from the generalism that is characterized by the ancestral AA type. Further investigations in combination with phylogenetic analysis would provide clear evidence for this hypothesis, i.e., evidence to judge if the emergence of modern AA types relies on convergent evolution, which has the advantage of adaptation to the hosts.

The ancestral AA type, AA02, was observed in all of the different *hsp65* code types (Table 2). This implies that AA02 had existed in the MAH (or its immediate ancestor) prior to the occurrence of the divergence of the *hsp65* gene in MAH. Although no NA type variations were found in any of the modern AA types (one-to-one correspondence between NA types and AA types), most of them were distributed in more than two *hsp65* sequevars (Table 2). The absence of sSNPs suggests that insufficient time has elapsed since the emergence of these variants to fix them in the genome. Therefore, it is unlikely that the modern AA types were present prior to the occurrence of the divergence of the *hsp65* gene in MAH. These two genes, MACPPE12 and *hsp65*, would diverge independently; thus the combination of these two genes can provide further discrimination of sub-groups. Indeed, 57 pig isolates with *hsp65* code type 1, which was characterized as predominant code type of pig isolates in our previous study (Iwamoto et al., 2012), were subclassified into 5 AA types; AA01 ($n = 26$), AA02 ($n = 4$), AA07 ($n = 12$), AA08 ($n = 11$), and AA09 ($n = 4$) (Table 2, and Table S1). This subclassification might be a useful approach when we compare the clinical significance at the strain levels.

In conclusion, the present study highlighted the variability of the MACPPE12 gene, which is absent in subspecies other than MAH. The MACPPE12 variants were classified into two groups: ancestral type (AA02) and modern types. AA02 reflects the general concept of MAH, i.e., ubiquitous host distributions and heterogeneity. The distribution of the modern types correlated with their major habitats (hosts). The divergence of the MACPPE12 gene and its distribution may be a good indicator to characterize MAH strains prevalent in certain areas. Further studies using global sample sets may shed light on the variation and distribution of the MACPPE12 gene at a global level.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2013.08.010.

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Simple Multiplex PCR Assay for Identification of Beijing Family *Mycobacterium tuberculosis* Isolates with a Lineage-Specific Mutation in *Rv0679c*

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The Beijing genotype of *Mycobacterium tuberculosis* is known to be a worldwide epidemic clade. It is suggested to be a possibly resistant clone against BCG vaccination and is also suggested to be highly pathogenic and prone to becoming drug resistant. Thus, monitoring the prevalence of this lineage seems to be important for the proper control of tuberculosis. The Rv0679c protein of *M. tuberculosis* has been predicted to be one of the outer membrane proteins and is suggested to contribute to host cell invasion. Here, we conducted a sequence analysis of the *Rv0679c* gene using clinical isolates and found that a single nucleotide polymorphism, C to G at position 426, can be observed only in the isolates that are identified as members of the Beijing genotype family. Here, we developed a simple multiplex PCR assay to detect this point mutation and applied it to 619 clinical isolates. The method successfully distinguished Beijing lineage clones from non-Beijing strains with 100% accuracy. This simple, quick, and cost-effective multiplex PCR assay can be used for a survey or for monitoring the prevalence of Beijing genotype *M. tuberculosis* strains.

The *Mycobacterium tuberculosis* Beijing genotype, first identified by van Soolingen et al. (1), is known to be a worldwide epidemic clade (2–4). Its possible resistance to BCG vaccination, in addition to its tendency to have a multidrug-resistant (MDR) phenotype, might give a selective advantage to the wide geographic distribution of the Beijing genotype strains (3, 5–7). Although some of the Beijing genotype strains show hypervirulence in animal infection models (7–9), neither the virulence factor nor the phenotypically specific factor of this lineage has been elucidated. The origin of the Beijing lineage is thought to be east Asia, where the prevalence of this clade is from around 40% to >90% (1, 3, 4, 10–13). However, in some other global areas, i.e., countries in the former Soviet Union and South Africa, the prevalence of the Beijing lineage has increased markedly in a short period, and some increases were suggested to be related to MDR (4, 11, 14). In those areas, higher clonality of the circulating strains was suggested, and most were categorized as being in the modern or typical Beijing clade, which is defined as a strain having one or two IS*6110* insertions in the noise transfer function (NTF) chromosomal region (11, 15). On the other hand, a higher variety of strains can be observed in east Asian countries. Especially in Japan and Korea, the majority of the strains belong to another cluster called the ancient or atypical Beijing clade (12, 16). Details regarding the higher pathogenicity of the Beijing lineage are controversial. Some studies have suggested that the modern Beijing clade is more prone to be pathogenic, tends to be drug resistant, and is likely able to escape from BCG vaccination (4, 8, 11, 14); however, some of the ancient Beijing clones were also shown to have higher pathogenicity (17) or a tendency toward acquiring drug resistance (16).

Since Beijing lineage prevalence has a great impact on the tuberculosis (TB) control program, several methods to distinguish this clade have been developed. First, van Soolingen et al. (1) identified this clade by its specific IS*6110* restriction fragment length polymorphism (RFLP) signatures. Soon after, these strains were shown to have a specific spoligotype pattern lacking spacer numbers 1 to 34, and this has been proposed as the definition of the clade (18, 19), since IS*6110* RFLP genotyping is time-consuming, and comparing results between laboratories is difficult. The deletion of spacers observed in the Beijing spoligotype is caused by the insertion of IS*6110* in the direct repeat (DR) region (18). Since this typical spoligotype pattern has become a specific marker of the Beijing genotype, some PCR methods to detect this specific deletion, named region of difference 207 (RD207), have been developed (20–22). In addition to RD207, another deleted region named RD105 was also shown to be a good marker for discrimination of the Beijing genotype, although this deletion is common for all the east Asian lineages, including the non-Beijing strains (10, 23); however, most of these published detection methods require expensive real-time PCR equipment and high-cost reagents (24). The conventional PCR assay targeting RD207 still seems to be at a disadvantage, since it relies on an unstable inser-

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tion sequence that is likely to be a target of homologous recombination.

Instead of unstable repetitive structures, single nucleotide polymorphisms (SNPs) were recently considered to be a robust target for defining the accurate position of a strain on the phylogenetic tree, since horizontal gene transfer or gene recombination between different strains is rare in the *M. tuberculosis* complex (MTC) (12, 24, 25). Filliol et al. (26) drew phylogenetic trees of the MTC using several typing methods and showed that the dendrogram drawn with SNPs most accurately reflected the true evolution of the MTC. Some of those SNPs are suggested to be specific to the Beijing or east Asian lineages. In a search for membrane proteins that are suitable for vaccine antigens and/or are targets for the specific detection of the MTC, we found a candidate protein encoded by the *Rv0679c* gene. This protein was expressed on the cell surface as a lipoarabinomannan-associated protein (27, 28), and the coding sequence has an SNP that seems to be specific to the Beijing clade. In this study, we confirmed the lineage specificity of this SNP and developed a simple and low-cost multiplex PCR assay to distinguish the Beijing lineage strains.

