used as a gold standard method with the

availability of large databases. Depending on the presence or absence of spacers with different sequences at the direct repeats locus, spoligotyping has been applied for the differentiation of various types of sublineages such as East Asian Indian (EAI), Beijing, Central Asian strain (CAS), Latin American Mediterranean (LAM), X, S, T. MIRU-VNTR typing using with 12, 15, and 24 loci, which contain identical repeat units, is a useful analysis for the large numbers of strains and it is comparable by using digital format [2]. The spoligotyping and MIRU-VNTR typing data are able to be compared with a different large number of strains by using web site databases [3,4]. Another genotyping method, large sequence polymorphism (LSP), differentiates members of MTBC into 7 lineages (such as Indo-Oceanic lineage, East Asian lineage, Central Asian lineage, Euro-American lineage, West African lineage 1, West African lineage 2 and Ethiopian lineage) [5,6]. It is useful for the study of evolution and the structured population related to the variable human hosts [5,7].

The Beijing genotype, belonging to East Asian lineage 2, has been found in East Asia with increasingly worldwide [8,9]. A strong association of the Beijing lineage with the drug resistance, including MDR and XDR has been observed in previous studies of other countries as well as Myanmar [10–12]. Moreover, several studies found out the association between high virulence, increased pathogenicity, higher risk of transmission, and age of host and the Beijing genotype [9,11].

Myanmar is included in 30 high burden countries of TB as well as MDR-TB, and reached on the 10th position of the incidence of TB according to the WHO report, 2017 [1]. The incidence rate of TB in Myanmar is 361 per 100,000 population. The estimated MDR-TB cases were 9,000 and cases of multidrug resistance/ rifampicin resistance tuberculosis (MDR/RR-TB) in 2016 were 13,000 with the 5.1% new cases and 27% of previously treated cases [1]. The previous studies, including published and unpublished ones, in Myanmar, pointed out the higher proportion of Beijing genotype strains in MDR-TB and XDR-TB [13,14].

Therefore, we aimed to perform genotypic characterization of the Beijing genotype strains in Myanmar in order to investigate the lineage characteristics, population structure and the trend of transmission among the community in Myanmar.

The present thesis consists of three chapters. The first chapter contains genotyping on MDR-MTB for the determination of lineages by spoligotyping and SNPs typing. The majority of the lineage was Beijing genotype particularly modern Beijing subtype, and all the Beijing strains were further differentiated by MIRU-VNTR typing. In addition, sequence analysis of drug resistance associated genes including *rpoB, rpoC, katG, gyrA* and *rrs* was carried out. The combinatorial analysis of the clustered isolates were done to identify the transmission events. The findings suggested that the Beijing genotype had a tendency to develop drug resistance and might be spreading in Myanmar that are major challenging to effective TB control.

In the second chapter, the rapid determination of the modern Beijing genotype by new multiplex PCR was developed for determination of the ancient or modern Beijing sublineage. The test accuracy was determined by sequencing for detection of the SNP at 1477596 position on the H37Rv genome. The complete accuracy in determination of ancient and modern Beijing genotype strains was established.

In the third chapter, MTB isolated from sputum samples of TB patients in Mawlamyine, Myanmar in 2016 were subjected to genotypic characterization, including detection of the drug resistance conferring genes, lineage determination by spoligotyping, identification of the Beijing lineage strains by multiplex PCR and further determination of modern Beijing subtype by newly developed multiplex PCR. Furthermore, the Beijing genotype strains were subjected by MIRU-VNTR typing in order to perform further discrimination. The majority of the Beijing genotype strains were modern subtype and had a

strong association with the mutations of the drug resistance associated genes. Phylogenetic analysis and minimum spanning tree (MST) analysis were done for the determination of the transmission pattern by using additional data from previous studies of Myanmar and Thailand. The tendency of spread of the Beijing genotype strains across the border between the countries had been observed.

Chapter I

Insight into multidrug-resistant Beijing genotype *Mycobacterium tuberculosis* isolates in Myanmar

Introduction

Tuberculosis (TB) is the leading cause of death by infectious disease worldwide, with an estimated 10.4 million new TB cases in 2016 [1]. Myanmar, situated in the South East Asian region, is one of the 30 highest TB burden countries with a TB incidence rate in 2016 of 361 per 100,000 population [1].

MDR-TB is defined as TB that is caused by *Mycobacterium tuberculosis* (MTB) resistant to at least the two most powerful anti-TB drugs, isoniazid (INH) and rifampicin (RFP). MDR-TB forms an estimated 19% of previously treated TB cases and 4.1% of new TB cases, with a global incidence of 600,000 MDR/RR-TB (RFP resistant TB) cases in 2016. Myanmar is also included in the top 20 countries that cumulatively account for 84% of the global multidrug-resistant TB (MDR-TB) burden. In Myanmar, there were 9,000 estimated cases of MDR-TB and 13,000 cases of MDR/Rifampicin resistant (RR)-TB in 2016, including 5.1% of new cases and 27% of previously treated cases [1]. MDR-TB relatively easily develops further drug resistance such as extensively drug resistant tuberculosis (XDR-TB) which is categorized when MDR-TB has additionally acquired resistance against a fluoroquinolone and an injectable aminoglycoside such as kanamycin or capreomycin. MDR-TB, XDR-TB and the development of additional drug resistance are major threats to the local and global control of TB; efficient strategies to overcome them are required.

Several genotyping methods have been applied for the differentiation of MTB for the purpose of molecular epidemiology. One of the genotyping methods, spoligotyping, is used to differentiate MTB strains into distinct families and reveal its population genetic structure and phylogeographical characteristics [15,16]. Spoligotyping is performed by detecting the presence of 43 unique spacer sequences in the direct-repeat region in the MTB genome with a PCR-based reverse-hybridization blotting technique. This method classifies MTB strains into different lineages/sublineages such as East-African Indian (EAI), Beijing, Central Asian (CAS), Latin American Mediterranean (LAM), Haarlem, X and T [15]. Genetic characterization using regions of difference (RD) further clarified the positions of each spoligotype family on the MTB complex phylogenetic tree [7]. Seven human-adapted lineages, which also show characteristic geographic distribution, have been identified by whole genome sequencing and show congruence with major spoligotypes as lineage 1 (EAI), lineage 2 (East-Asian lineage, Beijing), lineage 3 (CAS) and lineage 4 (LAM, Haarlem, X and T) [17].

The Beijing genotype has been considered as one of the most predominant genotypes of MTB. It is commonly found in Asia and is particularly dominant in East Asian countries such as China, Mongolia, Korea, and Japan [9,18–20]. It is also widely distributed around the world in regions other than Asia, with relatively high prevalence or numbers of outbreaks reported in areas such as the former Soviet Union, the U.S.A and South Africa [10,15,18,21–23]. The Beijing genotype has been reported to be more virulent than other MTB genotypes, showing higher pathogenicity as well as increased mortality in animal studies [9,24]. Another particular feature of the Beijing genotype is higher mutation rates that facilitate the emergence of drug resistance [25]. The strong association between the Beijing genotype and drug resistance was evidenced and the proportion of this lineage among MDR-TB and/or XDR-TB was higher than drug-susceptible TB [23–25]. These characteristics of increased drug

resistance are thought to promote the transmission, success and ultimate global distribution of the Beijing lineage.

In a previous study conducted in Myanmar, the most frequently observed MTB lineage was EAI (48.4%) and the Beijing type was the second (31.9%); however, the proportion of the Beijing lineage was significantly higher than EAI among drug resistant isolates [13]. In other surveys on drug resistant TB in Myanmar, the prevalence of the Beijing genotype in MDR or XDR-TB population was also found to be higher than that of total TB cases [14,26]. As the majority of Beijing-type isolates shows Spoligo-International-Type (SIT) pattern 1(SIT 1) and they might have the different repeat numbers in the MIRU-VNTR loci, additional typing method like mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) analysis was recommended for further discrimination of this genotype [20,27,28].

However, only a few phylogenetic analyses of the circulating MTB Beijing genotype in Myanmar applied additional typing methods. To develop greater insight into the emergence and transmission of drug resistant MTB in Myanmar, we have performed multiple genotyping analysis, including spoligotyping and MIRU-VNTR, along with targeted analysis of mutations associated with drug resistance on MTB clinical isolates from Myanmar with the Beijing genotype.

Materials and methods

Sample collection and drug susceptibility test

A total of 265 clinical isolates, identified as MDR-MTB by phenotypic drug susceptibility tests (DSTs), were used in this study, comprising 96 and 169 isolates collected in 2010 and 2012, respectively, by the Myanmar National TB program in Yangon. All the isolates were derived from different individuals. DST was carried out on Löwenstein-Jensen medium by the conventional proportional method with concentrations of 0.2, 2, 4, 40 μg/ml of isoniazid (INH), ethambutol (EMB), streptomycin (STR) and rifampicin (RFP), respectively. After 6 weeks incubation at 37℃, growth results were interpreted as susceptible or resistance.

DNA extraction

MDR-MTB colonies recovered from Löwenstein-Jensen media were suspended in distilled water and boiled for 20 minutes and the supernatant containing bacterial DNA was used for PCR.

Spoligotyping

Spoligotyping was carried out on all the MDR-MTB samples as described by Kamerbeek et. al. [16]. In brief, by using a primer pair, the direct repeat region was amplified and the PCR products were hybridized to a set of 43 oligonucleotide probes corresponding to each spacer which covalently bound to the membrane. The SIT type was determined from the spoligotyping data base SpolDB4[15].

MIRU-VNTR typing

Beijing genotype samples were subjected to MIRU-VNTR typing using 15 loci selected from Wang et al. [20] based on the high discriminatory power for the Beijing genotype isolates. These are based on Supply's standard 15-locus MIRU-VNTR [28], with ETR-C was replaced by MIRU39, and comprise: five mycobacterial interspersed repeat units (MIRU10, MIRU16, MIRU26, MIRU40, MIRU39); three exact tandem repeats (ETR-A, ETR-D, ETR-E); four Mtub group (Mtub04, Mtub21, Mtub39, Mtub30); and three MIRU-VNTR loci identified by Queen's University Belfast group (QUB26, QUB11b, QUB4156). The numbers of each tandem repeat were calculated from the PCR-product size by conventional gel electrophoresis. Experiments with ambiguous results were repeated for confirmation, and isolates which showed multiple bands in more than two loci, suggestive of mixed populations, were excluded from the analysis.

Gene mutation detection by sequencing

Discrimination of so-called "modern" or "ancient" Beijing sub-lineages was determined by the detection of a single nucleotide polymorphism (SNP) corresponding to position 1477596 on the H37Rv genome [29] by sequencing according to the procedure in Li et al [30].

Sequencing to detect mutations in genes associated with drug resistance was performed as previously described by Poudel et al. [31,32] targeting: rifampicin resistance determining region (RRDR) in *rpoB* for RFP; *katG* coding and *inhA* promoter regions for INH; quinolone resistance determining region (QRDR) in *gyrA* for fluoroquinolone; and 16S ribosomal RNA gene *rrs* for aminoglycoside. Mutations in *rpoC* were analyzed by sequencing a PCR amplicon (*rpoC* position: 1202-2202) produced by the following primer

pair: TB *rpoC* (1202-22): CGCTTTCCGATCTGCTCAAGG and TB *rpoC* (2202-183): GGCCATCGACACCGTCACGC. The PCR and sequencing conditions were same as for the protocol in the previous report [31]. The sequences were compared with the wild-type sequences of H37Rv using Bio-Edit software version 7.0.9 [33].

Data analysis

Allelic diversity (*h*) of each MIRU-VNTR locus was calculated by using the equation $h = 1 - \sum xi\ 2\ [n/(n - 1)]$, in which *n* is the number of isolates and *xi* is the frequency of the *i*th allele at the given MIRU-VNTR locus [20]. The results obtained from the MIRU-VNTR typing were analyzed by BioNumerics software version 6.6 (Applied Maths, Belgium). For clustering analysis, phylogenetic trees were calculated using the unweighted pair group method with arithmetic averages (UPGMA). Minimum spanning tree (MST) was constructed using the categorical coefficient according to the software's instructions. Clusters were defined as two or more isolates sharing an identical 15-loci MIRU-VNTR pattern, and the clustering rate was calculated using the formula number of clustered isolates / total number of isolates [34].

Results

Spoligotype and SNP type

The lineage distribution of the MDR-MTB isolates is shown in Table 1. Of the total MDR-MTB isolates, 79.2 % were classified as the Beijing lineage (210/265). The second most common lineage was EAI which accounted for 10.6 % (28/265) of the total isolates. All other isolates belonged to minor lineages with each percentage < 1.5 %. The SIT was identified according to the previous study [15]. Among the Beijing lineage, SIT 1 was the majority type and consisted of 204 isolates, while five isolates belonged to SIT 190 and one isolate was classed as SIT 621[15] (Table 2).

All the Beijing genotype isolates were subjected to sequencing to determine whether they were the "modern" or "ancient" subtype. Twenty-five out of the 210 Beijing isolates (11.9 %) had nucleotide C at position 1477596 and were revealed as the ancient type, while the remaining isolates (88.1 %) had nucleotide T at this position and were hence classed as modern type [29,35]. All the 25 ancient-type Beijing lineage isolates showed spoligotype SIT1 pattern, while SIT190 and SIT621 isolates were all included in the modern-type.

MIRU-VNTR

A total of 210 Beijing genotype MTB isolates were subjected to further differentiation by 15-locus MIRU-VNTR typing. The allelic diversity (*h*) of each locus was determined as shown in Table 3 [36]. High discriminatory power ($h > 0.6$) was detected in 3 loci, Qub26, MIRU26 and Qub11b, while poor discriminatory power ($h < 0.3$) was observed in 5 loci (QUB4156, Mtub30, Mtub39, ETR A, and MIRU16) [37].

Genetic diversity of the Beijing isolates was represented in a dendrogram as shown in Figure 1. A total of 144 MIRU-VNTR patterns were identified, with 36 clusters disclosed consisting of 102 isolates, while the remaining 108 patterns were unique. A cluster was defined as two or more patient isolates with identical MIRU-VNTR pattern. The number of isolates contained in each cluster ranged up to 8. The clustering rate was calculated as 48.6 % (102/210). Among the 36 clusters, 21 and nine clusters consisted of isolates solely from 2012 and 2010, respectively, while the remaining six clusters of 3 ~ 8 isolates were mixture in both years (Figure 1).

The same set of the MIRU-VNTR data is shown in a MST in Figure 2. Each node represents a MIRU-VNTR type and the size of the node indicates the number of the isolates in each cluster. All the modern-type Beijing isolates were connected in a large group, whereas ancient-type isolates formed small groups or were scattered (Figure 2). The clustering rate in the modern-type was 53.0 % (98/185), while it was 16.0 % (4/25) in the ancient-type. A cluster of three isolates, positioned in the middle of the modern-type grouping (circled blue in Figure 2), had the same MIRU-VNTR type with the successful Russian clone CC2 [21] as revealed by the comparison with the MIRU-VNTRplus web tool (http://www.miru-vntrplus.org/MIRU/index.faces) [4].

Sequence analysis of drug resistance-associated genes

For the Beijing genotype MDR-MTB isolates, drug resistance-associated mutations in CDS of *rpoB*, *katG*, *gyrA*, *rrs* and the *inhA* promoter region were analyzed (Table 4). The majority of *rpoB* gene mutations was Ser450Leu, accounting for 67.6 % of the isolates, while all other *rpoB* mutations accounted for less than 5.7 %. Among INH resistance mutations, *katG* Ser315Thr was found to be dominant (91.0 %), while promoter mutations *inhA*

accounted for 8.1 % of the total. The proportion of *rpoB* (Ser450Leu) among the modern and the ancient isolates were 69% (128/185) and 56% (14/25), respectively, and no significant difference was detected between these sub-genotypes. Similarly, the ratio of *katG* (Ser315Thr) among the modern isolates was 92% (170/185) and 84% (21/25) in ancient isolates, revealing no significant difference between them.

Mutations indicative of resistance against fluoroquinolones or injectable aminoglycosides were also found in *gyrA* or *rrs*, respectively (Table 4). Thirty-one isolates (14.8%) had a mutation, including double mutation in one, in the QRDR region of *gyrA*, with the most frequent mutation being A281G (Asp94Gly). Nine isolates (4.3%) had a mutation in *rrs*, with all but one being A1400G according to the rrs position of Suzuki et al. [38]; of these nine, eight were accompanied by a *gyrA* mutation. These genetic results would suggest these were XDR-MTB strains, accounting for 3.8% of MDR-MTB. The number of pre-XDR strains, having a mutation either in *gyrA* or *rrs*, was 24 (11.4 % of the MDR-MTB).

Combinatorial analysis on the clustered isolates

To identify possible MDR-MTB transmission events, isolates clustered by the MIRU-VNTR analysis were further analyzed by comparison of the acquired drug resistance mutations (Table 5). In the 102 isolates grouped into 36 clusters, the prevalence of the major *rpoB* mutation of C1349T (Ser450Leu) (69/102, 67.6%) was the same as in the total 210 isolates, although the ratio in each cluster was different. Among the 36 clusters, 26 clusters had two or more isolates that shared the same *rpoB* mutation, and most of these clusters (23/26) showing the major Ser450Leu substitution. Other than this, there were four *rpoB* mutations shared by isolates in the same cluster: Clusters No. 10, 13, 17 and 18 (Table 5). In Cluster 13, two isolates shared *rpoB* His445Arg also had an additional mutation C-15T in the

inhA promoter region as well as *katG* Ser315Thr. In Cluster 17, two isolates had the same deletion mutation of Asn437 in *rpoB,* and three isolates had the same *rpoB* substitution His445Tyr in Cluster 18.

Certain mutations in *rpoC* have been suggested to act as compensatory mutations to an RFP resistance-associated mutation in *rpoB* [39,40]. We therefore investigated the *rpoC* sequence, focusing in a region where compensatory mutations are frequently observed, in isolates having the same *rpoB* mutation in the same cluster (n=74). Twenty-three isolates had a mutation in the *rpoC* region (23/74, 31.1%) and all of them showed the *rpoB* Ser450Leu genotype. The majority of the isolates (15/23, 65.2% of *rpoC* mutations) were shown to harbor the T1448G (Val483Gly) mutation. Three pairs, each in the same clusters, had the *rpoB* Ser450Leu – *rpoC* Val483Gly combination (Table 5). In the combinatorial comparison with all the genotyping results including *gyrA* and *rrs* mutations, one pair in Cluster 33 showed a four-gene match, i.e. *rpoB* Ser450Leu, *katG* Ser315Thr, *gyrA* Asp94Gly and *rrs* A1400G, although all these mutations were the most frequent ones for each gene. Out of eight genetically XDR-MTB isolates, seven were found among the clustered isolates. The ratio of isolates having a mutation in *gyrA*, suggestive of XDR or pre-XDR, was higher in clustered groups (18/74, 24.3%) than in non-clustered groups (13/136, 9.6%), although all, except in the case of Cluster 33, did not share the same *gyrA* mutations in the same cluster. The diversity of the mutations in *rpoB*, *katG* and the *inhA* promoter region is also shown visually in a MST (Figure 3 and 4).

Discussion

This study aimed to determine the transmission of drug resistant TB in Myanmar, a high burden country for both TB and MDR-TB. As expected, based on previous studies in Myanmar [13,14,26], the majority of the MDR-MTB isolates were determined to be the Beijing genotype, although its ratio among the total TB cases was less than one third [13]. Among the Beijing strain types, the vast majority were determined to be the 'modern' genotype by identification of the modern Beijing lineage-defining SNP marker [29,35,41]. The modern Beijing genotype was reported to have a strong association with drug resistance and the spread of MDR-MTB. The majority of the globally distributed Beijing strains belong to the modern type, and they tend to cause outbreaks that generate large clusters that suggest recent transmission [21,23,27,42,43]. In our current study, no large cluster suggestive of a large MDR-MTB outbreak was observed; however, the clustering rate of the modern isolates was more than 50 %.

