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Research paper

Genotypes and genetic characters of *Mycobacterium tuberculosis* from Myanmar using three typing methods

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ABSTRACT

Knowledge on basic characteristics of *Mycobacterium tuberculosis* (MTB) is helpful to understand the disease epidemiology and support the prediction of clinical outcome of the disease. The aim of this study was to detect the genotypes and genotypic characters of clinical *Mycobacterium tuberculosis* (MTB) isolates from new and re-treatment rifampicin-resistant patients using three different genotyping methods. Mycobacterial interspersed repetitive units-variable number tandem repeat (MIRU-VNTR) typing was used to determine the diversity of 222 clinical isolates. Spoligotyping and IS*6110*-restriction fragment length polymorphism (RFLP) typing were also used to investigate the genetic characters of 105 MTB strains. Among the 15 genotypes detected by MIRU-VNTR, Beijing strains were the most prevalent of all strains (54.8%); new cases (40.5%) and retreatment cases (69.4%), followed by EAI strain. Spoligotyping categorized the strains into 11 lineages and 13 orphans whereas 96 different IS*6110* patterns were identified using RFLP method. The mode number of IS*6110* was 18 and 20. Higher band numbers were found in Beijing genotype ($p < 0.001$). Clustering rates by spoligotyping, MIRU-VNTR and IS*6110*-RFLP typing were 0.714, 0.004 and 0.085, respectively. Discriminatory powers of spoligotyping, MIRU-VNTR typing and IS*6110*-RFLP typing were 0.637, 1.000 and 0.997, respectively. Dominant Beijing genotype in both new and retreatment cases denoting that prevailing tuberculosis in Myanmar changed from EAI to Beijing lineage.

1. Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* complex (MTBC) remains the leading cause of death from infectious diseases which accounts for 10 million new cases and 1.3 million deaths in 2017. Myanmar is one of the 30 high burden countries in tuberculosis (TB), HIV/TB co-infection as well as multidrug-resistant TB (MDR-TB). Although Myanmar has met the Millennium Development Goal (MDG) 2015 targets for TB, by reducing half of the TB prevalence in 1995 by the year 2015, it still remains as a high TB burden country with an estimated TB incidence of 358 per 100,000 of population in 2017 (World Health Organization, 2016; World Health Organization, 2018).

Genotyping of MTB greatly improved understanding of the evolutionary history, population dynamics, outbreak confirmation, and patterns of dissemination of MTB including multidrug resistant. Moreover, it is also applicable in distinguishing endogenous reactivation and exogenous re-infection for probable clinical implications under certain circumstances (Allix-Béguec et al., 2010).

Among major *Mycobacterium tuberculosis* (MTB) lineages, Beijing genotype is known to be more virulent and suggested to be associated with drug-resistance, relapse, fast spread, infectious and fulminant TB infections (Coscolla et al., 2010; Bifani et al., 1996; Niemann et al., 2010; Lan et al., 2003; Sun et al., 2006; Huyen et al., 2013). The lineage distribution shows geographical localizations by which certain strains

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Table 1
Demographic data of newly diagnosed cases (2013) and retreatment cases (2015–16).

		New case n = 111	Retreatment case n = 111	Total n = 222
Age range (mean ± SD) year		15–83	18–71	15–83 (37.12 ± 12.86)
Sex	Male	78 (70.3%)	73 (65.8%)	151 (68.0%)
	Female	33 (29.7%)	38 (34.2%)	71 (32.0%)
DST	FLS	87 (78.4%)	–	87 (39.2%)
	MDR	18 (16.2%)	106 (95.5%)	124 (55.9%)
	Resistance other than MDR	6 (5.4%)	5 (4.5%)	11 (4.9%)
Laboratory of isolation	NTRL (Yangon)	96 (86.5%)	43 (38.7%)	139 (62.6%)
	UMTL (Mandalay)	15 (13.5%)	68 (61.3%)	83 (37.4%)
	Yangon			
Region of residence (State/Division)	Ayawaddy	1 (0.9%)	–	1 (0.5%)
	Kachin	3 (2.7%)	2 (1.8%)	5 (2.3%)
	Magwe	–	10 (9.0%)	10 (4.5%)
	Mandalay	4 (3.6%)	28 (25.2%)	32 (14.4%)
	Mon	–	1 (0.9%)	1 (0.5%)
	Nay Pyi Taw	5 (4.5%)	4 (3.6%)	9 (4.0%)
	Sagaing	2 (1.8%)	13 (11.7%)	15 (6.7%)
	Shan	1 (0.9%)	11 (9.9%)	12 (5.4%)
	Tanintheryi	–	2 (1.8%)	2 (0.9%)
	Yangon	95 (85.6%)	40 (36.0%)	135 (60.8%)

are more prevalent in some areas than others. Accordingly, Beijing genotype is prevalent in East Asia and has been reported to be expanding into other regions of the world (Borrell and Gagneux, 2009; European Concerted Action on New Generation Genetic M, 2006). Molecular characteristics and epidemiological investigation have been implemented as a tool for tuberculosis control and surveillances that enables us to gain insights about the disease transmission and to differentiate relapses and infections. Moreover, various researches revealed that certain genotypes are highly associated with drug resistance and virulence. Therefore, observing the prevalent genotype that is prevailing in each geographic region especially in high TB prevalent country might help to support the control program by providing data for the development of better management strategies.