MATERIALS AND METHODS

Preparation of genomic DNA from *M. tuberculosis* isolates. *M. tuberculosis* was isolated from the sputa or other clinical specimens of patients by conventional procedures using *N*-acetyl-L-cysteine (NALC)–NaOH. A total of 619 isolates obtained in Japan ($n = 145$), Bangladesh ($n = 122$), Nepal ($n = 110$), Myanmar ($n = 198$), and China (Heilongjiang Province, $n = 44$) were used in this study. Some of these isolates were the same as those in previous studies, and the details are described elsewhere (13, 29–31). Colonies grown on egg-based medium (either Ogawa or Löwenstein-Jensen medium) were resuspended in distilled water and boiled for 20 min, and the supernatant was used in the Bangladeshi and Myanmar samples. In the Japanese and Nepalese samples, colonies were suspended in 0.5 ml of 10 mM Tris-HCl, 1 mM EDTA (Tris-EDTA [TE] buffer [pH 8]), and 0.5 ml chloroform; 0.5 g glass beads of 0.17-mm diameter was added; and they were disrupted with a bead beater (MicroSmash; Tomy Seiko Co. Ltd., Tokyo, Japan). After centrifugation at 10,000 × *g* for 5 min, DNA in the supernatant was precipitated by ethanol, and the precipitated genomic DNA was resuspended in TE buffer for further use. In China, bacteria grown in a BACTEC *Mycobacterium* growth indicator tube (MGIT) (Becton, Dickinson and Company, Franklin Lakes, NJ) were used, and DNA was extracted by lysozymes and the phenol-chloroform method (13). All the DNA samples extracted in each country were brought to Japan, and the following steps were carried out in the Hokkaido University Research Center for Zoonosis Control. To determine the specificity of the method, DNAs extracted from five reference MTC strains (i.e., *M. tuberculosis* H37Rv, *Mycobacterium africanum* ATCC 25420, *Mycobacterium orygis* Z0001, *Mycobacterium microti* TC 89, and *Mycobacterium bovis* BCG Tokyo 172) and 30 nontuberculous mycobacterial (NTM) species, including *Mycobacterium avium*, *Mycobacterium intracellulare*, and *Mycobacterium kansasii*, were used.

Gene sequencing and comparison. A subset of 197 *M. tuberculosis* samples, 68 from Japan, 92 from Bangladesh, and 37 from Nepal, were chosen from the total 619 clinical isolates, and the *Rv0679c* gene fragment was amplified by PCR. The PCR mixture contained GoTaq PCR buffer (Promega Co., Madison, WI), 0.2 mM each deoxynucleoside triphosphate (dNTP), 0.3 μM each primers og0001 and og0002 (Table 1), 0.5 M betaine, 1 ng genomic DNA from *M. tuberculosis*, and 0.5 units of GoTaq polymerase. Amplification was carried out by applying 35 cycles of denaturation at 95°C for 10 s, annealing at 57°C for 10 s, polymerase reaction mixture at 72°C for 40 s, and a final extension at 72°C for 5 min. The amplified DNA fragment was subjected to sequence analysis with BigDye Terminator v3.1 (Life Technologies Co., Carlsbad, CA) reagents by a sequencer, the 3130 genetic analyzer (Life Technologies Co.), according to the manufacturer's protocol. The *Rv0679c* sequence was also compared with those of 80 whole-genome sequenced MTC strains registered in the GenBank (http://www.ncbi.nlm.nih.gov/GenBank/) or TB (http://genome.tbdb.org/annotation/genome/tbdb/MultiHome.html) (32) databases by the BLASTn algorithm (http://blast.ncbi.nlm.nih.gov/).

Genotyping. The spoligotype of *M. tuberculosis* clinical isolates was determined as described previously (33). Briefly, the DR region was amplified with a primer pair, and the PCR products were hybridized to a set of 43 spacer-specific oligonucleotide probes, which were covalently bound to the membrane. The spoligo-international type (SIT) was determined by comparing spoligotypes against the international spoligotyping database (SpolDB4) (3).

The detection of an RD105 deletion was performed by multiplex PCR in Beijing clones and by conventional PCR in east Asian strains other than those of the Beijing type, since the deletion pattern is different between those two groups (10). The reaction mixture consisted of GoTaq PCR buffer (Promega), 0.2 mM each dNTP, 0.3 μM (each) two or three primers (Table 1), 0.5 M betaine, 1 μl extracted DNA sample, and 0.5 units of GoTaq polymerase. The target was amplified by 35 cycles of denaturation at 95°C for 10 s, annealing at 55°C for 10 s, and extension at 72°C for 40 s, with a final extension at 72°C for 5 min. RD207 deletion was detected by two PCR assays described by Warren et al. (22), and TbD1 was detected by PCR using the Huard et al. (25) protocol (Table 1). The amplified DNA fragment was subjected to agarose gel electrophoresis with ethidium bromide (EtBr) to see the size of the band under a UV transilluminator.

The multilocus sequence type (MLST) was determined with 9 SNPs, which were described by Filliol et al. (26) and were selected for Beijing subtyping by Iwamoto et al. (16). Each locus was amplified with a primer pair (Table 1), and the product was subjected to sequencing. SNPs were detected by comparing the sequences with those of H37Rv (34). The sequence type (ST) was identified according to Filliol et al. (26).

Beijing lineage identification by multiplex PCR. Multiplex PCR for the identification of the Beijing lineage was performed under the following conditions. The PCR mixture, in a final volume of 15 μl, contained 1× PCR buffer (1.5 mM Mg; TaKaRa Bio, Inc., Shiga, Japan), 0.5 μl dNTP solution mix (10 mM each dNTP; New England BioLabs, Inc., Ipswich, MA), 0.5 μl each of Fw and R1 primers, 0.2 μl R2 primer (primer solutions in 10 μM; Table 1), 1.5 μl of 5 M betaine, 0.45 μl of 25 mM $MgCl_2$ (to make a final Mg concentration of 2.25 mM), 1 ng of sample DNA, and 0.5 units of TaKaRa Hot Start *Taq* polymerase (TaKaRa). Amplification was carried out with the first denaturation at 95°C for 1 min followed by 35 cycles of denaturation at 95°C for 10 s, annealing at 66°C for 10 s, extension at 72°C for 15 s, and the final extension at 72°C for 3 min. The amplicon was subjected to electrophoresis in a 2% agarose gel that included EtBr. DNA samples extracted from the isolate BCG Tokyo 172 and a well-characterized clinical isolate (Beijing OM-9) were used as controls for the non-Beijing and Beijing banding patterns, respectively. Sensitivity was determined with serially diluted genomic DNA obtained from these BCG and Beijing control strains. A specificity study was performed with genomic DNA samples (2 ng/μl each) from the MTC and NTM strains described above.

