Clonal Expansion of Both Modern and Ancient Genotypes of *Mycobacterium tuberculosis* in Southern Taiwan

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Abstract

We present the first comprehensive analysis of *Mycobacterium tuberculosis* isolates circulating in the Kaohsiung region of southern Taiwan. The major spoligotypes found in the 224 isolates studied were Beijing lineages (n = 97; 43.3%), EAI lineages (n = 72; 32.1%) and Haarlem lineages (n = 18; 8.0%). By 24 MIRU-VNTR typing, 174 patterns were identified, including 24 clusters of 74 isolates and 150 unique patterns. The combination of spoligotyping and 12-MIRU-VNTR revealed that 129 (57.6%) of the 224 isolates were clustered in 18 genotypes. Moreover, 63.6% (7/11) of infected persons younger than 30 years had a Beijing strain, which could suggest recent spread among younger persons by this family of TB strains in Kaohsiung. Among the 94 Beijing family (SIT1, SIT250 and SIT1674) isolates further analyzed for SNPs by mass spectrometry, the most frequent strain found was ST10 (n = 49; 52%), followed by ST22 (n = 17; 18%) and ST19 (n = 11; 12%). Among the EAI-Manila family isolates analyzed by region deletion-based subtyping, the most frequent strain found was RD type 1 (n = 63; 87.5%), followed by RD type 2 (n = 9; 12.5%). In our previous study, the proportion of modern Beijing strains (52.5%) in northern Taiwan was significantly higher than the proportion of EAI strains (11%). In contrast, in the present study, EAI strains comprised up to 32% of Beijing strains in southern Taiwan. In conclusion, both ‘modern’ (Beijing) and ‘ancient’ (EAI) *M. tuberculosis* strains are prevalent in the Kaohsiung region, perhaps suggesting that both strains are somehow more adapted to southern Taiwan. It will be interesting to investigate the dynamics of the lineage composition by different selection pressures.

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Introduction

Tuberculosis (TB) remains a worldwide healthcare concern and is characterized by the World Health Organization (WHO) as an epidemic. TB is a leading notifiable communicable disease on the island of Taiwan. In 2008, 14,265 cases (62.0/100,000) were reported, with the highest numbers of new TB patients occurring in Taipei City to obtain information on potential transmission and for formulation of infection-control policy.

Materials and Methods

Ethics statement

This study was approved by the Human Ethics Committee of the National Health Research Institutes, Taiwan (Code: EC0961103). Because of the retrospective nature, routine collec-
tion of clinical data in daily practice, and dislinkage of personal
government, the requirement to obtain informed consent was
waived by our institutional review board.

Mycobacterial strains and genomic DNA
A total of 224 samples were collected between 2006 and 2008
from 224 patients at the Kaohsiung Veterans General Hospital, a
large medical center that handles a substantial number of TB
patients referred from hospitals throughout Kaohsiung. All of the
patients were sputum microscopy positive and culture positive.
Mycobacterial genomic DNA was extracted from cultured cells as
described previously [2]. Briefly, mycobacterial colonies were
resuspended in 100–200 μl of distilled H₂O and incubated at 85°C
for 30 min. After centrifugation of the suspension, the supernatant
containing the DNA was removed and stored at −20°C until
further use. The study protocol was approved by the institutional
review board of the National Health Research Institutes, Taiwan.

Spoligotyping and spoligotype analysis
Spoligotyping was carried out according to the manufacturer’s
instructions (Isogen Bioscience B.V., Maarssen, The Netherlands).
The resulting spoligotypes were documented using a binary code