Up to date, various genotyping methods have been developed to investigate the genetic character of Mycobacterial isolates and each have their own advantage and disadvantage in technical or analytical ways. The widely used genotyping methods in TB epidemiology are: IS*6110*-restriction fragment length polymorphism (RFLP) typing, mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) typing and spoligotyping (spacer oligonucleotide typing) (Ei et al., 2016).

Yangon is the largest city in Myanmar. Previously, the major TB genotype in this region was reported as East African Indian (EAI) in 2009 (Phyu et al., 2009). However, recent report revealed that Beijing genotype became the predominant in Myanmar which included 80% of MDR-TB (Tun et al., 2017a). Having this background, the aim of this study was to identify the dominant genotypes and describe genetic characteristics of MTB isolates circulating the country both in new and retreatment drug-resistant cases.

2. Materials and methods

2.1. Mycobacterial strains

A total of 222 MTB isolates were cultured from sputum samples collected from 111 new cases registered in 2013 and 111 rifampicin (RIF) resistant retreatment cases those were enrolled under MDR-TB treatment program during 2015–2016. TB isolates from new cases were obtained from two sputum samples of each newly diagnosed pulmonary TB patients attending tuberculosis centers in Yangon or Mandalay. Sputum samples were cultured in Lowenstein Jensen (LJ) media (BD Difco, Sparks, USA) at the National Tuberculosis Reference Laboratory (NTRL), Yangon or the Upper Myanmar Tuberculosis Laboratory (UMTL), Mandalay (Global Laboratory Initiative, 2014). The two laboratories are major TB laboratories with culture facilities located in two largest cities of lower and upper Myanmar to which the TB cases from respective part of the country were referred for treatment monitoring. Sputum samples from retreatment cases were applied to GeneXpert MTB/RIF by National TB Program (NTP). RIF-resistant samples were cultured to recover MTB isolates. Cultured MTB isolates were included to the study without considering any other criteria to avoid selection bias.

2.2. DNA extraction

Two to three loopful of MTB colonies were suspended in 1 mL of distilled water and sterilized by heating at 99 °C for 20 min with vortex mixing shortly for 5 min interval. The tube was centrifuged at 13,000 × *g* for 5 min and the supernatant was retrieved. Genomic DNAs were extracted using the cetyltrimethyl ammonium bromide method (van Embden et al., 1993). DNAs were stored in 4 °C until used.

2.3. MIRU-VNTR typing

A standardized 24-loci MIRU-VNTR typing was performed (Supply, 2005). For each VNTR locus, 50 µL of PCR mixture containing HotstarTaq kit (Qiagen, Hilden, Germany) were subjected to thermocycling conditions. Amplicons were separated by 3% agarose gel-electrophoresis at 50 V for 2 h, visualized by using Geldoc imager (Bio-Rad, USA) and sizes determined by comparing with 100 plus base pair DNA ladder (Bioneer, Daejeon, Korea). The sizes of repeat sequences were converted to allelic numbers according to Supply (2005) and the patterns analyzed using MIRU-VNTR*plus* web application (Supply, 2005; Weniger et al., 2010). Similarity search was performed to compare with world's lineages. Dendrogram was generated using the UPGMA algorithmand minimum spanning wheel was developed (Michener and Sokal, 1957).

2.4. Spoligotyping

Spoligotyping was performed amplifying the spacers of direct repeat (DR) region using biotinylated primer DRa and DRb and it was applied to the membrane to which 43 oligo-probes were arrayed by using miniblotter followed by hybridization as described previously (Kamerbeek et al., 1997). Identical spoligotypes were considered as clusters while, non-clustered named as unique. All spoligotype patterns were translated into binary code and/or octal code. Then it was compared with the international spoligotype database (SITVIT2) of the Pasteur