RESULTS

Spoligotyping and MLST. A total of 619 clinical isolates were subjected to spoligotyping, and 393 were identified as being in the Beijing lineage and 226 as a non-Beijing group (Table 2). The non-Beijing group consisted of a variety of strains belonging to the following lineages: east African-Indian (EAI), central Asian (CAS), Latin American Mediterranean (LAM), Haarlem, S, T, X, and non-Beijing east Asian (3). Ninety-four of the Beijing isolates were subjected to MLST analysis and were subtyped into 8 sequence-type classes, namely, ST26, ST3, STK, ST25, ST19, ST10, ST22, and ST8, which are listed in evolutional order from ancient to modern Beijing types (16, 26).

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TABLE 1 Primers used in the study

Target	Primer name	Nucleotide sequence	Purpose	Reference
Rv0679c	og0001	CCGGGAACTAGGAATGGTAA	Sequencing	This study
	og0002	AGCAACCTCGCAATCTGAC	Sequencing	This study
	ON-1002 (Fw)	GTCACTGAACGTGGCCGGCTC	Multiplex PCR for Beijing type identification	This study
	ON-1258 (R1)[a]	TCGGTCACCGTTTTTGTAGGTGACCGTC	Multiplex PCR for Beijing type identification	This study
	ON-1127 (R2)	AGCAACCTCGCAATCTGACC	Multiplex PCR for Beijing type identification	This study
RD105	RD105-F (−239∼−218)	GGAAAGCAACATACACACCACG	Multiplex PCR for east Asian type determination[b]	This study
	RD105-R	AGGCCGCATAGTCACGGTCG	Multiplex PCR for east Asian type determination[b]	This study
	RD105-M (+304∼323)	TCCTGGGTGCCGAACAAGTG	Multiplex PCR for east Asian type determination[b]	This study
	RD105EA-F (−80∼−60)	TCGGACCCGATGGCTTCGGTG	PCR for east Asian type determination[c]	This study
	RD105EA-R (61∼42)	TGATCACGGTTCGCCCGCAG	PCR for east Asian type determination[c]	This study
RD207	RD207-1F (Warren)	TTCAACCATCGCCGCCTCTAC	PCR for Beijing type identification (set 1)	22
	RD207-1R (Warren)	CACCCTCTACTCTGCGCTTTG	PCR for Beijing type identification (set 1)	22
	RD207-2F (Warren)	ACCGAGCTGATCAAACCCG	PCR for Beijing type identification (set 2)	22
	RD207-2R (Warren)	ATGGCACGGCCGACCTGAATGAACC	PCR for Beijing type identification (set 2)	22
TbD1	TbD1F	CGTTCAACCCCAAACAGGTA	PCR for ancestral *M. tuberculosis* determination	25
	TbD1R	AATCGAACTCGTGGAACACC	PCR for ancestral *M. tuberculosis* determination	25
797736[d]	Beijing ST-1F	GACGGCCGAATCTGACACTG	MLST for Beijing lineage	This study
	Beijing ST-1R	CCATTCCGGGTGGTCACTG	MLST for Beijing lineage	This study
909164[d]	Beijing ST-2F	CGTCGAGCTCCCACTTCTTG	MLST for Beijing lineage	This study
	Beijing ST-2R	TCGTCGAAGTGGACGAGGAC	MLST for Beijing lineage	This study
1477596[d]	Beijing ST-3F	GTCGACAGCGCCAGAAAATG	MLST for Beijing lineage	This study
	Beijing ST-3R	GCTCCTATGCCACCCAGCAC	MLST for Beijing lineage	This study
1692067[d]	Beijing ST-5F	GATTGGCAACTGGCAACAGG	MLST for Beijing lineage	This study
	Beijing ST-5R	TGGCCGTTTCAGATAGCACAC	MLST for Beijing lineage	This study
1892015[d]	Beijing ST-6F	GCTGCACATCATGGGTTGG	MLST for Beijing lineage	This study
	Beijing ST-6R	GTATCGAGGCCGACGAAAGG	MLST for Beijing lineage	This study
2376133[d]	Beijing ST-7F	TCTTGCGACCCGATGTGAAC	MLST for Beijing lineage	This study
	Beijing ST-7R	GAGCGCAACATGGGTGAGTC	MLST for Beijing lineage	This study
2532614[d]	Beijing ST-8F	CCCTTTTCTGCTCGGACACG	MLST for Beijing lineage	This study
	Beijing ST-8R	GATCGACCTTCGTGCACTGG	MLST for Beijing lineage	This study
2825579[d]	Beijing ST-9F	CCTTGGAGCGCAACAAGATG	MLST for Beijing lineage	This study
	Beijing ST-9R	CTGGCCGGACGATTTTGAAG	MLST for Beijing lineage	This study
4137829[d]	Beijing ST-10F	CGTCGCTGCAATTGTCTGG	MLST for Beijing lineage	This study
	Beijing ST-10R	GGACGCAGTCGCAACAGTTC	MLST for Beijing lineage	This study

[a] Beijing-type specific mutation-detection primer. Underlined 2-base sequences at the 5′ end are not complementary sequences.
[b] This assay was used for Beijing genotype strains.
[c] This assay was used for non-Beijing genotype strains.
[d] This SNP nucleotide position on the *H37Rv* genome is according to references 26 and 34.

Sequence analysis of the *Rv0679c* gene of *M. tuberculosis* isolates. Nucleotide sequences of the full-length *Rv0679c* gene obtained from 197 clinical *M. tuberculosis* isolates collected in Japan, Bangladesh, and Nepal were compared with the *Rv0679c* sequence in *M. tuberculosis* H37Rv (34). Only a single nucleotide difference of cytosine to guanine at position 426, which leads to an amino acid change at codon 142 from Asn (AAC) to Lys (AAG), was detected in 87 isolates, all of which were identified as being in the Beijing lineage by spoligotyping and, supportively, by RD207 PCR (22) (data not shown). One Bangladeshi isolate showed a mixed peak of C and G at position 426 and was revealed as a mixed culture of Beijing and another strain by RD105 and RD207 detection PCR (Table 2). None of the non-Beijing isolates had the mutation, and vice versa. In public databases, 14 strains reported from several countries were revealed to have this mutation, and all were confirmed as being in the Beijing lineage by checking for the RD207 deletion *in silico* (18). None of the other 66 MTC strains, which were determined to be non-Beijing, had this mutation. The 498-bp *Rv0679c* sequence was well conserved among the MTC strains, and the following three strains in the database showed alterations: *M. tuberculosis* strains C and T17 and *Mycobacterium canettii* CIPT 140010059.