| SIT | Spoligotype | Label | No. of isolates | Prevalence (%) |
|-----|-------------|-------|----------------|---------------|
| 1   |             | Beijing | 91             | 40.63         |
| 250 |             | Beijing | 1              | 0.45          |
| 1311|             | Beijing | 3              | 1.34          |
| 1674|             | Beijing | 1              | 0.45          |
| 36  |             | H3     | 1              | 0.45          |
| 50  |             | H3     | 6              | 2.68          |
| 99  |             | H3     | 1              | 0.45          |
| 742 |             | H      | 3              | 1.34          |
| 946 |             | H      | 2              | 0.89          |
| 2092|             | H3     | 1              | 0.45          |
| 53  |             | T1     | 6              | 2.68          |
| 131 |             | T1     | 1              | 0.45          |
| 249 |             | LAM    | 3              | 1.34          |
| 917 |             | T1     | 1              | 0.45          |
| 926 |             | T1     | 1              | 0.45          |
| 2393|             | T1     | 2              | 0.89          |
| 52  |             | T2     | 2              | 0.89          |
| 848 |             | T2     | 1              | 0.45          |
| 1302|             | T2     | 1              | 0.45          |
| 19  |             | EAI2_Manila | 62          | 27.68         |
| 89  |             | EAI1_Nonthaburi | 1          | 0.45          |
| 483 |             | EAI2_Manila | 1          | 0.45          |
| 894 |             | EAI2_Manila | 1          | 0.45          |
| 2085|             | EAI2_Manila | 1          | 0.45          |
| 2098|             | EAI2_Manila | 1          | 0.45          |
| 2351|             | EAI2_Manila | 1          | 0.45          |
| 2097|             | EAI5    | 2              | 0.89          |
| 177 |             | LAM9    | 2              | 0.89          |
| 246 |             | undefined* | 2          | 0.89          |
| 523 |             | MANU-ancestor | 3          | 1.34          |
| 574 |             | T1      | 1              | 0.45          |
| 2587|             | undefined* | 2          | 0.89          |
| 684 |             | BOV_1   | 1              | 0.45          |

*Shared international type (SIT), international spoligotype database SITVITWEB (SITVITWEB, database. http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE).
Label representing spoligotype families as assigned in the international spoligotype database SITVITWEB.
Prevalence, representing the number of isolates with a common SIT relative to the total number of isolates from the same database (224) classified by SIT from Kaohsiung Veterans General Hospital (expressed as a percentile).
Undefined in SITVITWEB database.

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representing either a positive or a negative hybridization result (n and o, respectively) and analyzed using Excel software for grouping and ordering of the patterns. The SITVITWEB database [3] and a web-based computer algorithm, Spotclust [4], were used to assign new isolates to families, subfamilies and variants. SITVITWEB assigned names (shared types) were used whenever a spoligopattern was found in the database. Patterns not found in SITVITWEB were assigned to families and subfamilies by Spotclust. Spoligotypes described only once (non-clustered) in this study and in the SITVITWEB were designated as “orphan”. A cluster was defined as two or more isolates from different patients with identical spoligotype patterns.

PCR and MIRU analysis

PCRs were carried out using a PCR reagent system (Gibco-BRL). Sequences of the primers used for amplification of the 24 MIRU loci were selected according to descriptions in other studies [5]. Five microliters from fivefold-diluted DNA solutions were added to a final volume of 50 μl containing 0.2 μl of DNA polymerase (1 U); 0.2 mM each of dATP, dCTP, dGTP, and dTTP; 5 μl of PCR buffer; 0.4 μM (2 μM for locus 7) of primers; and 1 to 3.5 mM of MgCl₂. The primers and MgCl₂ concentrations used were as described by Mazars et al. [6]. The PCR fragments were analyzed by 2% agarose gel electrophoresis (Figure S1). The sizes of the amplicons were estimated by comparison with