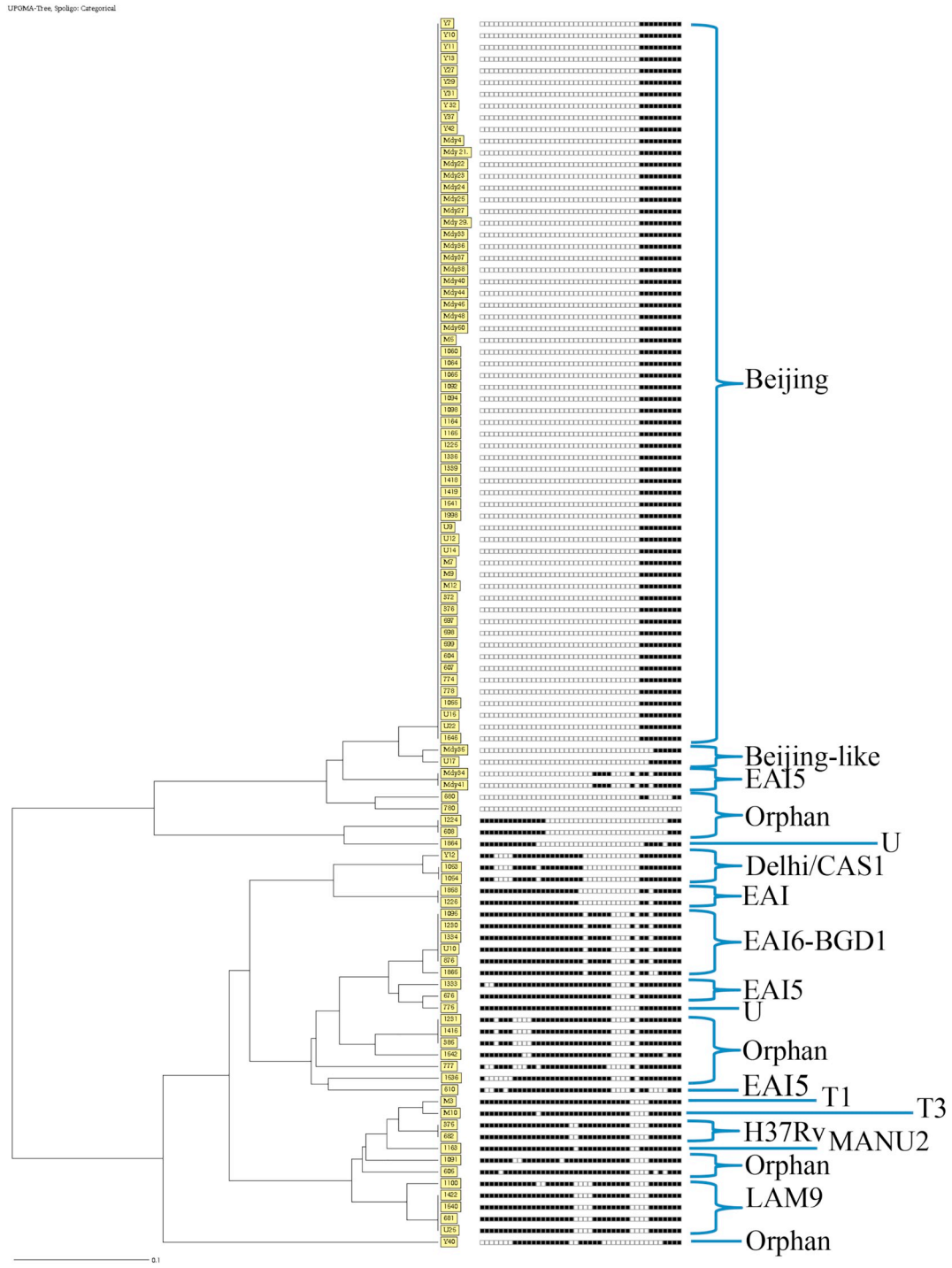

Fig. 1. Phylogenetic analysis of spoligotypes on 105 MTB strains (A subset of strains from total 222 isolates to investigate the genetic characters and compare and confirm the genotypes with MIRU-VNTR typing results).

Institute of laGuadalupe (http://www.pasteur-guadeloupe.fr:8081/SITVIT2 online version) (Couvin et al., 2019). In this database, spoligotype pattern shared by two or more patient isolates was designated as Spoligotype International Type (SIT), while "orphan" designates patterns reported for a single isolate.

2.5. IS6110 RFLP typing

Approximately 4.5 μg of MTB genomic DNA was digested overnight with *Pvu*II restriction endonuclease and the digested DNA was separated by agarose gel-electrophoresis. Then, Southern blot hybridization was performed according to the International Standard Typing method for MTB as described (van Embden et al., 1993). The IS*6110* band patterns were analyzed by Fingerprinting II software version 3.0 (Bio-Rad, CA, USA).

2.6. Data analysis

Data were analyzed using SPSS (Statistical Package for the Social Sciences) version 23 (IBM, USA). Chi square test were done with CI of

