The following full text is a publisher's version.

For additional information about this publication click this link.  
http://hdl.handle.net/2066/124314

Please be advised that this information was generated on 2017-08-17 and may be subject to change.
Investigation on *Mycobacterium tuberculosis* Diversity in China and the Origin of the Beijing Clade

Kanglin Wan¹,²,³, Jinghua Liu¹,²,³, Yolande Hauck¹,², Yuanyuan Zhang³, Jie Liu³, Xiuqing Zhao³, Zhiguang Liu³, Bing Lu³, Haiyan Dong³, Yi Jiang³, Kristin Kremer⁴,⁵, Gilles Vergnaud¹,²,⁶, Dick van Soolingen⁴, Christine Pourcel¹,²

¹Univ Paris-Sud, Institut de Génétique et Microbiologie, UMR 8621, Orsay, France, ²CNRS, Orsay, France, ³National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention/State Key Laboratory for Infectious Disease Prevention and Control, Beijing, People’s Republic of China, ⁴National Mycobacteria Reference Laboratory, National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands, ⁵Departments of Clinical Microbiology and Pulmonary Diseases, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands, ⁶DGA/MRIS- Mission pour la Recherche et l'Innovation Scientifique, Bagneux, France

**Abstract**

*Background:* Investigation of the genetic diversity of *Mycobacterium tuberculosis* in China has shown that Beijing genotype strains play a dominant role in the tuberculosis (TB) epidemic. In order to examine the strain diversity in the whole country, and to study the evolutionary development of Beijing strains, we sought to genotype a large collection of isolates using different methods.

*Methodology/Principal Findings:* We applied a 15-loci VNTR typing analysis on 1,586 isolates from the Beijing municipality and 12 Chinese provinces or autonomous regions. The data was compared to that of 900 isolates from various other worldwide geographic regions outside of China. A total of 1,162/1,586 (73.2%) of the isolates, distributed into 472 VNTR types, were found to belong to the Beijing genotype family and this represented 56 to 94% of the isolates in each of the localizations. VNTR typing revealed that the majority of the non-Beijing isolates fall into two genotype families, which represented 17% of the total number of isolates, and seem largely restricted to China. A small number of East African Indian genotype strains was also observed in this collection. Ancient Beijing strains with an intact region of difference (RD) 181, as well as strains presumably resembling ancestors of the whole Beijing genotype family, were mainly found in the Guangxi autonomous region.

*Conclusions/Significance:* This is the largest *M. tuberculosis* VNTR-based genotyping study performed in China to date. The high percentage of Beijing isolates in the whole country and the presence in the South of strains representing early branching points may be an indication that the Beijing lineage originated from China, probably in the Guangxi region. Two modern lineages are shown here to represent the majority of non-Beijing Chinese isolates. The observed geographic distribution of the different lineages within China suggests that natural frontiers are major factors in their diffusion.

**Citation:** Wan K, Liu J, Hauck Y, Zhang Y, Liu J, et al. (2011) Investigation on *Mycobacterium tuberculosis* Diversity in China and the Origin of the Beijing Clade. PLoS ONE 6(12): e29190. doi:10.1371/journal.pone.0029190

**Editor:** Igor Mokrousov, St. Petersburg Pasteur Institute, Russian Federation

**Received** July 29, 2011; **Accepted** November 22, 2011; **Published** December 29, 2011

**Copyright:** © 2011 Wan et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Funding:** This work was financially supported by the project “Transmission Mode of Tuberculosis” (2008ZX100/03-010-02) and “Warning Mode of Tuberculosis” (2008ZX10003-008) of National Key Program of Mega Infectious Diseases. Exchanges between European and Chinese laboratories were supported by The FP7 grant LSHP-CT-2005-012166 “Tuberculosis China.” The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

* E-mail: christine.pourcel@u-psud.fr

**Introduction**

Tuberculosis (TB) affects millions of people worldwide with an estimated global prevalence of 164 per 100,000 population. Although the incidence is believed to be generally slowly declining, this disease remains a major health problem in many countries. The average prevalence of TB in China amounts to 367 per 100,000 and this country has the highest absolute number of cases annually in the world. Among TB patients notified in China in 2009, slightly more than 30,000 (12%) were diagnosed and notified as multidrug resistant TB (MDR-TB) but this number may be underestimated [1]. BCG vaccine is not providing sufficient protection against tuberculosis and breakdown to disease, and this may in particular favor the emergence of new genotypes with enhanced virulence, such as the Beijing genotype, as shown in animal models and recently in humans in Vietnam [2,3,4].

To investigate the population structure of *M. tuberculosis* and define genetic lineages, several methods have been developed including spoligotyping [5,6], single nucleotide polymorphisms [7], variable number of tandem repeat (VNTR) [8,9,10,11], large sequence polymorphism (LSP) typing [12,13,14,15], partial [16,17] or whole genome [18] sequence analysis. Worldwide, nine superfamilies of *M. tuberculosis* strains with a preferred geographic distribution were described using spoligotyping [19]. Further studies based on LSP [12,15] and on the sequence of 89
genes [16] or whole genome sequencing [18] allowed the definition of 6 lineages globally highly congruent with spoligotyping defined lineages, with some exceptions. Lineage 4 (Euro-American) includes a number of spoligotype patterns which cannot be readily classified based on spoligotyping only. Lineage 2 contains, in addition to the Beijing family, strains with poorly informative spoligotypes [17].