| Table 2. Spoligotypes of 15 orphan strains identified in Spotclust. |
| Spoligotype | MIRU-VNTR typing | No. of isolates | Spotclust | Prevalence (%) |
|------------|-----------------|----------------|-----------|----------------|
| 000000000000171 | 4242233251735334544336112 | 1 | Beijing⁶ | 0.45 |
| 57777763720771 | 314222325153323224233282 | 1 | LAM | 0.45 |
| 00377747713771 | 46254326223432193232951 | 1 | EAT⁶ | 0.45 |
| 65777747713771 | 462543262234321103232951 | 1 | EAT⁶ | 0.45 |
| 00177777020771 | 31422232517323234233382 | 1 | H1 | 0.45 |
| 777777575720771 | 534228225173433544231981 | 1 | H1 | 0.45 |
| 775777575720771 | 31422232517332334233352 | 2 | H3 | 0.89 |
| 774000170020771 | 3142223251733233233392 | 1 | T3 | 0.45 |
| 77617777777761 | 52422722517342344321861 | 1 | Family33 | 0.89 |
| 524227225173424444231861 | | | | |
| 777777400037771 | 534227225173434344231881 | 1 | Family33 | 0.45 |
| 77777757577771 | 524225225173433441221881 | 1 | Family33 | 0.45 |
| 77777767777771 | 53422622517343344231881 | 1 | Family33 | 0.45 |
| 700000007760731 | 42422332573534544336112 | 1 | Family36 | 0.45 |

⁶: RD105 deletion.
⁷: TBD1 positive.

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| Table 3. Comparative analysis of the genotyping methods applied to study the clinical *Mycobacterium tuberculosis* strains circulating in Kaohsiung in southern Taiwan. |
| Typing methods, Strain group | Number of genotypes in total | No. of unique genotypes | No. of clusters | No. of strains in clusters | HGDI value |
|-----------------------------|-------------------------------|------------------------|----------------|---------------------------|-----------|
| Spoligotyping               |                               |                        |                |                           |           |
| All strains                 | 46                            | 29                     | 17             | 2-91                      | 0.758     |
| Beijing strain only         | 5                             | 3                      | 2              | 3-91                      | 0.063     |
| Non-Beijing strains         | 41                            | 26                     | 15             | 2-62                      | 0.768     |
| VNTR analysis, all strains  |                               |                        |                |                           |           |
| 12-MIRU-VNTR                | 91                            | 69                     | 22             | 2-52                      | 0.917     |
| 24-MIRU-VNTR                | 174                           | 150                    | 24             | 2-10                      | 0.995     |
| Spoligotyping+12-MIRU       |                               |                        |                |                           |           |
| All strains                 | 113                           | 95                     | 18             | 2-43                      | 0.937     |
| Beijing strains only        | 42                            | 33                     | 9              | 3-35                      | 0.863     |
| Spoligotyping+24-MIRU       |                               |                        |                |                           |           |
| All strains                 | 181                           | 159                    | 22             | 2-9                       | 0.996     |
| Beijing strains only        | 80                            | 72                     | 8              | 2-5                       | 0.994     |

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50- and 100-bp ladders. The MIRU copy number per locus was calculated by using the conventions described by Supply et al. [7].

Single-nucleotide polymorphism typing
PCR and extension primers were designed using MassArray Assay Design 3.1 software (Sequenom, San Diego, CA). PCRs contained, in a volume of 5 µL, 1 pmol of the corresponding primers, 10 ng genomic DNA, and HotStar reaction mix (Qiagen) in 384-well plates. PCR conditions were as follows: 94°C for 15 min, followed by 40 cycles of 94°C (20 s), 56°C (30 s), 72°C (60 s), and a final extension of 72°C for 3 min. In the primer extension procedure, each sample was denatured at 94°C, followed by 40 cycles of 94°C (5 s), 52°C (5 s), 72°C (5 s). The mass spectrum from time-resolved spectra was retrieved by using a MassARRAY mass spectrometer (Sequenom), and each spectrum was then analyzed using SpectroTYPER software (Sequenom) to perform the genotype calling. A detailed explanation of this methodology has been described [8].

Detection of RD deletions
EAI family strains in our collection were further classified by using PCR amplification to determine the RD deletion genotype. A primer set was used to check for the presence or absence of RD131ab, RD135, RD141, RD147c, and RD239. The PCR mixture consisted of 0.2 µg DNA template, 13.9 µL Q buffer, 5 µL 5× buffer, 4 µL 10 mM deoxynucleoside triphosphates, 1 µL of each primer (10 pmol/µL), 1 µL DMSO, and 0.6 µL Herculase II Fusion DNA polymerase (Stratagene, USA). Sterile water was used to dilute the mixture up to 25 µL. Regions amplified by PCR were sequenced to confirm the reaction. A detailed explanation of this methodology has been described [9].