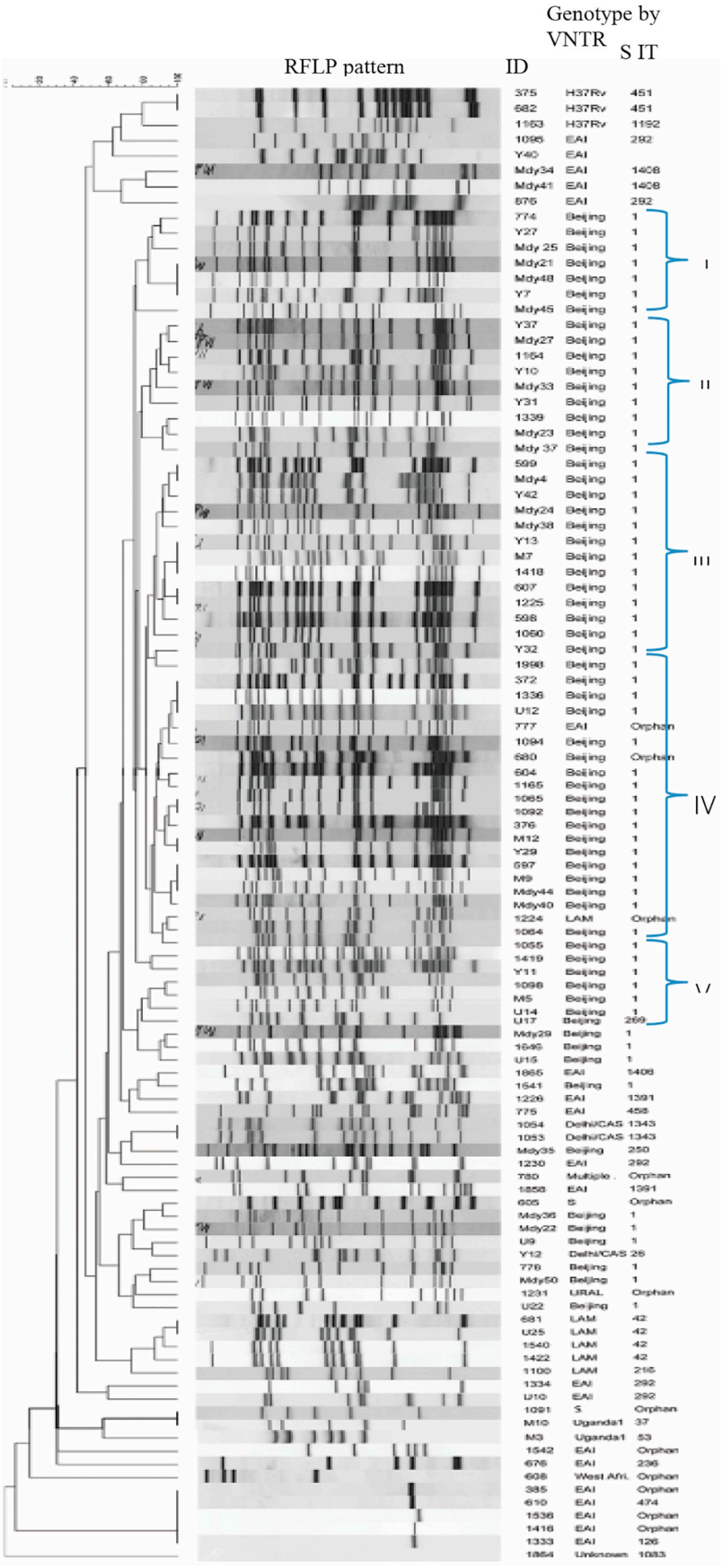

Fig. 2. Phylogenetic analysis of IS*6110* on 105 MTB strains. (A subset of strains from total 222 isolates to investigate the characters of IS*6110* and compare with the genotypes).

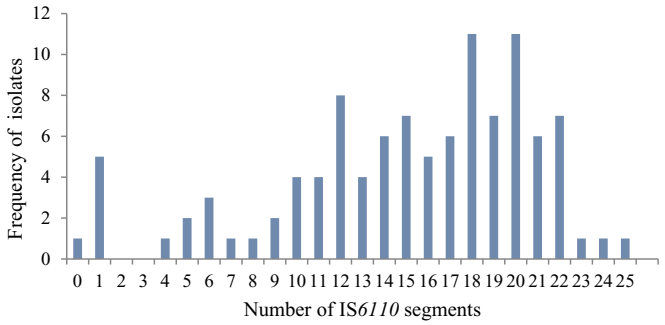

Fig. 3. The Frequency of isolates at the different copy numbers of IS*6110* segment.

Table 2
Genotypes of 222 MTB strains by 24 loci MIRU-VNTR typing (similarity distant < 0.7)

Genotypes	Newly diagnosed strains	Retreatment R-resistant strains	All strains
	n (%)	*n*(%)	*n* (%)
Beijing	45 (40.5)	77 (69.4)	122 (54.8)
EAI	32 (28.8)	5 (4.5)	37 (16.7)
LAM	8 (7.2)	1 (0.9)	9 (4.1)
H37Rv	6 (5.4)	–	6 (2.7)
West African-1	1 (0.9)	4 (3.6)	5 (2.3)
Delhi/CAS	2 (1.8)	2 (1.8)	4 (1.8)
S	2 (1.8)	2 (1.8)	4 (1.8)
Haarlem	1 (0.9)	2 (1.8)	3 (1.4)
Bovis	–	2 (1.8)	2 (0.9)
New-1	2 (1.8)	–	2 (0.9)
Uganda-1	1 (0.9)	1 (0.9)	2 (0.9)
Ural	1 (0.9)	–	1 (0.5)
Caprae	–	1 (0.9)	1 (0.5)
Ghana	–	1 (0.9)	1 (0.5)
West African-2	–	1 (0.9)	1 (0.5)
Multi matches	10 (9.0)	8 (7.2)	18 (8.1)
Unknown	–	4 (3.6)	4 (1.8)
Total	111 (100)	111 (100)	222 (100)

95% and *p* value of 0.05. Discriminatory power analysis was conducted for the clusters of isolates using Hunter-Gaston diversity index (HGDI) (Hunter and Gaston, 1988) by Discriminatory Power Calculation application in web http://insilico.ehu.eus/mini_tools/discriminatory_power/. The clustering rate was calculated using the following formula: clustering rate = $(n_c \_ c)/n$, where n_c is the total number of clustered isolates, *c* is the number of isolate clusters, and *n* is the total number of isolates in the sample (Wang et al., 2011).

2.7. Ethics approval

This study was approved by the Ethic Review Committee, Department of Medical Research, Yangon, Myanmar (23/Ethics 2015).

3. Results

3.1. Demographic characteristics

A total of 222 isolates were represented patients from different regions attending the two laboratories, 139 (62.6%) from NTRL and 83 (37.4%) from UMTL, and equal (50%) proportion of new and retreatment cases. Of which 151 (68%) were males and 71 (32%) were female. The mean age of the patients was 37.12 ± 12.86 years (range 15 to 83 years). The isolates were from patients residing in 9 out of 14 States and Regions and Nay Pyi Taw Union Territory of Myanmar. The demographic data of new and retreatment cases that were collected from 2013 and 2015–16 respectively were shown in Table 1.

3.2. Genotyping by spoligotyping

Spoligotyping was done on 105 strains, including 67 new TB and 38 retreatment cases. It showed 30 different spoligo-patterns identified in nine clusters of 84 isolates (2 to 62 in each cluster) and 21 unique patterns. Analysis of the results using SITVIT2 showed that 92 strains were clustered in to 11 lineages with 20 different SIT numbers and the rest 13 were orphans. Beijing genotype was the largest with 62 isolates (Fig. 1). Clustering rate of spoligotying was 0.714 and discriminatory power was 0.637.