The Beijing family was described for the first time as a genetically closely related genotype family in 1995, one of its characteristics being the absence of spacers 1 to 34 in the direct repeat (DR) locus [6]. In the early 1990s the ‘W’ strains, later shown to constitute a minor branch of the Beijing family, were associated with the spread of MDR-TB in North American cities [20,21]. In multiple areas in the world, such as Vietnam, Russia and South Africa the Beijing genotype was correlated with TB in young patients, and, hence, it is thought to be emerging rapidly [22,23,24]. Such observations raise the question of the origin of this family. Spoligotyping of *M. tuberculosis* bacteria in paraffin-embedded material from a hospital in Beijing demonstrated that the Beijing strains were already present in China in 1956 [25]. Later studies showed that these strains were mostly prevalent in East Asia [6,21,26] and the former Soviet Union [23,27,28,29], but also South Africa [30]. Recent reports also described a high prevalence of Beijing strains in Japan (70–80%) [31,32,33,34] but the largest percentages were observed in China with a prevalence of 93% in the Beijing municipality area [35,36,37].

Members of the Beijing genotype family have previously been identified and classified based upon the IS6110 insertion site (A1) in the origin of chromosome replication (oriC) [38,39]. Later, Beijing strains were subdivided into modern/Typical and ancient/Atypical sublineages based on the analysis of the NTF-1 locus [29] or IS6110 insertion profiles [40,41]. In addition to the characteristic deletion of the DR locus (affected by RD207), Tsolaki et al. described LSPs (RD105, RD142, RD150, and RD181) which further divided this family into monophyletic subgroups [42], the more ancestral event being the RD181 deletion. Some studies have shown that not all ancient/Atypical strains are RD181 intact (RD181 [+] which indicates that the RD181 deletion occurred before the insertion of an IS element in the NTF-1 locus [34,43]).

RD105 was deleted in all Beijing genotype strains but also in RD207 [+] strains belonging to lineage 2 [12,17]. It has been suggested that the Beijing family successfully expanded relatively recently from a single ancestor which presumably had selective advantages over other genotypes of *M. tuberculosis* [6,44,45]. Demographic factors may also be responsible for this predominance in particular geographic areas. Besides, the relative homogeneity of Asian populations at the anthropological level may contribute to the genetic conservation of *M. tuberculosis* strains in China, owing to co-evolution between the host and the pathogen [46,47]. Phylogeographic studies that considered the population structures of Beijing genotype strains and humans suggested that the Beijing lineage originated in Central Asia. However these studies were based on relatively small numbers of isolates from Chinese in Singapore [29].

To further explore the population structure of *M. tuberculosis* in China, geographically large surveys still need to be performed with a combination of genotyping approaches. VNTR typing is increasingly being used as a first line assay owing to its discriminatory power, to the relevance of the clustering it achieves, and to the availability of relatively large data sets [11]. Although a 24-VNTR loci protocol has been proposed to be the reference in standard typing, different combinations of VNTRs are used alone, or with spoligotyping or IS6110 restriction fragment length polymorphism (RFLP) [28,48,49,50,51,52,53,54]. When an appropriate collection of markers is applied, VNTR typing recognizes the major genotype families within the *M. tuberculosis* complex [17,43]. To obtain sufficiently informative analysis and robust clustering, it is necessary to use a panel of markers with different discrimination indexes and to combine the data with other typing approaches [17,53,56]. In the present survey involving 1,386 Chinese isolates among 2,346 previously analyzed by spoligotyping [37], we aimed to be able to describe unrecognized families and to search for potential ancestors of the Beijing genotype family.

### Results

#### *M. tuberculosis* diversity in China

Spoligotyping was not sufficiently discriminative for in depth analysis of the *M. tuberculosis* population in China [37] and we therefore decided to apply the VNTR genotyping technique, which provides a higher level of phylogenetic information [17]. In order to facilitate efficient genotyping of large number of isolates we selected a limited panel of highly typable VNTRs that would correctly cluster the bacteria into the main clades/genotype families. A first series of 98 isolates from the Beijing municipality and 4 different provinces were genotyped with 21 VNTRs (VNTR21Orsay) as previously described [10]. Clustering was performed with different combinations of VNTRs and we retained a set that was amenable to easy manual reading of agarose gel images while producing a sufficient degree of information (Figure S1 and Table S1). We furthermore eliminated markers that yielded technical problems in the amplification or visual gel image analysis such as Mtbu02 (9 bp repeat) or that were unstable such as Qub11a, or were not very informative such as Mtb12. The selected 15 VNTR loci forming the VNTR15China scheme were: ETR-A, ETR-B, ETR-C, ETR-D (alias MIRU04), ETR-E (alias MIRU31), MIRU10, MIRU16, MIRU23, MIRU26, MIRU27, MIRU39, MIRU40, Mtbu21, Mtbu30, Mtbu39. The 15 VNTRs of this scheme are included in the VNTR24 scheme described by Supplly et al. [11]. Eleven are shared with the VNTR15 scheme [11], and nine are in common with the earlier MIRU12 selection of loci [57]. We retained ETR-B (VNTR 2461), MIRU23 (VNTR 2531), MIRU27 (VNTR 3007) and MIRU39 (VNTR 4348) which present a lower level of allelic diversity but are useful to anchor the different lineages [17] and to make comparisons with published data sets. Thereafter VNTR15China was performed on all the collected isolates from nine regions and only the non-Beijing isolates for the Beijing municipality and the Fujian province. The total number of samples for which data were obtained was 1,386, of which 1,162 belonged to the Beijing family and 424 were non-Beijing according to the spoligotyping (Table 1 and Table S2) [37].

Clustering analyses were performed by UPGMA using the categorical coefficient, and 17 groups differing by a maximum of five VNTRs (cut-off value of 60%) were defined. The larger cluster corresponded to the Beijing isolates as confirmed by spoligotyping and three clusters showed the signature of isolates belonging to lineage 2 (which includes all spoligotype clades with deletion of spacers S33 to S36). Other clusters showed mutually recognisable spoligotype signatures. For 20 strains there was no concordance with the spoligotyping results. In particular, ten isolates with a spoligotype profile corresponding to Manu2 (absence of S33 and S34 [58]) were clustered with Beijing strains or with lineage 4 strains. These confusing findings suggest a superposition of both a Beijing and a lineage 4 profile as might result from mixed strains and were therefore excluded from further analysis.