Drug resistance testing
The proportional method for drug susceptibility testing (DST) of MTB was performed as described previously [10]. Briefly, for each drug a 1:10 dilution of standardized suspension was inoculated onto the control and drug-containing media. The extent of growth in the absence or presence of the drug was compared and expressed as a percentage. If growth at the critical concentration of a drug was >1%, the isolate was considered to be clinically resistant. 7H10 agar with 0.2 or 1 mg/L isoniazid (INH), 1 or 5 mg/L streptomycin was used.

Statistical analysis
The Hunter–Gaston equation [11], an application of Simpson’s index of diversity [12], was used to calculate the allelic diversity, or the ‘Hunter–Gaston discrimination index’ (HGDI), at each locus. The equation is:

\[ D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{N} a_j \]

where \( D \) is the index of discriminatory power, \( a_j \) is the number of strains in the population which are indistinguishable from the \( j \)th strain, and \( N \) is the number of strains in the population [13].

Results
Analysis of spoligotyping patterns
A total of 224 isolates from 224 patients (median age = 68 years; 72.8%, \( n = 163 \) were male) who were diagnosed with culture-confirmed TB were subjected to spoligotyping and MIRU-VNTR typing (Table S1). Molecular analysis showed that all of the TB cases were caused by \( M. \) tuberculosis, except one by \( M. \) bovis. Of the 224 isolates analyzed, spoligotypes from 209 isolates (93.3%) were classified according to SITVITWEB into one of 33 shared international types (SITs) (Table 1). Of these defined spoligotypes the most frequent strains found were Beijing SIT1 (40.6%) and East African-India (EAI)_2 Manila SIT19 (27.7%) (Table 1). Of the remaining 15 isolates that were not represented in SITVITWEB, 2 were EAI, 5 were Family-33, 4 were Haarlem, 1 was T, 1 was LAM, 1 was Family-36, and 1 was Beijing, based on Spotclust (Table 2).

Overall, the Beijing family was the most prevalent genotype, identified in 97/224 (43.3%) isolates, followed by the EAI family, identified in 72/224 (32.1%) isolates (Table 3). Among the 15 novel spoligotypes, 2 strains were found to be TBD1 positive and to lack DR spacers 29 to 34 and 36 but to possess spacer 33. Based on this result, these 2 new spoligotypes belong to the EAI family.

Comparative analysis of genotyping methods
We calculated the HGDI value, a measure of allelic diversity, for each of the different genotyping methods. For the Beijing strains, the HGDI value was 0.063 for spoligotyping and 0.863 for the 12-MIRU loci set. The best

| Table 4. Characteristics of the tuberculosis (TB) patients from whom the study’s Mycobacterium tuberculosis strains were isolated. |
|---|
| Genotype family | Beijing* | EAI | Haarlem | T | Family33 | Family36 | LAM | Manu_ancestor | Bovis |
| No. of isolates | 97 | 72 | 18 | 17 | 5 | 1 | 6 | 3 | 1 |
| (% | (43.3) | (32.1) | (8.0) | (7.6) | (2.2) | (0.4) | (2.7) | (1.3) | (0.4) |
| Sex | | | | | | | | | |
| male | 74 | 52 | 11 | 14 | 3 | 1 | 5 | 1 | 1 |
| female | 23 | 20 | 7 | 5 | 2 | 0 | 1 | 2 | 0 |
| Age (median) | 66 | 69.7 | 62.7 | 67 | 70.4 | 81 | 72.8 | 79 | 36 |
| ≤30 | 7 | 2 | 1 | 1 | 0 | 0 | 0 | 0 | 0 |
| 31–50 | 22 | 9 | 4 | 1 | 0 | 0 | 1 | 0 | 1 |
| 51–70 | 20 | 18 | 6 | 6 | 3 | 0 | 1 | 0 | 0 |
| >70 | 48 | 43 | 7 | 9 | 2 | 1 | 4 | 3 | 0 |


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discriminatory power was demonstrated by a combination of 24 loci VNTR analysis and spoligotyping: the HGDI value was 0.996 for all strains and was 0.994 for Beijing strains only (Table 3).