3.3. Genotyping by IS6110-RFLP typing

Ninety-six different IS*6110* patterns were detected by RFLP typing including 6 clusters of identical isolates (2 to 5 isolates in each cluster) and 90 unique patterns. In Beijing genotype, the IS*6110* patterns showed > 80% similarity that grouped into five large clusters of closer distance (Fig. 2). Clustering rate by RFLP typing was 0.085 and discriminatory power was 0.9973. Numbers of IS*6110* insertion segment ranges from 0 to 25 with the mode of 18 and 20 (11 numbers each). The frequency of isolates in insertion segment numbers was shown in Fig. 3.

Out of 105 isolates, 79 (75.2%) possessed high-copy-number (> 7) of IS*6110* segments. Seven isolates possessed copy number less than five. Of those, one isolate had four copy numbers, five had one copy number and one did not have any copy of IS*6110*. All of the Beijing strains possessed more than seven numbers of IS*6110*. Higher band numbers were found in Beijing genotype compared to non-Beijing type ($p < 0.0001$).Single copy strain in this study to overall isolates was 5/105 (4.76%). Among the 20 EAI strains, 12 were high-copy-number isolates and 8 were low-copy-number isolates. Five (25%) of EAI strains have only one copy number.

3.4. Genotyping by MIRU-VNTR typing

Analysis of 222 strains by MIRU-VNTR*plus* web application at similarity distant < 0.7 revealed the best match of 90% with the pre-existing MIRU-VNTR pattern of the global strains. Among 15 genotypes that were assigned by similarity search, Beijing was dominant genotype followed by EAI, LAM, H37Rv, West African-1, Delhi/CAS, S, Haarlem, Bovis, New-1, Uganda-1, Ural, Caprae, Ghana, and West African-2. Two percent could not be assigned by preexisting global lineages which we call it unknown and 8% have the same similarity distance with at least two of the pre-existing strains (multiple matches) (Table 2). When assigned to MtbC15–9 code, 10 were completely assigned to the strains in application. The rest isolates were submitted for MtbC15–9 code in web application. There was only one identical cluster of two strains and the 220 were found as unique codes (Supplementary table 1). Phylogenetic analysis showed that the strains were diverse in VNTR patterns and only one cluster with two identical isolates was detected (Fig. 4). Clustering rate by MIRU-VNTR typing was 0.004 and discriminatory power was 1.0.

The comparison of genotype determination by spoligotyping and 24 loci MIRU-VNTR typing revealed lineages detected were concordant in almost all of the strains expect for two isolates. Among the 13 orphan strains by spoligotyping, 12 could be assigned by MIRU-VNTR typing (Supplementary Table 2).

3.5. Distribution of clusters by minimum spanning tree using MIRU-VNTR data

The clusters of genotype families were graphically shown by minimum spanning tree mapping the major clusters and their genetic links using MIRU-VNTR*plus* software (Fig. 5). Each nodal point

