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Clonal Expansion of Multidrug-Resistant and Extensively Drug-Resistant Tuberculosis, Japan

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The emergence and spread of multidrug-resistant (MDR) and extensively drug-resistant (XDR) tuberculosis (TB) has raised public health concern about global control of TB. To estimate the transmission dynamics of MDR and XDR TB, we conducted a DNA fingerprinting analysis of 55 MDR/XDR *Mycobacterium tuberculosis* strains isolated from TB patients throughout Japan in 2002. Twenty-one (38%) of the strains were classified into 9 clusters with geographic links, which suggests that community transmission of MDR/XDR TB is ongoing. Furthermore, the XDR *M. tuberculosis* strains were more likely than the non–XDR MDR strains to be clustered (71% vs. 24%; p = 0.003), suggesting that transmission plays a critical role in the new incidence of XDR TB. These findings highlight the difficulty of preventing community transmission of XDR TB by conventional TB control programs and indicate an urgent need for a more appropriate strategy to contain highly developed drug-resistant TB.

The epidemic of drug-resistant tuberculosis (TB) has raised public health concern about the global control of TB. The World Health Organization estimated that 0.5 million cases of multidrug-resistant TB (MDR TB) (i.e., *Mycobacterium tuberculosis* resistant to ≥2 of the most potent TB drugs, rifampin and isoniazid) occurred in 2007 (*1*). Some countries have extraordinarily high rates of this disease, but the problem is universal, and the extent varies from 1 country to another.

Another recent alarming issue is the emergence of extensively drug-resistant TB (XDR TB) (i.e., *M. tuberculosis* with MDR plus resistance to any fluoroquinolone and >1 injectable drug, thus posing even greater management challenges than MDR TB alone). The treatment outcome of XDR TB is worse than that of simple MDR TB, and the death rate is particularly high among HIV-infected patients (*2*). Also, because XDR TB is much more expensive to manage in terms of prolonged medication and prolonged period of infectivity to other persons (*3*), it has the potential to exhaust human and financial resources of the public health system for TB control. Although this new life-threatening disease had been reported from 49 countries as of June 2008 (*4*), its transmissibility among immunocompetent persons is not well known (*5*).

In Japan, TB remains a major infectious disease; in 2008, a total of 19.4 cases/100,000 population were reported (*6*), and Japan is generally classified as a country with intermediate TB incidence. According to the most recent nationwide drug-resistance survey, the prevalence of MDR TB and XDR TB were 1.9% and 0.5%, respectively (*7*). Approximately one third of MDR and XDR (MDR/XDR) *M. tuberculosis* strains were isolated from new TB patients, implying ongoing transmission of MDR/XDR TB in Japan.

Our purpose was to evaluate the transmission dynamics of MDR/XDR TB by using strains from the most recent (2002) nationwide drug-resistance survey in Japan, an industrialized country with low HIV incidence and intermediate TB incidence. We did so by analyzing the MDR/XDR strains by molecular genotyping methods, i.e., insertion sequence *6110* restriction fragment length polymorphism (IS*6110*-RFLP), spacer-oligonucleotide genotyping (spoligotyping), and variable number tandem repeats (VNTR).

Materials and Methods

We used data and culture isolates obtained in the 2002 nationwide drug-resistance survey, as previously reported (*7*). Briefly, during June–November 2002, a total of 3,122

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clinical strains were collected from different patients who had started treatment in 99 hospitals throughout Japan. The number of patients enrolled represented 36.0% of all new reported TB cases during the study period. The sampling of the hospital was not randomized but was based on voluntary participation. The survey identified 60 MDR/XDR *M. tuberculosis* strains, 55 of which were analyzed in this study; the other 5 strains were unavailable for use in this study.

Patient Information

We used patient information collected from a standard data collection form in the drug-resistance survey in 2002 (*7*). The information included demographic data (age, sex, nationality, hospital, geographic area of the hospital), clinical data (history of TB treatment, site of TB disease, chest radiograph findings, underlying disease), and bacteriologic data (results of sputum smear test for acid-fast bacilli). Patients were classified as new if they had never been treated for TB for >4 weeks and as previously treated if they had ever been treated for TB for ≥4 weeks. The survey protocol conformed to the national guidelines for epidemiologic research (*8*).

Drug Susceptibility Testing

Drug susceptibility testing was performed at the Research Institute of Tuberculosis, Tokyo, by using the proportion method on standard 1% Ogawa egg-based slants (*7*) and the following drug concentrations: isoniazid 0.2 μg/mL, rifampin 40 μg/mL, streptomycin 10 μg/mL, ethambutol 2.5 μg/mL, ethionamide 20 μg/mL, kanamycin 20 μg/mL, cycloserine 30 μg/mL, *p*-aminosalicylic acid 0.5 μg/mL, and levofloxacin 1 μg/mL. Pyrazinamide susceptibility was tested by using MGIT AST (Becton Dickinson, Sparks, MD, USA) at a concentration of 100 μg/mL. All compounds were obtained from Sigma (St. Louis, MO, USA).

Definition of XDR TB

We defined XDR strains according to the World Health Organization definition of XDR (*1*) and drug availability for TB treatment in Japan. XDR strains were defined as *M. tuberculosis* strains with resistance to at least isoniazid, rifampin, levofloxacin, and kanamycin.

Molecular Genotyping

Three molecular genotyping methods based on IS*6110*-RFLP, spoligotyping, and VNTR were performed on the 55 MDR/XDR strains. IS*6110*-RFLP typing was performed according to the standardized protocol (*9*). The RFLP band patterns were compared by using the BioNumerics software package version 5.1 (Applied Maths, Kortrijk, Belgium). Strains with an identical band pattern were classified as an RFLP cluster. Spoligotyping was also performed according to the standard protocol (*10*). Classification of the spoligotype family was performed according to the international database, SpolDB4 (*11*). The VNTR analysis was conducted as described elsewhere (*12*) by using the standard 15 mycobacterial interspersed repetitive unit–VNTR proposed by Supply et al. (*13*), i.e., VNTRs 0424, 0577, 0580, 0802, 0960, 1644, 1955, 2163b, 2165, 2401, 2996, 3192, 3690, 4052, and 4156 plus 4 other loci, VNTRs 2074, 2372, 3155, and 3336. The latter 4 loci were added to increase discrimination for the Beijing family strains because of their high prevalence in Japan (*12*).

Statistical Analysis

Statistical analysis was performed with Epi Info software 3.51 (Centers for Disease Control and Prevention, Atlanta, GA, USA), by using χ^2 test or Fisher exact test for the comparison of proportions. A p value <0.05 was considered significant.

Results

Epidemiologic Characteristics of Patients

A total of 55 MDR/XDR cases were analyzed. The characteristics of patients with MDR/XDR TB are summarized and compared with those of patients with pansusceptible strains (n = 2,782) in Table 1. Patients with MDR/XDR TB were significantly more likely to be younger (odds ratio [OR] 5.69 for age 21–40 years; 4.11 for age 41–60 years) and foreigners (OR 6.41) and to have been previously treated (OR 10.55). All MDR/XDR TB patients had pulmonary disease, and these patients were significantly more likely to have cavitary lesions (OR 3.24) and to have positive sputum smear test results (OR 2.20).

Of the 55 MDR/XDR TB cases, 17 (31%) were XDR TB. We found no significant differences between patients with XDR TB and patients with MDR but not XDR (non–XDR MDR) TB. XDR TB patients tended to be female, although the difference was not statistically significant (p = 0.06, Fisher exact test).

Analysis by Spoligotyping

We performed spoligotyping to determine the population structure of the 55 MDR/XDR strains (Table 2). The most dominant spoligotype family in the MDR/XDR cases was the Beijing family genotype (62%, n = 34), followed by the T (13%, n = 7), Latino-American and Mediterranean (5%, n = 3), U (2%, n = 1), East-African Indian (2%, n = 1), and X (2%, n = 1) family genotypes. Eight (15%) strains were unclassified according to the database.