Characteristics of the TB patients

The average age (in years) of patients infected with each of the MTB genotypes was as follows: Beijing: 66; EAI: 69.7; Haarlem: 62.7; T: 67; Family-33: 70.4; LAM: 72.8 and Manu_ancestor: 79.

The composition of Beijing and EAI lineages in young TB patients was very different compared with that in older ones (Table 4 and Figure 1). The prevalence of Beijing strains decreased as the age of patients increased, whereas that of EAI gradually increased (Table 4). The prevalence of Beijing strains in different age groups (age ≤30, 31–50, 51–70 and >70) was 63.6%, 57.9%, 37.0%, and 41.0%, respectively (p = 0.055) whereas that of EAI was 18.2%, 23.7%, 33.3%, and 36.8%, respectively (p = 0.077). Furthermore, the age distributions across sampling years were compared within each genotype: the percentage of Beijing-infected patients ≤30 years old increased monotonically from 3.9% in 2006 to 9.3% in 2008, but no such increasing trend was observed for all non-Beijing genotypes alone or combined (Figure 1). Thus, the balance that normally exists between the proportion of Beijing and EAI lineages in older patients appears to be in flux in young TB patients (Figure 1). The MTB-infected young population is increasingly more likely to be infected with a Beijing strain than with any other strain as compared to the older population, which may suggest a possible recent spread of the Beijing genotype in young persons in Kaohsiung.

Subtyping of Beijing and EAI strains

From a collection of 97 Beijing strains, a total of 94 Beijing sublineages were identified by SNPs. The most frequent strain found was ST10 (52%), followed by ST22 (18%) and ST19 (12%), which all belong to modern sublineages.

Subtyping of Beijing and EAI strains was further analyzed by RD typing. The
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**Discussion**

The present study revealed Beijing strains to be the dominant TB pathogen in Kaohsiung in southern Taiwan (43.3% of cases), which is a lower percentage than what we recently observed in northern Taiwan (52.5% of cases) (p = 0.0377) [1]. A similar distribution trend was also recently observed by Huang et al. (Beijing strains: North, 53%; South, 27%; EAI strains: South, 20%) [14]. As its name implies and has generally has been accepted, the Beijing genotype originated in the Beijing area in the north of China [13,16] although a recent study suggested its hypothetical origin to be in the Guangxi province in the south of China [17], and strains of this family were found to be dominant not only in China (82%), but also in neighboring countries such as Indonesia (44%), Thailand (44%), Vietnam (53%), Japan (79%), and South Korea (91%) [16–21]. Our recent report of MTB isolates in northern Taiwan showed the most frequent Beijing sublineage there to be ST10 (53.3%), followed by ST19 (41.8%) and ST22 (14.5%) [8]; in the present study, we found a similar distribution of Beijing sublineages in Kaohsiung. In contrast to the distribution of modern Beijing family strains seen in Taiwan and other countries worldwide, the ancient Beijing strains are dominant in Japan [8,19,22]. We believe that the modern Beijing strains, with their high degree of transmissibility, are currently spreading throughout the world. It was previously reported that BCG vaccination favors the positive selection of modern Beijing strains [23]. Our results are consistent with this assertion.

Nearly 7% (15/224) of the spoligotypes characterized in the present study were absent from the SITVITWEB database, which suggests that a large number of genotypes prevalent in southern Taiwan is still not identified. Further analysis by Spotclust revealed the majority of these to be Haarlem strains and Family-33 strains.