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TABLE 2 *Rv0679c* multiplex PCR results compared with other typing results in 619 *M. tuberculosis* clinical isolates

Isolate origin	Spoligotype family[a]	RD207, RD105, or other typing methods[b]	Sequence type[c]	*Rv0679c* M-PCR type[d]	No. of isolates
Beijing or Beijing-like					393
Japan	Beijing	ND	26	Beijing	10
	Beijing	ND	3	Beijing	24
	Beijing	ND	STK	Beijing	13
	Beijing-like	$RD207^+$	STK	Beijing	1
	Beijing	ND	25	Beijing	3
	Beijing	ND	19	Beijing	9
	Beijing	ND	10	Beijing	12
	Beijing	ND	22	Beijing	4
	Beijing	ND	ND	Beijing	23
Bangladesh	Beijing	ND	26	Beijing	3
	Beijing	ND	10	Beijing	12
	Beijing	ND	22	Beijing	2
	Beijing	ND	8	Beijing	1
	Beijing	ND	ND	Beijing	29
	Beijing-like	$RD105^+$, $RD207^+$	ND	Beijing	1
Nepal	Beijing	ND	ND	Beijing	64
Myanmar	Beijing	ND	ND	Beijing	141
	Beijing-like	$RD105^+$, $RD207^+$	ND	Beijing	1
China (Heilongjiang)	Beijing	ND	ND	Beijing	40
Non-Beijing or undesignated/new[a]					216
Japan	Undesignated/new[e]	$RD105^+$, $RD207^-$	ND	Non-Beijing	29
	Others[f]	ND	ND	Non-Beijing	16
Bangladesh	—[g]	ND	ND	Non-Beijing	73
Nepal	—[h]	ND	ND	Non-Beijing	45
Myanmar	—[i]	ND	ND	Non-Beijing	51
China (Heilongjiang)	Undesignated/new	ND	ND	Non-Beijing	2
Mixed clone samples					6
Bangladesh	Undesignated/new	Mixed peak in sequence[j] $RD105^+$, $RD207^+$	ND	Beijing	1
Myanmar	Undesignated/new	$RD105^+$, $RD207^+$	ND	Beijing	2
	EAI2_NTB	$RD105^+$	ND	Beijing	1
	EAI5	$RD105^+$	ND	Beijing	1
China (Heilongjiang)	Undesignated/new	$RD105^+$	ND	Beijing	1
New spoligotype lacking spacers 1–34[k]					4
Japan	New	$RD105^+$, $RD207^{+k}$	ND	Beijing	1
Nepal	New	$RD105^-$, $TbD1^{+k}$	ND	Non-Beijing	1
Myanmar	New	$RD105^+$, $RD207^+$	ND	Beijing	1
China (Heilongjiang)	New	$RD105^+$, $RD207^+$	ND	Beijing	1

[a] Spoligotype labeling is according to SpolDB4 (3).
[b] A positive superscript indicates that a deletion was detected; a minus superscript indicates that the RD was not deleted or the region was intact. ND, not determined.
[c] Sequence type is according to reference 26.
[d] M-PCR, multiplex PCR.
[e] East Asian lineage.
[f] Including the clades LAM1, LAM9, T1, T2, T3, T3-Osaka, and new (other than the east Asian lineage).
[g] Including the clades EAI1_SOM, EAI2-MANILA, EAI3_IND, EAI5, EAI6_BGD1, EAI7_BGD2, EAI unidentified, CAS, CAS1-DHLHI, CAS2, LAM9, T1, T4, H1, H3, X1, X2, and undesignated/new.
[h] Including the clades EAI3_IND, EAI5, CAS, CAS1-DHLHI, LAM1, LAM5, T1, T2, T3, H3, S, and undesignated/new.
[i] Including the clades EAI2_MANILA, EAI2_NTB, EAI5, EAI6_BGD1, EAI7_BGD2, CAS1-DHLHI, LAM9, T1, T3, X2, S, and undesignated/new.
[j] Overlapped peak of C and G was observed at nucleic acid position 426.
[k] Details are described in Table 3.

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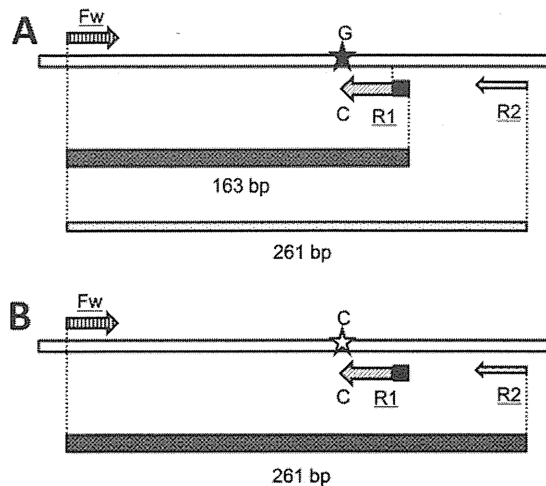

FIG 1 PCR primers and products of *Rv0679c*-targeting multiplex PCR for Beijing lineage discrimination. (A) In the Beijing sample, the 163-bp product is amplified more dominantly than is the 261-bp product. (B) In the non-Beijing sample, 163-bp product is not amplified because of the mismatch of the 3′ end of R1. Fw, forward primer; R1, reverse primer 1 (Beijing lineage specific); R2, reverse primer 2. Two-base noncomplement nucleotides at the 5′ end are shown by black squares.

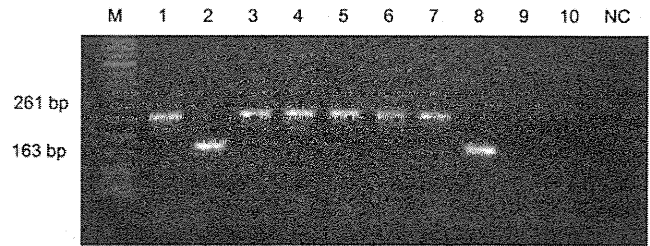

FIG 2 Electrophoresis results of the multiplex PCR products. Lane M, 50-bp ladder DNA size marker; lane 1, *M. bovis* BCG Tokyo 172 (non-Beijing lineage control) strain; lane 2, *M. tuberculosis* OM-9 strain (Beijing lineage control); lane 3, *M. tuberculosis* H37Rv; lane 4, *M. africanum* ATCC 25420; lanes 5–8, *M. tuberculosis* clinical isolates (lane 5, non-Beijing east Asian; lane 6, EAI; lane 7, LAM9; lane 8, Beijing); lane 9, *M. avium* strain JATA51-1; lane 10, *M. kansasii* JATA21-1; lane NC, negative control.