The proportion of the Beijing family, which is frequently reported to be associated with drug resistance (*14*), did not significantly differ between the non–XDR MDR *M.*

Table 1. Comparison of characteristics between TB patients with XDR TB and non-XDR MDR TB from the most recent (2002) nationwide drug susceptibility survey, Japan*

Characteristic	No. (%) cases				Odds ratio (95% confidence interval)	
	XDR, n = 17	Non–XDR MDR, n = 38	MDR/XDR, n = 55	Drug susceptible, n = 2,782	MDR/XDR vs. drug susceptible	XDR vs. non–XDR MDR
Age, y						
0–20	0	1 (3)	1 (2)	51 (2)	2.57 (–)	–
21–40	6 (35)	14 (37)	20 (36)	460 (17)	**5.69 (2.63–12.45)**	0.51 (0.12–2.18)
41–60	10 (59)	12 (32)	22 (40)	701 (25)	**4.11 (1.93–8.85)**	1
≥61	1 (6)	11 (29)	12 (22)	1,570 (56)	1	0.11 (0.00–1.11)
Sex						
M	9 (53)	30 (79)	39 (71)	1,973 (71)	1	1
F	8 (47)	8 (21)	16 (29)	809 (29)	1.00 (0.53–1.86)	3.33 (0.83–13.77)
Nationality						
Japanese	16 (94)	31 (82)	47 (85)	2,710 (97)	1	1
Foreigner	1 (6)	7 (18)	8 (15)	72 (3)	**6.41 (2.69–14.72)**	0.28 (0.01–2.64)
Treatment history						
New	8 (47)	17 (45)	25 (45)	2,498 (90)	1	1
Previous	9 (53)	21 (55)	30 (55)	284 (10)	**10.55 (5.93–18.83)**	0.91 (0.25–3.33)
Site of TB						
Pulmonary	17 (100)	38 (100)	55 (100)	2,687 (97)	–	–
Extrapulmonary	0	0	0	95 (3)	–	–
Chest radiograph finding						
Noncavitary	5 (29)	8 (21)	13 (24)	1,394 (50)	1	1
Cavitary	12 (71)	30 (79)	42 (76)	1,388 (50)	**3.24 (1.68–6.38)**	0.64 (0.15–2.84)
Sputum smear test result						
Negative	3 (18)	6 (16)	9 (16)	838 (30)	1	1
Positive	14 (82)	32 (84)	46 (84)	1,944 (70)	**2.20 (1.03–4.85)**	0.88 (0.16–5.23)
Complication						
None	6 (35)	21 (55)	27 (49)	1,344 (48)	1	1
Diabetes mellitus	4 (24)	7 (18)	11 (20)	438 (16)	1.25 (0.58–2.65)	2.00 (0.34–11.88)
Malignancy	3 (18)	2 (5)	5 (9)	180 (6)	1.38 (0.46–3.83)	5.25 (0.52–61.86)

*TB, tuberculosis; XDR, extensively drug-resistant; MDR, multidrug-resistant; MDR/XDR, MDR TB and XDR TB; –, not available. **Boldface** indicates significance.

tuberculosis strains and XDR *M. tuberculosis* strains (68% vs. 47%; p = 0.14), and distribution of the other spoligotype families did not differ significantly (data not shown).

Cluster Analysis by IS*6110*-RFLP

All 55 MDR/XDR strains were genotyped by IS*6110*-RFLP, and 21 (38%) were classified into 9 clusters, each with identical RFLP patterns (clusters 1–9) (online Appendix Figure, www.cdc.gov/EID/content/16/6/948-appF.htm). All members of each cluster belonged to the same spoligotype family. The remaining 34 strains exhibited unique RFLP patterns. Cluster size ranged from 2 to 4, and 7 clusters had 2 members, 1 with 3 members and 1 with 4 members. IS*6110* copy numbers ranged from 1 to 18. Four (7%) strains had <5 copies of IS*6110*, and these strains were discriminated as unique strains by the subsequent VNTR analysis. Of the 21 RFLP-clustered strains, 13 (62%) were classified as XDR *M. tuberculosis*, and 8 (38%) were isolated from new TB patients. Of the 17 XDR *M. tuberculosis* strains, 4 were resistant to all drugs tested, and 2 belonged to cluster 8. Among the 9 RFLP-clusters, 7 were from Japanese patients exclusively; the other 2 clusters were from both Japanese and foreign-born patients.

Cluster Analysis by VNTR

The results of the 19-locus VNTR (15 mycobacterial interspersed repetitive unit–VNTR + additional 4 loci) analysis showed that 7 of the 9 RFLP-clusters were identical according to the 19-locus VNTR (online Appendix Figure). A difference in only 1 locus was observed in the remaining 2 RFLP-clusters: 1 at VNTR1955 in cluster 6, and 1 at VNTR2163b in cluster 9 (online Appendix Figure).

Geographic Distribution of Hospitals with MDR/XDR TB Patients among Each Cluster

To estimate the possible clonal expansion of MDR/XDR TB in communities, we compared the geographic areas of patients' hospitals among each cluster (Table 3). The 55 MDR/XDR TB patients were treated by 23 hospitals, which were located in 16 of the 47 prefectures in Japan. Of the 9 clusters, 7 consisted of patients whose hospitals were located in the same or neighboring prefectures, and the remaining 2 clusters consisted of patients whose hos-

Table 2. Distribution of *Mycobacterium tuberculosis* spoligotype families among 55 persons with MDR/XDR TB, Japan*

Spoligotype family	No. (%) cases		
	XDR TB, n = 17	Non-XDR MDR TB, n = 38	MDR and XDR TB, n = 55
Beijing†	8 (47)	26 (68)	34 (62)
T	2 (12)	5 (13)	7 (13)
LAM	3 (18)	0	3 (5)
U	0	1 (3)	1 (2)
EAI	0	1 (3)	1 (2)
X	0	1 (3)	1 (2)
Unclassified‡	4 (24)	4 (11)	8 (15)

*MDR, multidrug-resistant; XDR, extensively drug-resistant; TB, tuberculosis; LAM, Latino-American and Mediterranean; EAI, East-African Indian.
†Includes Beijing-like strains.
‡Unclassified according to the SpolDB4.

pitals were located in the same region (i.e., a geographic and administrative area with several prefectures). Clusters with geographic links suggest the clonal expansion of MDR/XDR TB occurred in local areas. Although patients in clusters 4 and 7 were from the same hospital, further information about their contact was unavailable because an epidemiologic survey was not performed.

Characteristics of Clustered Cases

We analyzed the associations of genetic clustering by IS*6110*-RFLP with patients' characteristics and bacteriologic factors (Table 4). XDR TB occurred more frequently among the clustered cases than among the unique cases (OR 7.73), but differences between the 2 groups were not significant for any of the remaining factors i.e., age, sex, nationality, treatment history, site of TB disease, chest radiographic findings, sputum-smear test results, underlying disease, or member of the Beijing family genotype.

Discussion

Our study described the transmission dynamics of MDR/XDR TB in Japan on a national scale. Analysis of the 55 MDR/XDR TB cases showed that MDR/XDR TB was more frequent among younger patients, previously treated patients, and foreign-born patients than among patients with drug-susceptible TB (Table 1). In addition, the XDR TB cases, which represented 31% of the total MDR TB cases, were more likely to be associated with clustering than were the non–XDR MDR TB cases (Table 4), suggesting that ongoing transmission plays a critical role in new cases of XDR TB. We also found that the Beijing family genotype predominated in MDR/XDR *M. tuberculosis* (Table 2) and in drug-susceptible *M. tuberculosis* in this setting (*12*).

Although IS*6110*-RFLP analysis has been the standard for strain typing in studies of *M. tuberculosis* transmission, false clusters comprising strains without any epidemiologic link, and thus not reflecting clonal transmission, have been reported (*15–17*). This use of IS*6110*-RFLP analysis is especially problematic in area in which strains with stable RFLP patterns are endemic (*18,19*). In this context, because of its post hoc nature, a limitation of our study is its lack of information about epidemiologic links among clustered patients.

We therefore evaluated the genetic clonality of RFLP-clustered strains more rigorously by using the 19-locus VNTR. Our previous study demonstrated that most of the RFLP-clustered strains without epidemiologic links were discriminated by the 19-locus VNTR (*12*). In this study, the results correlated strongly between RFLP and VNTR in terms of cluster formation; 7 of the 9 RFLP-clusters had completely identical 19-locus VNTR profiles (online Appendix Figure). Each of the remaining 2 clusters contained a single-locus variant, i.e., the difference was at only 1 locus of 1 strain in each cluster. This minor difference in VNTR profile is highly likely to have occurred as a random variation in strains from the same source, as argued by several investigators (*13,20*). In addition to analyzing by RFLP and VNTR, we confirmed that all of the 9 clusters shared identical mutations on drug resistance genes for rifampin and isoniazid (i.e., *rpoB* and *katG*, respectively, data not shown).

Geographic distribution of the hospitals also supports the clonal expansion of MDR/XDR TB (Table 3). Isolation of most of the clustered strains from hospitals in the same or neighboring prefectures may indicate that transmission is occurring in the communities. Furthermore, we assume that some of the clustering in our study did not result from direct transmission among the members but rather resulted from exposure of the members to a common infection source or infection with different sources sharing a near ancestor. The discordance of drug resistance other than isoniazid and rifampin resistance among clusters implies that the stepwise acquisition of drug resistance had occurred chronologically during successive transmissions (online Appendix Figure). Thus, all these findings support the assumption of ongoing community transmissions of MDR/XDR TB.