The EAI lineage, the most ancient MTB lineage, was first described in Guinea-Bissau [24] and predominates in Southeast Asia, particularly in the Philippines (73%), in Myanmar and Malaysia (53%), and in Vietnam and Thailand (32%) [3]. After modern humans dispersed out of Africa about 60,000 years ago [25] they migrated to the Asian mainland aided by low sea levels during the last ice age [26]. Subsequent prehistoric migrations to islands of East Asia and the Pacific have been designated differently depending on whether they were traced by language, archeological remains, or genetic studies. Most of the native Pacific languages from near the African coast through to Polynesia are Malayo-Polynesian, a subgroup of the Austronesian language family [27]. The nine other subgroups of Austronesian are spoken only in Taiwan, suggesting that Taiwan is the origin of Austronesian [28]. The high prevalence of the EAI lineage in Southeast Asia and southern Taiwan, areas which also share related Austronesian languages, suggests a possible relationship between EAI spreading and migration of Austronesian-speaking peoples, but this hypothesis needs further investigation. EAI strains in northern Taiwan comprise about 11% of MTB isolates, but in southern Taiwan they comprise up to 32%. It is very interesting that, despite the same ethnic background, southern Taiwan has a higher proportion of EAI than northern Taiwan. One possible explanation for this difference could be the more tropical climate of southern Taiwan, which is similar to that of the Philippines and Malaysia. Perhaps EAI strains are better adapted for growth and transmission in high-temperature environments, but this remains to be determined. EAI is characterized by a low copy number of IS6110, absence of spacers 29–32 and 34 and presence of spacer 53 (by spoligotyping), presence of the TBD1 region [29], and the RD239 deletion [29].

Fitness is a genotype-by-environment interaction. *M. tuberculosis* lives in a complex environment within the human host where numerous adaptive pressures are applied. Both modern (Beijing) and ancestral (EAI) *M. tuberculosis* strains are prevalent in southern Taiwan, suggesting that both types are somehow adapted to the region. Reasons for the successful adaption of the EAI lineage in a given geographical area have to be explored by studying host–pathogen interactions and immune responses. It will be interesting to investigate the dynamics of lineage composition under different selection pressures. The apparent changing proportions of modern and ancient genotypes of *M. tuberculosis* in young TB patients in southern Taiwan might be because of natural selection, possibly skewed by the two major health-measures against tuberculosis: BCG vaccination and anti-tuberculosis treatment [30].

The aim of this study was to acquire data that can be used to enhance TB control activities through identification of previously unrecognized chains of transmission and by monitoring disease trends, including drug resistance phenotypes. Knowledge of these factors will enable more precise allocation of public health

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**Table 6. Mycobacterial genotype and drug resistance in patients with culture-confirmed tuberculosis.**

| Genotype family | No. of isolates | MDR (%) | Any one drug (%) | Sensitive to all drugs (%) |
|-----------------|----------------|---------|------------------|---------------------------|
| Beijing         | 97(43.3%)      | 4 (4.1) | 20 (20.6)        | 72 (74.2)                |
| EAI             | 72 (32.1%)     | 0       | 12 (16.7)        | 60 (83.3)                |
| T               | 17 (7.6%)      | 0       | 4 (23.5)         | 13 (76.5)                |
| Haarlem         | 18 (8.0%)      | 0       | 2 (11.1)         | 16 (88.9)                |
| Others          | 20 (8.9%)      | 1 (5)   | 6 (30)           | 13 (65)                  |
| Total           | 224            | 5 (2.2) | 44 (19.6)        | 175 (78.1)               |

*Others*, all genotype families with a frequency of less than 10 cases (LAM, MANU-ancestor, Family-33, Family36 and Bovis).

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most common typing pattern, type 1, without deletions, was shared by 63 of the 72 isolates (Table 5).
resources. Strain analysis, together with virulence studies, will also help in pinpointing isolates associated with higher morbidity and mortality, with the aim of directing efforts to limit the spread of those strains within the region. This study gives the first overview of the *M. tuberculosis* strains circulating in southern Taiwan. Based on a combination of spoligotyping and MIRU-VNTR, our data show that the Beijing strain has a high number of clusters in our sample population. The high prevalence of the Beijing genotype in the younger segment of the population warrants close attention to control policy and vaccination strategy.