In Figure 1 a minimum spanning tree shows the clustering of 401 Beijing isolates originating from the Jilin province and...
Guangxi autonomous region and of the 404 non-Beijing isolates (Table 1: the remaining Beijing isolates were not included to simplify the figure). Three clusters shown in green, red and blue (respectively indicated as China2, China3 and China4) had the lineage 4 spoligotype signature (absence of S33 to 36). The pink cluster corresponded to lineage 3 (CAS) strains, and the purple one may represent an ‘ancestral’ lineage or sub-lineage.

To further strengthen the assignation of isolates to known *M. tuberculosis* clades, the genotypes of Chinese isolates were compared to those of a large collection of isolates from other countries worldwide (616 from our own database in Orsay, Table S3 which can also be queried at http://mlva.u-psud.fr; and 186 from the VNTRplus database held at http://www.miru-vntrplus.org/) in which the major clades of the *M. tuberculosis* complex are represented. The nomenclature of the spolDB4 and of the MIRU-VNTR database was used to identify the clusters. The result indicated that China2 is not represented in the reference data set used here, and that China3 seemed to be almost entirely restricted to China (Figure S2). These genotypes accounted for 17% of the total Chinese collection of isolates (Table 2). In contrast China4 is found worldwide.

**Distribution of clades in the different Chinese regions**

Clustering of VNTR15China typing data of all isolates was performed for each location separately in order to better evaluate the importance of the major clades in the collection and to show the occurrence of the newly identified groupings (Table 2). The distribution of isolates in Xinjiang and Guangxi is shown on Figure S3 and Figure S4, respectively. A group of isolates specific

---

**Table 1.** Number of isolates genotyped by VNTR21Orsay or VNTR15China in each province.

| Origin* | Beijingb | non-Beijing | Total |
|---------|-----------|-------------|-------|
| (21 VNTRs) |           |             |       |
| Anhui (AH) | 24 6 | 30 | |
| Beijing (BJ) | 19 0 | 19 | |
| Fujian (FJ) | 12 9 | 21 | |
| Hunan (HN) | 14 4 | 18 | |
| Jiangsu (JS) | 8 2 | 10 | |
| Total | 77 21 | 98 | |
| (15 VNTRs) |           |             |       |
| Beijing (BJ) | 0 8 | 8 | |
| Fujian (FJ) | 0 44 | 44 | |
| Gansu (GS) | 58 14 | 72 | |
| Guangxi (GX) | 113 89 | 202 | |
| Hunan (HN) | 64 27 | 91 | |
| Jilin (JL) | 288 38 | 326 | |
| Shaanxi (SX) | 78 16 | 94 | |
| Sichuan (SC) | 65 37 | 102 | |
| Xinjiang (XJ) | 134 61 | 195 | |
| Xizang (XZ) | 173 18 | 191 | |
| Zhejiang (ZJ) | 112 51 | 163 | |
| Total | 1085 | 403 | 1488 |
| All isolates | 1162 | 424 | 1586 |

*aIncludes municipality, provinces and autonomous regions. bBased on spoligotyping.

doi:10.1371/journal.pone.0029190.t001

---

**Table 2.** Distribution of *M. tuberculosis* China 2 and China 3 isolates in nine regions.

| Origin* | Nbr of isolates | China 2 isolates | Rate (%) | China 3 isolates | Rate (%) |
|---------|----------------|-----------------|----------|-----------------|----------|
| Gansu | 72 | 2 | 2.77 | 4 | 5.55 |
| Guangxi | 202 | 25 | 12.3 | 33 | 16.3 |
| Hunan | 91 | 12 | 13.18 | 11 | 12.08 |
| Jilin | 326 | 15 | 4.60 | 15 | 4.60 |
| Shaanxi | 94 | 4 | 4.25 | 8 | 8.51 |
| Sichuan | 102 | 19 | 18.63 | 13 | 12.74 |
| Xinjiang | 195 | 12 | 6.15 | 23 | 11.79 |
| Xizang | 191 | 5 | 2.61 | 8 | 4.18 |
| Zhejiang | 163 | 20 | 12.27 | 19 | 11.65 |
| Total | 1436 | 114 | 7.93 | 134 | 9.33 |

*The number of isolates from Anhui, Beijing, Fujian and Jiangsu was too low to be included in this table.

doi:10.1371/journal.pone.0029190.t002
The Guangxi autonomous region possesses the lowest percentage of Beijing-family strains and a larger diversity in non-Beijing strains. The analysis revealed the existence of a group of seven isolates, clustering together with the Beijing and lineage 3 (CAS) strains (purple in Figure 1 and circled in Figure S4). Table 3 shows the spoligotypes of all the isolates from this group, as well as one from Sichuan and one from Zhejiang. An analysis of different regions of genomic deletions was performed showing that ThD1 and RD105 were deleted in all isolates of this grouping (data not shown). This is the signature of lineage 2 strains. A preliminary study of the DR locus by PCR revealed the presence of an additional group of spacers not detected by spoligotyping, and which is characteristic of Beijing family strains (spacers 49 to 50 [59]). These characteristics strongly suggest that this group of 9 strains, 7 of which were found in the Guangxi region, might represent the ‘ancestor’ of the whole Beijing family. Nine strains from Xinjiang (Figure S3) and three isolates from Tibet showed a typical lineage 3 (CAS) spoligotype pattern and clustered by VNTR typing with lineage 3 CAS-Delhi isolates from other geographical origins (Figures 1 and S2). Isolates with a typical lineage 1 (EAI) spoligotype signature (deletion of spacer 29–32 and 34) were found only in Fujian province and were distributed into two clusters.

Strains XJ06036 and XJ06002 clustered with LAM isolates (members of lineage 4). One strain from Jilin province clustered with M. bovis.