A high proportion (71%, 12/17) of the XDR strains were involved in clusters, a finding consistent with the results of a hospital-based study in Osaka, Japan (*21*). Of the 12 clustered cases, 4 were new cases and 8 were among previously treated patients. The clustered XDR TB cases among the new cases strongly indicated that these persons had been primarily infected with XDR *M. tuberculosis*

Table 3. Geographic distribution of hospitals among each cluster of MDR and XDR TB, Japan*

Cluster no.	Patient ID	Type of TB	Hospital	Geographic link of the hospitals in clusters
1	DR43	MDR	A	
	DR42	XDR	B	A and B located in same prefecture
2	DR14	MDR	C	
	DR53	MDR	D	C located 103 km from D
3	DR54	XDR	D	
	DR58	XDR	D	
	DR18	XDR	E	D and E located in same prefecture
4	DR03	MDR	F	
	DR04	MDR	F	Same hospital
5	DR11	MDR	G	
	DR10	MDR	H	G located 221 km from H
6	DR51	XDR	D	
	DR50	XDR	D	
	DR55	MDR	D	
	DR16	XDR	E	D and E located in same prefecture
7	DR38	XDR	D	
	DR39	MDR	D	Same hospital
8	DR13	XDR	I	
	DR56	XDR	D	I and D located in neighboring prefectures
9	DR12	XDR	I	
	DR49	XDR	D	I and D located in neighboring prefectures

*MDR, multidrug-resistant; TB, tuberculosis; XDR, extensively drug-resistant.

strains. Also, TB may have developed in the clustered XDR TB patients with previous TB treatment as a result of exogenous reinfection with XDR TB, as described in a report of fatal TB in a patient infected with an MDR strain when his disease had been almost cured (*22*).

One possible explanation for the high clustering rate of XDR TB is that new cases of XDR TB are more likely to be caused by transmission than by acquisition of multiple drug resistance as a result of treatment failure. Shah et al. reported that patients with XDR TB were more likely than those without XDR TB to be infectious in terms of duration and proportion of sputum smear positivity (*3*). Furthermore, at least 142 non–XDR MDR TB and 180 XDR TB patients were reported to be in Japan as of 2000 (*23*). All were culture positive, and a considerable number were treated as outpatients despite their infectiousness and drug resistance. Thus, we assume that some of those patients with chronic MDR/XDR TB may have played a role as a source of transmission, as described in this study.

Because these findings and our cluster analysis suggest that the current TB control strategy is insufficient to prevent community transmission of MDR/XDR TB, more detailed investigations of MDR/XDR TB transmission based on contact tracing are urgently needed to improve the infection control strategy, including an isolation policy for highly infectious patients with refractory drug-resistant strains. At the same time, ethical issues, such as the human rights of these patients, should be carefully considered.

Only a few cases of XDR TB transmission have ever been reported. A large group of XDR TB cases, mainly among HIV-infected patients, was reported in South Africa (*2*). Another study in South Africa reported 12 cases of exogenous reinfection with XDR TB (*24*). In Iran, DNA fingerprinting analysis suggested 2 outbreaks of XDR TB involving 12 patients in 1 family and their close contacts (*25*). In Norway, a patient who was lost to follow-up has been transmitting an XDR *M. tuberculosis* strain for 12 years, and that transmission has ultimately resulted in 15 XDR TB cases (*26*). Consistent with these previous reports, our study has added novel evidence for clonal expansion of XDR *M. tuberculosis* strains even in an industrialized country with intermediate TB incidence and low incidence of HIV.

Another limitation of this study is a possible sampling bias, which could be caused by the following factors. First, the sampling of the survey participants was not randomized but was based on voluntary participation of the hospitals, which may be likely to include more serious TB cases (*7*). Second, the sampling fraction is low (38%) and the study period is short (6 months), either of which may produce a biased subset of the total cases. In addition, the low sampling fraction and the short study period may lead to reduced clustering of cases (*27*,*28*). A more complete understanding of transmission dynamics of MDR/XDR TB requires a real-time DNA fingerprinting method such as VNTR on a nationwide scale.

Four strains from the 55 MDR/XDR TB cases were identified as totally drug-resistant strains (*29*), indicating that they were resistant to all 10 drugs tested (online Appendix Figure). Of these strains, 2 in 1 cluster (cluster 8) were from new cases, and both patients were women in their 20s who were full-time workers and had no underlying disease.

Table 4. Comparison of MDR/XDR TB patients and bacteriologic characteristics between clustered and nonclustered cases by IS*6110*-RFLP, Japan*

Characteristic	No. (%) cases		Odds ratio (95% confidence interval)
	Clustered, n = 21	Unique, n = 34	Clustered vs. unique
Age, y			
0–20	1 (5)	0	–
21–40	8 (38)	12 (35)	0.96 (0.23–3.95)
41–60	9 (43)	13 (38)	1
≥61	3 (14)	9 (26)	0.48 (0.08–2.83)
Sex			
M	12 (57)	27 (79)	1
F	9 (43)	7 (21)	2.89 (0.75–11.45)
Nationality			
Japanese	19 (90)	28 (82)	1
Foreigner	2 (10)	6 (18)	0.49 (0.06–3.18)
Treatment history			
New	8 (38)	8 (24)	1
Previous	13 (62)	26 (76)	0.50 (0.13–1.90)
Site of TB			
Pulmonary	21 (100)	34 (100)	–
Extrapulmonary	0	0	–
Chest radiograph finding			
Noncavitary	6 (29)	7 (21)	1
Cavitary	15 (71)	27 (79)	0.65 (0.15–2.71)
Sputum smear test result			
Negative	4 (19)	5 (15)	1
Positive	17 (81)	29 (85)	0.73 (0.14–3.86)
Complication			
None	9 (43)	18 (53)	1
Diabetes mellitus	5 (24)	6 (18)	1.67 (0.32–8.78)
Malignancy	2 (10)	3 (9)	1.33 (0.13–12.94)
XDR TB			
No	9 (43)	29 (85)	1
Yes	12 (57)	5 (15)	**7.73 (1.84–34.83)**
Beijing family genotype			
No	6 (29)	15 (44)	1
Yes	15 (71)	19 (56)	1.97 (0.57–7.46)

*MDR, multidrug-resistant; XDR, extensively drug-resistant; TB, tuberculosis; IS*6110*-RFLP, insertion sequence *6110* restriction fragment length polymorphism –, not available. **Boldface** indicates significance.

Both patients were registered in the same area (no further data available); TB as formidable as totally drug-resistant TB can affect otherwise healthy young adults even on a mass basis.

The results of this study showed that transmission of MDR TB, especially XDR TB, is currently occurring in communities of Japan. Further studies to prospectively identify the transmission route through contact tracing and real-time DNA fingerprinting methods on a population basis are required. Although the MDR/XDR TB problem is not great in Japan, our findings highlight the relevance of proper infection control, as well as effective treatment, to further contain highly developed drug-resistant TB.

Acknowledgments

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SHORT COMMUNICATION

Beijing family *Mycobacterium tuberculosis* isolated from throughout Japan: phylogeny and genetic features

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SUMMARY

To estimate the current population genetic structure of *Mycobacterium tuberculosis* in Japan, phylogenetic traits were analysed for 237 Beijing family strains isolated from tuberculosis patients throughout the country. Unlike previous reports from other countries, the ancient Beijing sublineage was predominant throughout Japan. Clustering analysis based on JATA-VNTR (Japan Anti-Tuberculosis Association variable numbers of tandem repeats), a specialised set of VNTR for the discrimination of Japanese *M. tuberculosis* strains, revealed high similarity of the modern Beijing sublineage strains, irrespective of their geographic origin. JATA-VNTR might be useful for the phylogenetic classification in populations where ancient Beijing strains are frequently isolated.

KEY WORDS: *Mycobacterium tuberculosis*; Beijing family; VNTR; phylogeny

THE BEIJING FAMILY is a lineage of *Mycobacterium tuberculosis* that has been defined using various genotyping methods.[1,2] This lineage, although reportedly predominating throughout eastern Asia,[1] is disseminated worldwide.[3] In Japan, located at the far eastern end of Eurasia, Beijing family strains are highly prevalent (about 75%).[4,5] The Beijing family is classifiable phylogenetically into two sublineages according to the absence or presence of insertion sequence (IS) *6110* insertion in the NTF genome region: the ancient (atypical) and modern (typical) sublineages.[6] The modern sublineage has been considered to be more virulent and to have higher fitness to human hosts than the ancient sublineage. However, irrespective of the worldwide dissemination of the modern sublineage, the predominance of the ancient sublineage in Japan has been inferred from a population study of *M. tuberculosis* Beijing family strains isolated in two Japanese cities.[5]

In the present study, we performed phylogenetic analyses of Beijing family isolates collected from all over Japan based on genotyping methods to validate the particularity of the population genetic structure throughout the country.