In strain C, the C185T SNP was observed, and in T17, a cytosine was inserted at position 92. In *M. canettii* CIPT 140010059, two SNPs and a codon insertion, ACC at position 154, were observed.

Beijing lineage identification by multiplex PCR. Multiplex PCR was developed targeting the Beijing-specific SNP on *Rv0679c*, employing a primer with the mutated nucleic acid at the 3′ end of the sequence (primer R1; Fig. 1 and Table 1); the optimal reaction conditions were determined as described in Materials and Methods. With this system, a bright band of 163 bp was observed as an amplified product of the primers Fw and R1 in the Beijing genotype samples (Fig. 1A and 2). An additional band of 261 bp, which is the product of primers Fw and R2, can be seen depending on the conditions, although it is always significantly thinner than the 163-bp band because of the low R2-primer concentration (see Materials and Methods). In contrast, only the 261-bp band is observed in a non-Beijing genotype sample (Fig. 1B and 2). Since the sequences of the primers are specific to the MTC, no amplification occurs in the absence of MTC genomic DNA (Fig. 2, data for *M. avium* and *M. kansasii*). A total of 619 clinical isolates obtained in the five Asian countries of Japan, Bangladesh, Nepal, Myanmar, and China were subjected to this Beijing lineage-identifying multiplex PCR, and the results were compared with their spoligotypes. All the isolates determined as having a Beijing or Beijing-like genotype by the SpolDB4 ($n = 393$) were determined to be in the Beijing lineage by the multiplex PCR (Table 2). On the other hand, no samples that included only non-Beijing genotype DNA ($n = 216$) were identified as being in the Beijing lineage. Twenty-nine non-Beijing east Asian lineage strains, which were suggested by a characteristic spoligotype having spacer 34 and were defined by RD105 detection, were determined to be non-Beijing by the multiplex PCR. Six isolates that showed a discrepancy between their spoligotype and the multiplex PCR result were further determined by RD207 or RD105 detection PCR and were revealed to be a mixture of Beijing and other subtype strains (mixed clone samples, Table 2). Four samples from different countries had confusing spoligotypes that lacked spacers 1 to 34 and additionally lacked some of the spacers from 35 to 43. These samples could also be identified correctly (Tables 2 and 3). The minimum detection limits were 100 and 1,000 cells per reaction in the Beijing genotype and BCG strains, respectively (data not shown).

DISCUSSION

In this study, we demonstrated that the SNP of C to G at position 426 in the *Rv0679c* gene is specific to the Beijing genotype strains. We developed a new multiplex PCR using this SNP to identify Beijing lineage isolates. This PCR assay successfully distinguished Beijing genotype strains from others, including the non-Beijing east Asian strains, with 100% accuracy. The Beijing lineage genotype is usually identified by spoligotyping, specific patterns of IS*6110* RFLP, or the detection of RD207, which is led by an insertion of IS*6110* in the DR region. However, spoligotyping is well known to show gene conversions, and strains having no genetic relationship sometimes show the same spoligotype (3, 26). Fenner et al. (35) reported pseudo-Beijing strains that had a typical Beijing spoligotype even though they actually belonged to the CAS family. This type of confusion seems to occur especially in areas that have a higher prevalence of principal genetic group 1 (PGG1) lineages, including the EAI, CAS, and east Asian lineages, since PGG1 strains usually possess spacers 35 and 36, which are lacking in PGG2 and PGG3 strains (3, 36). In other areas, mixed infections of more than two strains sometimes disrupt correct spoligotyping by showing mixed spacer patterns. The Manu1-SIT100 and Manu2-SIT54 types, which lack the spacers 34 or 33 and 34, respectively, are known to be producible by the mixture of Beijing family and T1 strains (3, 37). In this study, we found that some samples showed discrepant results between *Rv0679c* multiplex PCR and spoligotyping that determined a strain to be of the Beijing genotype by multiplex PCR, despite having another spoligotype. Using RD105 and RD207 detection methods, all of these samples were confirmed to be a mixture of Beijing and another strain. This type of mixed culture is sometimes observed in countries with a higher TB burden, where a coinfection of more than two strains is not rare (22). Some of the spoligopatterns of those samples showed faint positive spacers, suggesting the mixed presence of other strains. Even clear and correct spoligotypes can sometimes lead to misjudgments. In the current study, some samples showed only one to several spacers to be positive in the Beijing spacer area,

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TABLE 3 Typing result comparison in clinical isolates having confusing spoligotype patterns

Sample (identification)	Spoligotype pattern	Spoligotype family[a]	*Rv0679c* M-PCR type	Detection type RD207 set 1[b]	RD207 set 2[b]	RD105	TbD1	Final typing result
Japan (O-05-44)	□□■	New	Beijing	−	+[c]	+	−	Beijing
Nepal (no. 51)	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■□□□□	New	Non-Beijing	−	−	−	+	Ancestral[d]
Myanmar (no. 95)	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■	New	Beijing	+	+	+	ND	Beijing
China (2460)	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■□□□□□□□	New[e]	Beijing	+	+	+	ND	Beijing

[a] These patterns were not found in the SpolDB4 list.
[b] PCR sets 1 and 2 in reference 22.
[c] A faint correctly sized band and an additional band of a different size were observed.
[d] Ancestral type of *M. tuberculosis* strain possessing TbD1 region (25).
[e] The spoligotype pattern of this sample has been reported in reference 13.

namely, from spacers 35 to 43. Most were identified as being of the Beijing genotype by multiplex PCR, while one was judged to be a non-Beijing strain. All Beijing genotype-positive results were confirmed by RD105 and RD207 PCRs, and the non-Beijing isolate was revealed as an "ancestral type," which involves EAI but not the Beijing lineage, by TbD1 detection (Table 3) (25). These examples support the high specificity and applicability of this SNP-targeting PCR. The disadvantages of IS*6110* RFLP and RD207 detection have already been described above. RD207-detection PCR did not work as expected in the sample that lost spacers 1 to 42 (Japan O-05-44; Table 3), suggesting that some additional reconstruction had occurred at the IS*6110* insertion site of the DR region. SNPs in MTC genomes can provide robust lineage information, whereas repetitive elements, such as direct repeats in the DR region, the mycobacterial interspersed repetitive unit (MIRU) tandem repeats (38), or IS*6110*, are prone to alteration. One hundred percent concordance of the PCR results with the genetically confirmed Beijing type is not surprising because of the rigidity of the SNPs in the MTC (25, 26). Of the 393 Beijing family isolates, 94 were subtyped by MLST and consisted of 8 STs covering a wide range of the Beijing family, from ancient to modern types (Table 2). This suggested that a specific mutation in *Rv0679c* seemed to have occurred in the Beijing lineage at the same time as the RD207 deletion event.