### The origin of the Beijing clade

The 1,162 Beijing isolates genotyped in this study represented 56 to 94% of the isolates in the different parts of China. Only 21 spoligotypes were observed among these isolates, whereas VNTR15China typing identified 472 genotypes. Two hundred and eleven clusters were observed when a cut-off value of 90% was used (1–2 alleles differences between genotypes). One genotype accounted for 16% of the isolates (183) from all origins, except for the Guangxi autonomous region. Figure 2 shows the genotypes distribution in the different regions using different colors. Interestingly, the isolates from Guangxi autonomous region shown in yellow are mostly found in two clusters. This may be explained Interestingly, the isolates from Guangxi autonomous region shown in yellow are mostly found in two clusters. This may be explained. This is the signature of lineage 2 strains. A preliminary study of the DR locus by PCR revealed the presence of an additional group of spacers not detected by spoligotyping, and which is characteristic of Beijing family strains (spacers 49 to 50 [59]). These characteristics strongly suggest that this group of 9 strains, 7 of which were found in the Guangxi region, might represent the ‘ancestor’ of the whole Beijing family. Nine strains from Xinjiang (Figure S3) and three isolates from Tibet showed a typical lineage 3 (CAS) spoligotype pattern and clustered by VNTR typing with lineage 3 CAS-Delhi isolates from other geographical origins (Figures 1 and S2). Isolates with a typical lineage 1 (EAI) spoligotype signature (deletion of spacer 29–32 and 34) were found only in Fujian province and were distributed into two clusters.

**Table 3. Spoligotype of ancestral strains.**

| Strain      | Spoligoprofile       | SIT⁵                           |
|-------------|----------------------|--------------------------------|
| GX06002     | 111111111111111111   |                                |
| GX06030     | 111111111111111111   | 246                            |
| GX06040     | 111111111111111111   | 246                            |
| GX06121     | 111111111111111111   |                                |
| GX06145     | 111111111111111111   |                                |
| GX06162     | 111111111111111111   |                                |
| GX06203     | 011111111111111111   |                                |
| ZJ06098     | 111111111111111111   |                                |
| SC06005     | 111111111111111111   |                                |

⁵: 1, presence of the spacer; 0, absence of the spacer.

Discussion

A survey on the strain diversity in Beijing municipality and 12 provinces and autonomous regions

This is the first extended, detailed study on the genetic diversity of *M. tuberculosis* covering a significant part of China using several genotyping approaches. It describes not only the population structure of *M. tuberculosis*, including the presence of genotype families not or rarely found elsewhere in the world, but also provides information on the possible origin of Beijing genotype strains. There was an obvious genetic diversity in the *M. tuberculosis* strains isolated from the different parts of China, although the main epidemic strain cluster in the different locations is formed by the lineage 2 Beijing family (RD105 [−] and RD207 [−]) isolates.

As previously shown by spoligotyping performed on the same samples [37], the highest density of Beijing strains was observed in the Northern and Western part of China, including Xizang (Tibet) with the exception of Xinjiang autonomous region which is mostly populated by Uyghurs. The region with the second highest prevalence of Beijing strains was the central part of China, e. g. between Yellow River and Yangtze River, and the lowest was observed in southern China (Figure 3). The VNTR typing added to the spoligotyping results revealed a high degree of polymorphism in this family. The second more abundant lineage in China is lineage 4 with three main subgroups of which two are nearly exclusive for China (China2 and China3) according to available VNTR typing data and one is found worldwide (China4). In Xinjiang another lineage 4 subgroup specific for this region was observed. These subgroups could only be revealed by VNTR typing as their spoligotype is not specific [37]. A small group of isolates belonging to lineage 3 (CAS) observed in Xinjiang and Xizang (Tibet), both close to India where isolates of this family are very abundant. In Xinjiang, three isolates clustered with isolates of the LAM clade, frequently found in Latin American and African countries but also in Russia and Central/Eastern Asian countries [60]. However, the spoligotyping profile is unclassified and does not show the signature of typical LAM isolates (deletion of S21 to S24).

Interestingly, the only isolates belonging to lineage 1 (EAI) were found in Fujian province, although such strains are very abundant in other countries of Eastern Asia, like Vietnam [22]. No isolate clustered by VNTR15China typing with isolates of ancestrally branching-out Manu lineages, although spoligotypes characteristic of these clades could be occasionally observed. The most likely explanation is the presence of mixed infections with Beijing and non-Beijing strains such as previously reported in Taiwan [61]. Indeed superposition of lineage 2 and lineage 4 spoligotypes (the two most frequent lineages in China) would create a typical but artefactual Manu spoligotype (deletion of spacer 33).
The Beijing family

The results obtained on the basis of VNTR15China typing were in agreement with spoligotyping data. However, although the majority of Beijing genotype strains aggregated into a large homogenous group, some showed more polymorphism as seen for example in the Guangxi autonomous region (Figure S4). It is assumed that the more clonal and more frequent modern/Typical Beijing strains that are emerging in several parts of the world [21, 44] are derived from ancient/Atypical Beijing. Contemporary representatives of these 'ancient' lineages show a higher degree of genetic diversity [62]. Interestingly, Guangxi, in which the lowest rate of Beijing strains was found, is also the region where the largest proportion of RD181 [+] Beijing isolates occurred (16.5%, Table 4) and where a relatively large number of 'ancestral' RD207 [+] strains were observed. Some of these strains have a complete spoligotype pattern (Table 3 and [63]). They are thought to be representatives of lineages branching out before the emergence of the Beijing genotype family. Flores et al. first showed that a proportion of strains with such an ancestral spoligotype were RD105 [−], and that they originated from East Asia, Vietnam, China and Laos [63]. In Japan a high incidence of Beijing family strains exists with a high level of ancient/Atypical RD181 [+]
strains within the Beijing genotype [34,64,65,66]. A proportion of
5.5% Beijing family strains are RD181 [+\(^{+}\)] on average across
Japan [34], and this percentage raises to 10% (48/498) in the
Chiba prefecture [66]. Interestingly Kang et al. [34,43] report a
remarkable proportion of 45.3% RD181 [+\(^{+}\)] strains among 64
Beijing family strains from diverse geographic origin in South
Korea. Few publications investigate RD105 [\(-\)] RD207 [+\(^{+}\)]
isolates, because these strains which belong to lineage 2 and are
very close to the Beijing genotype are not readily identified as such
by spoligotyping. Yokoyama et al. report the presence of 12 RD207
[\(+\)] isolates among a total of 510 lineage 2 isolates (2.35%) in the
Chiba prefecture [66]. This can be compared to the ratio of 7/115
60.0% observed in the Guangxi region.