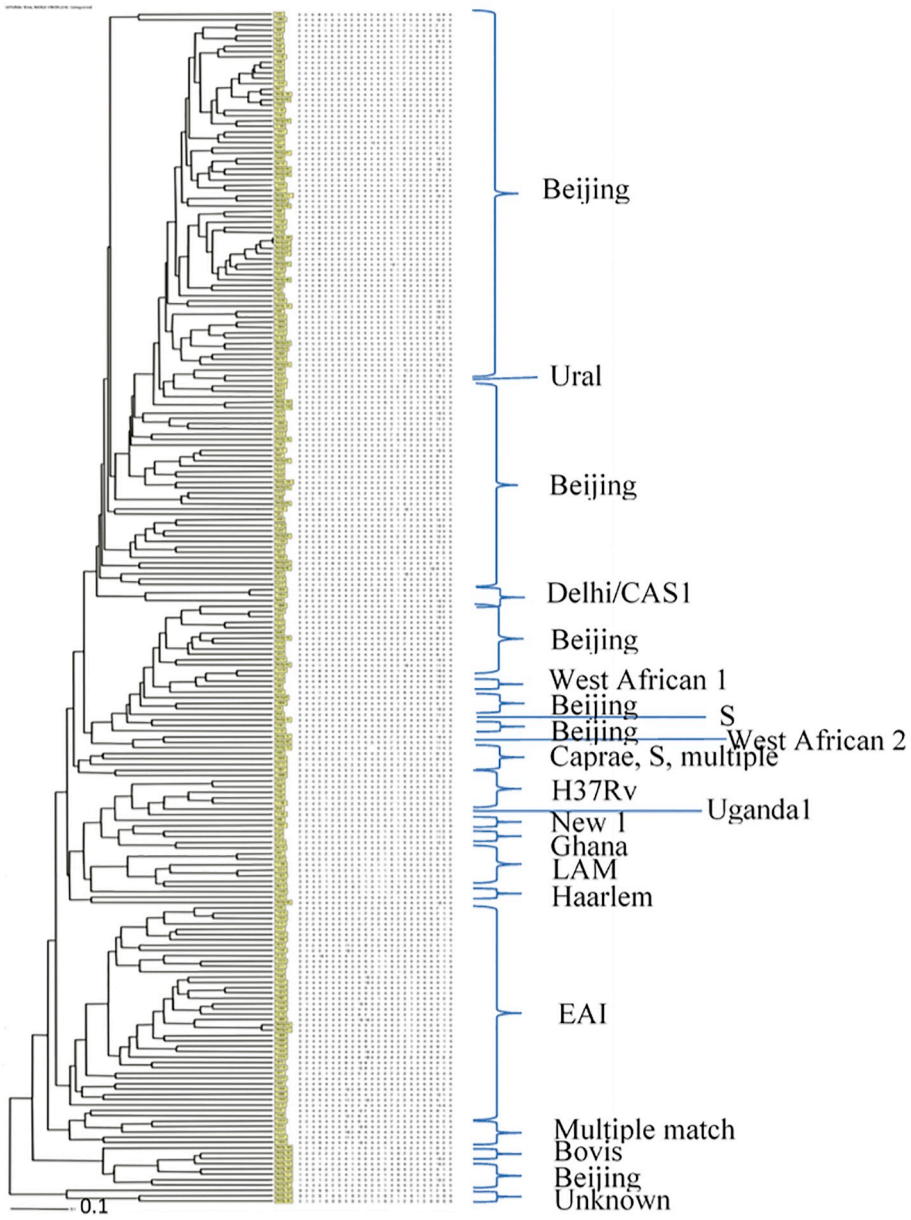

Fig. 4. Phylogenetic analysisof MTB strains using UPGMA of MIRU-VNTR typing.

represents particular VNTR pattern and the size of each nodal point was related to the number of strains within that VNTR type. There was one identical cluster of two strains in Beijing family and three complexes which comprises of strains that were one VNTR locus different from each other.

3.6. Genotype diversity of MTB isolates from different states/regions using MIRU-VNTR

The genotype distribution of MTB in different geographic regions of Myanmar showed that Beijing lineage was the top prevalent in all the regions except Tanintheryi Region (Southernmost part of Myanmar) where the two collected strains were EAI (Fig. 6).

3.7. Analysis of genotypes in demographic and clinical characters

Comparison of Beijing and non-Beijing genotype distribution in different age groups, and gender of new and retreatment clinical types were shown in Table 3. There was no significant change according to increasing age group and sex difference in both groups. On the other hand, Beijing genotype was higher in retreatment cases when compared to non-Beijing genotype (< 0.001).

4. Discussion

Myanmar is one of the high TB and MDR-TB burden country which has shared a large border with two countries of highest TB incidence in the world: India and China (World Health Organization, 2017). In Myanmar, Yangon and Mandalay are the most crowded cities, dwelling diverse ethnic groups from all corners of the country as well as from foreign countries, mainly, India, China, Bangladesh and Pakistan (Myanmar Central Statistical Organization, 2012).

Knowledge on genotype distribution of pathogen and distinct characters of the specific genotype in relation to clinical data in specific region is will be helpful in prediction of clinical outcome of the disease which in turn supports planning the disease prevention and control