Regarding gene mutations associated with INH resistance, the *katG* Ser315Thr substitution accounted for more than 90 %; this is a well-known low-fitness cost substitution and MTB that acquire this mutation maintains efficient transmission [44–46]. The high prevalence of the *katG* Ser315Thr mutation may suggest an ongoing spread of INH resistant strains harboring this mutation. Among the *rpoB* mutations that confer RFP resistance, the major Ser450Leu substitution is also suggested to be of a low-fitness cost. These low-fitness-cost mutations were observed in higher ratios in the modern-type isolates than the ancient-type in our study, though the difference was not significant. The combination of *rpoB* Ser450Leu with *katG* Ser315Thr is reported to be the most likely transmissible MDR-MTB genotype [42,47]. As this mutation combination was seen in more than half the MDR-MTB isolates in our current study, this finding is of great concern for TB control in Myanmar.

Mutations in a specific region of *rpoC* are thought to act as a compensatory mechanism for the lost functional activity caused by *rpoB* mutations [39,40]. Screening for such compensatory *rpoC* mutations revealed that they were also found in a high ratio (1/3) in the clustered isolates, suggesting those were transmissible MDR-MTB. Although a high ratio (65.2 %) of *rpoC* T1448G (Val483Gly) was observed among the *rpoC* compensatory mutations, those isolates were scattered in the different clusters on the MST (Figure 5) and a low possibility of the clonal expansion of a specific MDR-MTB clone was suggested. As T1448G (Val483Gly) is one of the major *rpoC* mutations [39,40], the predominance might occur if the fitness cost of this compensatory mutation was low enough. Moreover, an additional mutation might have occurred in *rpoB*, *rpoC* or an associated gene of the parent clone that caused *rpoC* T1448G (Val483Gly) to be selected as the most fit compensatory mutation of *rpoB* (Ser450Leu) in the descendants. These questions will be answered once these isolates are analyzed by whole genome sequencing.

Furthermore, possible XDR or pre-XDR isolates were detected at a significantly higher frequency in the clustered group than in the non-clustered group ($p < 0.01$). Mutations in *gyrA* suggest that the patients had not been cured by the standard TB treatment and had been treated for MDR-TB, suggesting that some of the patients might have been originally infected with an MDR-MTB strain. Some isolates shared not only the MIRU-VNTR pattern but a rare *rpoB* mutation, or combination of specific mutations, suggesting a direct transmission of the particular MDR-MTB clone. The isolates collected in the same year tended to form clusters indicating transmission of MDR-MTB or a potential sampling bias. Unfortunately, we could not get detailed patient information, e. g. residence, treatment history or patient contact history, in this retrospective study. Further studies are needed to clarify the situation of MDR-MTB transmission by analyzing primary MDR-TB cases as well as a comparison of the clustering pattern of MDR-MTB with that of drug-susceptible isolates.

However, the current data may indicate that we still can detect possible MDR-MTB transmission events from MTB genotypic information [48].

In the MST constructed by the 15-loci MIRU-VNTR, all the modern isolates were branch-connected and formed a large tree, suggesting they were genetically closely related (Figure 2). As the ancient genotype isolates, having different MIRU-VNTR patterns from each other and modern genotype isolates, showed higher diversity, it suggested that this genotype had spread in Myanmar earlier than the modern type, and that the latter was introduced into Myanmar at a later date. The cluster at the center of the MST (circled blue line in Figure 2) could be the original genotype of modern Beijing isolates introduced in Myanmar. Intriguingly this cluster had the same MIRU-VNTR as CC2 (clonal complex 2) identified by Merker et al. [21]. CC2 includes the well-known clone W148, which has been spreading around East-Europe and Russia in the form of MDR-MTB. Although the characteristic IS6110-RFLP pattern had been checked and the Myanmar isolates were not deemed to be part of W148 by Mokrousov et al. [49], our data suggests that the Myanmar modern Beijing strains described in our study and the notorious Russian strain should share a recent common origin. The pattern of the adjacent cluster (also consisting of three isolates, shown in Figure 2) was just one locus (QUB26) different in the 15-loci MIRU-VNTR, and it was also observed in the center of the phylogenetic trees constructed from modern Beijing isolates from Shanghai, China [50] and Nepal [32] (Maharjan et al., unpublished data), suggesting it was the original genotype of the clone expanding in these areas. Those clones might belong to the original clonal complex CC3 or CC1 that are spreading throughout Central Asia [21]. In any case, the ancestor of the Myanmar modern Beijing genotype was a descendent of the widely spreading clones. Thus, there is little wonder that the Myanmar MTB Beijing isolates possess similar characteristics of high transmissibility and being prone to develop drug resistance. The level of the similarity and evolutionary relationship among these clones will

be resolved once they are subjected to whole-genome sequence analyses [43]. Recently, Ates et al. [51] describe that modern Beijing strains have a genetic lesion in the PPE38 gene that inactivates a type VII secretion system and leads to increased virulence of the lineage; this increased virulence may contribute to the comparative success of the Beijing lineage compared to others.

In summary, the majority of the MDR-MTB isolates in Myanmar were shown to belong to the modern Beijing genotype and most of the drug resistance seemed to be acquired in each patient under the pressure of TB treatment. They were genetically diverse, with no dominant cluster observed that would have been suggestive of an MDR-TB outbreak. However, genetic analysis of the isolates indicated the spread of INH resistant clones possessing the *katG* Ser315Thr substitution as well as revealing MDR-MTB transmission cases. To reach the TB-related targets set out in the United Nations Sustainable Development Goals, TB incidence must be reduced to 75/100,000 in Myanmar. To achieve these targets, our work shows that it will be necessary to carry out continuous surveillance of drug resistant TB, especially focusing on the modern Beijing genotype MTB, so as to develop a clear picture of prevalence, transmission and evolution of the TB epidemic in Myanmar.

Summary

Myanmar is a high tuberculosis (TB) burden country designated by WHO with a high multidrug-resistant (MDR)-TB burden. A significantly high prevalence of the Beijing genotype of *Mycobacterium tuberculosis* (MTB) among MDR-MTB has been reported previously. To explore an association between the prevalence of the Beijing genotype and MDR-TB in Myanmar, we performed detailed genetic characterization of TB clinical isolates.

A total of 265 MDR-MTB clinical isolates collected in 2010 and 2012, were subjected to spoligotyping, mycobacterial interspersed repetitive unit–variable number tandem repeat (MIRU-VNTR) analysis, SNP typing and drug resistance-associated gene sequencing including *rpoC* to detect potential compensatory evolution.

Beijing genotype isolates were differentiated by 15-loci MIRU-VNTR. Of the total MDR-MTB isolates, 79.2 % (210/265) were the Beijing genotype, the majority of which were the “modern” subtype in which a high clustering rate (53.0 %) was observed. High prevalence of *katG* Ser315Thr, and genetic evidence of XDR and pre-XDR and compensatory mutations in *rpoC* were observed among clustered isolates.

MDR-MTB strains of the Beijing genotype might be spreading in Myanmar and present a major challenge to TB control in this country.

Table 1: Spoligotyping results of MDR-MTB isolates (n=265)

Clade*	No. of isolates	Percentage (%)
Beijing	210	79.2
EAI	28	10.6
X2	4	1.5
CAS	3	1.1
Beijing like	2	0.8
H37Rv	2	0.8
Harlem like	1	0.4
LAM	1	0.4
S	1	0.4
T1	1	0.4
Undesignated	12	4.5
Total	265	100.0

*Clades are according to SpolDB4 (Brudey et al., 2006)

Table 2: MDR-MTB Beijing isolates with different Spoligo international types (SIT) (n=210)

		Beijing sub genotype	No. of isolates	Percentage (%)
	SIT 1	Modern	179	85.2
		Ancient	24	11.4
Beijing	SIT 190	Modern	6	2.8
		Ancient	0	0
	SIT 621	Modern	1	0.48
		Ancient	0	0
Total			210	

Table 3: MIRU-VNTR allelic diversity of Beijing genotype isolates (n=210)

TR*	**MIRU-VNTR locus**														
	MIRU 10	MIRU 16	MIRU 26	MIRU 31	MIRU 40	Mtub 04	Mtub 21	Mtub 39	ETR-A	QUB 11b	QUB 26	MIRU 4	Mtub 30	QUB 4156	MIRU 39
0		1		3	1				3			50			
1		1			8		3					10			
2	61	208	1		11	5	2	8	3		3	150	6	175	12
3	139			6	158	15	42	200	2	10			6	16	139
4	10			40	32	172	23	2	202	3	1		194	19	58
5			20	158		17	126			118	5		3		1
6			100	3		1	8			60	44				
7			81				3			17	89		1		
8			8				3			1	68				
9										1					
10															
*h***	**0.48**	**0.02**	**0.62**	**0.40**	**0.41**	**0.32**	**0.59**	**0.09**	**0.07**	**0.60**	**0.67**	**0.49**	**0.15**	**0.29**	**0.48**

(number of isolates)

* TR: Tandem repeat number in each MIRU-VNTR locus

**Allelic diversity: $h = 1 - \Sigma xi^2 [n/(n - 1)]$

Table 4: Drug resistance associated mutations in *rpoB, katG, gyrA, rrs* genes and the promoter region of *inhA* in Beijing isolates (N=210)

Gene	Amino acid position	Nucleotide change		Amino acid change		Number of isolates	Ratio (%)
		From	To	From	To		
rpoB	429	CAG	CGG	Gln	Arg	1	0.5
	430	CTG	CCG	Leu	Pro	2	1.0
	432	CAA	CCA	Gln	Pro	2	1.0
	432	CAA	CTA	Gln	Leu	1	0.5
	435	GAC	TAC	Asp	Tyr	1	0.5
	435	GAC	GTC	Asp	Val	5	2.4
	437	AAC	deletion	Asn	-	2	1.0
	445	CAC	CTC	His	Leu	3	1.4
	445	CAC	CGC	His	Arg	5	2.0
	445	CAC	AAC	His	Asn	1	0.5
	445	CAC	GAC	His	Asp	9	4.3
	445	CAC	TAC	His	Tyr	12	5.7
	450	TCG	TAC	Ser	Tyr	1	0.5
	450	TCG	TTG	Ser	Leu	142	67.6
	450	TCG	TTC	Ser	Phe	2	1.0
	452	CTG	CCG	Leu	Pro	4	1.9
	435/437	GAC/AAC	TAC/GAC	Asp/Asn	Tyr/Asp	1	0.5
	435/452	GAC/CTG	GCC/CCG	Asp/Leu	Ala/Pro	1	0.5
	445/452	CAC/CTG	AAC/CCG	His/Leu	Asn/Pro	2	1.0
	430/435/437	CTG/GAC/AAC	CCG/TAC /GAC, CAC	Leu/Asp/Asn	Pro/Tyr/Asp,His	1	0.5
	None	None		None		12	5.7
katG	285	GGC	CGC	Gly	Arg	1	0.5
	315	AGC	AAC	Ser	Asn	5	2.4
	315	AGC	ACC	Ser	Thr	191	91.0
	None	None		None		13	6.2
inhA	-15	C	T			16	7.6
	-34	C	T			1	0.5
	None	None				193	91.9
gyrA	90	GCG	GTG	Ala	Val	6	2.9
	94	GAC	GCC	Asp	Ala	2	1.0
		GAC	AAC	Asp	Asn	2	1.0
		GAC	GGC	Asp	Gly	16	7.1
		GAC	TAC	Asp	Tyr	4	1.9
	90/94	GCG/GAC	GTG/GGC	Ala/Asp	Val/Gly	1	0.5
	None	None		None		179	85.2
rrs	1400*	A	G			8	3.8
	1401*	C	T			1	0.5
	None	None				201	95.7

**rrs* position is according to Suzuki et al (1988).

Table 5: Genetic characteristics of clustered isolates

ID	Genotype			Putative amino acid substitutions and confering mutations in drug resistance-associated genes[d]									Cluster
	SIT [a]	M/A [b]	MIRU-VNTR[c]	*rpoB* amino acid	*rpoB* nucleotide	*rpoC amino acid*	*katG amino acid*	*katG* nucleotide	*inhA*	*gyrA amino acid*	*gyrA* nucleotide	*rrs*	
M4-120	1	M	235534534572424	Ser 450 Leu	C 1349 T	Val 483 Gly	Ser 315 Thr	G 944 C	WT	WT	WT	WT	**1**
M4-66	1	M	235534534572424	Ser 450 Leu	C 1349 T	WT	Ser 315 Thr	G 944 C	WT	WT	WT	WT	
M1-58	1	M	236534534572424	Ser 450 Leu	C 1349 T	WT	Ser 315 Thr	G 944 C	WT	WT	WT	WT	**2**
M4-128	1	M	236534534572424	Ser 450 Leu	C 1349 T	WT	Ser 315 Thr	G 944 C	WT	WT	WT	WT	
M4-139	1	M	236534534572424	Ser 450 Leu	C 1349 T	Val 483 Gly	Ser 315 Thr	G 944 C	WT	Asp 94 Asn	G 280 A	WT	
M4-162	1	M	236534534572424	Ser 450 Leu	C 1349 T	WT	Ser 315 Thr	G 944 C	WT	Asp 94 Gly	A 281 G	C 1401 T	
M4-165	1	M	236534534572424	Ser 450 Leu	C 1349 T	WT	Ser 315 Thr	G 944 C	WT	WT	WT	WT	
M4-3	1	M	236534534572424	WT	WT	-	Ser 315 Thr	G 944 C	WT	WT	WT	WT	
M4-116	1	M	236534534562424	Ser 450 Leu	C 1349 T	WT	Ser 315 Thr	G 944 C	WT	WT	WT	WT	**3**
M4-167	1	M	236534534562424	Ser 450 Leu	C 1349 T	WT	Ser 315 Thr	G 944 C	WT	WT	WT	WT	
M4-168	1	M	236534534562424	Ser 450 Leu	C 1349 T	WT	Ser 315 Thr	G 944 C	WT	WT	WT	WT	
M1-50	1	M	235434534572424	Ser 450 Leu	C 1349 T	Val 483 Gly	Ser 315 Thr	G 944 C	WT	WT	WT	WT	**4**
M4-62	1	M	235434534572424	Ser 450 Leu	C 1349 T	WT	Ser 315 Thr	G 944 C	WT	Ala 90 Val	C 269 T	WT	
M4-64	1	M	235434534572424	Ser 450 Leu	C 1349 T	WT	Ser 315 Thr	G 944 C	WT	WT	WT	WT	
M4-108	1	M	337534534572424	Ser 450 Leu	C 1349 T	-	Ser 315 Thr	G 944 C	WT	Asp 94 Tyr	G 280 T	WT	**5**
M4-48	1	M	337534534572424	Gln 432 Leu	A 1295 T	-	Ser 315 Thr	G 944 C	WT	WT	WT	WT	
M4-75	1	M	336544534572424	His 445 Arg	A 1334 G	-	Ser 315 Thr	G 944 C	C -15 T	Ala 90 Ala/Val, Asp 94 Gly/Asp	C 269 C/T, A 281 G/A	WT	**6**
M4-77	1	M	336544534572424	Leu 452 Pro	T 1355 C	-	Ser 315 Thr	G 944 C	C -15 T	WT	WT	WT	
M4-88	1	M	335544534562424	Ser 450 Leu	C 1349 T	WT	Ser 315 Thr	G 944 C	WT	WT	WT	WT	**7**
M4-89	1	M	335544534562424	Ser 450 Leu	C 1349 T	Val 483 Gly	Ser 315 Thr	G 944 C	WT	WT	WT	WT	
M1-2	1	M	336514524582424	Ser 450 Leu	C 1349 T	Asn 698 Ser	Ser 315 Thr	G 944 C	WT	Ala 90 Val/Ala	C 269 T/C	WT	**8**
M1-5	1	M	336514524582424	Ser 450 Leu	C 1349 T	Ile 522 Thr	Ser 315 Thr	G 944 C	C -15 T	WT	WT	WT	
M4-145	1	M	236535534662424	Ser 450 Leu	C 1349 T	WT	Ser 315 Thr	G 944 C	WT	Asp 94 Asn	G 280 A	A 1400 G	**9**
M4-73	1	M	236535534662424	Ser 450 Leu	C 1349 T	WT	Ser 315 Thr	G 944 C	WT	Asp 94 Gly	A 281 G	WT	
M1-60	1	M	237535534662424	Asp 435 Val	A 1304 T	-	Ser 315 Thr	G 944 C	WT	Asp 94 Gly	A 281 G	WT	**10**
M4-117	1	M	237535534662424	Ser 450 Leu	C 1349 T	WT	Ser 315 Thr	G 944 C	WT	WT	WT	WT	
M4-12	1	M	237535534662424	Ser 450 Leu	C 1349 T	WT	Ser 315 Thr	G 944 C	WT	WT	WT	WT	
M4-125	1	M	237535534662424	Ser 450 Phe	C 1349 T, G 1350 C	WT	Ser 315 Thr	G 944 C	WT	WT	WT	WT	
M4-152	1	M	237535534662424	Ser 450 Phe	C 1349 T, G 1350 C	WT	Ser 315 Thr	G 944 C	WT	Asp 94 Gly	A 281 G	A 1400 G	
M4-126	1	M	237535534662424	Leu 430Leu/Pro, Asp 435 Tyr, Asn 437 Asp/His	T 1289 T/C, G 1303 T, A 1309 G/C	-	Ser 315 Thr	G 944 C	WT	WT	WT	WT	