Based on the observations described in the present work it is
tempting to speculate that TB has the longest history in the South
part of China and that Beijing strains have emerged from there.
Whole genome sequencing of key isolates as identified by the
present investigation may reveal whether the ‘ancestral’ RD207
[\(+\)] lineages which can be found in the Guangxi province are likely
candidates to represent the lineage from which the ancient/
recent clonal modern Beijing strains developed. Such an analysis
will also facilitate more phylogenetic studies on the genetic
relatedness between different M. tuberculosis lineages that determine
the current TB epidemic worldwide.

Taking all studies on the prevalence of Beijing genotype strain
in China together, the conclusion is that there is a significant
diversity in clinical M. tuberculosis isolates from China. Beijing
family strains, representing 56 to 94% of the isolates in each of the
12 studied regions, is the main prevalent genotype. The subgroups
of lineage 4 of which two are mainly found in China (China2 and
China3) might be emerging and deserve specific attention as they
might possess particular characteristics. Some strains, presumably
representing ancestors of the whole Beijing genotype family, were
found mainly in Guangxi autonomous region. Further studies on
the composition of the genome of these strains and of those in
other regions of the world should give clues about their origin and
about the mechanisms underlying the enhanced capacity to gain
resistance and restore fitness recently acquired by the Beijing
sublineage.

It is hoped that the snapshot of the M. tuberculosis diversity in
China as investigated here will serve as a reference for future
investigations, and help evaluate the temporal and geographic
dynamics of the emergence and disappearance of lineages in China.

**Materials and Methods**

**Ethics statement**

The study obtained approval from the Ethics Committee of
National Institute for Communicable Disease Control and
Prevention, Chinese Center for Disease Control and Prevention.
The patients with TB included in the present research protocol
were given a Subject information sheet and they all gave written
informed consent to participate in the study.

**Collection and identification of clinical isolates in China**

From the period 2005 to 2007, 2,346 M. tuberculosis isolates were
randomly collected from sputum samples of TB confirmed patients
institutes for TB control and cure, as well as TB hospitals
distributed in each region included in the study [37]. We tried to
equally divide the collected isolates over both sexes and from
different age categories (although patients aged 0–16 were
underrepresented). The total number of isolates when the collect
ended was different in the different regions (Table 1). All isolates
were analyzed by spoligotyping and a subset of 1,586 M. tuberculosis
clinical strains were retained for more detailed molecular typing.
The isolates studied in the present work were from the Beijing
municipality (BJ) and the following 12 different provinces or
autonomous regions: Anhui (AH), Fujian (FJ), Gansu (GS),
Guangxi (GX), Hunan (HN), Jiangsu (JS), Jilin (JL), Shanxi (Sx),
Sichuan (SC), Xinjiang (X), Xizang/Tibet (XZ), Zhejiang (ZJ).
Only few isolates could be recovered from the Anhui and
Jiangsu province during the time of this investigation. The strain
identity code starts with the indicated initials.

The patients were diagnosed on the basis of a Ziehl-Neelsen
smear-positive sputum and/or showed signs of pulmonary tubercu-
losis on X-ray. M. tuberculosis was cultured on Lowenstein-Jensen
or Coletso medium. Growing acid fast bacilli were identified according
to conventional biochemical procedures (PNB/TCH differential
medium) and growth characteristics, and the drug susceptibility was
assessed by the proportional drug susceptibility test.

DNA extracts were prepared by suspending approximately
10 mg wet bacterial cells in 100 μl of sterile distilled water and
subsequent heating at 80°C to 100°C for 30 min to kill and lyse the
cells [67]. Only few isolates could be recovered from the Anhui and
Jiangsu province during the time of this investigation. The strain
identity code starts with the indicated initials.

**PCR amplification**

Two microliters of lysates obtained from the cultured M.
tuberculosis strains were added to 15 μl of PCR mixture, which
included 1.5 μl dNTP mix (2 mM each), 1.5 μl 10×Buffer
(including 25 mM MgCl2), 3 μl 5 M Betain, 1.0 μl primers (a
10 μM mix of each of the lower and upper primers), 0.5 μl DNA
polymerase (5 U/μl), and 3 μl distilled water, to obtain a 15 μl
total volume. The PCR amplification cycles consisted of: 3 min
94°C for DNA denaturation; 35 cycles: 30 seconds at 94°C
for DNA denaturation, 1 min at 62°C for primer annealing and
30 second at 70°C for primer extension; following by a last cycle
of 10 min at 72°C for primer extension. PCR products were
analyzed by electrophoresis on a 2% agarose gel.
Variable Number of Tandem Repeat (VNTR) typing

PCR amplification of 21 VNTR loci and electrophoresis of products on agarose gels was carried out as described in a previous report [68]. The VNTR15China assay comprised the following markers: ETR-A, ETR-B, ETR-C, ETR-D (MIRU04), ETR-E (MIRU31), MIRU10, MIRU16, MIRU23, MIRU26, MIRU27, MIRU39, MIRU40, Mtub21, Mtub30, Mtub39. To compare the informativity of the VNTR21Orsay and the VNTR15China assays a selection of 98 isolates was made in five provinces in which the prevalence of Beijing genotype was 56 to 94%. The 15 VNTRs are present in the VNTR24 scheme described by Supply et al. [11]. Comparison with the gold standard IS6110-RFLP genotyping method was not performed.