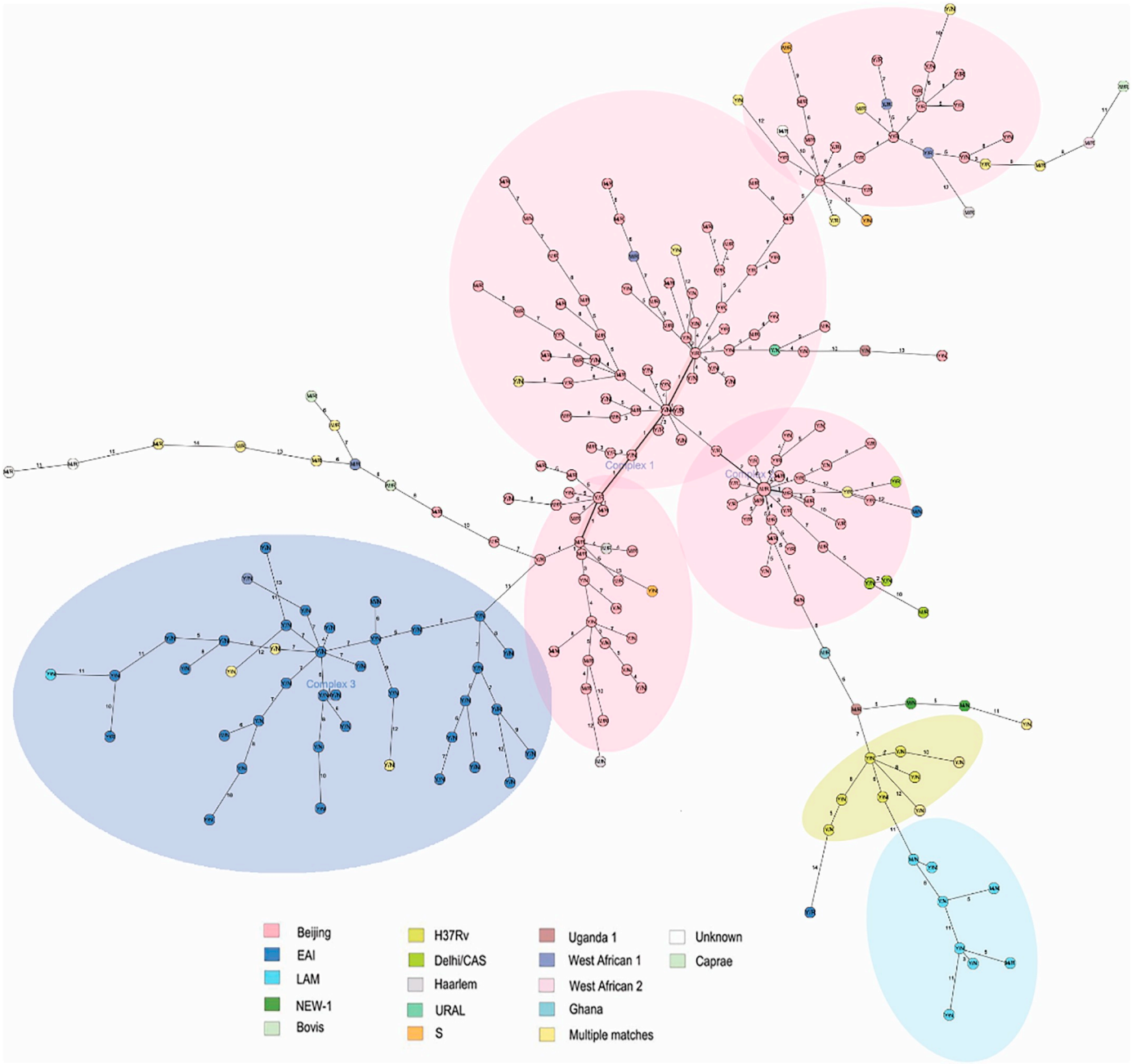

Fig. 5. Minimum spanning tree of 222 MTB strains using 24-loci MIRU-VNTR. Different clusters are shown in different colors, pink color represents Beijing family isolates, purple represents EAI family, light-blue represents LAM family and greenish-yellow represents H37Rv. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

strategies.

Aiming to provide the baseline data of clinical MTB strains circulating in Myanmar, genotype and its distinct characters were identified in this study. Accordingly, genotypes of 222 clinical strains were determined by internationally accepted 24-loci MIRU-VNTR typing. Similar to Asian and South-East Asian countries like as China, and Thailand where Beijing genotype is dominant (Van Soolingen et al., 1995; Srilohasin et al., 2014; Lu et al., 2014), Beijing was the most prevalent genotype in Myanmar; being 54.8% of all strains, 40.5% of new cases, 69.4% of retreatment cases (Table 2). Reports from a decade ago revealed that EAI family was the most prevalent in Yangon region (Phyu et al., 2009; Phyu et al., 2003; Tun et al., 2017b). However, in this study, more than half of the strains from Yangon Region being Beijing family suggested that Myanmar MTB strains are changing over time from EAI to Beijing. Moreover, Beijing strains were dominant in almost all of the regions (Fig. 6) and in newly diagnosed cases in which the majority was registered as drug susceptible new cases (Tables 1 and 2).

This observation might support the previous finding that progression to active disease was higher in individuals infected with Beijing lineage although the transmission of MTB lineages was similar (de Jong et al., 2008).

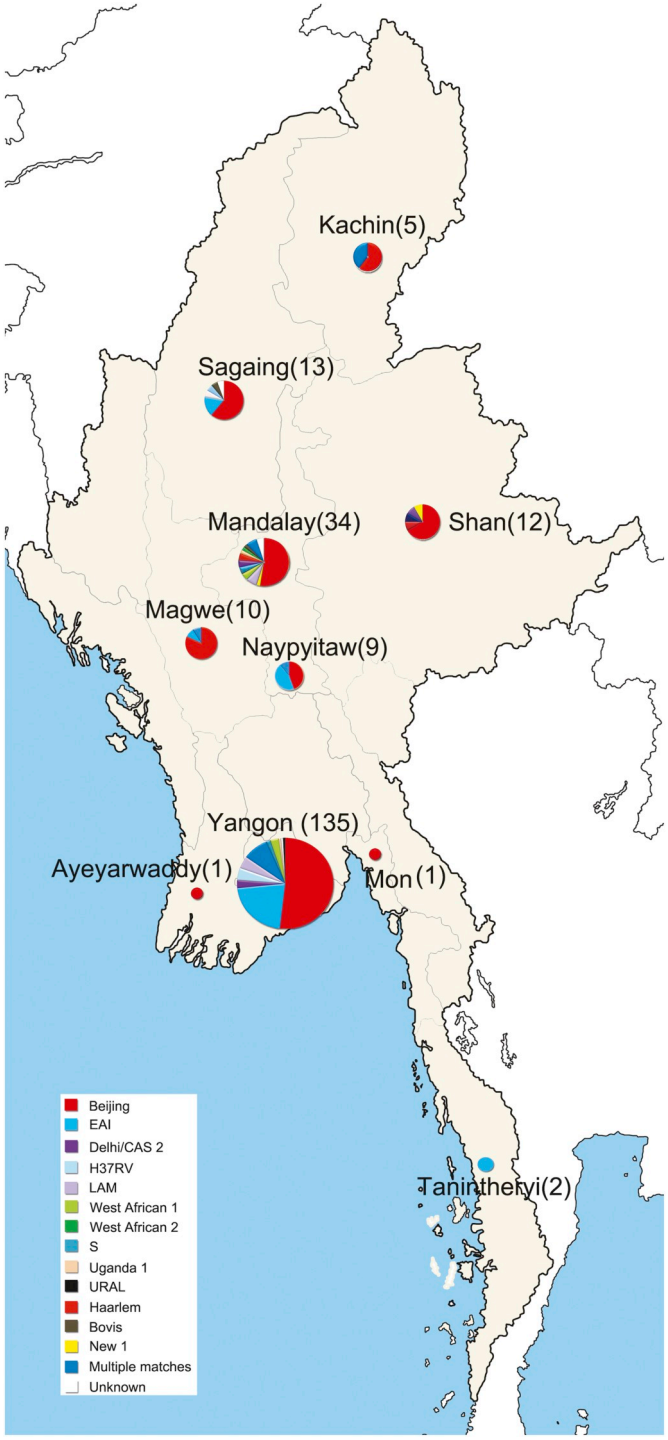

Fig. 6. Distribution of MTB MIRU-VNTR genotypes in Myanmar. Total number information is shown for each region.