M4-171	1	M	237535534662424	Ser 450 Leu	C 1349 T	WT	Ser 315 Thr	G 944 C	WT	WT	WT	WT	
M4-37	1	M	237535534662424	Leu 430 Pro	T 1289 C	-	Ser 315 Thr	G 944 C	WT	WT	WT	WT	
M1-16	1	M	236435534662424	WT	WT	WT	Ser 315 Thr	G 944 C	WT	WT	WT	WT	**11**
M1-45	1	M	236435534662424	WT	WT	WT	Ser 315 Thr	G 944 C	WT	WT	WT	WT	
M4-30	1	M	337534534572423	WT	WT	WT	Ser 315 Ser/Thr	G 944 G/C	WT	WT	WT	WT	**12**
M4-34	1	M	337534534572423	WT	WT	WT	WT	WT	WT	WT	WT	WT	
M4-118	1	M	337534534672423	Ser 450 Leu	C 1349 T	-	Ser 315 Thr	G 944 C	WT	WT	WT	WT	**13[e]**
M4-43	1	M	337534534672423	His 445 Arg	A 1334 G	WT	Ser 315 Thr	G 944 C	C -15 T	WT	WT	WT	
M4-44	1	M	337534534672423	His 445 Arg	A 1334 G	WT	Ser 315 Thr	G 944 C	C -15 T	WT	WT	WT	
M1-61	1	M	337534534682423	Ser 450 Leu	C 1349 T	Val 483 Gly	Ser 315 Thr	G 944 C	WT	Asp 94 Gly	A 281 G	WT	**14**
M1-73	1	M	337534534682423	Ser 450 Leu	C 1349 T	WT	Ser 315 Thr	G 944 C	WT	WT	WT	WT	
M4-136	1	M	337534534682423	His 445 Tyr	C 1333 T	-	Ser 315 Thr	G 944 C	WT	WT	WT	WT	
M4-53	1	M	336534534682433	Ser 450 Leu	C 1349 T	WT	Ser 315 Thr	G 944 C	WT	WT	WT	WT	**15**
M4-57	1	M	336534534682433	Ser 450 Leu	C 1349 T	Val 483 Gly	Ser 315 Thr	G 944 C	WT	WT	WT	WT	
M4-18	1	M	336534534652423	Ser 450 Leu	C 1349 T	Val 483 Gly	Ser 315 Thr	G 944 C	WT	WT	WT	WT	**16**
M4-19	1	M	336534534652423	Ser 450 Leu	C 1349 T	WT	Ser 315 Thr	G 944 C	WT	WT	WT	WT	
M4-21	1	M	336534534652423	Ser 450 Leu	C 1349 T	WT	Ser 315 Thr	G 944 C	WT	WT	WT	WT	
M4-84	1	M	335544534672423	Asn 437 deletion	AAC 1309-1311 del	WT	Ser 315 Thr	G 944 C	WT	WT	WT	WT	**17**
M4-85	1	M	335544534672423	Asn 437 deletion	AAC 1309-1311 del	WT	Ser 315 Thr	G 944 C	WT	WT	WT	WT	
M4-41	1	M	237534634572423	His 445 Tyr	C 1333 T	WT	Ser 315 Thr	G 944 C	WT	WT	WT	WT	**18**
M4-42	1	M	237534634572423	His 445 Tyr	C 1333 T	WT	Ser 315 Thr	G 944 C	WT	WT	WT	WT	
M4-45	1	M	237534634572423	His 445 Tyr	C 1333 T	WT	Ser 315 Thr	G 944 C	WT	WT	WT	WT	
M4-76	1	M	237544534372423	Ser 450 Leu	C 1349 T	Val 483 Gly	Ser 315 Thr	G 944 C	WT	WT	WT	WT	**19**
M4-82	1	M	237544534372423	Ser 450 Leu	C 1349 T	Val 483 Gly	Ser 315 Thr	G 944 C	WT	Asp 94 Gly	A 281 G	WT	
M1-41	190	M	236434634582423	Ser 450 Leu	C 1349 T	Val 483 Ala	Ser 315 Thr	G 944 C	WT	WT	WT	WT	**20**
M1-42	190	M	236434634582423	Ser 450 Leu	C 1349 T	Pro 434 Glu	Ser 315 Thr	G 944 C	WT	WT	WT	WT	
M1-31	1	M	236434534382423	Ser 450 Leu	C 1349 T	Val 483 Gly	Ser 315 Thr	G 944 C	WT	WT	WT	WT	**21**
M1-32	1	M	236434534382423	Ser 450 Leu	C 1349 T	Val 483 Gly	Ser 315 Thr	G 944 C	WT	WT	WT	WT	
M1-43	1	M	336434534682423	Ser 450 Leu	C 1349 T	-	Ser 315 Thr	G 944 C	WT	WT	WT	WT	**22**
M4-146	1	M	336434534682423	His 445 Tyr	C 1333 T	-	Ser 315 Thr	G 944 C	WT	WT	WT	WT	
M4-65	1	M	336434534682423	Asp 435 Tyr	G 1303 T	-	Ser 315 Thr	G 944 C	WT	WT	WT	WT	
M1-13	1	M	236434534682423	His 445 Leu	A 1334 T	-	Ser 315 Asn	G 944 A	WT	WT	WT	WT	**23**
M1-34	1	M	236434534682423	Asp 435 Val	A 1304 T	-	Ser 315 Thr	G 944 C	WT	WT	WT	WT	
M1-17	1	M	336433134582423	Ser 450 Leu	C 1349 T	WT	Ser 315 Thr	G 944 C	WT	WT	WT	WT	**24**
M1-40	1	M	336433134582423	Ser 450 Leu	C 1349 T	WT	Ser 315 Thr	G 944 C	WT	WT	WT	WT	
M1-55	1	M	438524534582523	Ser 450 Leu	C 1349 T	WT	WT	WT	C -15 T	WT	WT	WT	**25**
M1-56	1	M	438524534582523	Ser 450 Leu	C 1349 T	WT	WT	WT	C -15 T	WT	WT	WT	
M1-52	1	M	437524434560423	Ser 450 Leu	C 1349 T	-	Ser 315 Thr	G 944 C	WT	WT	WT	WT	**26**
M1-53	1	M	437524434560423	WT	WT	-	Ser 315 Thr	G 944 C	WT	Asp 94 Tyr	G 280 T	WT	
M4-119	1	M	337534334570423	Asp 435 Ala, Leu 452 Pro	A 1304 C, T 1355 C	-	Ser 315 Thr	G 944 C	WT	WT	WT	WT	**27**
M4-131	1	M	337534334570423	Ser 450 Leu	C 1349 T	WT	Ser 315 Thr	G 944 C	WT	WT	WT	WT	

M4-134	1	M	337534334570423	Ser 450 Leu/Ser	C 1349 T/C	-	Ser 315 Thr	G 944 C	WT	Ala 90 Val	C 269 T	WT	
M4-154	1	M	337534334570423	Ser 450 Leu	C 1349 T	WT	Ser 315 Thr	G 944 C	WT	WT	WT	WT	
M4-155	1	M	337534334570423	His 445 Leu	A 1334 T	-	Ser 315 Thr	G 944 C	WT	WT	WT	WT	
M4-156	1	M	337534334570423	Ser 450 Leu	C 1349 T	Asn 698 Ser	Ser 315 Thr	G 944 C	WT	WT	WT	WT	
M4-107	1	M	337534334560423	His 445 Asn, Leu 452 Pro	C 1333 A, T 1355 C	-	Ser 315 Thr	G 944 C	WT	WT	WT	WT	**28**
M4-32	1	M	337534334560423	Ser 450 Leu	C 1349 T	-	Ser 315 Thr	G 944 C	WT	WT	WT	WT	
M4-38	1	M	337534334560423	His 445 Leu	A 1334 T	-	Ser 315 Thr	G 944 C	WT	WT	WT	WT	
M1-66	1	M	337534334560323	His 445 Asp	C 1333 G	-	Ser 315 Thr	G 944 C	WT	WT	WT	WT	**29**
M1-69	1	M	337534334560323	Ser 450 Leu	C 1349 T	-	Ser 315 Thr	G 944 C	WT	WT	WT	WT	
M4-90	1	M	336544334570423	Ser 450 Leu	C 1349 T	WT	Ser 315 Thr	G 944 C	WT	WT	WT	WT	**30**
M4-92	1	M	336544334570423	Ser 450 Leu	C 1349 T	WT	Ser 315 Thr	G 944 C	WT	WT	WT	WT	
M4-142	1	M	336534334570423	Ser 450 Leu	C 1349 T	WT	Ser 315 Thr	G 944 C	WT	Asp 94 Gly	A 281 G	A 1400 G	**31**
M4-159	1	M	336534334570423	Ser 450 Leu	C 1349 T	Lys 445 Arg	Ser 315 Thr	G 944 C	WT	WT	WT	WT	
M4-69	1	M	336534334570423	Ser 450 Leu	C 1349 T	Asn 698 Ser	Ser 315 Thr	G 944 C	WT	WT	WT	WT	
M4-98	1	M	336534334570423	Ser 450 Leu	C 1349 T	WT	Ser 315 Thr	G 944 C	WT	WT	WT	WT	
M1-19	1	M	336434334570423	Ser 450 Leu	C 1349 T	WT	Ser 315 Thr	G 944 C	WT	WT	WT	WT	**32**
M1-20	1	M	336434334570423	Ser 450 Leu	C 1349 T	WT	Ser 315 Thr	G 944 C	WT	WT	WT	WT	
M1-22	1	M	336434334570423	Ser 450 Leu	C 1349 T	WT	Ser 315 Thr	G 944 C	WT	WT	WT	WT	
M4-59	1	M	336434334570423	Ser 450 Leu	C 1349 T	Val 483 Gly	Ser 315 Thr	G 944 C	WT	Ala 90 Val	C 269 T	A 1400 G	
M4-60	1	M	336434334570423	Ser 450 Leu	C 1349 T	WT	Ser 315 Thr	G 944 C	WT	WT	WT	WT	
M4-63	1	M	336434334570423	Ser 450 Leu	C 1349 T	Leu 516 Pro	Ser 315 Thr	G 944 C	WT	WT	WT	WT	
M4-130	1	M	337544534580423	Ser 450 Leu	C 1349 T	WT	Ser 315 Thr	G 944 C	WT	Asp 94 Gly	A 281 G	A 1400 G	**33**
M4-141	1	M	337544534580423	Ser 450 Leu	C 1349 T	WT	Ser 315 Thr	G 944 C	WT	Asp 94 Gly	A 281 G	A 1400 G	
M4-150	1	M	337544534580423	Ser 450 Leu	C 1349 T	Val 483 Gly	Ser 315 Thr	G 944 C	WT	WT	WT	WT	
M4-5	1	M	337544534580423	WT	WT	-	WT	WT	WT	WT	WT	WT	
M4-105	1	M	337544534570423	Ser 450 Leu	C 1349 T	-	Ser 315 Thr	G 944 C	WT	WT	WT	WT	**34**
M4-31	1	M	337544534570423	WT	WT	-	WT	WT	WT	WT	WT	WT	
M4-109	1	A	335534334762243	Ser 450 Leu	C 1349 T	Val 483 Gly	Ser 315 Thr	G 944 C	WT	WT	WT	WT	**35**
M4-110	1	A	335534334762243	Ser 450 Leu	C 1349 T	Val 483 Gly	Ser 315 Thr	G 944 C	WT	WT	WT	WT	
M4-83	1	A	336443434781442	Ser 450 Leu	C 1349 T	WT	Ser 315 Thr	G 944 C	WT	WT	WT	WT	**36**
M4-94	1	A	336443434781442	Ser 450 Leu	C 1349 T	WT	Ser 315 Thr	G 944 C	WT	WT	WT	WT	

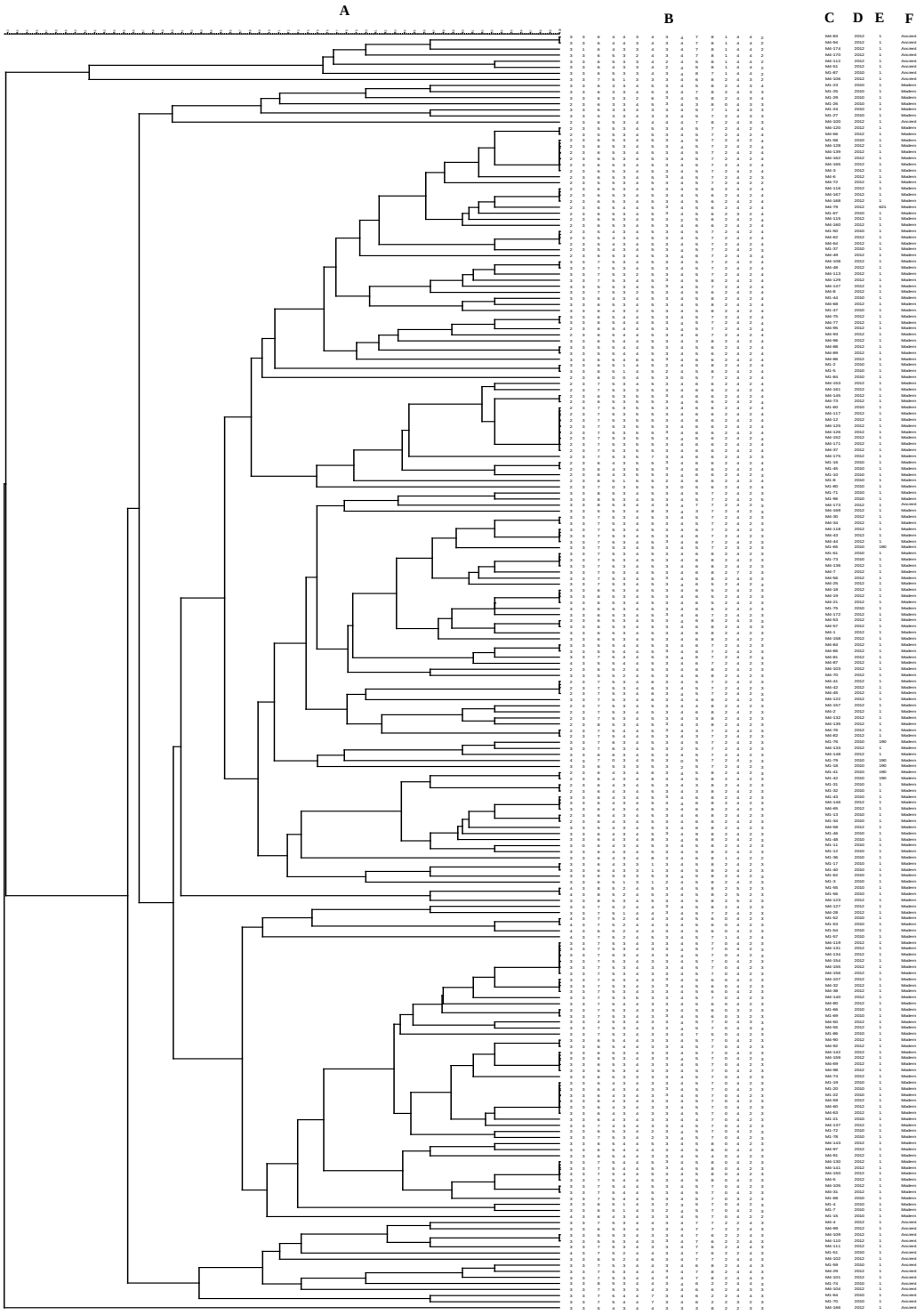
[a]SIT: Spoligo-International Type

[b]M: "modern" type, A: "ancient" type

[c]Order of loci: MIRU10, MIRU16, MIRU26, MIRU31,MIRU40,Mtub04, Mtub21, Mtub39, ETRA, QUB11b, QUB26, MIRU04, Mtub30,QUB4156, and MIRU39.

[d]Amino acid substitutions are shown in the codon number. WT: wild-type sequence, -: not determined, a/b: mixture of a and b

[e]Cluster 13: Blue circled cluster in Figure 2 with the same MIRU-VNTR pattern as 100-32 in MIRU-VNTRplus (http://www.miru-vntrplus.org/MIRU/index.faces).



Sample collected in 2012
Sample collected in 2010
N=210

Figure 1: Dendrogram of the Beijing genotype MDR-MTB isolated in 2010 and 2012

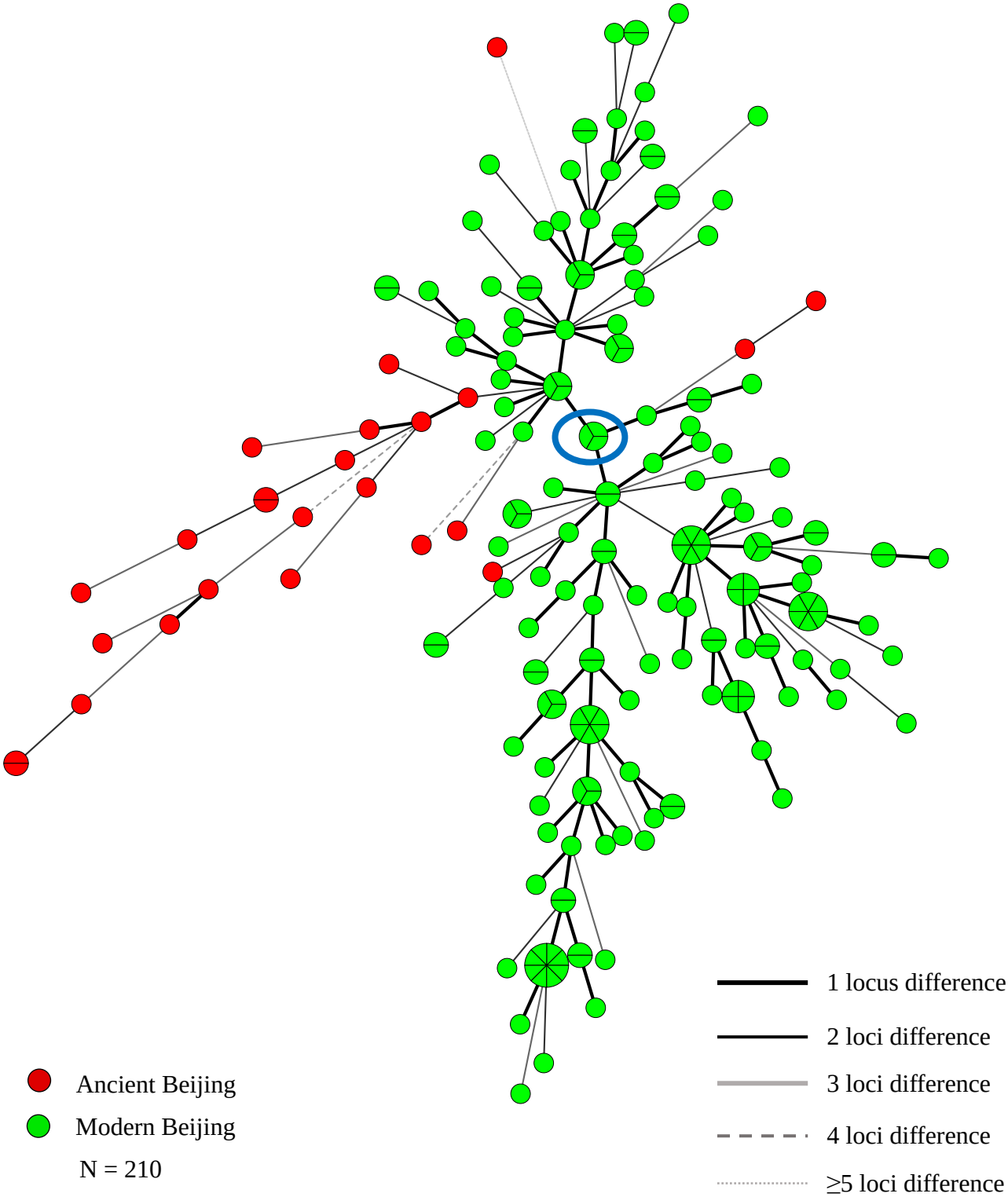
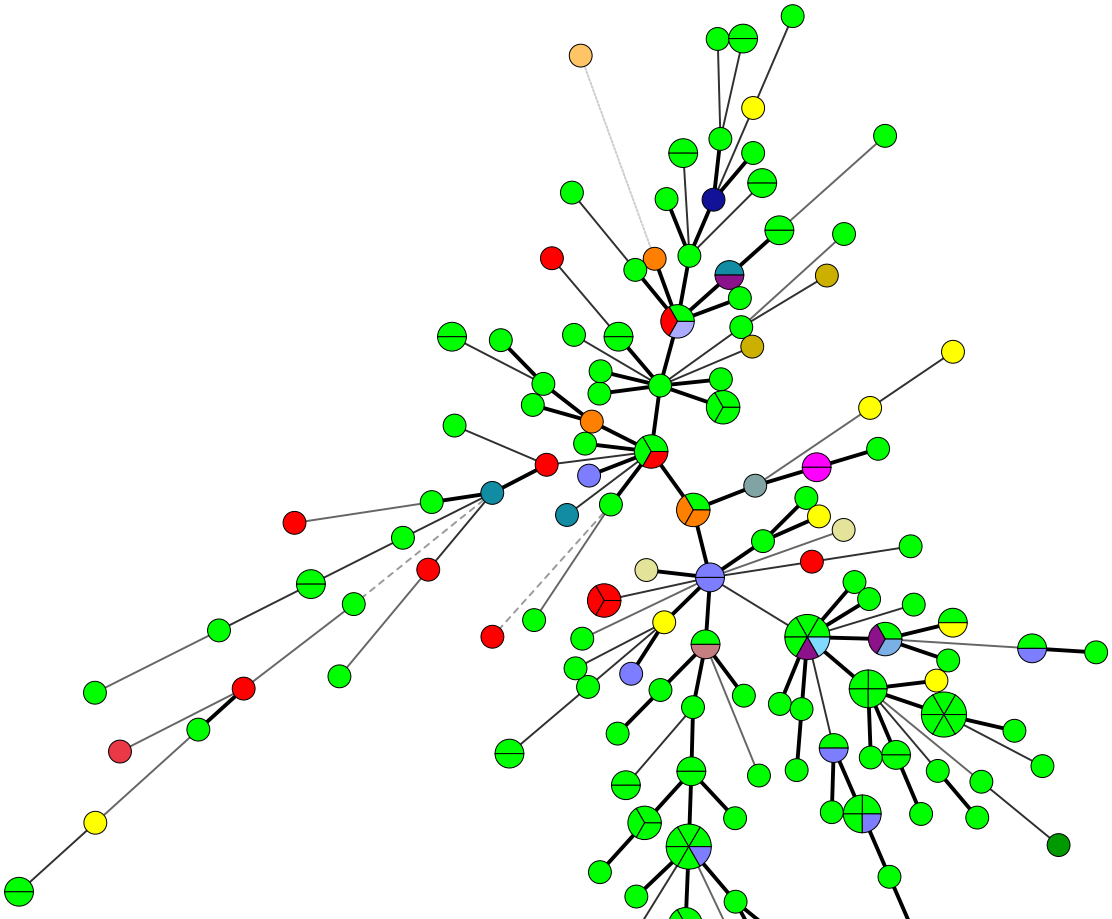