Region of Deletion analysis

The regions of deletion (RD) 105 and 181 were investigated using primers localized on both sides of the regions as described by Tsolaki et al. [69]. In addition primers were designed that were localized inside RD181: RD181Int_L 5′ TAACAGCAGTGG-GACCAAGC 3′ and RD181Int_R 5′ GACTGCGGGTCT-TAGTCTGGG 3′. TbD1 was investigated using primers described by Brosch et al. [15].

Data management and analyses

Gel images were analyzed using the BioNumerics software package (version 6.5; Applied-Maths, Sint-Martens-Latem, Belgium) as previously described [68,70]. The number of repeats in each allele was deduced from the amplicon size. The resulting data were analyzed with BioNumerics as a character data set. Clustering analysis was done using the categorical parameter and the unweighted pair group method with arithmetic averages coefficient. The minimum spanning tree [71] was constructed with the following options: (i) in case of equivalent solutions in terms of calculated distances, the selected tree was the one containing the highest number of links between genotypes differing at only one locus ("Highest number of single locus variants" option); (ii) the creation of hypothetical types (missing links) reducing the total length of the tree was allowed. Hunter-Gaston Index (HGDI) [72] is calculated by the equation:

\[
HGDI = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} n_j (n_j - 1)
\]

For comparison we used data from the MIRU-VNTRplus database at [http://www.miru-vntrplus.org/][73], and the VNTR...
profiles of 616 isolates genotyped in the Institute of Genetics and Microbiology, Paris Sud University http://mlva.u-psud.fr. Part of these strains were previously described [10,74,75]. The remaining samples were isolated by M. Fabre and C. Soler in Percy hospital as part of TB surveillance in several African and Asian countries (publication in preparation).

Nomenclature of superfamilies/lineages

The nomenclature of lineages currently used for describing the *M. tuberculosis* complex reflects the different methods which have been applied to characterize the complex over decades of investigations. We have used in the present study the classification of Comas et al. which takes advantage of large sequence polymorphisms and sequence analysis to define 6 main lineages [12,17]. We also refer to superfamilies which are based upon spoligotyping as defined by Fliol et al. [19]: the ancestrally branched East Africa and India (EAI) clade, the Central Asia (CAS) clade, the Beijing family, the Latin America and Mediterranean (LAM) family, the West-African 1 (AFR11) and West-African 2 (AFR12) clades. Lineages 1, 2, 3, 5 and 6 described by Comas et al. include respectively EAI, Beijing, CAS, AFR12 and AFR11. Lineage 4, also called Euro-american includes the LAM, Haarlem, T, X and other families characterized by deletion of spacers S33 to S36. Animal-adapted species of MTBC are clustered in a separate lineage called *M. bovis* phylogenetically closest to lineage 6 strains. Beijing family strains are subdivided according to the presence/absence of RD181 into ancient and modern Beijing isolates.

Supporting Information

Table S1 Comparison between MLVA21*Orsay* and MLVA15*China*. The table indicates the diversity index and confidence interval for the 22 VNTR loci as estimated in the 98 isolates test panel. It also indicates which loci are included in MLVA21*Orsay* and MLVA15*China*.

Table S2 MLVA15*China* profile of 1586 Chinese isolates. The table provides the MLVA15*China* typing data in 1586 chinese isolates. The H37Rv profile as deduced from *in silico* analysis of refseq sequence NC_000962 is also indicated for data compatibility purposes.

References

1. WHO (2010) Global tuberculosis control 2010: epidemiology, strategy, financing. Geneva: WHO, Available: http://wwwwhoint/tb/country/en/index.html.
2. Lopez B, Aguilar D, Orozco H, Burger M, Espitia G, et al. (2003) A marked difference in pathogenesis and immune response induced by different *Mycobacterium tuberculosis* genotypes. Clin Exp Immunol 133: 30–37.
3. Grode L, Seiler P, Baumann S, Hess J, Brinkmann V, et al. (2005) Increased vaccine efficacy against tuberculosis of recombinant *Mycobacterium bovis* bacille Calmette-Guérin mutants that secrete listeriolysin. J Clin Invest 115: 2472–2479.
4. Kremer K, van-der-Werf MJ, Au BK, Anh DD, Kam KM, et al. (2009) Vaccine-induced immunity circumvented by typical *M. tuberculosis* complex based on tandem repeat typing. BMC Microbiol 9: 116.
5. van Soolingen D, Qian L, de Haas PE, Douglas JT, Traore H, et al. (1995) Predominance of a single genotype of *Mycobacterium tuberculosis* for diagnosis and epidemiology. J Clin Microbiol 33: 3034–3036.
6. Frothingham R, Meeker-O’Connell WA (1998) Genetic diversity in the *Mycobacterium tuberculosis* complex based on variable numbers of tandem DNA repeats. Microbiology 144: 1109–1196.
7. Supply P, Alix C, Lejean S, Cardoso-Oelemann M, Rusch-Gerdes S, et al. (2006) Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of *Mycobacterium tuberculosis*. J Clin Microbiol 44: 4498–4510.
8. Gagneux S, DeRiemer K, Van T, Kato-Maeda M, de Jong BC, et al. (2006) Variable human mini-mlst-like regions in the *Mycobacterium tuberculosis* genome. Mol Microbiol 61: 762–771.
9. Le Flèche P, Fabre, M Denoua F, Koeck JL, Vergnaud G (2002) High resolution, on-line identification of strains from the *Mycobacterium tuberculosis* complex based on tandem repeat typing. BMC Microbiol 2: 37.
10. Gagneux S, DeRiemer K, Van T, Kato-Maeda M, de Jong BC, et al. (2006) Variable host-pathogen compatibility in *Mycobacterium tuberculosis*. Proc Natl Acad Sci U S A 103: 2689–2693.
11. Comas D, Schaub L, Kolb A, van Agterveld M, van Sooilingen D, et al. (1997) Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. J Clin Microbiol 35: 907–914.
12. Supply P, Allix C, Lejean S, Cardoso-Oelemann M, Rusch-Gerdes S, et al. (2006) Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of *Mycobacterium tuberculosis*. J Clin Microbiol 44: 4498–4510.
13. Alland D, Lacher DW, Hazbon MH, Motivola AS, Qi W, et al. (2007) Role of large sequence polymorphisms (LSPs) in generating genomic diversity among clinical isolates of *Mycobacterium tuberculosis* and the utility of LSPs in phylogenetic analysis. J Clin Microbiol 45: 39–46.
Brosch R, Gordon SV, Marmiesse M, Brodin P, Buchrieser C, et al. (2002) A new evolutionary scenario for the Mycobacterium tuberculosis complex. Proc Natl Acad Sci U S A 99: 3684–3689.