METHODS

Previously isolated 325 TB strains collected from 42 of 47 prefectures in Japan were used for this study (Supplemental Figure).[4]* Each prefecture provided 3–10 strains with no epidemiological links for the collection. These included 240 (73.8%) Beijing family strains which had been certified using the spacer-oligonucleotide (spoligo) genotyping method.[2] VNTR (variable numbers of tandem repeats) analyses were performed as described previously.[4] Three strains were excluded from the study due to the problematic genotypes of VNTR, such as amplification of multiple bands in some VNTR loci. Using a computer programme (Bionumerics, Applied Maths, Sint-Martens-Latem, Belgium), a minimum spanning tree (MST) analysis was performed. The rules for constructing trees were the same as those in previous reports.[5,7] The creation of hypothetical types was allowed for the reconstruction of the tree based on the genotypes of JATA-VNTR to compensate for its high diversity in the population.

RESULTS

Predominance of the ancient Beijing sublineage throughout Japan

All 237 Beijing strains were subjected to phylogenetic classification. Figure 1 shows that two genomic deletions, region of difference (RD) 181 and RD150, are

* Supplemental Figure is available in the online version of this article at http://www.ingenta connect.com/content/iuatld/ijtld/2010/00000014/00000009/art00022

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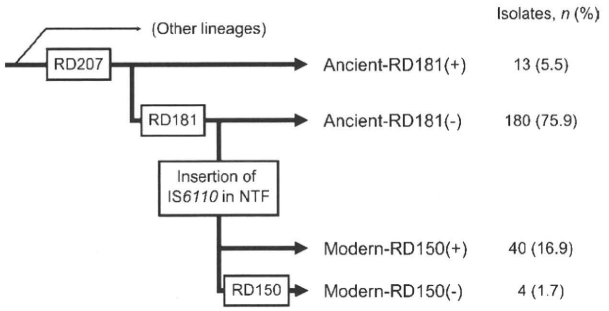

Figure 1 Schematic phylogenetic tree of the *M. tuberculosis* Beijing family. Text boxes show putative times at which genetic events (deletions of three RDs and IS*6110* insertion in the NTF) occurred during the evolution of the lineage. We defined Beijing family strains according to their particular spoligotypes (corresponding to the deletion of RD207) in the study. The distributions and proportions of the Beijing family isolates analysed in the study are also described. RD = region of difference; IS = insertion sequence.

known to have occurred during the evolutionary process of the lineage.[5,8] They are therefore classifiable into four genetic subgroups: ancient (RD181[+]), ancient (RD181[−]), modern (RD150[+]) and modern (RD150[−]). These genetic characteristics were analysed using the polymerase chain reaction, as in previous reports.[5] The results are presented in Figure 1. The ancient sublineage (n = 193, 81.4%) predominated over the modern sublineage (n = 44, 19.6%). Isolates belonging to the ancient sublineage were identified from all 42 prefectures (data not shown). Through RD classification, strains exhibiting RD181(+) and RD150(−) were less prevalent in our population. These results demonstrate that the ancient (RD181[−]) sublineage is the most dominant strain throughout Japan, in agreement with the hypothesis of the predominance of the ancient Beijing sublineage in the country.[5]

A population-based study recently revealed that the proportion of modern Beijing strains isolated from younger TB patients was greater than that obtained from elderly patients in Japan.[9] The endemic prevalence of the ancient Beijing sublineage throughout Japan several decades ago might have foreshadowed the current predominance of the sublineage. It is necessary to give constant attention to the population

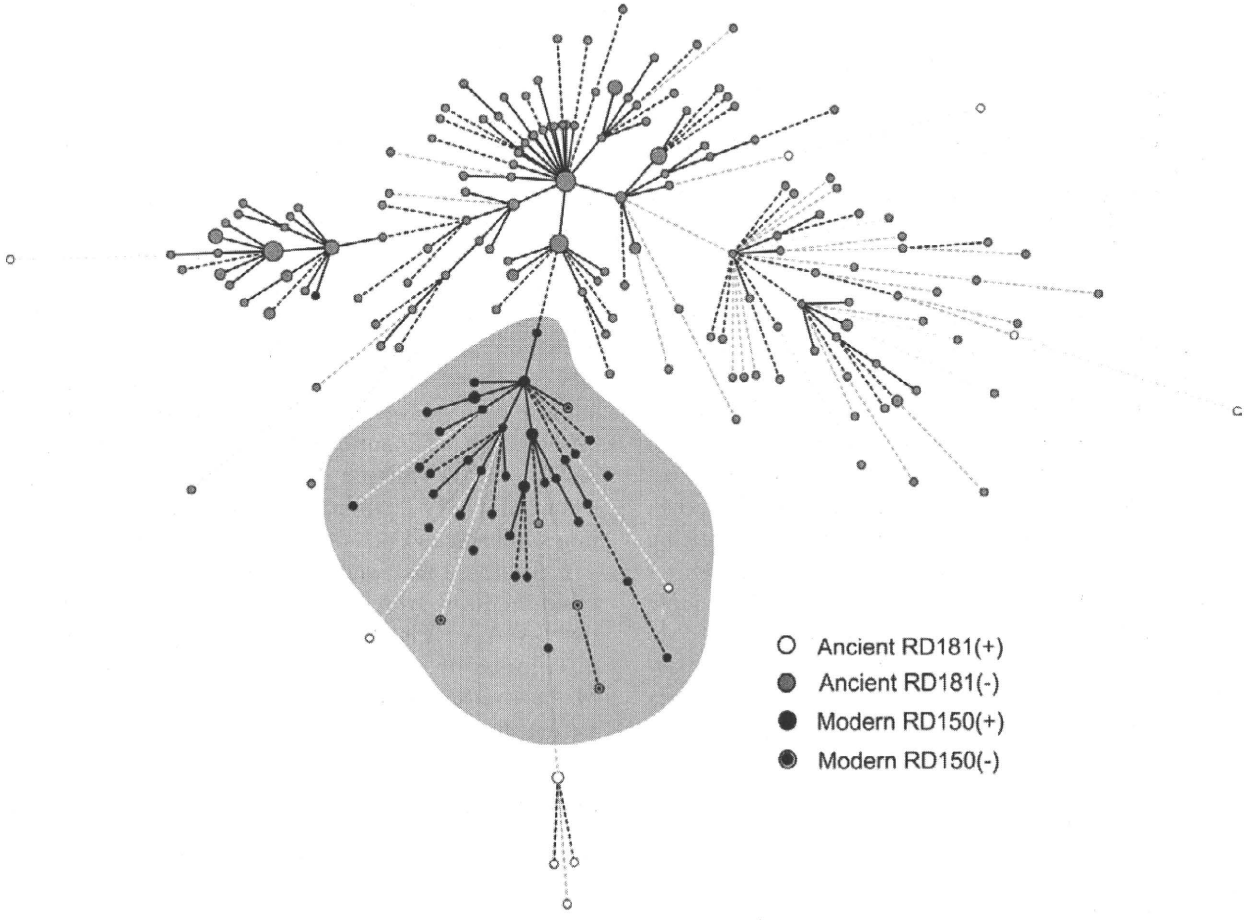

Figure 2 Minimum spanning tree of 237 *M. tuberculosis* Beijing isolates based on a global standard 15-MIRU-VNTR. Each circle corresponds to a different type identified using VNTR genotyping. Heavy lines connecting two VNTR types signify that they are single-locus variants, thin lines connect double-locus variants, and dotted lines (black) connect triple-locus variants. Gray dotted lines represent the most likely connection between two types differing by more than three VNTR loci. Sublineages of each genotype are represented respectively, as shown in the inset. Modern Beijing strains are observed to gather in the tree (gray shaded area). RD = region of difference; JATA = Japan Anti-Tuberculosis Association; VNTR = variable number of tandem repeats.