Rv0679c is an MTC-specific gene, as shown by Cifuentes et al. (27), and no significantly similar sequence was detected by an NCBI BLASTn search in the GenBank database. Thus, this multiplex PCR assay can be used for the identification of the MTC, as well as for the differentiation of Beijing and non-Beijing lineages (Fig. 2). The Beijing mutation detection primer (R1; Fig. 1 and Table 1) was designed to have two additional noncomplement bases at the 5′ end to block the second amplification by the PCR product that produces the 261-bp fragment with an outer R2 primer. Additionally, the higher concentration and melting temperature of the R1 primer compared to those of the outer R2 primer increase the Fw-R1 product more than the Fw-R2 product. With these techniques, the Beijing band (163 bp) can be shown to be significantly brighter than the non-Beijing band (261 bp) when the sample is derived from Beijing lineage *M. tuberculosis* strains (Fig. 2). The relatively higher annealing temperature of 66°C gave good contrast of those two bands and prevented nonspecific amplifications. Modified *Taq* or other polymerases that have 3′-to-5′ exonuclease activity should be avoided, since those enzymes can trim the mutated nucleotide at the R1 primer end. It is recommended to check the PCR conditions using positive controls for Beijing and non-Beijing types (i.e., BCG) every time (Fig. 2). The detection limit of 100 to 1,000 copies per reaction might be relatively high; however, it can be improved by about 10 times by increasing the PCR cycle number to 40, although the necessity of identifying the MTC lineage in direct clinical specimens seems to be low.

In papers featuring SNPs as epidemiological markers, synonymous mutations are usually selected to avoid the effect of evolutional pressure (26). However, both SNPs for the differentiation of PGG1, PGG2, and PGG3 were nonsynonymous mutations in *katG* and *gyrA* (36), and so far, they have provided robust differentiation results. In the MTC, nonsynonymous mutations on functional genes can be observed in a relatively higher frequency than in other bacteria because of extremely reduced purifying selection pressure (39). Thus, nonsynonymous mutations can be preserved unless they are significantly disadvantageous. Indeed, 100% of the Beijing family strains in the current study could be identified with this nonsynonymous mutation, suggesting that it at least has no adverse effect on those strains. The function of the Rv0679c protein is still unclear, although its expression on the cell surface has been confirmed (27, 28). Cifuentes et al. (27) reported that the surface-localized Rv0679c protein contributed to the *M. tuberculosis* invasion of host cells and proposed the protein as a vaccine candidate. The substituted amino acid at position 142 was located in the C-terminus region of the protein, which was included in the "high-activity binding peptide" to target cells (27). Thus, this highly conserved nonsynonymous SNP, which results in an amino acid substitution with different characteristics (Asn → Lys), might have some biological meaning in explaining Beijing lineage pathogenicity. Since BCG vaccine strains, as well as other non-Beijing strains, have Rv0679c-Asn142, this substitution might affect the antigenicity of the Beijing bacterial surface and might contribute to the possible evasion of BCG-derived immunity. Further investigation of the association of the Rv0679c Asn142Lys substitution with Beijing strain outer membrane characteristics and antigenicity is ongoing.

In conclusion, a simple, robust, and low-cost multiplex PCR assay for the detection of Beijing lineage *M. tuberculosis* strains was successfully developed using a Beijing-specific SNP on *Rv0679c*. This PCR assay can be used in local laboratories to monitor the prevalence of the Beijing genotype, and this is strongly recommended to control this possibly highly pathogenic and drug resistance-prone sublineage.

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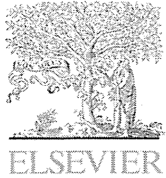

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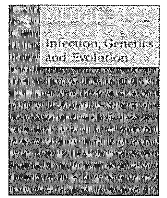

Short communication

Evolutionary robust SNPs reveal the misclassification of *Mycobacterium tuberculosis* Beijing family strains into sublineages

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ABSTRACT

Genotypic classification in *Mycobacterium tuberculosis* has greatly contributed to the comprehension of phylogenetic and population genetic relationships. It is, therefore, necessary to verify the robustness of the genetic markers for phylogenetic classification. In this study, we report some examples of homoplasy for two molecular markers, the IS*6110* insertion at the NTF region, and a single nucleotide polymorphism (SNP) at locus 909166, through genotyping of 1054 Beijing family strains. Our data revealed that a small fraction of strains traditionally classified into modern sublineages by IS*6110* insertion at NTF actually belong to an ancient sublineage. We also proved that the robustness of branches in the evolutionary tree established using the putative homoplasious SNP 909166 is relatively low. Our findings highlight the importance of validating genetic markers used to establish phylogeny, evolution, and phenotypic characteristics.

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1. Introduction

Recent progress in research on genetic diversity of *Mycobacterium tuberculosis* has brought new evidence that the outcome of infection and disease may differ depending on the genotype, lineage, and sublineage of the strain involved (Coscolla and Gagneux, 2010; Malik and Godfrey-Faussett, 2005). Both large sequence polymorphisms (LSPs), and single nucleotide polymorphisms (SNPs) have facilitated comprehension of phylogenetic and population genetic relationships (Comas and Gagneux, 2009; Comas et al., 2009), because they exhibit minimal rates of homoplasy. Moreover, highly discriminatory markers, including multilocus variable number of tandem repeats (VNTRs), are widely used to track particular strains in the community (Comas et al., 2009).

The Beijing family of *M. tuberculosis* strains is the most frequently analyzed lineage (Coscolla and Gagneux, 2010), and various genetic markers have been developed for subdividing the species into the Beijing family and its sublineages (Dou et al., 2008; Faksri et al., 2011; Filliol et al., 2006; Hanekom et al., 2007; Kremer et al., 2004; Luo et al., 2012; Mokrousov et al., 2002, 2005; Tsolaki et al., 2005; Wada et al., 2009b). On the other hand, some recent reports have indicated the risk of misclassification of Beijing family strains based on traditional molecular markers due to their homoplasy (Faksri et al., 2011; Fenner et al., 2011). We therefore decided to investigate the robustness of sublineage categorization using two molecular markers that have already shown their usefulness for evolutionary studies, namely: (i) the IS*6110* insertion at the NTF region, which is traditionally used to divide the family into modern (typical) and ancient (atypical) sublineages (Mokrousov et al., 2005), and (ii) SNP locus 909166, which is 1 of the 10 SNPs in a set used to classify the family into Filliol's sequence types (STs) (Filliol et al., 2006).