Hershberg R, Lipatov M, Small PM, Sheffer H, Niemann S, et al. (2008) High frequency of drug resistance in Mycobacterium tuberculosis driven by genetic drift and human demography. PLoS Biol 6: e311.

Comas I, Honolkas S, Niemann S, Gagneux S (2009) Genotyping of genetically monomorphic bacteria: DNA sequencing in Mycobacterium tuberculosis highlights the limitations of current methodologies. PLoS One 4: e7815.

Comas I, Chakraborty T, Small PM, Galagan J, Niemann S, et al. (2010) Human T cell epitopes of Mycobacterium tuberculosis are evolutionarily hyperconverged. Nat Genet 42: 496–503.

Fillol I, Driscoll JR, van Soolingen D, Kremer K, van Soolingen D (2002) Worldwide assessment of Beijing family in a population-based study of the genetic diversity of Mycobacterium tuberculosis: W-Beijing family strains. Trends Microbiol 10: 45–52.

Glynis JR, Whiteley J, Bilani PJ, Kremer K, van Soolingen D (2002) Worldwide occurrence of Beijing/W strains of Mycobacterium tuberculosis: a systematic review. Emerg Infect Dis 8: 347–353.

Driscoll JR, van Soolingen D, Kremer K, et al. (2002) Spoligotyping Patterns. J Clin Microbiol 40: 4294–4298.

Kremer K, Glynis JR, Lilbeek T, Niemann S, Kurepina NE, Kremer K, et al. (2004) Definition of the Beijing/W lineage of Mycobacterium tuberculosis on the basis of genetic markers. J Clin Microbiol 42: 4040–4049.

Mokrousov I, Jiao WW, Valcheva V, Azayova A, Otten T, et al. (2006) Rapid detection of the Mycobacterium tuberculosis Beijing genotype and its ancient and modern sublineages by IS6110-based inverse PCR. J Clin Microbiol 44: 2851–2856.

Tosdal AG, Gagneux S, Pym AS, Gouget de la Salomone YO, Kreiswirth BN, et al. (2003) Genomic deletions classify the Beijing/W strains as a distinct genetic lineage. J Clin Microbiol 41: 3185–3191.

Kang HY, Wada T, Iwamoto T, Maeda S, Murase Y, et al. (2010) Phylogeographical particularity of the Mycobacterium tuberculosis Beijing family in South Korea based on international comparison with surrounding countries. J Clin Microbiol 48: 1191–1197.

Parwati I, van Crevel R, van Soolingen D (2010) Possible underlying mechanisms for successful emergence of the Mycobacterium tuberculosis Beijing genotype strains. Lancet Infect Dis 10: 105–111.

Wirth T, Hildebrand F, Allie-Begue C, Wolfelning F, Kubica T, et al. (2008) Origins, spread and demography of the Mycobacterium tuberculosis complex. PLoS Pathog 4: e1000160.

Oota H, Kitano T, Jin F, Yuasa I, Wang L, et al. (2002) Extreme mtDNA homogeneity in continental Asian populations. Am J Phys Anthropol 118: 146–155.

Caws M, Thwaites G, Dunstan S, Hawk TR, Lam NT, et al. (2008) The influence of host and bacterial genotype on the development of disseminated disease with Mycobacterium tuberculosis. PLoS Pathog 4: e1000304.

Soumounos RS, Quitakura T, Schupp J, Palmer EG, et al. (2003) Molecular Typing of Mycobacterium tuberculosis by using nine novel variable-number tandem repeats across the Beijing family and low-copy-number IS6110 isolates. J Clin Microbiol 41: 4224–4230.

Fillol I, Fordinand S, Negropontis E, Josa L, Castro R (2000) Molecular Typing of Mycobacterium tuberculosis based on variable number of tandem DNA repeats used alone and in association with spoligotyping. J Clin Microbiol 38: 2520–2524.

Sola C, Fillol I, Legrand E, Lejean S, Lecht C, et al. (2003) Genotyping of the Mycobacterium tuberculosis complex using MIRUs: association with VNTR and spoligotyping for molecular epidemiology and evolutionary genotypes. Infect Genet Evol 3: 125–133.

Kremer K, Au BK, Yi PC, Stooke R, Supply P, et al. (2003) Use of Variable-Number Tandem-Repeat Typing To Differentiate Mycobacterium tuberculosis Beijing Family Isolates from Hong Kong and Comparison with IS6110 Restriction Fragment Length Polymorphism Typing and Spoligotyping. J Clin Microbiol 41: 314–320.