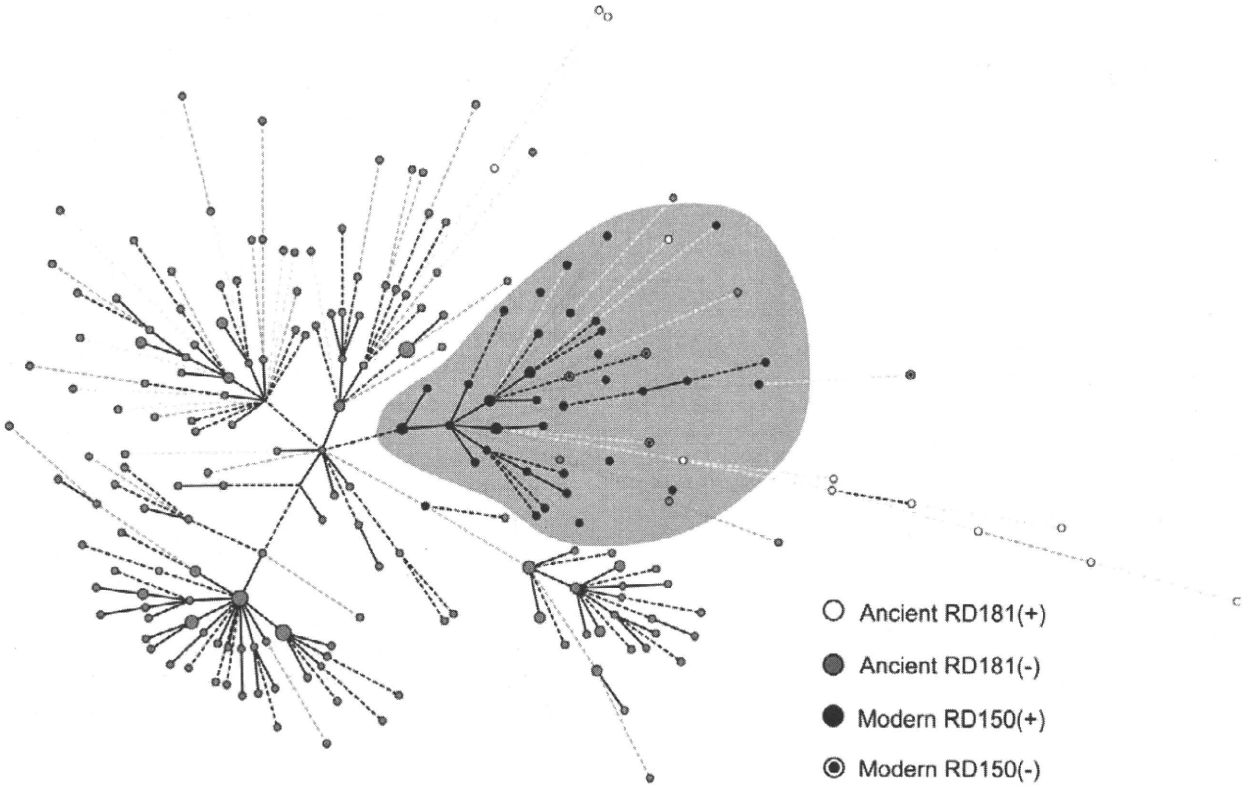

Figure 3 Minimum spanning tree of 237 *M. tuberculosis* Beijing isolates based on 12-JATA-VNTR. Each circle corresponds to a different type identified using VNTR genotyping. Heavy lines connecting two VNTR types signify that they are single-locus variants, thin lines connect double-locus variants, and dotted lines (black) connect triple-locus variants. Gray dotted lines represent the most likely connection between two types differing by more than three VNTR loci. Sublineages of each genotype are represented respectively, as shown in the inset. Modern Beijing strains are observed to gather in the tree (gray shaded area). Knots without circles show hypothetical types created during the tree reconstruction. RD = region of difference; JATA = Japan Anti-Tuberculosis Association; VNTR = variable number of tandem repeats.

structure dynamics to evaluate the emergence of the modern Beijing sublineage in Japan.

JATA-VNTR clustering by minimum spanning tree

We then reconstructed putative phylogenetic trees of the 237 Beijing strains based on their VNTR types using the MST algorithm (Bionumerics, Applied Maths). Reportedly, an international set of VNTR comprising 15 loci (15-mycobacterial interspersed repetitive unit [MIRU]-VNTR)[10] offered a phylogenetically reliable topology for Beijing family strains in Japan.[5,7] In this study, 44 modern Beijing family strains were obtained from 25 separate prefectures in Japan (data not shown). Strains were located in a tight branch in the tree irrespective of their origin (Figure 2), which concorded with those reported in an earlier paper.[5]

Murase et al. proposed 12 loci of VNTR (JATA-VNTR) as a localised set for Beijing family strains specifically for high discrimination of domestic isolates in Japan.[4] These loci were selected according to their high diversity. We reconstructed an MST tree based on JATA-VNTR to assess whether the set specialised for discriminatory power can also be phylogenetically informative (Figure 3). Results show that, in addition to being more dispersed than 15-MIRU-VNTR, modern Beijing strains were also observed to gather in the topology of the tree based on JATA-VNTR. These results indicate that the modern Beijing subfamily is likely to be genetically stable not only by 15-MIRU-VNTR but also by the highly discriminate JATA-VNTR. Thirteen strains belonging to the ancient (RD181[+]) sublineage, the most ancestral Beijing sublineage in our population study, were located in a dispersed fashion, which means that their genotypic relations were not clear. These results suggest that JATA-VNTR might be suitable for both reliable discrimination and phylogenetic interpretation of *M. tuberculosis* Beijing strains in populations where ancient Beijing strains have been spread.

CONCLUSION

Results of phylogenetic analyses performed in this study support the view that an ancient Beijing sublineage has predominated throughout Japan. The JATA-VNTR genotypes of our population of Beijing strains offered a phylogenetically reasonable topology of the clustering tree—reconstructed using the MST algorithm—as a global standard 15-MIRU-VNTR. The set of JATA-VNTR might be highly suitable for a

local standard of TB genotyping in Japan and other populations from which ancient Beijing strains are frequently isolated.

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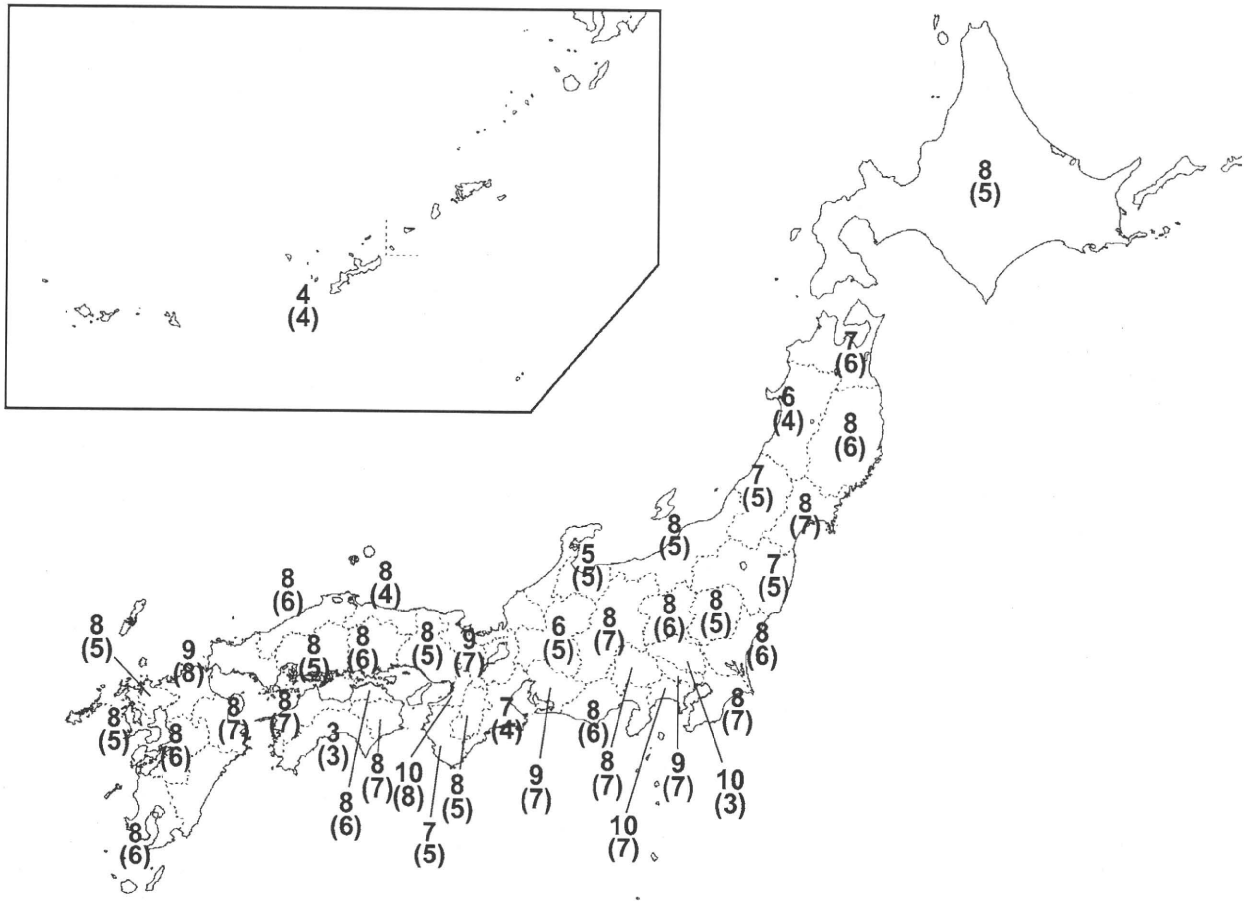
RÉSUMÉ