Figure 2: Minimum spanning tree analysis of the ancient and modern Beijing genotype MDR-MTB isolates



Color	No. of isolates	Mutations
	142	*rpoB* Ser 450 Leu
	12	*rpoB* His 445 Tyr
	12	*rpoB* WT
	9	*rpoB* His 445 Asp
	5	*rpoB* Asp 435 Val
	5	*rpoB* His 445 Arg
	4	*rpoB* Leu 452 Pro
	3	*rpoB* His 445 leu
	2	*rpoB* Leu 430 Pro
	2	*rpoB* Ser 450 Phe
	2	*rpoB* His 445 Asn, Leu 452 Pro
	2	*rpoB* Asn 437 deletion
	2	*rpoB* Gln 432 Pro
	1	*rpoB* Leu 430 Pro, Asp 435 Tyr, Asn 437 Asp/His
	1	*rpoB* Asp 435 Tyr, Asn 437 Asp
	1	*rpoB* Asp 435 Ala, Leu 452 Pro
	1	*rpoB* Asp 435 Tyr
	1	*rpoB* His 445 Asn
	1	*rpoB* Gln 429 Arg
	1	*rpoB* Gln 432 Leu
	1	*rpoB* Ser 450 Tyr

1 locus difference

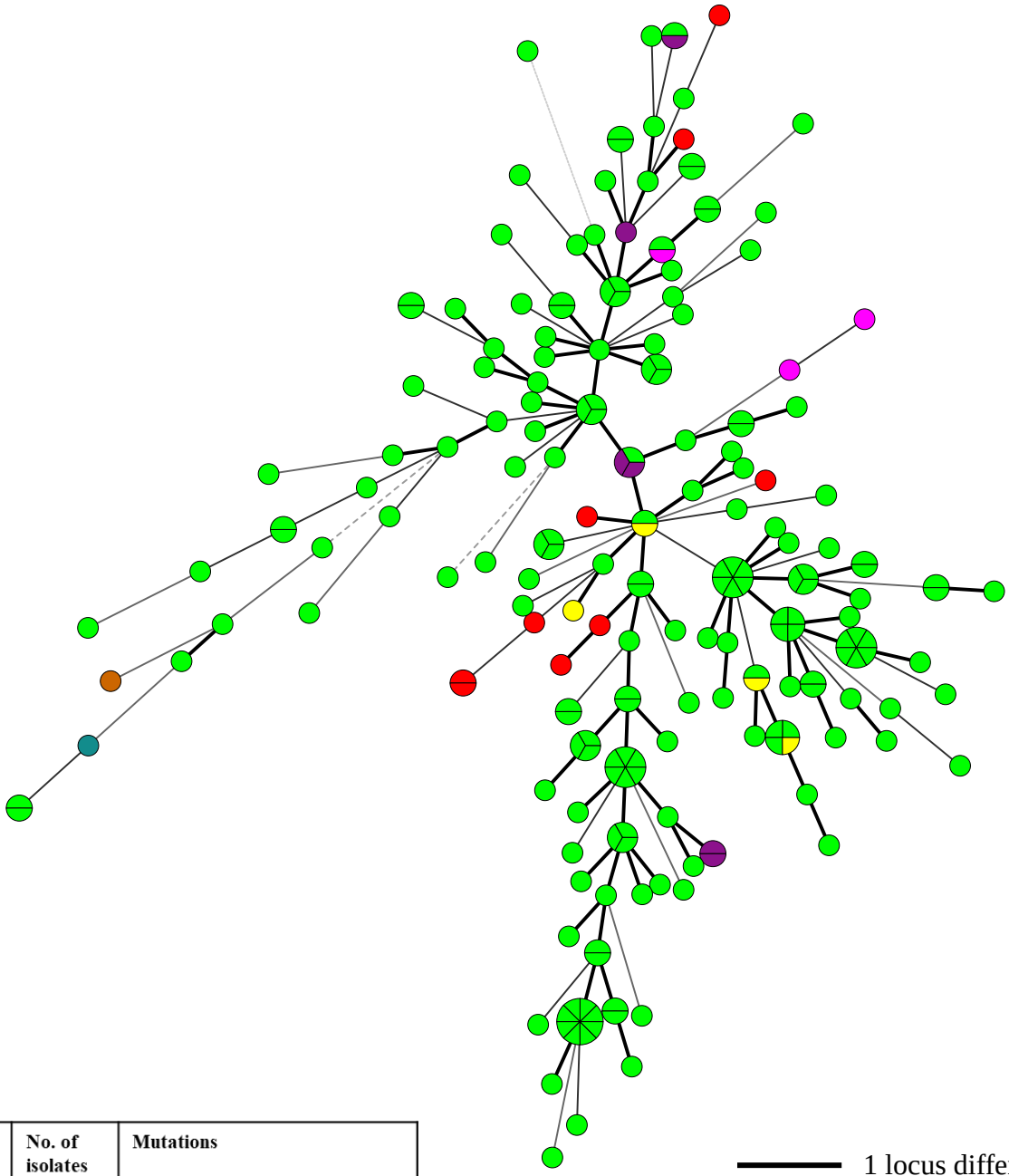
2 loci difference

3 loci difference

4 loci difference

≥5 loci difference

Figure 3: Minimum spanning tree analysis of the Beijing genotype MDR-MTB isolates showing *rpoB* gene mutations (N=210)



Color	No. of isolates	Mutations
	186	*katG* Ser 315 Thr, *inhA* WT
	9	*katG* WT, *inhA* C-15 T
	6	*katG* Ser 315 Thr, *inhA* C-15 T
	4	*katG* WT, *inhA* WT
	3	*katG* Ser 315 Asn, *inhA* WT
	1	*katG* Ser 315 Thr, *inhA* C-34 T
	1	*katG* Gly 285 Arg, *inhA* C-15 T

Figure 4: Minimum spanning tree analysis of the Beijing genotype MDR-MTB isolates showing *katG* & *inhA* gene mutations (N=210)

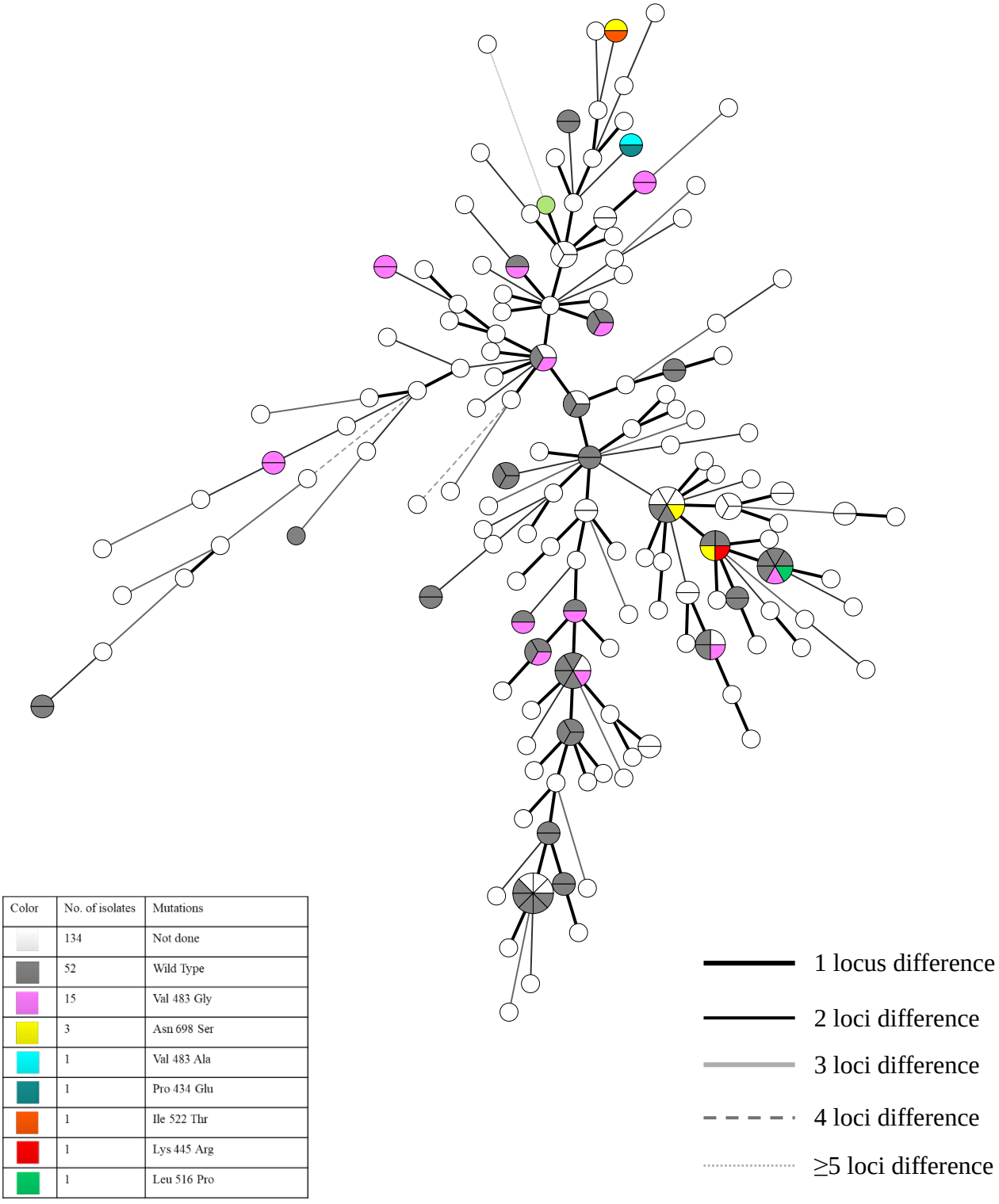


Figure 5: Minimum spanning tree analysis of the Beijing genotype MDR-MTB isolates showing *rpoC* gene mutations (N=210)

Chapter II

Differentiation of ancient and modern Beijing strains by multiplex PCR assay

Introduction

Mycobacterium tuberculosis (MTB) is slow growing bacteria and because of this nature, it is difficult to identify rapidly. The management and control of tuberculosis (TB) is somewhat difficult because of delay in diagnosis. The typical tuberculosis is found as an infection in the lung and the mode of transmission is via airborne that favors the easy spread. Therefore, there may be chances of spreading to susceptible persons before getting confirmed diagnosis. The high TB burden countries have been facing many obstacles to control disease including rapid detection of disease.

MTB complex (MTBC) is highly conserved compared with the other species and has 99.9% of similarity in gene. However, it has diversity according to the geographical factor, pathogenicity and virulence of the disease in certain region or community. The genetic changes of MTB are related to bacterial factors, environmental factors and human factors concerning with the usage of antibiotics rarely relying on the plasmid factors as other bacteria.

MTB varies with the different lineages according to the phylogeographical condition. A variety of genotyping methods can differentiate the organisms into different lineages and sublineages. The commonly used genotyping are large sequence polymorphism (LSP), clustered regularly interspersed short palindromic repeats (CRISPRs) based genotyping so called spoligotyping and VNTR-based genotyping (MIRU-VNTR) typing, and SNPs typing [52].

The Beijing genotype, belonging to the East Asian Lineage (also called Lineage 2), was firstly found in the region of Beijing, China and Mongolia in 1995. It is well known lineage which can be found at highly prevalent in China, Mongolia, Russia and South East Asia regions but a few were in Europe and America continents [9,53]. This Beijing genotype strain has high pathogenic, high virulent properties, associations with drug resistance, and potential spreading in young age [18] even though several studies showed controversial findings of the association with the drug resistance and age [54–56]. A study referred to a link between this lineage strain and extensively drug-resistant tuberculosis (XDR-TB) [57]. The clinical and epidemiological characteristics of the Beijing genotype have become challenging for the management and control of TB.

The Beijing lineage is determined by spoligotyping and IS6110 restriction fragment length polymorphism (RFLP) profile characteristics. The Beijing is monophyletic clade and is also determined by regions of difference (RDs) including RD 105, RD 181, RD 150, RD 142 deletion, and synonymous single nucleotide polymorphism (sSNP) position [58]. Spoligotyping has been used as a method of choice for the establishment of a diagnosis and transmission tracking as well as phylogenetic and population genetic studies. The absence of the spacers 1-34 with at least 3 of the 35-43 spacer hybridization in the 43 spacer analysis is a key finding to the Beijing genotype [16]. It is useful for the definition of the Beijing genotype as well as rapid determination of strains of MTB even though it has less discriminatory power compared with other genotypings [59]. Several genotyping methods of identification of the Beijing genotype have been developed and employed for the differentiation of lineages of MTB [6,60]. However, there were limitations for determination of the Beijing genotype because of the unstable nature of the direct repeat (DR) region of *Mycobacterium tuberculosis* [61].

The Beijing lineage is differentiated into ancient, modern and W subfamilies according to the IS*6110* insertion at NTF region. The modern Beijing strain, also known as

typical Beijing strain, is widely distributed throughout the region of the world with higher ratio among the sublineages of the Beijing and has a relation with resistance to anti-TB drug, high pathogenicity and selective pressure with BCG vaccination [62]. The high proportion of the modern Beijing strains had been detected in studies in China [30]. Moreover, it had been found out with the higher chance of cluster formation than ancient strains [42]. Evolution of the Beijing lineage depends on the mechanisms of IS6110 transposition, gene deletion of the regions of difference (RD), synonymous and non-synonymous mutation and mutations in mismatch repair gene [58]. The ancient Beijing is observed with the absence of IS*6110* insertion and mainly found out in Japan and Korea [63]. W Beijing strain, which is predominant in the United States of America, has been found with two insertions of IS*6110* sequence in noise transfer region (NTF) of the genome [59].

The drug resistant tuberculosis, especially multidrug resistant tuberculosis (MDR-TB) is becoming increasing year by year. The possible clonal transmission with the predominance of specific patterns of drug resistance in relation with the geographically dominant strains, for example Beijing genotype strains, in a specified region was discovered worldwide [46]. Once the dominant strains in the specific region were confirmed, the identification in rapid and reliable diagnostic method is preferable in order to control TB effectively and prevent further transmission.

Previous studies in chapter I indicated that the drug resistance associated mutations on the genes were higher in the Beijing lineage particularly modern Beijing subtype than other lineages. There was a suggestion of *katG* gene mutation which give the higher chance of development of other drug resistances, such as ethambutol (EMB) resistance, indicating more chance of developing mutations at the other genes [45]. The previous studies in Myanmar as well as other studies also found out that the drug resistance was strongly associated with the

Beijing lineage [13,14,64]. The drug resistance development of MTB has become an obstacle to effective TB control.

Although MTBC have almost the same gene of 99.9% identical, the single nucleotide polymorphism (SNPs) are observed in many strains [65]. SNPs are useful for genetic classification of MTB, identification of sublineages, detection of transmission, detection of drug resistance, and determination of the outbreak. The influence of anti-TB drugs on the evolution of Beijing genotype strains had been found out in higher proportions [66]. According to the study of Filliol et al. indicated that SNP based phylogenetic tree analysis was successful in grouping of the clade to get accurate assignment of the clades [29]. Nakajima et al.[67] indicated that some SNPs were seemed to be specific to the Beijing clade.

The influence of strain variations in the disease outcome and their impacts on the management of TB were discovered. Therefore, rapid and accurate identification of the lineage is crucial for the determination of possible outcomes and the appropriate management. The aim was to establish time-saving authentic identification of the Beijing modern lineage in one step in order to get rapid strain characterization to provide rapid diagnosis and thereby, effective treatment in a timely manner especially in high burden setting of the Beijing MDR strains.

Materials and methods

1. Sample collection

Three hundred and thirty one MTB isolates were collected from different sources, including 281 samples in Myanmar and 50 samples in Japan. Smear positive sputum samples from pulmonary TB patients (both new and retreatment cases) were collected in Myanmar in 2010, 2012, and 2016. The sputum samples were decontaminated by N-acetyl-L-cysteine NaOH and inoculated on Löwenstein-Jensen (L-J) medium and incubated for 6-8 weeks at 37º C. Four samples of common respiratory tract bacteria pathogens of non-mycobacterium species (*Staphylococcus aureus, Streptococcus pneumoniae, Pseudomonas aeruginosa, Klebsiella pneumoniae*), and 21 samples of non-tuberculous *Mycobacterium* (NTM) species were used for specificity of the method. Two reference strains (*M. bovis* BCG Tokyo and *M. africanum* ATCC 25420) were used to determine the specificity of the method.

2. DNA preparation

The samples were treated by different methods. For former Myanmar samples, colonies grown on medium were suspended in distilled water and boiled for 20 min and the supernatant was used for further investigations in former Myanmar samples. The later samples in Myanmar were done as follows. TE buffer consisting of 10mM TrisHCL pH (8.0) and 1mM EDTA in 2ml screw-cap vial, one-fourth of which was filled with 0.5 g glass beads. Mycobacterial cells were disrupted by shaking with 0.5 ml chloroform on a cell disrupter for 1min. After centrifugation, the DNA in the upper layer was concentrated by ethanol precipitation and dissolved in 100ml TE buffer and DNA will re-suspended in nuclease free water. For Japanese samples, the DNA extraction was done according to the previous study [67].

3. Genotyping

3.1. Determination of the Beijing genotype by Rv0679c multiplex PCR

The Beijing genotype was determined by multiplex PCR according to Nakajima et al. [67]. The *Rv0679C* gene was amplified by PCR and gelelectrophoresis results of PCR products were compared with the standard Beijing strains and *M. bovis* BCG strains.

3.2. Determination of the Beijing genotype by Spoligotyping

Spoligotyping was performed according to Kamerbeek et al. [16]. In a brief explanation, the DR regions were amplified and the spacers were hybridized to the membrane. According to the presence and absence of spacers, the lineage strains had been determined [59]. The data were compared on SITVIT WEB database [3].

3.3. Differentiation of Ancient and Modern Beijing genotype by multiplex PCR

The resulting Beijing gentype strains as well as non-Beijing genotype strains were further differentiated by a newly developed ancient-modern Beijing type differentiation multiplex PCR. The PCR mixture contained 3 ul of 5x GoTaq buffer (green), 0.2 ul of dNTP (10mM each), 0.5 ul of $MgCl_2$ (25mM), 1.5 ul of 5M betaine, 0.4 ul of forward primer, 0.5 ul and 0.1 ul of reverse primer 1 and 2 respectively (Table 6), 0.1 ul of GoTaq, 7.7 ul of distilled water and 1 ul of DNA sample in a final volume of 15 ul. The thermal cycle reaction parameters included denaturation at 95ºC for 60 sec, followed by 35 cycles of 95ºC for 10 sec, 55 ºC for 10 sec, 72 ºC for 10 sec, with the final extension step at 72 ºC for 3 min. After amplification, PCR products were visualized with the 2% agarose S gel electrophoresis by staining of 10% gel red in the 1x TAE buffer. The products were done electrophoresis for 18 min at 110V, then the bands were visualized under UV light.

3.4. Determination of ancient and modern Beijing strains by sequencing to detect the SNP

The sequencing for determination of ancient and modern Beijing strains at position 1477596 on the H37Rv genome [29] was performed in order to confirm the results of multiplex PCR according to the previous study by Li et al. [30]. The resulting sequences were analyzed by using BioEdit software [33].