Smittipan N, Billamas P, Pilahtanongpirun M, Thong-On A, Temu MM, et al. (2005) Polymorphism of variable-number tandem repeats at multiple loci in Mycobacterium tuberculosis. J Clin Microbiol 43: 5034–5035.

Cowen LS, Deml I, Monson T, Wand P, Temporado D, et al. (2005) Evaluation of a two-step approach for large-scale, prospective genotyping of Mycobacterium tuberculosis isolates in the United States. J Clin Microbiol 43: 509–515.

Murase Y, Mizutani S, Sugawara I, Kato S, Maeda S (2006) Promising loci of variable numbers of tandem repeats for typing Beijing family Mycobacterium tuberculosis. J Med Microbiol 55: 873–880.

Buchrieser C, Riquet J, Rousset F, Pujol-Borrell C, Le Guen M, et al. (2007) A new strategy for molecular epidemiology of Mycobacterium tuberculosis. J Clin Microbiol 45: 3602–3610.

Sanchez-Gutierrez MA, Gagneux S, Pym AS, Pym M, Cámara A, et al. (2006) M. tuberculosis complex: a systematic review. Clin Microbiol Rev 19: 586–625.

Brudey K, Driscoll JR, Rijoulet L, Prodinger WM, Gori A, et al. (2006) Mycobacterium tuberculosis complex genetic diversity: mining the fourth international spoligotyping database (SpoDB4) for classification, population genetics and molecular epidemiology. BMC Genomics 6: 523.

Lindstedt BA (2005) Multiple-locus variable number tandem repeats analysis for Mycobacterium tuberculosis. J Clin Microbiol 43: 4591–4595.

Cowan LS, Diem L, Monson T, Vand P, Temporado D, et al. (2005) Multilocus variable-number tandem repeat analysis for genetic fingerprinting of pathogenic bacteria. Electropherose 26: 2567–2568.

Vergnaud G, Pourcel C (2006) Multiple Locus VNTR Typing (Variable Number of Tandem Repeat) Analysis. In: Stackebrandt E, ed. Molecular Identification, Systematics, and Population Structure of Prokaryotes. Berlin Heidelberg: Springer-Verlag, pp 83–104.

Supply P, Lejean S, Savine E, Kremer K, van Soolingen D, et al. (2001) Automated high-throughput genotyping for study of global epidemiology of Mycobacterium tuberculosis based on mycobacterial interspersed repetitive units. J Clin Microbiol 39: 3353–3357.

Rehn K, Hoolander M, Olsson L, Bosque MJ, et al. (2010) High-resolution typing by integration of genome sequencing data in a large tuberculosis strain collection. J Clin Microbiol 48: 3401–3410.
64. Wada T, Isamoto T, Maeda S (2009) Genetic diversity of the *Mycobacterium tuberculosis* Beijing family in East Asia revealed through refined population structure analysis. FEMS Microbiol Lett 291: 35–43.

65. Millet J, Miyagi-Shiohira C, Yamane N, Mokrousov I, Rastogi N (2011) High-resolution MIRU-VNTRs typing reveals the unique nature of *Mycobacterium tuberculosis* Beijing genotype in Okinawa, Japan. Infect Genet Evol.

66. Yokoyama E, Hachisu Y, Hashimoto R, Kishida K (2010) Concordance of variable-number tandem repeat (VNTR) and large sequence polymorphism (LSP) analyses of *Mycobacterium tuberculosis* strains. Infect Genet Evol 10: 915–918.

67. van der Zanden AG, Kremer K, Schols LM, Caimi K, Cataldi A, et al. (2002) Improvement of differentiation and interpretability of spoligotyping for *Mycobacterium tuberculosis* complex isolates by introduction of new spacer oligonucleotides. J Clin Microbiol 40: 4628–4639.

68. Fabre M, Koeck JL, Le Fleche P, Simon F, Herve V, et al. (2004) High genetic diversity revealed by variable-number tandem repeat genotyping and analysis of hsp65 gene polymorphism in a large collection of “*Mycobacterium canettii*” strains indicates that the *M. tuberculosis* complex is a recently emerged clone of “*M. canetti*”. J Clin Microbiol 42: 3248–3255.

69. Tsolaki AG, Hirsh AE, DeRiemer K, Enciso JA, Wong MZ, et al. (2004) Functional and evolutionary genomics of *Mycobacterium tuberculosis*: insights from genomic deletions in 100 strains. Proc Natl Acad Sci U S A 101: 4863–4870.

70. Le Fleche P, Hauck Y, Otteniante L, Prieur A, Denoeud F, et al. (2001) A tandem repeats database for bacterial genomes: application to the genotyping of *Terrinia pestis* and *Bacillus anthracis*. BMC Microbiol 1: 2.

71. Feil EJ, Li BG, Anensen DM, Hanage WP, Spratt BG (2004) eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. J Bacteriol 186: 1518–1530.

72. Hunter PR, Gaston MA (1988) Numerical index of the discriminatory ability of typing systems: an application of Simpson’s index of diversity. J Clin Microbiol 26: 2465–2466.

73. Allie-Requere C, Harsman D, Weniger T, Supply P, Niemann S (2008) Evaluation and strategy for use of MIRU-VNTRplus, a multifunctional database for online analysis of genotyping data and phylogenetic identification of *Mycobacterium tuberculosis* complex isolates. J Clin Microbiol 46: 2692–2699.

74. Hauck Y, Fabre M, Vergnaud G, Soler C, Pourcel C (2009) Comparison of two commercial assays for the characterization of rpoB mutations in *Mycobacterium tuberculosis* and description of new mutations conferring weak resistance to rifampicin. J Antimicrob Chemother 64: 259–262.

75. Fabre M, Hauck Y, Soler C, Koeck JL, van Ingen J, et al. (2010) Molecular characteristics of “*Mycobacterium canettii*” the smooth *Mycobacterium tuberculosis* bacilli. Infect Genet Evol 10: 1165–1173.