In conclusion, Beijing and EAI are the prevalent lineages in Kaohsiung in southern Taiwan. One can assume that TB in this geographic region is shaped by the clonal expansion of both modern and ancient TB strains. The apparent changing proportions of modern and ancient genotypes of *M. tuberculosis* in young TB patients of Kaohsiung might be because of BCG vaccine selection. In spite of the presence of highly transmissible lineages like Beijing, there is no significant association with drug resistance in any of the lineages.

References

1. Dou HY, Tseng FC, Lin CW, Chang JR, Sun JR, et al. (2008) Molecular epidemiology and evolutionary genetics of *Mycobacterium tuberculosis* in Taipei. BMC Infect Dis 8: 170.

2. Kolk AH, Schuitena AR, Kuijper S, van Leeuwen J, Hermans PW, et al. (1992) Detection of *Mycobacterium tuberculosis* in clinical samples by using polymerase chain reaction and a nonradioactive detection system. J Clin Microbiol 30: 2567–2573.

3. Demary C, Liens B, Burguière T, Hill V, Covrin D, et al. (2012) SFTVTDWEB - A publicly available international multimarker database for studying *Mycobacterium tuberculosis* genetic diversity and molecular epidemiology. Infect Genet Evol 12:755–766.

4. Vitol I, Driscoll J, Kreiswirth B, Kurepina N, Bennett KP (2006) Identifying *Mycobacterium tuberculosis* complex strain families using spoligotypes. Infect Genet Evol 6: 491–504.

5. Supply P, Allix C, Lesjean S, Cardoso-Oelemann M, Rusch-Gerdes S, et al. (2006) Proposal for standardization of optimized mycobacterial interrepeat repetitive unit-variable-number tandem repeat typing of *Mycobacterium tuberculosis*. J Clin Microbiol 44: 4499–4510.

6. Mazars E, Lesjean S, Banuls AL, Gilbert M, Vincent V, et al. (2001) High-resolution minisatellite-based typing as a portable approach to global analysis of *Mycobacterium tuberculosis* molecular epidemiology. Proc Natl Acad Sci U S A 98: 1901–1906.

7. Supply P, Mazars E, Lesjean S, Vincent V, Gicquel B, et al. (2000) Variable human minisatellite-like regions in the *Mycobacterium tuberculosis* genome. Mol Microbiol 36: 762–771.

8. Chang JR, Lin CH, Tsai SF, Su IJ, Tseng FC, et al. (2010) Genotypic analysis of *Mycobacterium tuberculosis* clinical isolates in Wuhan, China. J Med Microbiol 59: 2456–2466.

9. Simpson EH (1949) Measurement of diversity. Nature 163: 688.

10. Struchlis MJ (1990) Consensus guidelines for appropriate use and evaluation of microbial epidemiologic typing systems. Clin Microbiol Infect 2: 2–11.

11. Huang SF, Su WJ, Dou HY, Feng JY, Lee YC, et al. (2012) Association of *Mycobacterium tuberculosis* genotypes and clinical and epidemiological features - a multi-center study in Taiwan. Infect Genet Evol 12: 20–37.

12. Simpson EH (1949) Measurment of diversity. Nature 163: 688.

13. Struelens MJ (1996) Consensus guidelines for appropriate use and evaluation of microbial epidemiologic typing systems. Clin Microbiol Infect 2: 2–11.

14. Glynn JR, Whiteley J, Bifani PJ, Kremer K, van Soolingen D (2002) Worldwide occurrence of Beijing/W strains of *Mycobacterium tuberculosis*: a systematic review. Emerg Infect Dis 8: 843–849.

15. Mokrousov I, Le HM, Otten T, Lan NN, Vynokurov B, et al. (2005) Origin and primary dispersal of the *Mycobacterium tuberculosis* Beijing genotype: clues from human phylogeography. Genome Res 15: 1357–1364.