Pour estimer la structure génétique de la population actuelle de *Mycobacterium tuberculosis* au Japon, on a analysé les traits phylogéniques de 237 souches de la famille Beijing isolées chez des patients tuberculeux dans l'ensemble du pays. A la différence de ce qui a été rapporté antérieurement dans d'autres pays, l'ancien sous-lignage Beijing est prédominant dans l'ensemble du Japon. Une analyse du regroupement en grappes basé sur les nombres variables de répétition en tandem de l'Association Japonaise de la Tuberculose [JATA-VNTR]), un ensemble de VNTR spécialisé pour la discrimination des souches japonaises de *M. tuberculosis*, a révélé une forte similarité des souches du sous-lignage Beijing moderne, quelle que soit leur origine géographique. Le JATA-VNTR pourrait être utile pour la classification phylogénique dans les populations où les anciennes souches Beijing sont isolées fréquemment.

RESUMEN

Con el propósito de evaluar la estructura de la actual población genética de *Mycobacterium tuberculosis* en el Japón, se analizaron las características filogenéticas de 237 cepas de la familia Beijing, provenientes de pacientes tuberculosos de todo el país. A diferencia de los informes de otros países, en todo el pais predominó el antiguo sublinaje Beijing. Se llevó a cabo un análisis de conglomerados con base en la colección de genotipos de la Asociación contra la Tuberculosis del Japón (JATA), que es un conjunto específico de perfiles con número variable de repeticiones en tandem (VNTR), utilizados en la diferenciación de las cepas japonesas de *M. tuberculosis*. Este estudio puso en evidencia una gran similitud de las cepas del sublinaje Beijing moderno, independientemente de su origen geográfico. La colección de genotipos de la JATA podría ser útil en la clasificación filogenética en poblaciones de las cuales se aíslan con frecuencia cepas de la antigua familia Beijing.



Supplemental Figure The quantities of *M. tuberculosis* strains analysed in this study are represented according to their respective origins (prefectures). Numbers in parentheses show the number of strains belonging to the Beijing family.

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Phylogeographical particularity of the *Mycobacterium tuberculosis* Beijing family in South Korea based on international comparison with surrounding countries

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To understand the domestic population structure of *Mycobacterium tuberculosis* clinical isolates in the Republic of Korea, we genotypically analysed 80 isolates obtained from various geographical origins in the country. Of these, 64 (80.0 %) isolates were identified as Beijing family strains. It is particularly interesting that their phylogenetic classification, based on the ancient/modern separation and the presence/absence of the genomic region RD181, revealed a majority of the ancient (RD181 +) subfamily in the population. The 15 loci of variable number of tandem repeat(s) of mycobacterial interspersed repetitive units (15-MIRU-VNTR) were also analysed. Combination with the previous VNTR data reported from surrounding countries revealed that the topology of the minimum spanning tree was linked tightly not to the geographical origins of the patients but to the phylogenetic characteristics of the isolates. These results show that the phylogeographical distribution of the *M. tuberculosis* Beijing family around far-eastern Asia could be estimated using international accumulation and comparison of VNTR genotyping data.

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INTRODUCTION

The Beijing family, a lineage of *Mycobacterium tuberculosis*, is well known for its highly endemic prevalence around East Asian countries and as a causative agent of tuberculosis (TB) (van Soolingen *et al.*, 1995). In the Republic of Korea (South Korea), TB is still a major public health concern, with 34 157 (70.3 per 100 000) registered new TB patients in 2008 and 2376 deaths attributable to TB in 2007 (Korea Centers for Disease Control & Prevention, 2008). More than 70 % of *M. tuberculosis* strains isolated from Korean pulmonary TB patients belong to the Beijing family. 'K strain' (Park *et al.*, 2000), one of the Beijing family strains, has been reported as the cause of a severe outbreak of TB in South Korea (Kim *et al.*, 2001). Although their phylogenetic position in the Beijing family lineage has been unclear, Shamputa *et al.* (2010) reported recently that K strains show genetic diversity by some genotyping methods, even in isolates obtained from a single hospital.

The detailed phylogenetic variation of the Beijing family has been unveiled by various genetic markers such as single nucleotide polymorphisms, regions of difference and variable number of tandem repeat (VNTR) loci (Filliol *et al.*, 2006; Tsolaki *et al.*, 2005; Wada & Iwamoto, 2009). A notable characteristic of the lineage is the insertion of IS*6110* in a genomic region named NTF (Mokrousov *et al.*, 2005; Plikaytis *et al.*, 1994). This phylogenetic marker can classify the Beijing family into two distinct sublineages: ancient and modern. The modern Beijing sublineage has been more predominant than the ancient sublineage throughout the world, including countries surrounding South Korea (Bifani *et al.*, 2002; Dou *et al.*, 2008; Kremer *et al.*, 2009; Mokrousov *et al.*, 2005, 2006; van Soolingen & Kremer, 2009). However, Wada *et al.* (2009) reported that the ancient Beijing sublineage has been observed to be exceptionally predominant in Japan.

Abbreviations: MIRU-VNTR, variable number of tandem repeats of mycobacterial interspersed repetitive units; MST, minimum spanning tree; TB, tuberculosis.

Supplementary tables giving information on and genotypic profiles of the isolates analysed in this study are available with the online version of this paper.

The goal of this study was to elucidate the phylogenetic distinctiveness and the genetic diversity of Beijing family strains around the Republic of Korea, including K family strains, for international comparison with strains from surrounding countries. For this purpose, we analysed a collection of *M. tuberculosis* isolates obtained from public health centres in the country using well-known phylogenetic markers and an international standard set of VNTR loci (Supply *et al.*, 2006).

METHODS

Bacterial isolates. Eighty *M. tuberculosis* clinical isolates were analysed in this study. They were randomly selected from smear-positive and culture-positive pulmonary TB patients who were under 46 years of age with no epidemiological links during 2006. All patients were primary cases. Information such as geographical origin, age and sex of the patients, date of isolation and drug susceptibility for each of the 80 isolates is presented in Supplementary Table S1 in JMM Online. The geographical distribution of the population is presented in Fig. 1.

Identification of Beijing family strains and their phylogenetic subdivision. The Beijing family strains were defined by the deletion of RD207 in this study (Tsolaki *et al.*, 2004). The deletion of RD207 corresponds with the absence of signals 1–34 in spacer oligonucleotide (spoligo) genotyping, which is the most standard definition of the lineage (van Soolingen *et al.*, 1995). The presence or absence of RD207 was analysed using PCR according to a previous report (Warren *et al.*, 2004).

The classification of the ancient and modern subfamilies of the 64 Beijing family isolates was determined according to a previous report (Wada *et al.*, 2009). The presence or absence of RD181 was also verified as described previously (Tsolaki *et al.*, 2004). The genomic deletion of RD181 has been considered to have occurred during evolution of the ancient Beijing sublineage (Tsolaki *et al.*, 2004; Maeda *et al.*, 2010). The sets of primer sequences were described in these previous reports. They were designed to detect insertions or deletions of regions by the difference in sizes of amplified DNA fragments.

IS*6110* DNA fingerprinting. For all 80 isolates, DNA isolation and IS*6110* RFLP typing were performed as described previously (Park *et al.*, 2000). The IS*6110* RFLP patterns were compared with UPGMA using the Dice coefficient to find RFLP clusters (GelCompar v. 5.1; Applied Maths). An RFLP cluster was defined by the identification of two or more isolates with identical band patterns. K family strains were identified according to the previously reported definition (Kim *et al.*, 2001; Park *et al.*, 2000).