2. Materials and methods

We obtained 1340 *M. tuberculosis* isolates from newly diagnosed tuberculosis (TB) in Kobe, Japan, between 2002 and 2009. Of these, 909 isolates overlapped with the isolates which were previously used for population structure analysis (Iwamoto et al., 2009). In total, 1054 isolates were identified as Beijing family based on spoligotyping (Kamerbeek et al., 1997), and subsequent confirmation by LSPs (RD181 and/or RD105) and SNPs described below. These isolates included 67 Beijing variant spoligotyping patterns that lack some of the spacers between 35 and 43. These 67 isolates were confirmed by RD181 and/or RD105 analysis as belonging to the Beijing family (Tsolaki et al., 2004). All the Beijing family strains were classified into ancient and modern Beijing sublineages based on the presence of IS*6110* in the NTF region (Mok-

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Table 1
Distribution of 1054 Beijing family strains in each sublineage according to SNPs.

Beijing sublineages	SNPs										No. (%) of strains	IS*6110* insertion in the NTF region	Subgroup
	1548149	1692069	797736	2825581	1892017	4137829	1477596	2376135	2532616	909166			
a. Classification based on phylogeny using published SNPs													
ST11	G	A	C	T	T	C	C	A	G	C	4(0.4%)	None	Ancient
ST26	G	A	C	T	T	C	C	A	G	T	68(6.5%)	None	Ancient
STK	G	A	T	G	T	C	C	A	G	T	167(15.8%)	None[d]	Ancient
ST3	G	A	T	G	T	C	C	A	G	C	271(25.7%)	None[d]	Ancient
ST25	G	A	T	G	C	T	C	A	G	C	10(0.9%)	None	Ancient
ST19	G	A	T	G	C	T	C	A	G	T	288(27.3%)	None[d]	Ancient
ST10	G	A	T	G	C	T	T	A	G	T	196(18.6%)	NTF:: IS6110	Modern
ST22	G	A	T	G	C	T	T	G	A	T	50(4.7%)	NTF:: IS6110	Modern
	1548149	1692069	797736	2825581	1576481	1892017	4137829	1477596	2376135	2532616			
b. Classification based on phylogeny using published SNPs and the additional SNP at position 1576481													
ST11/ST26	G	A	C	T	T	T	C	C	A	G	72(6.8%)	None	Ancient
STKr[c]	G	A	T	G	T	T	C	C	A	G	175(16.6%)[a]	None[d]	Ancient
ST3r[c]	G	A	T	G	G	T	C	C	A	G	263(25.0%)[b]	None[d]	Ancient
ST25/ST19	G	A	T	G	G	C	T	C	A	G	298(28.2%)	None[d]	Ancient
ST10	G	A	T	G	G	C	T	T	A	G	196(18.6%)	NTF:: IS6110	Modern
ST22	G	A	T	G	G	C	T	T	G	A	50(4.7%)	NTF:: IS6110	Modern

[a] Twelve isolates originally classified as ST3 were re-classified as STKr by using SNP 1576481 instead of SNP 909166.
[b] Four isolates originally classified as STK were re-classified as ST3r by using SNP 1576481 instead of SNP 909166.
[c] Previously STK and ST3 sublineages were renamed STKr and STK3r according to the definition of the new 10-SNP set, respectively.
[d] Three isolates horboring IS*6110* insertion in the NTF region belonged to the STKr, ST3r and ST19 sublineages, respectively.

rousov et al., 2005; Wada et al., 2009b). They were also classified into ST sublineages using Filliol's 10 SNPs (Filliol et al., 2006; Hanekom et al., 2007; Iwamoto et al., 2008). In addition to the 10-SNP set, SNP locus 1576481 recently introduced in the panel as the replacement with SNP 909166 (Wada et al., 2012), was also analyzed to assess the homoplasious behavior of SNP 909166. We negated the existence of "Pseudo-Beijing strains" (Fenner et al., 2011) in our sample set by the detection of RD105 (Tsolaki et al., 2004) for all of the strains classified as ST11 and ST26 sublineages using SNP genotyping (Iwamoto et al., 2008; Iwamoto et al., 2012). All strains were subjected to Supply's optimized 15-locus variable number of tandem repeats (15-MIRU-VNTR) analysis (Supply et al., 2006; Iwamoto et al., 2012).

3. Results and discussion

Recently, Faksri et al. (2011) pointed out that three of the traditional 10-SNP set (SNPs 909166, 1548149, and 4137829) did not represent unique, irreversible events. Although they noted that the robustness of branches harboring these reversible SNPs is relatively low, this has not yet been formally evaluated using large number of sample sets. Of these three SNPs, in particular, SNP 909166 has played a key role in facilitating division of strains into the STK and ST3 sublineages that are predominant in Japan (Iwamoto et al., 2008, 2009; Wada et al., 2009b). Therefore, evaluation of the robustness of the sublineage categorization for evolutionary studies is imperative.

The results obtained on the classification of 1054 Beijing family strains using published SNPs and the additional SNP at position 1576481 are summarized in Tables 1a and b. When we applied SNP 1576481, which is expected to compensate for the probable homoplasious behavior of SNP 909166 (Wada et al., 2012), to our sample set, all of the 1054 Beijing family isolates could be classified into sublineages without considering the reversibility of the SNP (Table 1b).The striking difference of this classification from that using SNP 909166 (Table 1a) confirmed that SNP 1576481 (T to G) is evolutionarily a more informative SNP than the former SNP.

Given this result, we aimed to verify the robustness of classification of sublineages using SNP 909166 (a reversible SNP), in comparison with classification using SNP 1576481 (an irreversible SNP). The results revealed a discrepancy in the classification of 16 isolates (16/438 [3.7%]) between these two SNPs (Tables 1a and b). Specifically, 12 isolates originally classified as ST3 isolates were re-classified as STKr, and four isolates originally classified as STK isolates were re-classified as ST3r, when SNP 1576481 was used (Table 1b). A minimum spanning tree (MST), based on 15-MIRU-VNTR which included phylogenetically informative loci for Beijing family strains (Wada and Iwamoto, 2009a; Faksri et al., 2011), was consistent with the classification using SNP 1576481 (Fig. 1). These results strongly suggest that the robustness of the branches identified using SNP 909166 is relatively low, while an evolutionarily more reliable classification for STKr and ST3r was possible using SNP 1576481. Although we could not verify the robustness of classification using the other two reversible SNPs, 1548149 and 4137829, in our sample set, their utilization as genetic markers may be reconsidered using the same approach.