16. Wan K, Liu J, Hauk Y, Zhang Y, Zhao X, et al. (2011) Investigation on *Mycobacterium tuberculosis* diversity in China and the origin of the Beijing clade. PLoS One 6: e29190.

17. Wan K, Liu J, Hauk Y, Zhang Y, Zhao X, et al. (2011) Investigation on *Mycobacterium tuberculosis* diversity in China and the origin of the Beijing clade. PLoS One 6: e29190.

18. Li WM, Wang SM, Liu YH, Shen GM, et al. (2005) Molecular epidemiology of *Mycobacterium tuberculosis* in China: a nationwide random survey in 2000. Int J Tubercul Lung Dis 9: 1314–1319.

19. Iwamoto T, Fujiyama R, Yoshida S, Wada T, Shirai C, et al. (2009) Population structure dynamics of *Mycobacterium tuberculosis* Beijing strains during past decades in Japan. J Clin Microbiol 47: 3340–3343.

20. Shamputa IC, Lee J, Allix-Beguec C, Cho EJ, Lee JI, et al. (2010) Genetic diversity of *Mycobacterium tuberculosis* isolates from a tertiary care tuberculosis hospital in South Korea. J Clin Microbiol 48: 387–394.

21. Han H, Wang F, Xiao Y, Ren Y, Yang L, et al. (2007) Utility of mycobacterial interspersed repetitive unit typing for differentiating *Mycobacterium tuberculosis* isolates in Wuhan, China. J Med Microbiol 56: 1219–1223.

22. Iwamoto T, Yoshida S, Suzuki K, Wada T (2008) Population structure analysis of the *Mycobacterium tuberculosis* Beijing family indicates an association between certain sublineages and multidrug resistance. Antimicrob Agents Chemother 52: 3805–3809.

23. Kramer K, van-den-Werf MJ, Au BK, Ahn DD, Kam KM, et al. (2009) Vaccine-induced immunity circumvented by typical *Mycobacterium tuberculosis* Beijing strains. Emerg Infect Dis 15: 335–339.

24. Gagneux S, DeRiemer K, Van T, Kato-Maeda M, de Jong BC, et al. (2006) Genotypic analysis of *Mycobacterium tuberculosis* in Japan. J Clin Microbiol 47: 3340–3343.

25. Liu H, Prugnolle F, Manica A, Balloux F (2006) A geographically explicit genetic structure dynamics of *Mycobacterium tuberculosis* Beijing strains. Proc Natl Acad Sci U S A 103: 2069–2074.

26. Han H, Figgie W, Lee J, Allix-Beguec C, Cho EJ, Lee JI, et al. (2010) Genetic diversity of *Mycobacterium tuberculosis* isolates from a tertiary care tuberculosis hospital in South Korea. J Clin Microbiol 48: 387–394.

27. Kevin OP, John ET (2008) Environmental setting of human migrations in the circum-Pacific region. Journal of Biogeography 35: 1–21.

28. Gray RD, Jordan EM (2006) Language trees support the express-train sequence of Australasian expansion. Nature 440: 1032–1035.

29. 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.

30. van Soolingen D, Kremer K, Borgdorff M (2001) *Mycobacterium tuberculosis* Beijing genotype, Thailand—reply to Dr. Prodinger. Emerg Infect Dis 7: 763–764.

Supporting Information

Figure S1 The quality test of MIRU-VNTR typing. Three loci was amplified and analyzed by electrophoresis using a 2% agarose gel. M, size markers; C, H37Rv control; lane 1–20; clinical isolates. (TIF)

Table S1 24-MIRU-VNTR, spoligotyping and drug resistant profiles of 224 clinical isolates in this study. (XLS)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: HYD IJS. Performed the experiments: JRC YYY TSH. Wrote the paper: HYD JRC YYC TSH. Contributed reagents/materials/analysis tools: JRC YYC CHL TSH JRS WFH. Analyzed the data: TSC.

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