VNTRs. The standard 15 VNTR loci for routine epidemiological discrimination (hereinafter, 15-MIRU-VNTR) were also analysed for all 80 isolates (Supply *et al.*, 2006). Their copy number was calculated from their size and assigned according to the number of repeats for each locus, and in agreement with published allelic tables (Iwamoto *et al.*, 2007). The accuracy of the size of amplified PCR fragments was confirmed using a capillary electrophoresis system (SV1210; Hitachi High Technologies). A VNTR cluster was defined by the identification of all 15 loci in two or more isolates.

Minimum spanning tree (MST). A MST was generated based on the 15-MIRU-VNTR types using software (Bionumerics v. 4.6; Applied Maths) for clustering analysis. We used the reconstruction rules as follows. A categorical coefficient was selected. The priority rule was set such that the type that had the highest number of single-locus variants would be linked first. Creation of hypothetical types was not allowed. The VNTR types of Beijing family strains from neighbouring countries were retrieved from previous reports (Jiao *et al.*, 2008; Wada *et al.*, 2009). All 15-MIRU-VNTR types published in these reports were incorporated into the construction of the MST tree.

Fig. 1. Nationwide distribution of the *M. tuberculosis* strains used for this study. The 80 *M. tuberculosis* isolates were obtained from new pulmonary TB patients registered in public health centres across South Korea in 2006.

RESULTS

Phylogeographical specificity of the Beijing family in South Korea

To elucidate the population structure of *M. tuberculosis* in South Korea, we identified 64 (80.0 %) Beijing family strains from 80 isolates obtained from various geographical origins (Fig. 1) and subdivided them into three phylogenetic sublineages [ancient (RD181+), ancient (RD181−) and modern]. A high proportion of the lineage is concordant with previous reports from South Korea and surrounding East Asian countries (Mokrousov *et al.*, 2006; Park *et al.*, 2000, 2005; van Soolingen *et al.*, 1995; Yun *et al.*, 2009). Phylogenetic subdivision of the 64 Beijing isolates revealed a higher proportion of the ancient Beijing sublineage (46 strains; 71.9 %) than the modern sublineage

(18 strains; 28.1 %). The ancient (RD181 +) sublineage, having diverged from the evolutionary process towards the modern Beijing sublineage before the deletion of RD181, was observed to be predominant (29 strains; 45.3 % of the Beijing strains) in the population. This study identified 13 K family strains (16.3 % of all; Fig. 2): all were found to belong to the ancient (RD181 +) sublineage.

Genotypic diversity of isolates in South Korea

We analysed all 80 isolates by 15-MIRU-VNTR to investigate the detailed genotypic diversity of the population. This genotyping method has been used as a standard discrimination tool for *M. tuberculosis* because of its high resolution among isolates from cosmopolitan origins (Supply *et al.*, 2006). The VNTR profiles and other genotypic characteristics analysed in this study are combined and listed in Supplementary Table S2. The genotyping was able to classify our 80 isolates into 71 distinct genotypes. There were four clusters, which comprised 16 (20.0 %) isolates. The clustering rate was slightly higher than that of RFLP genotyping (10 isolates; 12.5 %), which was concordant with the previous reports on the Beijing family (Iwamoto *et al.*, 2007; Yokoyama *et al.*, 2007). Six of 13 K family strains belonging to two RFLP clusters were separated further into unique genotypes using 15-MIRU-VNTR (Fig. 3).

Recently, it was reported that cluster modelling of 15-MIRU-VNTR genotypes of the *M. tuberculosis* Beijing strains was highly concordant with their phylogenetic subdivision into some sublineages (Wada & Iwamoto, 2009). MST clustering analysis was performed for the 64 Beijing strains of our population (Fig. 4). The tree topology was observed to be highly associated with the sublineage classification.

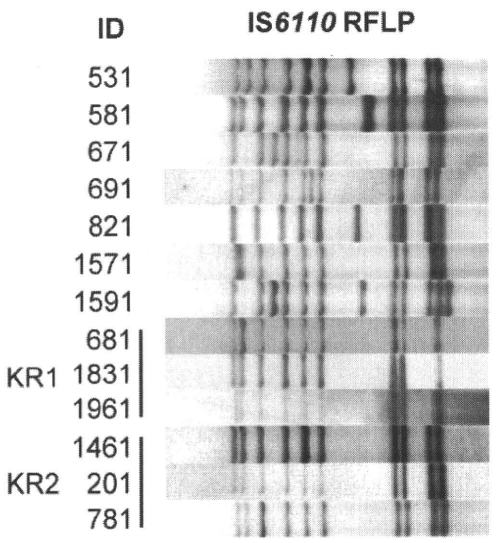

Fig. 2. IS*6110* RFLP patterns of the K family isolates identified in this study. Two clusters were detected in these 13 isolates (KR1, 681, 1831 and 1961; KR2, 1461, 201 and 781).

Comparison of VNTR profiles with those of neighbouring countries

The MST clustering of 15-MIRU-VNTR genotypes was sufficient to visualize the phylogenetic differences among the Beijing family successfully in our study (Fig. 4), which prompted us to make an international comparison of the 15-MIRU-VNTR types with those of strains from the neighbouring countries (China and Japan) reported previously. The 202 15-MIRU-VNTR types of Beijing strains from Japan (Wada *et al.*, 2009) and the 64 types from Beijing City, China (Jiao *et al.*, 2008), were combined with our current data to construct a mixed clustering tree using MST (Fig. 5). Results showed that three branches including types of strains from South Korea were readily apparent, two of which included mostly South Korean types (indicated by arrowheads). Both these branches comprised strains belonging to the ancient (RD181 +) sublineage, isolated in Japan and South Korea. A remaining branch included types from all three countries (indicated by an arrow). The results of the ancient/modern classification in the previous report by Wada *et al.* (2009) and this study showed that this branch was occupied by modern Beijing strains.

DISCUSSION

In general, the ancient Beijing sublineage has been considered to be an atypical Beijing genotype which has been only rarely observed (Bifani *et al.*, 2002; Milan *et al.*, 2004; Strauss *et al.*, 2008). The sole exception has been the population study of Beijing family strains from Japan (Wada *et al.*, 2009). In this study, we found that the ancient sublineage was predominant in South Korea. Contrary to the situation in Japan, it is unique that the ancient (RD181 +) sublineage was the most prevalent in our 80 isolates. This sublineage has been reported to be a minor component of the Beijing family population, even in Japan (Maeda *et al.*, 2010). Therefore, our data also revealed the particularity of the population structure of the *M. tuberculosis* Beijing family in South Korea. These observations suggest that the distribution of the Beijing family sublineages is broadly variable in different regions. It still remains unclear whether the higher prevalence of the sublineages in certain areas has been caused by the difference of fitness or by occasional clonal expansion in the past.

K family strains, derivatives of the Beijing family, have been isolated frequently in both a population-based study and outbreaks of TB in South Korea (Kim *et al.*, 2001; Shamputa *et al.*, 2010). In this study, they were also detected with a high clustering rate (46.2 %) using RFLP genotyping (Fig. 2) despite no epidemiological link. They belonged to the ancient (RD181 +) sublineage. Therefore,

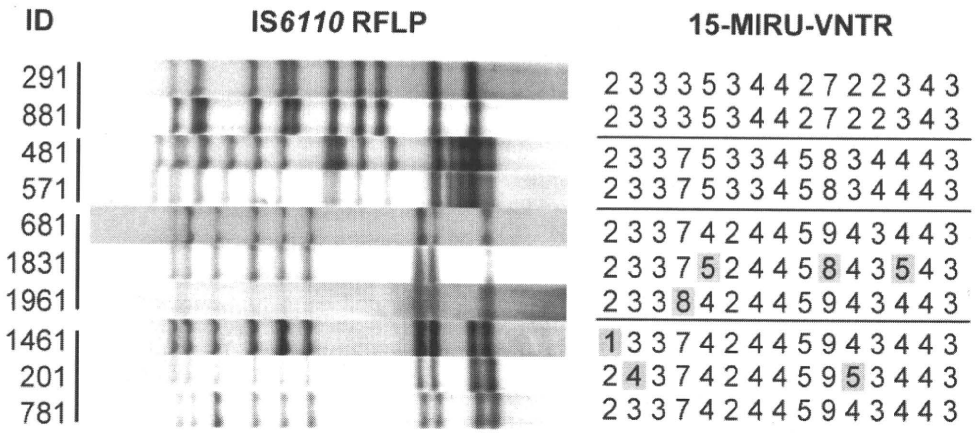

Fig. 3. 15-MIRU-VNTR profiles of 10 isolates belonging to four clusters by IS*6110* RFLP genotyping. The order of the VNTR loci is: MIRU 04, MIRU 10, MIRU 16, MIRU 26, MIRU 31, MIRU 40, ETR A, ETR C, QUB-2163b, QUB-26, QUB-4156, Mtub04, Mtub21, Mtub30 and Mtub39. The alleles that differed from those of other isolates in the respective clusters are shaded.

the domestic prevalence of K family strains may be related to the predominance of the sublineage in the country. The 15-MIRU-VNTR genotyping was able to classify the RFLP clusters as single-locus to three-locus variants comprising the K family strains (Fig. 3). This result suggests that frequent isolation of K family strains in South Korea may be caused not by a recent expansion of a single strain but by endemic fixation in the past. The genetic diversity within the K family strains should be analysed in more detail to uncover the history of their prevalence.