The IS*6110* insertion in the NTF region is well established as a genetic marker for grouping Beijing family strains into "modern" and "ancient" sublineages (Mokrousov et al., 2002, 2005). Although all of the modern-type strains in our study (ST10 and ST22) as classified by SNPs, certainly possessed an IS*6110* insertion in the NTF region, we also found three ancient strains (3/1,054, 0.3%) harboring an IS*6110* insertion in the NTF region (Table 2). Since there was no double peak in the VNTR profiles, mixed infection was eliminated (Table 2). For these three ancient strains, IS*6110* insertion in the NTF region was identified by PCR amplification and its sequence analysis. IS*6110* insertion sites in the NTF region in three strains exhibited distinctive patterns (data not shown). When we investigated the IS*6110* insertion sites at NTF region in the 10 modern-type strains, they were identified in the same position but different from that of the three ancient strains (data not shown). These results suggested that IS*6110* insertion in the NTF region occurs independently in these ancient strains. They belonged to the ST3r, ST19, and STKr sublineages, based on SNP data. These isolates presented sublineage-specific VNTR allele profiles, as previously described (Wada and Iwamoto, 2009a) (Table 2), which supports their assignment as ancient types. This result provided clear evidence that some of the ancient type isolates could be misclassified as modern type under the current definition, i.e., presence of

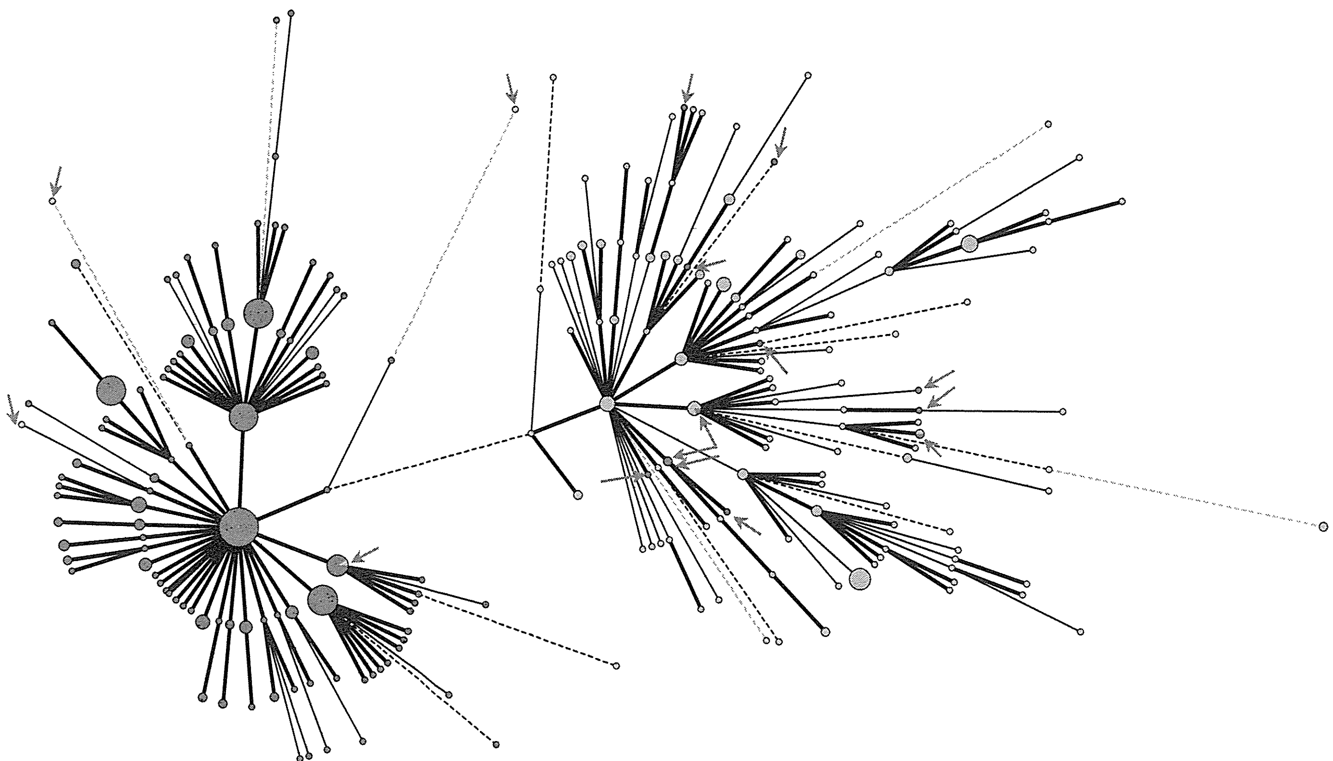

Fig. 1. A minimum spanning tree based on 15-MIRU-VNTR genotyping of 271 ST3 (red) and 167 STK (green) sublineage isolates, which were categorized using SNP 909166. Red arrows show 16 misclassified isolates. Twelve ST3 and 4 STK sublineages, as defined by SNP 909166, belonged to the STK and ST3 branches, respectively.(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
15-MIRU-VNTR alleles in the three isolates harboring IS*6110* insertion in NTF region.

No.	Years	Beijing sublineages	IS*6110* insertion in the NTF region	15-MIRU-VNTR loci allele profile*														
				MIRU 4	MIRU 10	MIRU16	MIRU 26	MIRU 31	MIRU 40	ETR A	ETR C	QUB-11b	QUB-26	QUB-4156	Mtub04	Mtub21	Mtub30	Mtub39
1	2007	ST3r	NTF:: IS*6110*	2	**1**	3	7	4	3	4	4	**7**	8	**5**	4	3	4	3
2	2009	ST19	NTF:: IS*6110*	2	3	4	7	5	3	4	4	7	2	**5**	3	3	4	3
3	2009	STKr	NTF:: IS*6110*	2	3	3	7	5	3	1	4	**3**	7	**4**	4	3	**2**	3

* Boldface data indicate specific VNTR alleles observed in the ancient phylogenetic sublineage of Beijng family as previously reported (Wada and Iwamoto, 2009a).

IS*6110* in the NTF region. These rare exceptions can be negligible in population-based studies. However, when exploring strain-specific differences in experimental or clinical phenotypes, we need to be careful of these rare but important exceptions to the classification of modern type Beijing.

In conclusion, we demonstrated homoplasious events in two molecular markers: the IS*6110* insertion in the NTF region and SNP 909166. Our findings highlight the importance of validating such genetic markers used to establish phylogeny, evolution, and phenotypic characteristics. With the tremendous increase in whole-genome sequence data, verification of the robustness of polymorphic loci has become very important for establishing the valid SNP-based classification scheme for *M. tuberculosis* strains.

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