Results

Identification of the Beijing lineage by multiplex PCR and spoligotyping

Two hundred and eighty one isolates from Myanmar and 50 isolates from Japan were subjected to multiplex PCR targeting the Beijing specific SNP on Rv0679c to identify the Beijing lineage. Among isolates from Myanmar, 250 isolates were identified as the Beijing lineage strains and confirmed by spoligotyping results. There was no discrepancy in the determination of the Beijing and non-Beijing strains.

Determination of the ancient and modern Beijing sublineages by multiplex PCR

The identified 300 Beijing isolates, 4 isolates of common respiratory tract bacteria pathogens of non-mycobacterium species, and 21 strains of non-tuberculous *Mycobacterium* (NTM) were subjected to the newly developed multiplex PCR targeting at the position 1477596 on H37Rv genome to determine the ancient Beijing or modern Beijing subtype [68]. The PCR sizes were estimated either 142 bp size for modern Beijing strains or 232 bp for ancient Beijing strains by comparing with 50 bp DNA ladder size (Figure 6, 7). The negative finding of NTM strains and non-tuberculous Mycobacterium species indicated 100% specificity of the test (Table 7) (Figure 8).

Confirmation of the results of multiplex PCR differentiation of ancient and modern Beijing sublineages

The results obtained from multiplex PCR for ancient and modern Beijing differentiation were confirmed by the detection of SNP at position 1477596 on the H37Rv

genome sequencing. The randomly selected 45 modern Beijing type samples and existing 31 ancient Beijing samples from Myanmar, 8 modern Beijing samples and 42 ancient Beijing Japanese samples were selected for the confirmation. The key mutation, SNP, C 121 T was detected in all modern sublineage of the Beijing genotype and no SNP was observed in ancient sublineage strains. There was no discrepancy result between the multiplex PCR and SNP sequencing for the detection of modern Beijing strains (Table 8).

Discussion

The molecular epidemiology of TB has been employed for the determination of strain specific characteristics, evolution in a regional as well as global distribution and specific phylogenetic characters with the purpose of management of TB. It has been applied to track the outbreaks and evaluation of drug resistance TB among the community [55].

SNPs has been used as genetic markers to reveal phylogenetic information for the determination of structured population, including strong correlation with the genotype and phenotype of strains [69–71]. By using SNP markers, the emerging strains are able to be identified due to their property of the less likely to mutate and converge [72]. Several SNPs targeting on replication, repair and recombination genes were established to determine the lineages [73]. Particularly for the East Asian (Lineage 2), SNPs has been used as markers by a number of previous studies to clarify the evolutionary pathway of the Lineage 2 [35].

The Beijing genotype strain, belonging to the East Asian lineage, is regarded as a family having conserved gene [74]. Higher virulence and pathogenicity were observed in these strains. Moreover, it has potential to develop drug resistance and association with the younger age group. The determination of the Beijing genotype was determined by RFLP typing which was laborious and time consuming [16]. The presence or absence of IS*6110* insertion determines the Beijing lineage into ancient, modern, and W branch Beijing subtypes [75]. The dominant strains were modern Beijing strains in most of the regions of the world [43], whereas, ancient strains were mainly dominant in Japan and Korea [76,77]. In addition to RFLP typing, spoligotyping and MIRU-VNTR typing of 15 and 24 loci were widely used methods for the determination of the lineages [18]. However, these methods have limitation to distinguish the Beijing genotype into further subtyping.

Multiplex PCR for the differentiation of the Beijing and non-Beijing strains was successfully developed by Nakajima et al. [67] based on single nucleotide change. The single nucleotide polymorphism of nucleotide difference of cytosine to guanine in the position 426 of the region of Rv0679c gene was able to discriminate and identify with the full rate of accuracy except for the isolates with mixed infections. It is useful for the monitoring of the prevalence of the Beijing genotype in the local region [67].

In this study, we use H37Rv MTB strain genome region of Rv1316c gene responsible for non-essential gene for in vitro growth [78]. The multiplex PCR targeted a point mutation at the position 1477596 in the H37Rv MTB genome, in which the nucleotide C is substituted by T in the modern Beijing strains. According to the size difference of multiplex PCR products, the modern Beijing subtype was determined when the band size was at 142 bp sized position and ancient subtype at 232 bp band sized position (Figure 6, 7). The sensitivity and specificity of the test were found 100% respectively.

Even though determination of the ancient or modern Beijing strains had been developed by detection of several SNPs in other previous studies, SNPs in several genes were necessary to be used for identification [58,70]. This newly developed method for the rapid detection of the Beijing subtypes has an advantage of targeting on the only one gene.

Although the proportion of the Beijing strains was in the second most common strains, it represents the largest proportion in the MDR-TB proportion in Myanmar (Chapter I). The association with the drug resistance, especially MDR-TB was confirmed by studies in Myanmar [13,14,26]. Among MDR-TB cases, modern Beijing strains were in majority (Chapter I and III).

The MDR-TB of modern Beijing subtype in Myanmar showed high prevalence of *katG* Ser315Thr, and the genetic evidence of XDR and pre-XDR and compensatory

mutations in *rpoC* among the clustered isolates (Chapter I). It had a tendency to have a high clustering rate, suggestive of ongoing transmission. Another study of our group observed that the Beijing genotype had a strong correlation with mutations of *rpsl* gene, responsible for streptomycin resistance, among the isolates collected in Myanmar [79]. In such a case, our new developed method can detect the modern Beijing strain in one step PCR, which provides the rapid determination of a virulent strain in a prompt manner that help effective TB control in a country like, Myanmar, which is included in 30 high burden countries of TB.

Regarding problems with TB are throughout the world and increasing the burdens along with the development of drug resistance, contributing diseases such as HIV, diabetes and contributing socioeconomic factors [10]. The development of one step detection method is required for the early detection of modern Beijing strains in highly prevalent settings such as in Myanmar.

As a conclusion, the application of simple, robust, and low-cost multiplex PCRs for the detection of the Beijing genotype and the further determination of the Beijing subtypes helps to determine high virulence and drug resistance prone strains in a timely manner for the further interventions of the control of TB especially in the high TB and MDR-TB burden regions.

Summary

Tuberculosis (TB) is the major leading cause of death by infectious disease worldwide. In addition, multidrug resistant TB (MDR-TB) become a burden to combat the disease. *Mycobacterium tuberculosis* (MTB) is a slow growing bacteria and it takes longer time to get diagnosed. Phylogenetic lineages of MTB has been disseminated globally. Among the lineages, the Beijing genotype strains show high transmission rate, high pathogenicity, high virulence and association with the drug resistance mutations. Particularly modern Beijing genotype strains have been found out with the higher ratio among the sublineages of the Beijing genotype especially in Asia including Myanmar and China. We aimed to develop the rapid method of ancient and modern Beijing sublineages of MTB, especially in regions where these strains are more prevalent. A multiplex PCR targeting at the position of 1477596 on H37Rv genome to determine the ancient and modern Beijing strains was developed by visualization of gel electrophoresis results. The rapid determination of a highly virulent strain, especially Beijing strains is necessary to develop in order to get a rapid diagnosis for the effective management of TB, especially in high burden countries like Myanmar where the Beijing genotype MDR-TB is predominant.

Table 6. Sequences of oligonucleotides used as a primer for multiplex PCR

Primer	**Target**	**Sequence (5’ – 3’)**	**Location**
Forward primer	1477596	GTCGACAGCGCCAGAAAATG	1477476-1477495
Reverse primer 1		CCCGATGGGGCTATCGATGGTG	1477596-1477617
Reverse primer 2		GTGCTGGGTGGCATAGGAGC	1477688-1477707

Table 7. Summary of results of multiplex PCR for differentiation of modern and ancient Beijing strains

Samples	No. of Samples	PCR products		Interpretation
		142 bp	232bp	
Samples from Myanmar	250	219	-	Modern
		-	31	Ancient
Samples from Japan	50	6	-	Modern
		-	44	Ancient
BCG-Tokyo-172	1	-	1	Ancient
***M. africanum* ATCC25420**	1	-	1	Ancient
S. aureus	1	-	-	Not Detected
S. penumoniae	1	-	-	Not Detected
P. aeruginosa	1	-	-	Not Detected
K. pneumoniae	1	-	-	Not Detected
M. abscessus	1	-	-	Not Detected
M. asiaticum	1	-	-	Not Detected
M. chelonae	1	-	-	Not Detected
M. fortuitum	1	-	-	Not Detected
M. szulgai	1	-	-	Not Detected
M. gordonae	1	-	-	Not Detected
M. intermedium	1	-	-	Not Detected
M. intracellulare	1	-	-	Not Detected
M. kansasii	1	-	-	Not Detected
M. peregrinum	1	-	-	Not Detected
M. terrae	1	-	-	Not Detected
M. scrofulaceum	1	-	-	Not Detected
M. shimoidei	1	-	-	Not Detected
M. simiae	1	-	-	Not Detected
M. smegmatis	1	-	-	Not Detected
M. lentiflavum	1	-	-	Not Detected
M. malmoense	1	-	-	Not Detected
M. marinum	1	-	-	Not Detected
M. xenopi	1	-	-	Not Detected
M. mucogenicum	1	-	-	Not Detected
M. nonchromogenicum	1	-	-	Not Detected
M. avium	1	-	-	Not Detected

Table 8. Detection of modern and ancient Beijing strains by multiplex PCR and SNP detection sequencing

		SNP (C 1477596 T)		Total
		Modern Beijing	**Ancient Beijing**	
Multiplex PCR	***Modern Beijing**	53	0	53
	***Ancient Beijing**	0	73	73

*Sensitivity and specificity were 100% respectively.

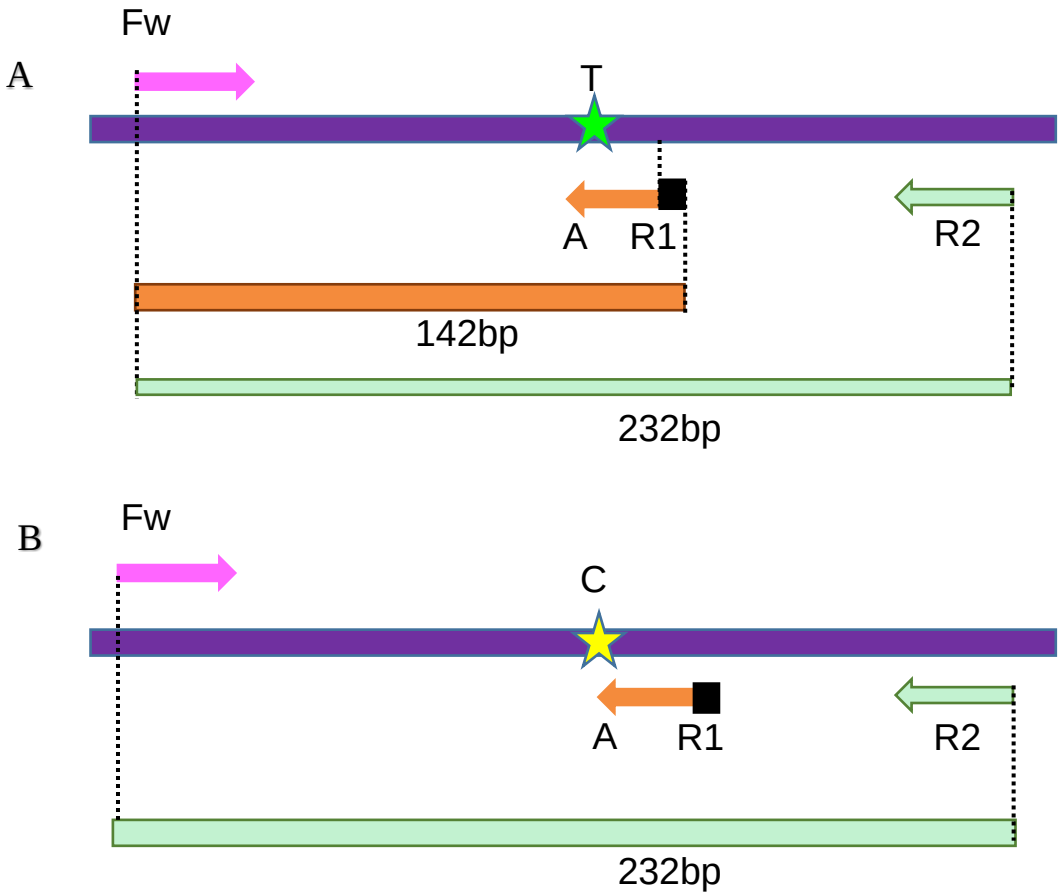


Figure 6: PCR primer and products at the position of 1477596 on H37Rv genome targeting multiplex PCR for Beijing subtype discrimination. (A) In the modern Beijing sample, 142-bp product is amplified more dominantly than the 232-bp product. (B) In the ancient Beijing or other MTC sample, 142-bp product is not amplified because of the mismatch of the 3'end of R1. Fw, forward primer; R1, reverse primer 1 (modern Beijing specific); R2, reverse primer 2.

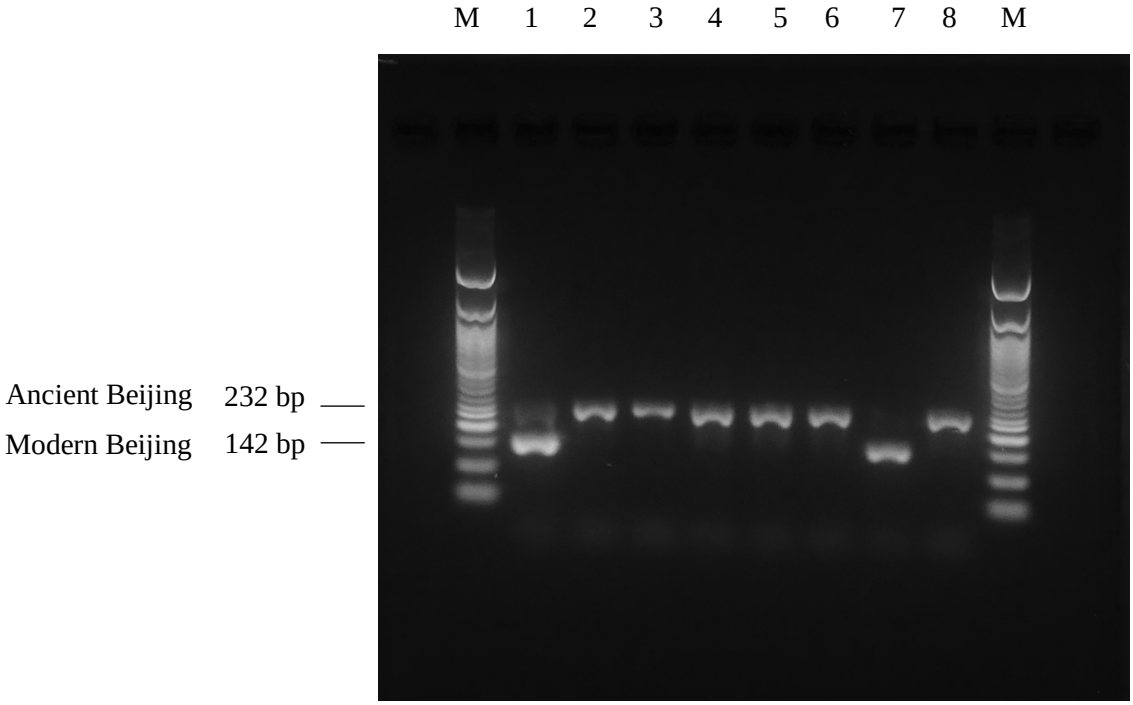


Figure 7: Multiplex PCR for differentiation of the ancient and modern Beijing strains. Lane M: 50 bp ladder DNA size marker; Lane 1: modern Beijing strain (control); Lane 2: Ancient Beijing stains (control); Lane 3: Sample 1; Lane 4: Sample 2; Lane 5: Sample 3; Lane 6: Sample 4; Lane 7: Sample 5; Lane 8: Sample 6.

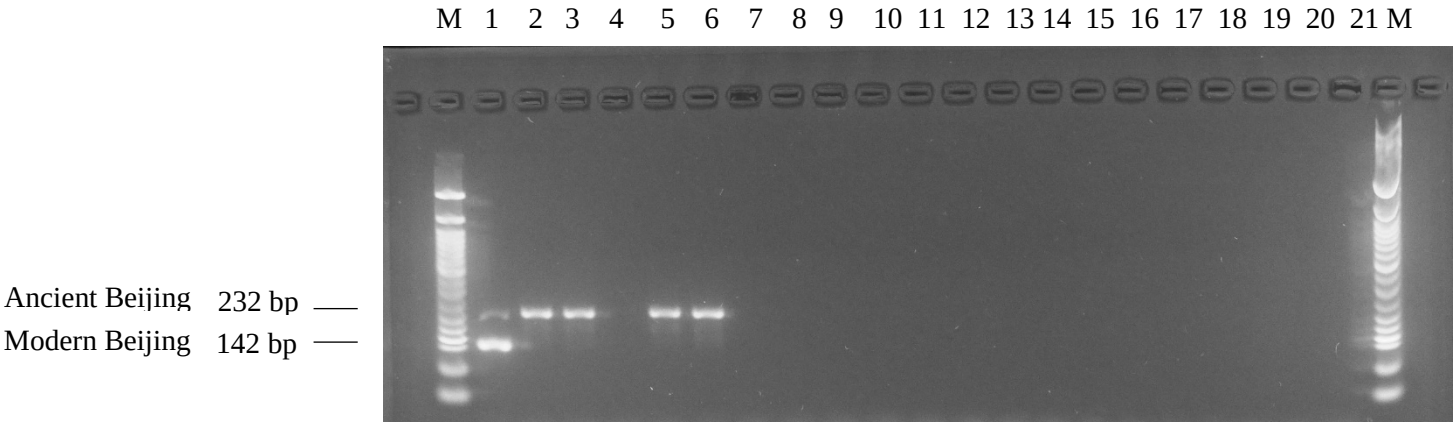


Figure 8: Multiplex PCR for differentiation of the ancient and modern Beijing strains.

Lane M: 50 bp ladder DNA size marker, Lane 1: modern Beijing strain (control); Lane 2: Ancient Beijing stains (control); Lane 3: Sample 1 (ancient Beijing strain), Lane 4: Sample 2 (*M. abscessus*); Lane 5: *M. bovis* BCG-Tokyo-172, Lane 6: *M. africanum* ATCC 25420; Lane 7: *S. aureus*; Lane 8: *S. penumoniae*, Lane 9: *P. aeruginosa*; Lane 10: *K. pneumoniae*; Lane 11: *M. abscessus*, Lane 12: *M. asiaticum* , Lane 13: *M. chelonae*, Lane 14: *M. fortuitum*, Lane 15: *M. szulgai*, Lane 16: *M. gordonae* , Lane 17: *M. intermedium* , Lane 18: *M. intracellulare*, Lane 19: *M. kansasii*, Lane 20: *M. peregrinum*, Lane 21: *M. terrae*

Chapter III

Transboundary spread of high risk Beijing genotype *Mycobacterium tuberculosis* strains in Mawlamyine near Myanmar-Thailand border area

Introduction

Tuberculosis (TB) is still a major public health problem globally as well as in Myanmar which is involved in 30 high burden countries of TB. It has one of three main infectious diseases targeted to get control in Myanmar until now [80]. According to the WHO report in 2016, the incidence rate of TB in Myanmar is 361/100,000 population. Moreover, Myanmar is one of the 30 high burden countries of multidrug resistant tuberculosis (MDR-TB) indicating 5% of new cases and 27% of previously treated cases [1]. Emergence of drug resistant TB is an obstacle to achieve national tuberculosis program (NTP) goal that is to reduce morbidity, mortality and transmission of TB until it is no longer a public health problem [80]. The drug resistance becomes weighted more for the management of the disease control. The additional burden is the development of TB in HIV infected population. In 2015, the TB cases were 1.4 million amongst people living with HIV globally. Many people with HIV die with TB without being diagnosed. The risk of developing TB was estimated to be between 16-27 times higher in people living with HIV than among those without HIV infection [81].