Combining our data with the reported VNTR types of strains from the surrounding countries for MST clustering analysis revealed that the tree topology (the branch formation) was consistent with the phylogenetic classification of strains, irrespective of their origins (Fig. 5). The types of strains originating in China (Beijing) reported by Jiao *et al.* (2008) were concentrated in the branch of the modern Beijing sublineage of South Korea and Japan. Although their data did not include information on sublineages of strains, the result is consistent with those

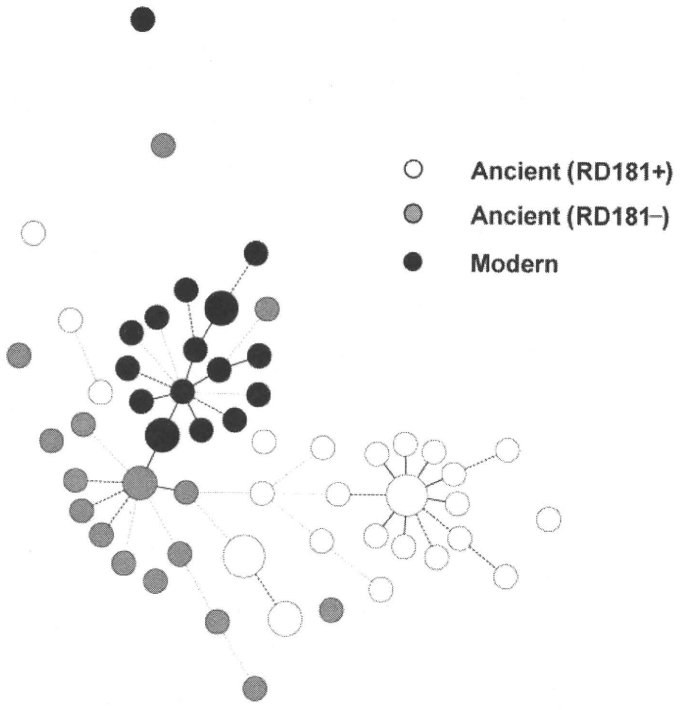

Fig. 4. Minimum spanning tree based on the 15-MIRU-VNTR genotypes of the 64 *M. tuberculosis* Beijing isolates. The 56 circles depicted correspond to the different types discriminated by the 15-MIRU-VNTR genotyping. Their sizes correspond to the number of isolates with a particular genotype. They were coloured according to the phylogenetic sublineages: ancient (RD181+), ancient (RD181–) and modern. Heavy lines connecting two types denote single-locus variants; thin lines connect double-locus variants; and dotted lines (black) connect triple-locus variants. The grey dotted lines represent the most likely connection between two types differing by more than three VNTR loci.

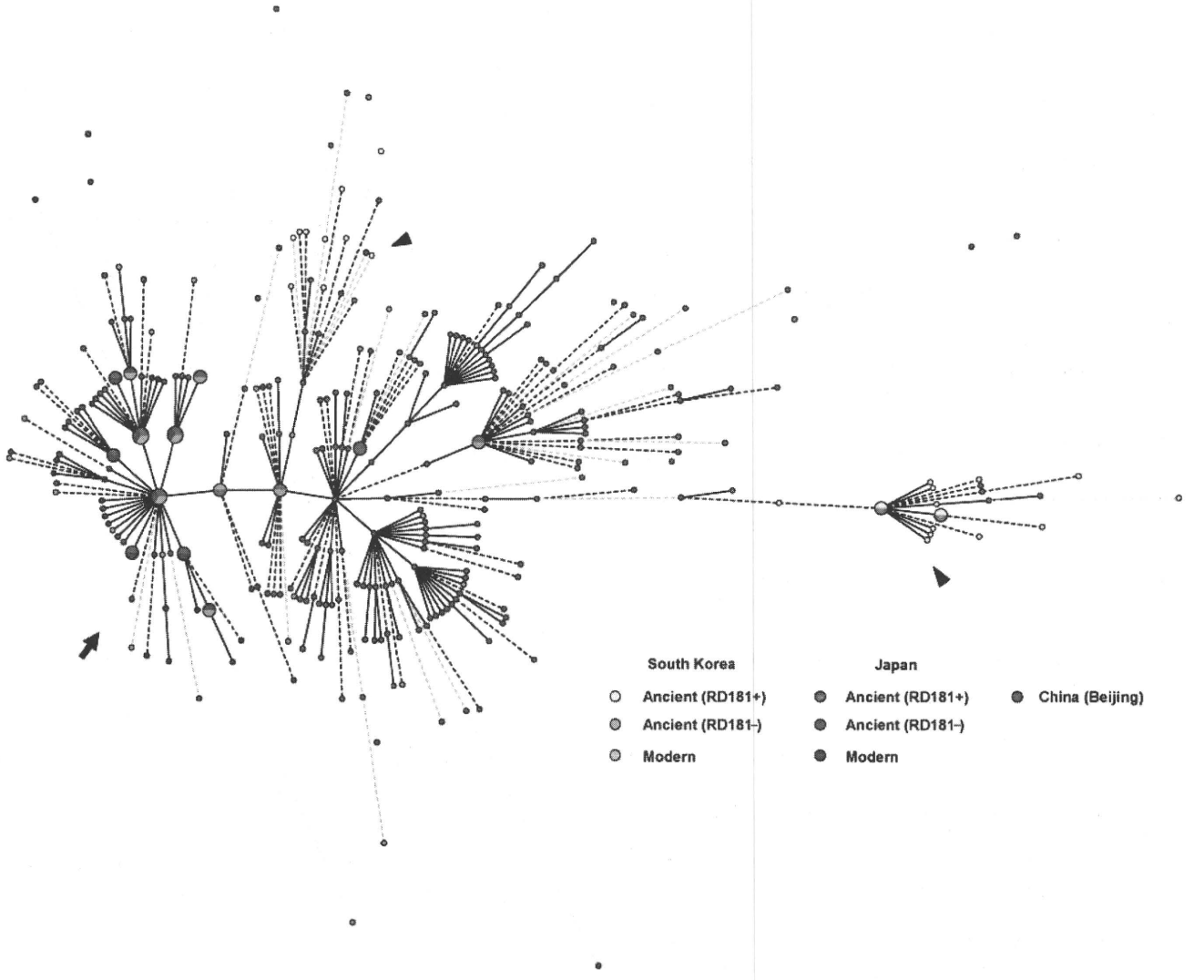

Fig. 5. Minimum spanning tree based on 15-MIRU-VNTR genotypes of the *M. tuberculosis* Beijing family from three East Asian countries. The 297 circles depicted correspond to the different types discriminated by the 15-MIRU-VNTR genotyping. The origin of each genotype is represented by different colours. The phylogenetic information of strains belonging to each genotype (from Japan and South Korea) is also indicated in colour. The designation of lines in the tree is the same as in Fig. 4. Two arrowheads point to clustering branches where genotypes originating from South Korea gather tightly. An arrow indicates a branch including genotypes of strains from all three countries.

of previous reports indicating that most of the isolated *M. tuberculosis* strains in the Beijing area (76–93 %) belonged to the modern Beijing sublineage (Mokrousov *et al.*, 2006; van Soolingen & Kremer, 2009). These results mean that similarity of 15-MIRU-VNTR types has been preserved in the ancient/modern sublineages of the Beijing family over the three East Asian countries. The phylogenetic validity of similar VNTR types must be verified in *M. tuberculosis* lineages because these genotypes are generally highly homoplasic. However, it is notable that similarity of VNTR types of the Beijing family has been observed based not on their geographical origins but in a phylogenetic manner. One merit of VNTR genotyping is its convenience for comparison of data from different countries (Allix-Béguec *et al.*, 2008; Mokrousov, 2008). Worldwide data accumulation of VNTR types of *M. tuberculosis* may shed light on the microevolution and genetic diversity of the species. Such global characterization of the phylogeographical distribution of the Beijing family may be useful in providing fundamental information about the ongoing worldwide expansion of the lineage.

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