The virulence of strains varies as some strains are more virulent than other, accordingly a number of researches have reported that Beijing strains were associated with extra-pulmonary, meningeal-TB or HIV-confection and drug resistance (Coscolla et al., 2010). A multi-country study has reported an increase in the number of TB cases due to Beijing lineage over time in European countries when combining data from all the countries, although not for individual countries (European Concerted Action on New Generation Genetic M, 2006). As result, a spread of this strain in certain regions will be an alarm to concerned bodies in developing essential diseases prevention and control strategies.

MIRU-VNTR typing methods have better discriminatory power and showed that the strains in Myanmar were diverse in which all the tested 222 strains revealed single cluster of two identical VNTR pattern (MtbC 22687-32) and the rest possessed unique MIRU-VNTR patterns. Previous reports also suggested that Beijing family/strains were less likely to be clustered (Zhang et al., 2012). From minimum spanning tree, it was vividly seen that the VNTR polymorphism was very high. Even in the large clusters of Beijing, EAI and LAM genotypes, MIRU-VNTR could separate into small branches. The large branches that arise between large clusters were formed by strains those were usually prevalent in geographic regions far from Myanmar such as New1, Uganda1, West African1 and unidentified strains (multiple matches and unknown). The great diversity of the strains observed may reflect the natural diversity in Myanmar that may be the consequence of re-activation of latent MTB infections or increased global travels which introduce new strains rather than transmission of the indigenous strains.

Using spoligotyping method, Beijing and non-Beijing types were clearly differentiated. There was a high cluster rate (0.714) and a moderate discriminatory power (0.637) in which 65 of 105 strains was Beijing genotype. The lineage determination using MIRU-VNTR typing and spoligotyping were concordance except for two strains which were shown as different genotypes. Clusters that appeared as identical using spoligotyping were shown to be diverse in VNTR pattern suggested that MIRU-VNTR method has better discriminative power. Moreover, 13 strains which were already identified as orphans in SITVIT2 being absence of same pattern in SIT, were categorized in to 12 strains by MIRU-VNTR typing.

Although it is labor demanding and time consuming to grow confluent colonies to obtain enough amount of DNA, IS*6110*-RFLP typing is accepted as gold standard for MTB genotyping. Characterization of IS*6110*-RFLP band pattern, Beijing genotype showed > 80% similarity and clustered into 5 groups with higher similarity providing the fact that the arising TB strains were descendants of closely related TB strains that may be originated or imported to Myanmar. When the copy numbers are investigated, all Beijing strains possessed high-copy-numbers ($p < 0.001$) i.e. more than seven copies and > 70% possessed 12 to 22 copy numbers with the mode number of 18 and 20 (Fig. 3) which was similar to strains from China (Park et al., 2000). Single copy strain in this study to overall isolates was 5/105 (4.76%) which is lower than the proportion (21% ± 5%) found in South East Asia region: Thailand, Vietnam and Malaysia, but higher than that in China (1.5%) and Korea (0.5%) (Park et al., 2000). A quarter of EAI strains (5/20) possessed single IS*6110* and the proportion was similar to the finding from previous study in Myanmar (40/150, 26.67%) (Phyu et al., 2009).

The identical spoligo pattern of 62 Beijing strains could be differentiated into 61 different RFLP patterns including one identical cluster of two isolates. The identical clusters generated by SpolDB4 in non-Beijing strains coincided with the clusters by IS*6110* RFLP but VNTR allelic loci were different from two to ten loci in those strains. This might be greater polymorphism of VNTR which detect genetic variants to differentiate similar strains, or due to lower polymorphism of IS*6110* and direct repeats region in detecting clonal expansion of the same strains when compared to VNTR.

In conclusion, the dominant genotype of MTB strains from Myanmar has been changing over time from EAI to Beijing strain alarming the all health personals in TB management and control.

Declaration of Competing Interest

There is no conflict of interest.

Table 3
Comparison of demographic data and genotyping analysis of new and retreatment TB cases.

Demographic characters	New Case				Retreatment case			
	Beijing	Non-Beijing	Total	P value	Beijing	Non-Beijing	Total	P value
Age group (year)								
< 25	12 (57.1)	9 (42.9)	21 (100)	p = 0.385	16 (72.7)	6 (27.3)	22 (100)	p = 0.514
25–39	15 (39.5)	23 (60.5)	38 (100)		28 (62.2)	17 (37.8)	45 (100)	
40–55	14 (38.9)	22 (61.1)	36 (100)		27 (75.0)	9 (25.0)	36 (100)	
> 55	5 (31.3)	11 (68.7)	16 (100)		6 (75.0)	2 (25)	8 (100)	
Sex								
Male	32 (41.0)	46 (59.0)	78 (100)	p = 0.891	48 (65.8)	25 (43.2)	73 (100)	p = 0.252
Female	14 (42.4)	19 (57.6)	33 (100)		29 (76.3)	9 (23.7)	38 (100)	
Clinical type	46 (41.4)	65 (58.6)	111 (100)		77 (69.4)	34 (30.6)	111 (100)	p < 0.001

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2019.104005.

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