Genotyping methods have been employed for the determination of the transmission pattern, and detection of the risk factors concerning with the epidemiological study. Restriction fragment length polymorphism (RFLP) typing of *IS6110* insertion sequence, spoligotyping and MIRU-VNTR typing methods are commonly used for the epidemiological study of TB. *IS6110* RFLP typing had been the gold standard for the differentiation of MTB

strains, but it had less discriminatory power in strains which had less than 6 numbers of insertion sequence. Spoligotyping shows less discriminatory power than MIRU-VNTR typing.

The lineage 2, the Beijing genotype *M. tuberculosis* strains, first described in 1995 by van Soolingen et al., are predominant in Asian region especially, in China, Mongolia and the southeast Asia region [9]. Beijing genotype strain is defined as on the basis of highly conserved spoligotyping and the characteristic *IS6110* RFLP pattern. [9]. The Beijing strains were found out with the properties of the higher chance of transmission, virulence and pathogenicity than other lineages, and is found more among the younger population. It has an association with the drug resistance, especially multidrug resistant tuberculosis (MDR-TB).

Nakajima et al. [67] developed a multiplex PCR method of identification of the Beijing genotype by SNPs in *Rv0679c* gene. It had been suggested to have a specific mutation in the *Rv0679c* gene by the time of occurrence of RD207 deletion. Rv0679c protein is highly conserved membrane protein of *M. tuberculosis* and it is translocated across the cytoplasmic membrane which is suggested to be associated with the pathogenicity. A single nucleotide difference of cytosine to guanine at position 426 (codon 143 position from Asn (AAC) to Lys (AAG)) was determined as the Beijing lineage. This multiplex PCR assay can be used to identify MTC and differentiated into the Beijing and non-Beijing lineage.

The Beijing lineage has been classified into modern and ancient strains which were determined by the insertion of *IS6110* into the noise transfer function (NTF) chromosomal region. The ancient, also called the atypical strains were observed mostly in Korea and Japan [63]. The modern strains, so called typical strains, showed one or more insertion of *IS6110* [82] and had been observed more virulent than the ancient strains and the cytotoxicity of this strains had been found significantly higher than the ancient strains [83].

Myanmar is situated in the south East Asia region, bordered by Thailand and Laos to the east, China to the north and northeast, India and Bangladesh to its west. The population is about 53 million [84]. Mawlamyine is situated in Mon state, the southern region of Myanmar. According to the 2014 Myanmar population and housing census, the population in Mon state was 2,054,393 and the percentage of male was 48.6% and female was 51.94%. The population density was 167.1 per Km^2 and the urban population is 28% [85].

The proportion of smear TB positive in Mon state is 4.7% in 2010. According to the sentinel surveillance in Myanmar in 2008, the HIV prevalence among new TB patients was 11.1% in nation and 13.33% in Mawlamyine [86]. The BCG coverage is 89% in the whole country while 92% in Mon state in 2008 [87].

The findings that we discovered in Chapter I indicated that there was an association between the drug resistance and the Beijing genotype MDR-MTB strains and a potential transmission between two countries, Myanmar and Thailand. Even though the genotypic characteristics of the Beijing genotype strains in Myanmar were divergent, some outbreak patterns were observed among the population in the region of Myanmar and Thailand border area. We aimed to determine the prevalence of strains among TB population, characteristics of the Beijing genotype in this region, and to trace the transmission pattern among the community.

Materials and methods

Study population and sputum sample

Total 123 sputum samples were collected from the pulmonary tuberculosis patients attending Mon and Kayin state National TB program, Mawlamyine, Myanmar, a region near to the Thailand and Myanmar border, from July to October in 2016. Mawlamyine, the fourth largest city in Myanmar, is situated in the southeast part of Myanmar. The clinical-epidemiological and demographic data of individual patient was collected. The identification number of the study, patient particularity such as age, sex, address, city of origin, previous history of TB, history of TB contact, number of households, associated disease such as diabetes and HIV infection were included in the demographic data of the patients. The data were collected in a separated data sheet with a research purpose and the format was pre-designed by the time of ethical reviewing.

The sputum samples were decontaminated by using N-Acetyl L-Cysteine-Sodium Hydroxide (NALC-NaOH) followed by centrifuging with 3000 x g for 15 minutes. The supernatant was discarded and smear was prepared for usual microscopic examination under fluorescence microscope with rhodamine auramine staining.

Primary isolation and drug susceptibility assay

The pellet was suspended in 1ml of 1x phosphate buffered saline and primary mycobacterial isolation was done on Löwenstein-Jensen medium. Drug susceptibility tests (DST) were done by using first-line anti-TB drugs such as isoniazid, rifampicin, streptomycin and ethambutol by proportion methods on Löwenstein-Jensen medium. DSTs were performed according to World Health Organization guidelines with the following criterial

drug concentrations: isoniazid (0.2μg/ml), rifampicin (40μg/ml), ethambutol (2μg/ml) and streptomycin (4μg/ml).

DNA extraction

DNA was extracted from bacterial colonies from Löwenstein-Jensen medium slant. The colonies were firstly suspended in TE buffer consisting of 10mM Tris HCL (pH 8.0) and 1mM EDTA in a 2ml screw-cap vial, one-fourth of which was filled with 0.5g glass beads. Mycobacterial cells were disrupted by shaking with 0.5ml chloroform on a cell disrupter for 1 min. DNA in the upper layer was concentrated by ethanol precipitation after centrifuging. Then it was dissolved in 100 ml TE buffer and DNA was re-suspended in nuclease-free water and the DNA concentration was determined by spectrophotometry in a Nanodrop 100 (Thermo Scientific, USA). The DNA solution was stored at -20℃ until use.

Genotypic characterization of *M. tuberculosis*

The extracted DNA was transported to the Hokkaido University Research Center for Zoonosis Control for genotypic characterization of MTB isolates.

Detection of drug resistance associated gene mutations

The drug resistance associated genes of *rpoB* and *katG* gene were amplified and sequenced according to the previous studies [31,32]. Sequencing of *rpoB* gene was done both for the identification of the *Mycobacterium tuberculosis* complex and for the detection of drug resistant conferring mutations. PCRs were done by making a total volume of 20 μl mixture

containing GoTaq buffer, 25mM of deoxynucleoside triphosphates, 25mM of $MgCl_2$, 5M betaine, 10μM of forward and reverse primers, 1U of GoTaq DNA polymerase and 1μl of DNA samples. The reactions were carried out under the condition of denaturation at 96°C for 60 s, followed by amplification at 96°C for 10 s, 55°C for 10 s, 72°C for 30 s and final extension at 72°C for 300 s. The PCR products was confirmed by 1.5% agarose gel electrophoresis. The PCR products were sequenced according to the manufacturer's protocol by using forward primer of each gene and BigDye Terminator v 3.1 cycle sequencing kit (Life Technologies Corp., CA) using an ABI Prism 3130*xl* Genetic Analyzer (Life Technologies Corp.,). The resulting sequences were compared to wild-type sequences of *M. tuberculosis* H37Rv using Bio-Edit software (version 6.6).

Lineage identification by spoligotyping

Spoligotyping was performed to all isolates as described by Kamerbeek et al. [16]. In a brief, the direct repeat region was amplified with the primers, and the resulting PCR products were hybridized to a set of 43 spacers-specific oligonucleotide probes, which were immobilized on the membrane. The data were analyzed and the spoligotyping international type (SIT) were determined by using database of SITVITWEB [3,88].

Multiplex PCR for the identification of the Beijing genotype

For the identification of the Beijing genotype, Rv*0679c* gene detection was done by multiplex PCR according to the previous studies [67]. In a brief, PCR was done to amplify Rv*0679c* gene fragment. The PCR mixture containing GoTaq PCR buffer (Promega Co., Madison, WI), 0.2mM each deoxy nucleoside triphosphate (dNTP), 0.3 M each primer, 05

μM betaine, 1 ng genomic DNA from *M. tuberculosis*, and 0.5 units of GoTaq polymerase. The reaction was carried out by denaturation at 95˚C for 10s, annealing at 72˚C for 40s and a final extension at 72˚C for 5 min. The products were visualized by gel electrophoresis methods using 50 bp DNA ladder as a marker. *Mycobacterium bovis* and the known Beijing genotype *Mycobacterium tuberculosis* were used as positive control for the determination of the Beijing and non-Beijing genotype.

Multiplex PCR for determination of modern and ancient Beijing subtypes

For the differentiation of modern and ancient Beijing subtype, the newly developed multiplex PCR methods as mentioned in chapter II was performed.

MIRU-VNTR typing of the Beijing genotype *Mycobacterium tuberculosis*

The procedure of MIRU-VNTR was conducted by standard method of Supply et. al. [28]. MIRU-VNTR typing was performed by PCR amplification using 15 loci which were used based on high discriminatory power for the specific Beijing family. MIRU-VNTR markers comprise of 5 mycobacterial interspersed repeat units (MIRU10, MIRU16, MIRU26, MIRU40, MIRU39), 4 exact tandem repeats (ETR-A, ETR-C, ETR-D, ETR-E), 4Mtub group (Mtub04, Mtub21, Mtub39, Mtub30) and 2 MIRU-VNTR loci identified by Queen's University Belfast group [20]. For the size of PCR products, conventional gel electrophoresis was performed with the use of appropriate molecular size markers. The copy number of different alleles was assigned to the reference table. Some experiments were repeated for the confirmation of the results.

MIRU-VNTR typing was performed on 40 isolates which had been identified as the Beijing *M. tuberculosis*. The 15 MIRU-VNTR loci selected for this typing had been used according to a study done by Wang et. al [20].

Data analysis

The results obtained from MIRU-VNTR typing were analyzed by the Bionumeric software version 6.6; Applied Maths. For clustering analysis, the results were calculated by using the UPGMA (unweighted pair group method with arithmetic averages) according to the instructions by manufacturer. The cluster was defined as when two or more isolates, obtained from different patients with the same MIRU-VNTR patterns, whereas non-clustered pattern from each individual were referred as unique. Regarding with the MIRU-VNTR typing data, Minimum spanning tree (MST) analysis was conducted by using the categorical coefficient.

The Hunter-Gaston discrimination index (HGDI) for 15 MIRU-VNTR loci sets was used for the estimation of the discriminatory power of the genotyping methods and the diversity of the molecular markers by using $\mathbf{HGDI} = \mathbf{1} - \frac{\mathbf{N}}{\mathbf{(N-1)}} \sum_{j=1}^{s} \boldsymbol{n_j} \left(\boldsymbol{n_j} - \mathbf{1}\right)$, where N is the total number of strains, n_j is the number of the *j* genotype, and *s* is the MIRU-VNTR locus genotype number [89].

Statistical analysis

For statistical analysis χ^2 test and Fisher's exact test were used to assess the difference between groups in binary variables. Odds ratios (OR) were obtained from univariate logistic regression adjusted for age, gender, history of previous TB, history of household TB contact.

Ethical Review

The ethical consent was applied for the conduction the study by defending at the Ethical Review Committee, Department of Medical Research, Ministry of Health and Sports, Myanmar. The proposal was applied and the review and discussion were done at the Department of Medical Research, Myanmar.

Results

Clinical-epidemiological and demographic characteristics

The demographic and clinical characteristics of the patients are indicated in Table 9. The average age of the patient was 46 years (ranging from 13-85 years). The most common age group was 45-54 year-old group which was 23% of the total. The smallest age group was 5-14 year old accounted for 1%. Male TB patient exceeded female patient by the ratio of 3.23. Fourteen percent of the patients were found previously treated and all were smear positive cases. Twenty-three percent of the patients had a previous history of household TB contact and 7% revealed previous TB contact apart from household contact (Table 9). Age, gender, history of previous TB, history of household TB contact, and history of outside TB contact were found no significant association between the Beijing lineage.

Sequencing of drug resistance-associated genes

rpoB gene sequencing for rifampicin resistance determination

According to *rpoB* gene sequencing, the sequences of nontuberculous mycobacterium (NTM) strains were detected in 2 isolates and they were excluded for the further analysis. The majority of the isolates showed wild type *rpoB* gene, which was accounted for 96.3% (104/108) of the total 108 MTB isolates (Table 10). Among the remaining isolates 2.8% (3/108) of the isolates showed a mutation of Ser 450 Leu and 0.9% (1/108) of the total isolates showed the mutation of Ser 450 Phe in *rpoB* gene.

katG gene detection for isoniazid resistance determination

Of total 108 MTB isolates, the majority of the *katG* gene were wild type in 92% (101/108). Ser 315 Thr mutation was accounted for 7% (8/108) and Thr 376 Met mutation was found in 1% (1/108) of the isolates.

The overall drug resistance in the sample population

There were 2 out of 108 isolates (2% of the total isolates) had both *rpoB* and *katG* mutation, genetically indicating MDR-TB. The mono-resistant *rpoB* and *katG* were observed 2% (2/108) and 6% (7/108) respectively. The mutations of *rpoB* and *katG* genes were detected only in the Beijing genotype in this study**.** The distribution of MTB families with drug resistance indicated that there was a significant association between the Beijing genotype and drug resistance responsible mutations of *rpoB* and *katG* genes (Table 10).

Distribution of *Mycobacterium tuberculosis* clade by spoligotyping

Of the total 110 isolates, 79% (87/110) of isolates were classified in 26 recognized spoligotype international type (SITs) and 20.9% (23/110) isolates were found as orphan isolates without SITs (Table 11). Most frequent clade was Beijing SIT 1, which was 31.8% (35/110), followed by orphan type 20.9% (23/110) in which 13 isolates were in clusters and 10 isolates had a unique pattern of spoligotyping. The third common was EAI6-BGD (SIT292), which was 9.1% (10/110). The other clades were less than 5.5%. Beijing (SIT190) and Beijing (orphan) lineage were found 0.9% (1/110) (Table 11).

Confirmation of the Beijing genotype and further differentiation of the modern and ancient Beijing subtype

The Beijing genotype was determined by multiplex PCR at the position 1477596 on H37Rv genome. Forty Beijing genotype strains were identified in which one orphan strain determined by spoligotyping was detected as Beijing strain by multiplex PCR. The Beijing genotype strains were further differentiated by the new multiplex PCR on *Rv1316c* gene resulting in 83% (33/40) belonged to the modern Beijing subtype.

MIRU-VNTR Typing

Fifteen MIRU-VNTR loci were used to differentiate the Beijing genotype MTB strains. The allelic diversity (*h*) of the 40 Beijing genotype MTB isolates were calculated for the specific MIRU-VNTR loci showing the variation from 0 to 0.65. The discriminatory power was regarded as high (HGDI >0.6), moderate (0.3≤ HGDI ≤0.6) and poor (HGDI <0.3) as described by Sola et al. [37]. The allelic diversity for loci QUB26, MIRU10, QUB11b, QUB4156 showed high discriminatory power and the individual diversity indices were 0.65, 0.63, 0.60, 0.60 respectively. MIRU31, MIRU04, ETRA, Mtub39, MIRU16, and Mtub30 loci showed less discriminating power with the discriminating index of 0.19, 0.19, 0.1, 0.14, 0.05, and 0. Mtub30 loci showed no discriminatory power for the typing strains in this study and it was suggested to exclude it for future use of loci in typing (Table 12).

Determination of the transmission pattern of the Beijing genotype MTB strains

The cluster analysis was performed with Bionumeric software to determine the transmission pattern of the Beijing genotype. There were total 32 MIRU-VNTR patterns comprising 8 clustered patterns and 24 unique patterns. A cluster was defined as two or more

isolates having identical MIRU-VNTR pattern. Two isolates were contained in each cluster comprising total 16 isolates in 8 clusters. The remaining 24 isolates showed a unique pattern of MIRU-VNTR typing. The proportion of clustering was 14.5% (16/110) in this study population (Figure 9).

Discussion

This study indicated the population structure of *M. tuberculosis* in Mawlamyine, Myanmar region. The lineage ratio was similar to the previous studies in Myanmar and the majority was EAI that is followed by Beijing lineage while the other lineage showed minority of less than 9.1%. Twenty three orphan isolates, in which 22 isolates and 1 isolate belonging to the EAI lineage and Beijing lineage respectively, showed 20.9% in the proportion and it is higher than the previous study which was 13.87% of total lineage [13]. In a previous study of drug resistance in new pulmonary TB cases in Myanmar from 2010 to 2012, the resistance to at least one drug was recorded as 15.8% of the total isolates, 4% of multidrug resistance and 7.4% of mono-resistance (unpublished data). In this study, MDR-TB was 2% of the total and mono-resistance was found 8% of the total isolates. The different findings between this study and the previous study might depend on the different geographic region and the size of sampling. The Beijing lineage was mainly found out even though a larger proportion of the lineage in overall TB cases was EAI. The majority of the Beijing strains were modern Beijing subtype (83%) and this finding agreed with the previous study in chapter I. A large cluster of the Beijing lineage strains were further discriminated by MIRU-VNTR typing by using 15 loci.

The 15 MIRU-VNTR loci using in this study was appropriate for the discrimination of the Beijing genotype in Myanmar [20]. Of total 40 Beijing isolates, 8 clusters, containing 2 identical MIRU-VNTR patterned isolates in each cluster, were observed. The nature of the Beijing genotype strains in this study was divergent and the probability of transmission among the community seemed less likely by clustering analysis. It was suggested that the chances of outbreaks were few in this study. According to MST analysis of MIRU-VNTR typing, mutations of the drug resistance conferring genes of *rpoB* and *katG* in two MDR-MTB isolates had different mutations with the different MIRU-VNTR patterns (Figure 12). It

was indicated the less transmission of MDR-MTB in the community and the drug resistance seemed to develop independently in each patient because of selective pressure of anti-TB drugs.

The significant association with the Beijing genotype and MDR-TB as well as XDR-TB had been observed in the previous studies in Myanmar [13,14]. The mutations of *rpoB* and *katG* genes were found only in the Beijing genotype while other lineages showed no mutation in this study (Table 10). This finding pointed out that there was a significant association of mutations in both *rpoB* and *katG* genes and the Beijing genotype (p-value<0.01). Even though the findings of an association between the drug resistance and the Beijing genotype has been regarded as controversial in some studies, it was acceptable to the Beijing strains in Myanmar [90]. Almost all mutations were found in the modern Beijing subtype except one ancient Beijing subtype, which had *katG* gene mutation Thr376Met. The Beijing genotype strains showed a higher chance of mutation in the drug resistance association genes with the tendency to transmit from one host to another. An association with drug resistance in the Beijing genotype indicated that the prevalence of drug-resistant Beijing strain is necessary to emphasize for the control of TB.

When comparing with the Beijing strains of Thailand study [91] by analyzing with the same 14 MIRU-VNTR loci, the identical MIRU-VNTR patterns were found out in two clusters even though they were not in the largest cluster representing the biggest outbreak (Figure 10).

The minimum spanning tree analysis of MIRU-VNTR typing with the previous Beijing strains indicated that some strains belonged to the same MIRU-VNTR patterns with the strains from previous study (Figure 11). It was suggested to be the persistence of certain strains in the community. However, it was necessary to clarify whether the actual

transmission among the population because some genotype matching might not infer the recent transmission [92].

Risk factors, including age, sex, the previous health conditions (such as HIV infection, diabetes, and immune deficiency), occupation, such as health care personnel, and people who have different living conditions such as homeless, incarcerated persons, contribute to prone to get TB infection. The recent transmission had been commonly observed linking with young age and male sex [93]. The most common age group was from 45-54 years age group and most of TB patients were over 25 years old, the most working age groups, indicating that it might have impacts on the socioeconomic aspect of the country. The male TB patients were observed more with the ratio of 3.2.

However, there was no significant association between the Beijing genotype strain infection and the following patient clinical, demographic data such as gender, age groups, the past TB history, the history of having contact with household TB patients and the outside TB contact, and the smear positivity (Table 9).

The cross-border people migration is common between the Thailand and Myanmar. A study indicated the statistics of increasing prevalence of TB in migrant people as well as the MDR-TB issues among the non-local people in Thailand. The movement of migrant people between the region of Myanmar and Thailand, create the gateway for TB transmission. One of the important factors is the development of the higher defaulter rate among the refugees leading to a public health threat. A study indicated that a movement of people to the place of the working area for less than 3 months was one of the risk factors for the development of treatment defaulter [95]. We need to emphasize on TB control not only in the local region but also for the mobile population crossing between the border of Myanmar and Thailand.

The Beijing strains showed a tendency to spread continuously among the community. It was suggested to be an association of the Beijing genotype and drug resistance. However, it was difficult to determine by only this study. It is necessary to conduct more research on the region-wide distribution in Myanmar as well as the border region between Myanmar and Thailand.

The presence of insufficient networking and collaboration at a local level, unstructured referral mechanisms were obstacles to control TB. Among several obstacles, lack of information on TB services provided by other organizations and patient loss to follow up were more common on the side of Myanmar than Thailand [96]. Some regions in Myanmar still need to improve the practical application of policies at the local level to overcome the tuberculosis burden.

This study was temporal in nature and it was conducted only for 4 months. It didn't represent the overall population of TB patients in that area. However, this study indicated an association with the Beijing and the drug resistant conferring gene mutations. It is still necessary to carry out further study to indicate the importance of strong association with the Beijing genotype and the drug resistance. Moreover, it is also necessary to prove the evidence of the higher chance of cross-border transmission between Myanmar and Thailand by conducting more studies.

A study of genotypic characterization of *M. tuberculosis* provides the understanding of prevalence and diversity of the strains in the community of the south eastern region of Mawlamyine, Myanmar. It is still necessary to continue to study the dominant strains and the cross-border outbreak strains between Myanmar and Thailand in order to provide effective treatment, better control and preventive effort to TB in this region.

Summary

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (MTB) is the leading cause of death by infectious disease. Myanmar is one of the countries included in both 30 high burden countries of TB and multidrug resistant TB. The association between the Beijing genotype, particularly modern Beijing subtype, and multidrug resistance has been found in many studies including in Myanmar.

This study was aimed to determine the transmission dynamic of the Beijing genotype MTB strains by genotyping methods. One hundred and twenty three sputum samples were collected from tuberculosis patients from Mawlamyaing, Myanmar, in 2016. Mutations of *rpoB* and *katG* genes were detected for the determination of resistance of rifampicin and isoniazid. A total of 40 Beijing genotype was identified by multiplex PCR and further subtyped by the newly developed multiplex PCR for the differentiation of the modern and ancient Beijing subtype. Among these, most of the Beijing strains belonged to modern Beijing subtype (83%) and had a strong association between the drug resistant associated mutations. The Beijing genotype strains were further discriminated by MIRU-VNTR typing.

The finding of 6 clusters (total 12 isolates) and 23 unique patterns in phylogenetic analysis suggested that the Beijing genotype strains in this study area were diverse. However, 2 isolates were observed similar to the genotype pattern of Thailand large outbreak strain when compared with the strains from Thailand near the border area of Thailand and Myanmar.

VNTR set used in this study was suitable for the discrimination of the Beijing genotype strains. The finding of highly diverse Beijing genotype MTB can be inferred that the outbreak is less likely to occur. However, some outbreaks between Myanmar and Thailand region were discovered. This study contributes to strengthen the effective measures for the better treatment and prevention of TB in Myanmar.

Table 9: Demographic information of tuberculosis patient from Mawlamyine, Myanmar

(N=110)

	Beijing		Non-Beijing			
Patient data	**No.**	**Ratio (%)**	**No.**	**Ratio (%)**	**OR**	**P-value**
Age:						
≥55 years	12	11%	21	19%	-	
<55 years	28	25%	46	42%	1.2	0.645
Unknown	-	-	3	3%		
Gender:						
Female	12	11%	14	13%	-	
Male	28	25%	56	51%	0.6	0.235
History of previous TB						
Yes	6	6%	8	7%		
No	24	22%	52	47%		
Not known	9	8%	10	9%		
History of household TB contact:						
Yes	10	9%	15	14%	-	
No	29	26%	55	50%	1.3	0.667
Unknown	1	1%	-	-	ND	
History of outside TB contact:						
Yes	4	4%	4	4%	-	
No	36	33%	61	55%	0.5	0.405
Unknown	-	-	5	5%	ND	
History of HIV infection						
Yes	3	3%	3	3%		
No	24	22%	50	45%		
Unknown	11	10%	17	15%		
No answer	2	2%				
History of diabetes						
Yes	9	8%	11	10%		
No	28	56%	56	51%		
Unknown	2	5%	3	3%		
No answer	1	1%				
Smear positivity						
Sputum smear +	17	15%	23	21%		
Sputum smear ++	6	5%	18	16%		
Sputum smear +++	17	15%	29	26%		

ND: Not done

Table 10: Drug resistance associating mutations of *rpoB* and *katG* gene *Mycobacterium tuberculosis* clinical isolates from Mawlamyine, Myanmar (N=108)

Gene	Affected codon	Nucleotide change		Amino acid change		Number	Ratio (%)	Clade
		From	**To**	**From**	**To**			
rpoB*	450	TCG	TTG	Ser	Leu	3	2.8%	Beijing
	450	TCG	TTC	Ser	Phe	1	0.9%	Beijing
	None	None		None		104	96.3%	
katG*	315	AGC	ACC	Ser	Thr	8	7%	Beijing
	376	ACG	ATG	Thr	Met	1	1%	Beijing
	None	None		None		99	92%	

*The mutations in both *rpoB* and *katG* genes were significantly associated with the Beijing lineage. (p-value <0.001)

Table 11: The distribution of spoligotype pattern of 110 *Mycobacterium tuberculosis* **clinical** isolates from Mawlamyine, Myanmar

Clade	SIT	No. of isolates	Percentage
Beijing	1	35	31.8
	190	1	0.9
	250	2	1.8
	-	1	0.9
CAS	203	1	0.9
EAI1_SOM	48	4	3.6
EAI1_SOM	730	1	0.9
EAI2_MANILLA	19	3	2.7
EAI2_MANILLA	1478	2	1.8
EAI2 Nonthaburi	89	6	5.5
EAI3_IND	11	2	1.8
EAI3__IND	1956	1	0.9
EAI5	236	2	1.8
EAI5	340	1	0.9
EAI5	517	2	1.8
EAI5	1886	2	1.8
EAI6_BGD1	292	10	9.1
EAI6_BDG1	591	2	1.8
EAI6_BDG1	1417	2	1.8
EAI7_BGD2	96	1	0.9
LAM9	177	1	0.9
H3	742	1	0.9
T1	966	1	0.9
T3	1426	1	0.9
U	1083	1	0.9
U	1226	1	0.9
Orphan*	-	23	20.9

*One orphan strain was determined as Beijing clade by multiplex PCR

Table 12: MIRU-VNTR allelic diversity of Beijing genotype isolates (n=40)

TR*	**MIRU-VNTR locus**														
	MIRU 10	MIRU 16	MIRU 26	MIRU 31	MIRU 40	Mtub 04	Mtub 21	Mtub 39	ETR-A	QUB 11b	QUB 26	MIRU 4	Mtub 30	QUB 4156	MIRU 39
0												2			
1					4							2			
2	6					2		1			1	36		23	3
3	17	39	3		32	2	1	38	3					9	23
4	17	1		4	4	32	7	1	37	4			40	7	14
5			1	36		4	27			7				1	
6			4				3			24	8				
7			25				2			4	21				
8			7							1	9				
9											1				
10															
h**	**0.63**	**0.05**	**0.58**	**0.19**	**0.35**	**0.35**	**0.52**	**0.10**	**0.14**	**0.60**	**0.65**	**0.19**	**0**	**0.60**	**0.56**

* TR: Tandem repeat number in each MIRU-VNTR locus

**Allelic diversity: $h = \mathbf{1} - \frac{\mathbf{N}}{(\mathbf{N-1})} \sum_{j=1}^{s} \boldsymbol{n_j} \left(\boldsymbol{n_j} - \mathbf{1}\right)$

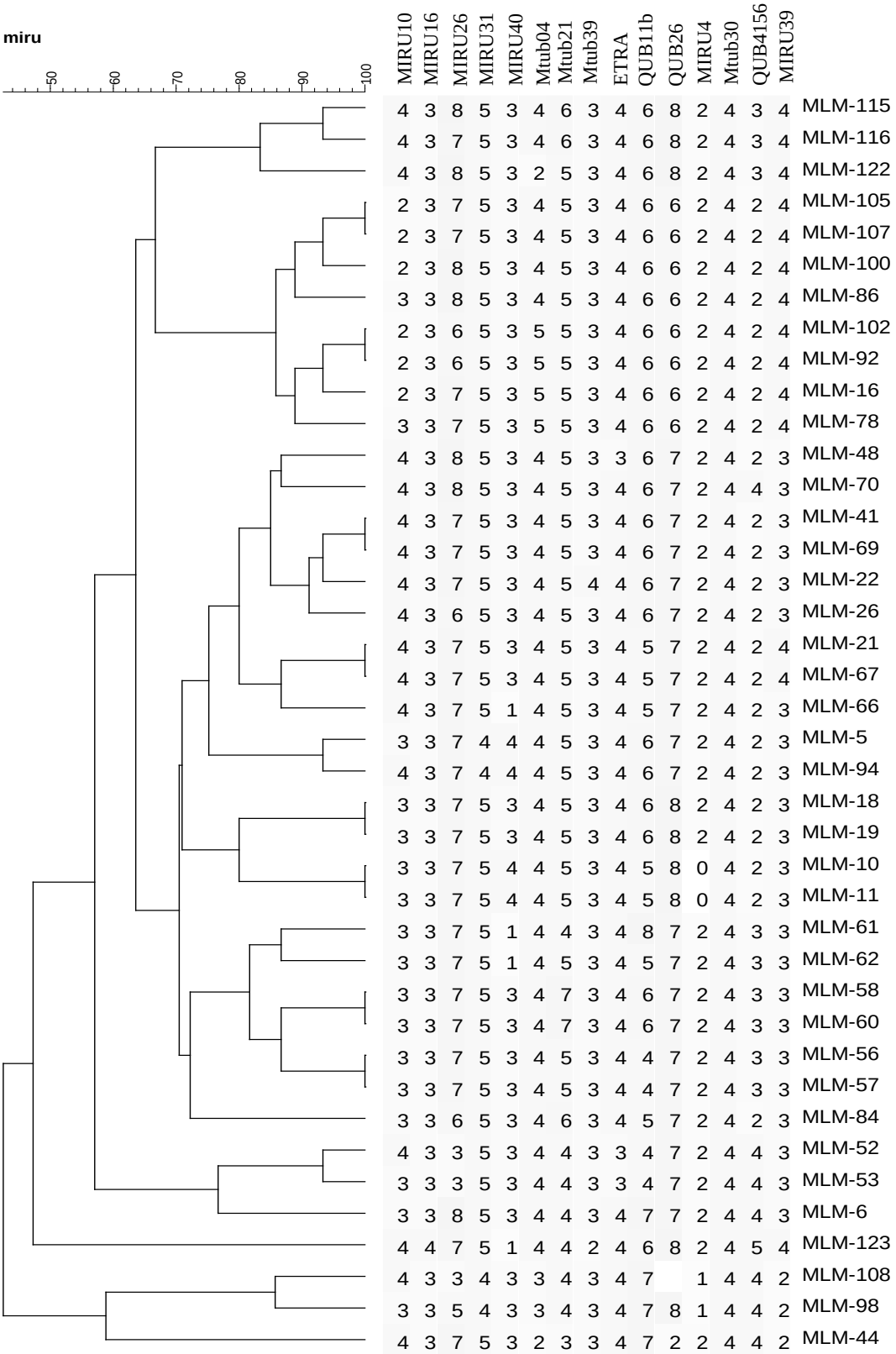


Figure 9: Dendrogram of the MIRU-VNTR typing of 40 Beijing genotype strains

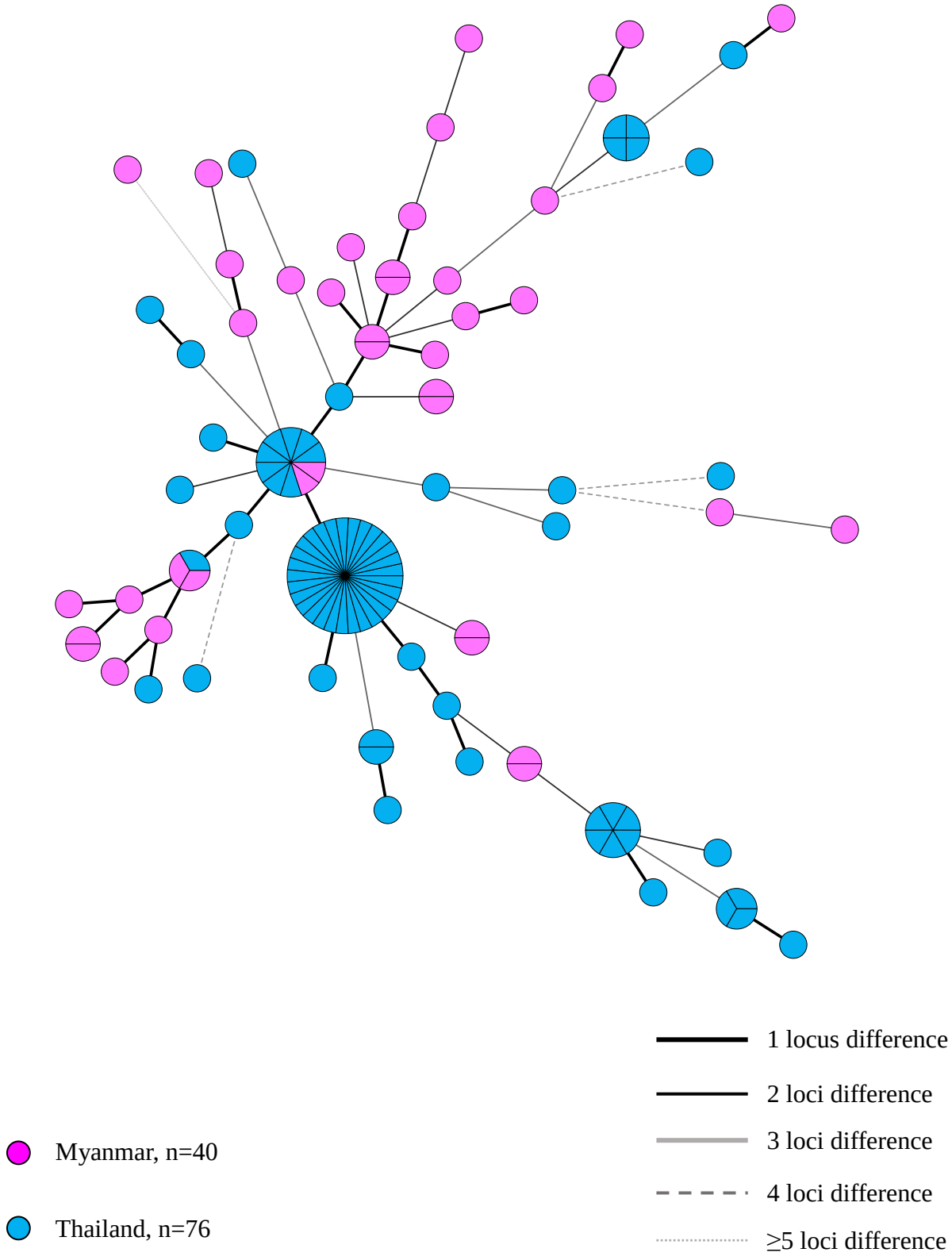


Figure 10: Minimum spanning tree analysis of MIRU-VNTR typing of the Beijing genotype strains of this study and Thailand study[27]

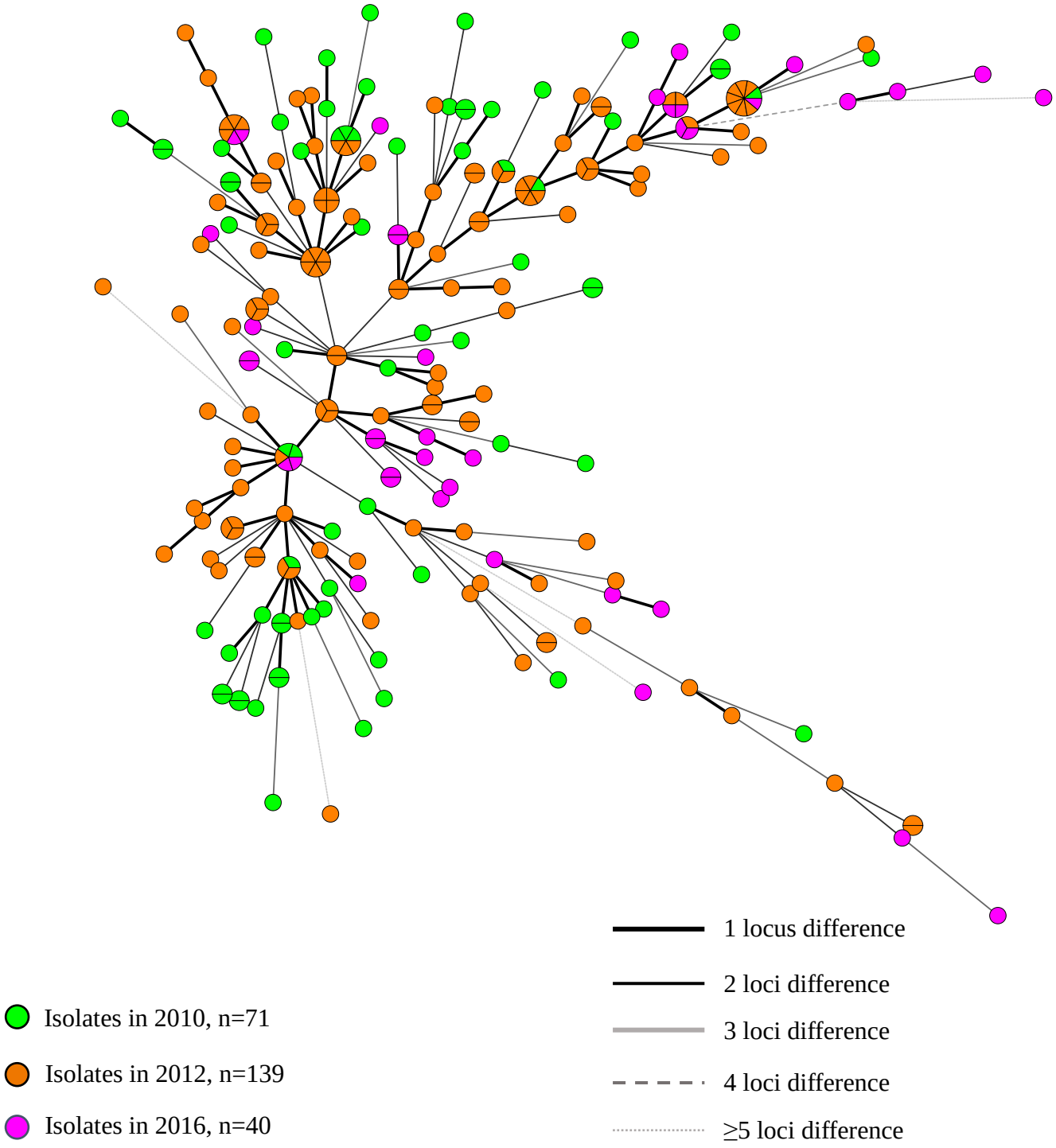
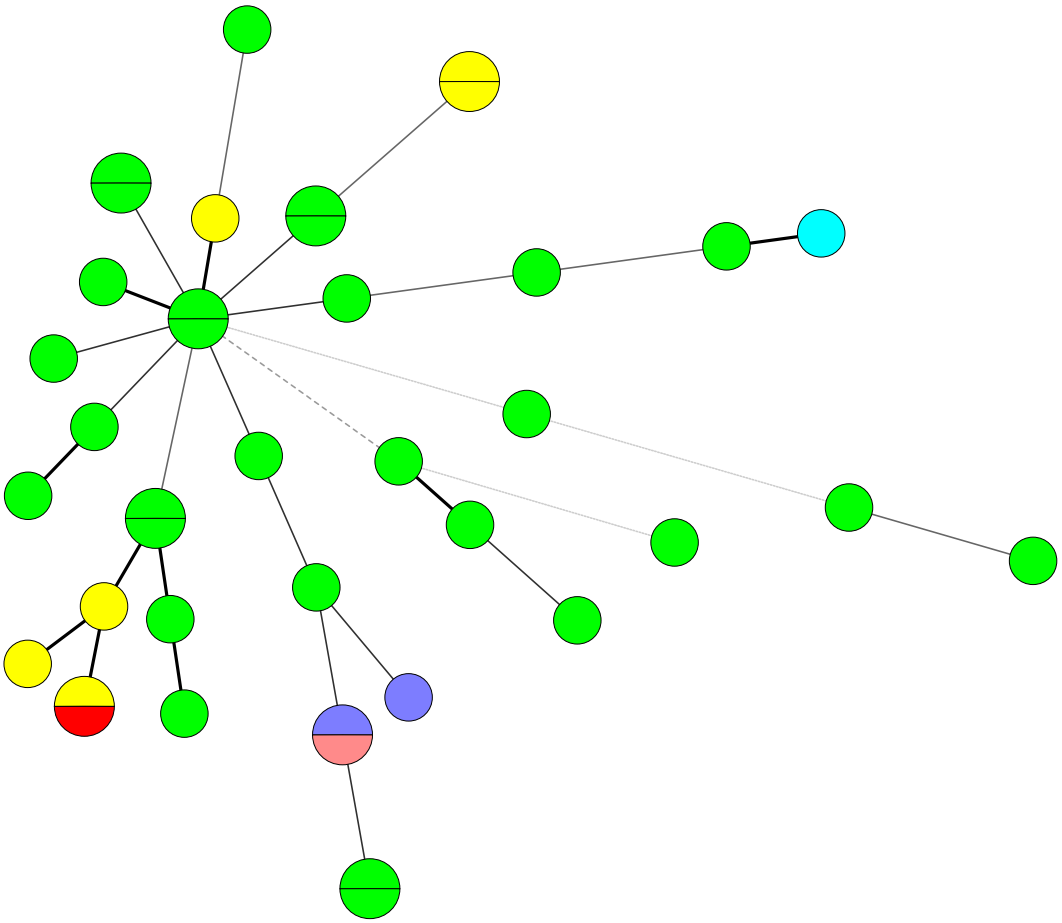


Figure 11: Minimum spanning tree analysis of the Beijing genotype strains of current and previous study



	rpoB & katG	Number
	Wild type, Wild type	29
	Ser 315 Thr, Wild type	6
	Wild type, Ser 450 Leu	2
	Ser 315 Thr, Ser 450 Phe	1
	Thr 376 Met, Wild type	1
	Ser 315 Thr, Ser 450 Leu	1

Figure 12: Minimum spanning tree analysis of the Beijing genotype strains showing *rpoB* and *katG* mutations

DISCUSSION

Myanmar included in 30 high burden countries of TB as well as MDR-TB regarding with WHO TB report 2017. It has a 10th position among the highest TB burden countries and is a neighbor country of top high burden countries, India and China.

My first study emphasized Beijing genotype MDR-TB in Myanmar. The majority of the lineage in Myanmar was EAI in total TB isolates. However, the Beijing lineage strains were nearly 80% proportion of MDR-MTB in the study population. The modern Beijing sublineage were found mostly in Myanmar and had a strong association with the drug resistance as well as spread even though there were controversial findings of these associations by other studies [97].

Regarding with the drug resistance conferring genes, *katG* Ser315Thr mutation in isoniazid (INH) resistance and *rpoB* Ser450Leu mutation in rifampicin resistance consisted in major proportion of drug resistance conferring mutation and the combination of these two mutations were the most likely transmissible MDR-TB [47].

The majority of MDR-MTB strains were modern Beijing strains with divergent property, showing few large outbreaks. Thus, there was a suggestion that the drug resistance developed in each patient individually instead of transmission among the community. However, there was an indication of the silent spread of INH resistant clones by determination of transmission cases revealed by the genetic analyses of the isolates. The genetic changes during the evolution process determines the virulence of the strains and severity of disease [66]. The understanding of this contributes to performing effective control and management of TB.

As a second study, a new multiplex PCR method was developed for the determination of ancient and modern Beijing sublineages as a rapid and easy determination method. It was

aimed to develop especially for the high prevalence of Beijing strains in a region. Although the Beijing lineage is the second most lineages in all TB cases in Myanmar, it is the most commonly found lineage in MDR-TB cases in the previous studies, particularly modern Beijing sublineage strains, which indicated the properties of notorious strains with high transmissibility and high risk of developing drug resistance. There was 100% accurate in the determination of the Beijing sublineages. This method may contribute to determine in a timely manner in order to get rapid diagnosis and effective management to control further implication of TB. Moreover, this method is able to use for all the settings where there is a high prevalence of the Beijing lineage of both modern and ancient subtypes.

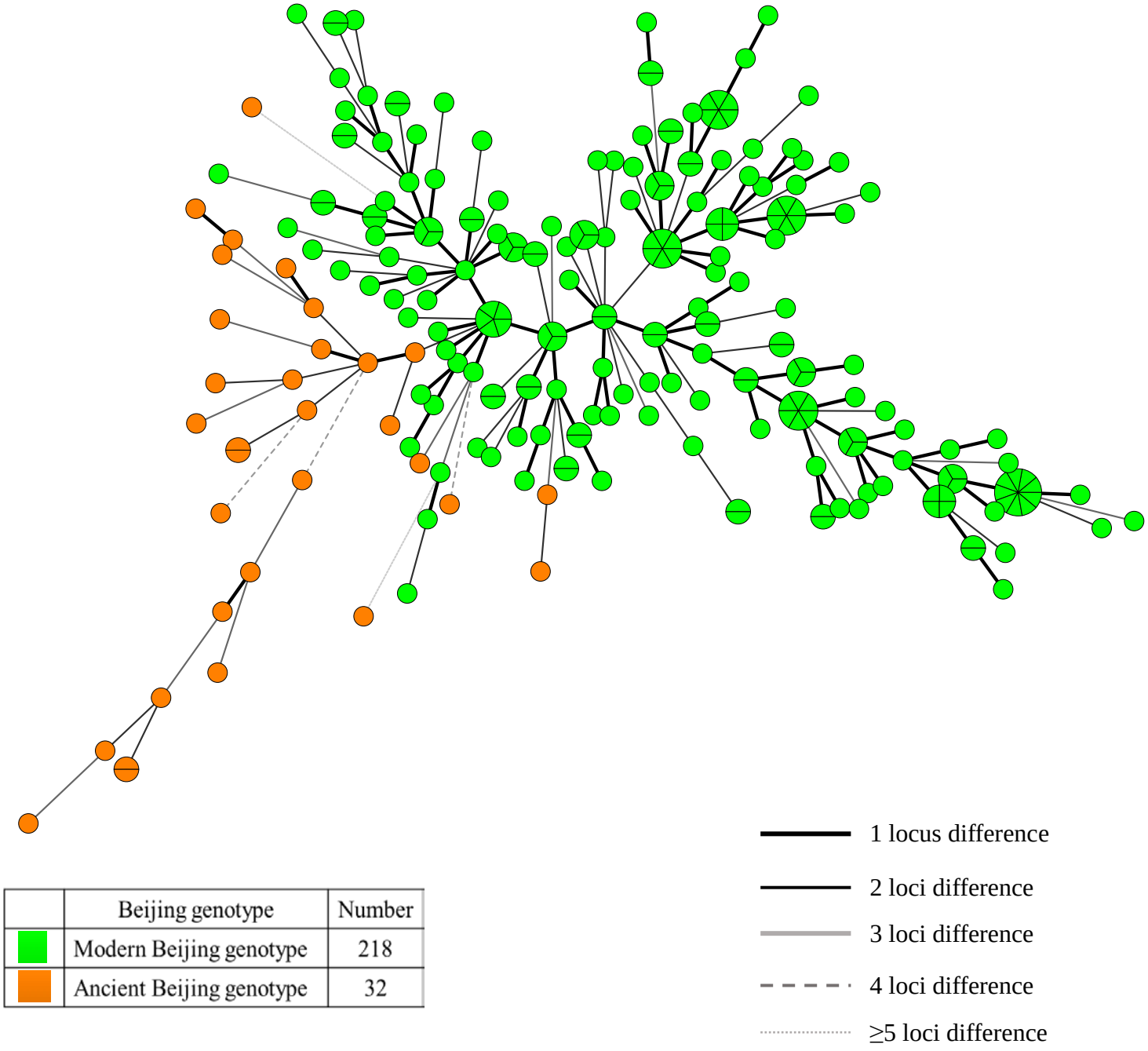
In chapter III, it was aimed to determine the population structure of a particular region, Mawlamyaing, situated in the south-east part of Myanmar near to the Myanmar-Thailand border. The mostly found lineage was EAI lineage which was followed by the Beijing lineage only in which the drug resistance mutations were detected. Majority is modern Beijing sublineage accounted for 83% (33/40) of the Beijing genotype strains. In comparing by minimum spanning tree analysis with the Beijing strains from Thailand, the evidence of transmission across the border of two countries was found out. Even though there was a large outbreak in Thailand region, it was rarely found in Myanmar. The same MIRU-VNTR pattern of the isolates collected in different years in Myanmar was found out in the same clusters with the Thailand isolates suggestive of successive spreading of particular strains among the community of two different countries.

When we looked at the modern and ancient Beijing genotype patterns, genotypic distribution were different from each other and the modern Beijing strains were seemed to form more clustered than the ancient strains (Figure 13).

In the overall MIRU-VNTR analysis of the isolates collected in 2010, 2012 and 2016, the same genotype patterns forming clusters were observed and it was indicated that certain strains were persistent to spread (Figure 14). Interestingly, a cluster containing of all three year isolates and Thailand isolates revealed that it belonged to the CC3 of Thailand isolates of the previous study [21].

Regarding the analysis of drug resistance determination and MIRU-VNTR typing on the Beijing lineage, there was a strong indication that the drug resistance developed independently in each patient. According to the findings, it was suggested to emphasize the strengthening of TB treatment and control strategy of Myanmar.

The finding contributes to the strengthening of the prevention and control strategies as well as policies of TB control in Myanmar to get effective TB control in a region-wide as well as across the border between the countries. Particularly on the modern Beijing strains, for which a prompt action should be undertaken by rapid detection method, effective treatment, and control strategies.



	Beijing genotype	Number
	Modern Beijing genotype	218
	Ancient Beijing genotype	32

Figure 13: Minimum spanning tree analysis of the Beijing genotype ancient and modern subtypes

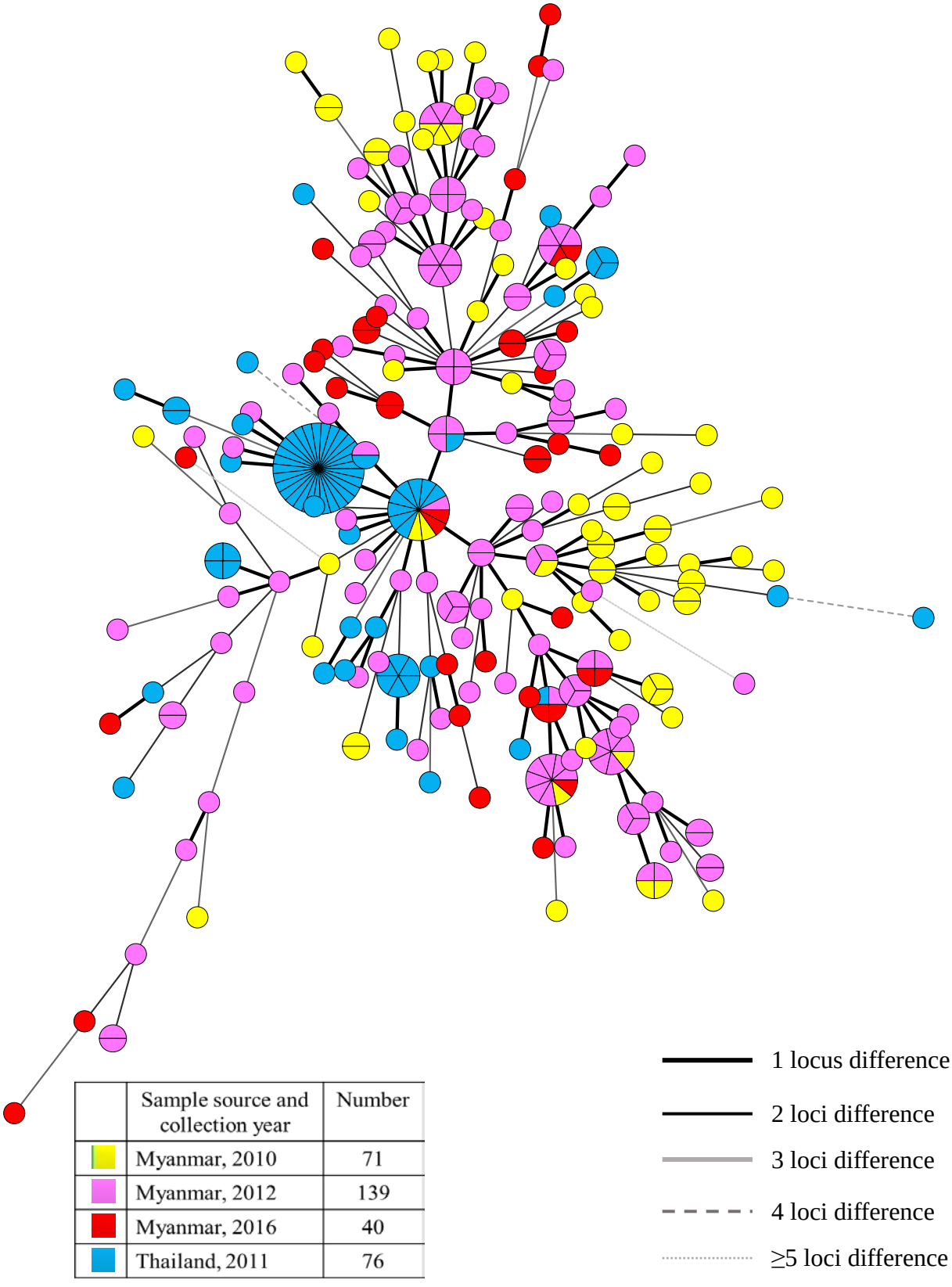


Figure 14: Minimum spanning tree analysis of the Beijing genotype strains from Myanmar and Thailand[27]

CONCLUSION

TB is threatening the world with the highest cause of death by infectious disease. High TB burden countries, including Myanmar, suffer from this disease with much impact. Moreover, MDR-TB, TB with HIV infection and other associated diseases cause more burden and delay in the management and control of TB. The present study contributes to the understanding of a transmission, drug resistance pattern and the predominant lineage in Myanmar via the molecular characterization approach.

In my chapter I, the lineage distribution of MTB in Myanmar and a strong association of the Beijing lineage with the drug resistance, especially MDR-TB, was revealed. Among the Beijing lineage strains, the modern Beijing strains were diverse.

In the chapter II, a new multiplex PCR for differentiation of ancient and modern Beijing sublineage strains was developed with the purpose of rapid detection, especially for the regions or countries, including Myanmar, where the Beijing genotype strains were predominant. This may contribute for the rapid detection of the virulent strains in a timely manner in order to manage the treatment, prevention and control of TB effectively.

In my last chapter, the Beijing genotype strains in Mawlamyaing, Myanmar also showed a strong relation with the drug resistance and showed a trace of tendency to spread among the community especially cross border transmission between the two countries, Myanmar and Thailand.

My study on this thesis pointed out the importance of the Beijing genotype especially modern Beijing strains in Myanmar. It is necessity to strengthen the drug treatment strategy to provide effective treatment to TB patients individually, and to prevent further development of drug resistance and transmission among the community in local, countrywide as well as inter-country level.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my supervisor Prof. Yasuhiko Suzuki for accepting me as a student in his wonderful laboratory, continuous support of my PhD study and related research, for his guidance with the great warm heart, patience, encouraging and immense knowledge from very beginning to the end. His guidance helped me in all the time to gain more knowledge throughout my study.

I would like to express my hearted thankful to Asso. Prof. Chie Nakajima for her great support in gaining knowledge, soft, gentle and kind guidance throughout the whole study and great effort to support me throughout my study. Her wonderful support encourages me to move forward my study more and more.

I would also like to show my sincere thanks which go to Prof. Hideaki Higashi and Prof. Norikazu Isoda for their great and invaluable guidance for my study. They provide me a lot of support for the continuation of my study with great advice.

I would like to show my sincere gratitude to Dr. Khin Saw Aye, Deputy Director General, Department of Medical Research, Myanmar, who kindly introduced and encouraged me to apply to study my PhD in Hokkaido University under the guidance of Prof. Yasuhiko Suzuki. I would like to express my many thanks to Dr. Kyaw Zin Thant, Director General, Department of Medical Research, Myanmar for kindly allow me to study abroad for a long term with greater consideration for the future prospect.

I would also like to express my thanks to Dr. Wah Wah Aung, Director, Department of Medical Research, Myanmar, for her great support, time and effort for my study to go smoothly to perform all my research work especially at the time of field epidemiology study. I would like to thank Dr. Mu Mu Shwe, Deputy Director, Department of Medical Research, Myanmar, for her kind contribution in sample preparation. I would also like to show my

thanks to Dr. Si Thu Aung, Deputy Director/Program Manager, National TB Program, Myanmar, and Dr. Wint Wint Nyunt, Consultant Microbiologist. National TB Reference Laboratory, Aung San, Yangon for their kind permission to conduct research on TB patients. I would like to show my sincere thanks to Dr. Thin Thin Ye, State TB Officer, Mon State, National TB Program, Myanmar, for her kind permission and great effort in obtaining sputum samples from TB patients.

I am indebted to Dr. Satoshi Mitarai, Dr. Akiko Takaki, Dr. Hiroyuki Yamada, Dr. Yoshiro Murase, Akio Aono, Kinyou Chikamatsu, Yuriko Igarashi for their kind contribution of knowledge and experience regarding with the TB research to broaden my knowledge of TB at Research Institute of Tuberculosis/Japan Anti-tuberculosis Association.

I would like to thank to Yuki Maki, Machiko Terashima from leading program for their kind effort to support my field epidemiology study, internship and Zoonosis Control expert examination and so on.

I would like to thank all members of Bioresources; Ms. Midori Yoshida, Ms. Haruka Suzuki, Ms. Miki Nakagawa, Ms. Yayoi Kameda for their kind support for my study, especially to Ms. Yukari Fukushima who kindly guide my laboratory work. My real thanks to Ms. Yuko Hidaka, who gives continuous support and, encourages me with a kind heart to move on in my study.

My thanks go to Dr. Ajay Poudel, Dr. Kanjana Changkaew, Dr. Siriporn Kongsoi, Dr. Ruchirada Changkwanyeun, Dr. Marvin Ardeza Villaneuea, Dr. Tomoyuki Yamaguchi, Dr. Hassan Diab, Ms. Conscilliah Menda, Dr. Jeewan Thapa, Dr. Yogendra Shah, Ms. Nan Aye Thida Oo, Ms. Charitha Mendis, Mr. Jong-Hoon Park, Mr. Yuki Ouchi, Mr. Kentaro Koide, Ms. Dipti Shrestha, Ms. Ruttana Pachanon, Mr. Thoko Flav Kapalamula, Ms. Mwangala Lonah Akapelwa, Ms. Risa Tsunoda, Ms. Wimorat Tanomsridachchai, Mr.

Lawrence P. Belotindos, Ms Precious Bwalya, Mr. Eddie Solo, Mr. Bhagwan Maharjan, Ms. Janisara Rudeeaneksin, Ms. Manyando Simbotwe and other members and friends of CZC for sharing with their great experience.

Last but not the least, I would like to thank my family, especially my parents for raising me up to be myself today and my sisters and my niece for their funny support to me.

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