Table 1
SNPs among the *M. avium* subsp. *hominissuis* in comparison with prototype strain *M. avium* subsp. *hominissuis* strain 104 (MAH strain 104).

Amino acid type	Nucleic acid type	Nucleotide at the base pair position of strain MAH strain 104[a]																						Total number of:[b]			
		4*	66	88*	272*	468	558	571*	605*	715*	733	744	752*	765	822	829*	831	834	866*	867	868*	870	877*	924	978	1000	1005	1150*	1161	1163*	1192*	1330*	sSNP	nsSNP	Isolates (*n* = 334)	Genotypes
AA01	NA01	C	A	G	A	T	T	G	C	C	C	G	G	G	A	C	G	T	G	G	G	C	G	G	G	G	A					31		12	
AA02	NA02	C	A	G	A	T	T	G	C	C	C	G	G	G	A	C	G	T	G	G	G	C	G	G	G	G	G	0	1	130	86				
AA02	NA03	C	A	G	A	C	T	G	C	C	T	G	G	G	A	C	G	T	G	G	G	C	G	G	G	G	G	2	1	8	6				
AA02	NA04	C	A	G	A	C	T	G	C	C	C	G	G	G	A	C	G	T	G	G	G	C	G	G	G	G	G	1	1	3	3				
AA02	NA05	C	G	G	A	C	T	G	C	C	C	G	G	G	A	C	G	T	G	G	G	C	G	G	G	G	G	2	1	2	2				
AA02	NA06	C	G	G	A	T	T	G	C	C	C	G	G	G	A	C	G	T	G	G	G	C	G	G	G	G	G	1	1	1	1				
AA02	NA07	C	A	G	A	T	T	G	C	C	C	G	G	G	A	C	G	T	G	G	G	C	G	T	G	G	G	1	1	1	1				
AA02	NA08	C	A	G	A	C	C	G	C	C	C	G	G	G	A	C	G	T	G	G	G	C	G	C	G	G	G	2	1	1	1				
AA03	NA09	C	G	G	A	A	T	C	G	C	C	G	G	G	G	T	C	T	G	G	G	C	G	C	G	G	G	5	4	89	44				
AA04	NA10	C	A	G	A	T	T	G	T	C	C	G	G	G	A	C	G	T	G	G	G	C	G	C	G	G	G	0	2	7	6				
AA05	NA11	C	A	G	A	T	T	G	C	C	C	G	G	G	A	C	G	T	G	G	G	C	G	C	G	A	A	1	3	1	1				
AA06	NA12	C	G	G	A	A	T	C	G	C	C	G	G	G	A	C	G	T	G	G	G	C	G	C	G	G	G	2	2	1	1				
AA07	NA13	C	G	G	G	A	C	C	G	C	C	G	G	G	G	T	G	T	G	G	A	C	G	C	G	G	G	5	2	19	14				
AA08	NA14	C	A	G	G	G	C	C	G	C	C	G	G	G	G	T	G	T	G	G	G	C	T	A	C	G	G	5	4	19	9				
AA09	NA15	T	A	G	A	T	C	C	G	C	C	G	G	G	G	T	G	T	G	G	G	C	G	C	A	G	G	3	4	10	5				
AA10	NA16	C	G	A	A	T	C	C	G	C	C	G	G	T	G	G	G	T	C	T	G	C	G	C	A	G	G	5	5	3	3				
AA11	NA17	C	A	G	A	T	T	A	C	T	C	G	G	G	A	C	G	T	G	G	G	C	G	C	G	G	G	0	3	1	1				
AA12	NA18	C	G	A	A	T	C	G	C	C	C	G	G	G	G	T	G	T	G	G	G	C	G	C	G	G	G	3	3	1	1				
AA13	NA19	C	A	G	A	C	C	G	C	C	C	A	G	A	A	G	T	G	A	A	G	C	G	C	G	A	G	7	4	6	3				

[a] Asterisks denote nonsynonymous position. AA01 and NA01 is the sequevars for MAH strain 104.
[b] sSNP, synonimous SNP; nsSNP, nonsynonymous SNP; genotypes were defined by 19-VNTR.

level (AA types) (Table 1). Among the 13 AA types, only AA02 showed variation in the nucleic acid sequence, i.e., it matched seven different NA types (NA02 to 08) that possessed various synonymous SNPs (sSNPs) (Table 1). All other AA types were associated in a one-to-one correspondence with NA types. These results suggest that AA02 has a longer history than the other AA types in MAH. We, therefore, classified AA02 as the ancestral type of MACPPE 12, whereas the other AA types were classified as modern types. When we used DnaSP, the minimum number of recombination events for the MACPPE12 gene in the sample set was estimated to be 3. The unrooted phylogeny for the gene sequences determined by SplitsTree4 demonstrated complex web-like topology (Fig. 1).

3.3. Comparison of the divergence of MACPPE12 in different isolation sources

The ancestral AA type of MACPPE12, AA02, was observed in all of the different isolation sources in this study (Table 2). In contrast, the modern types varied according to the isolation source. AA01 and AA07-09 were mostly present in pigs; AA03, in Japanese humans and bathroom samples; and AA13, in Korean humans. The topological positions of these AA types in the unrooted tree, except for that of AA01, were distinct from the position of the ancestral type (AA02) (Fig. 1).When we compared variation of MACPPE12 AA types with that of the *hsp65* gene sequevar, which consists of only sSNPs in this sample set, the AA02 type was observed in all 11 *hsp65* code types (Table 2). This would be natural due to the ancestral feature of AA02. Apart from the ancestral type, most of the modern types were distributed across more than two *hsp65* code types and not in a single lineage. This data strongly suggested that these two genes diverged independently, not in parallel to each other, during the history of MAH. It would be noteworthy that 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).

A further finding in this study is the commonality and differences between Japanese and Korean human clinical isolates. A high prevalence of *ISMav6*, which was reported in the genetic characterization of MAH in Japan (Ichikawa et al., 2009), was also observed in Korean isolates (42/77, 54.5%) as was reported by (Niimi et al., 2012). The characteristics of Korean isolates was demonstrated by their high prevalence of *hsp65* sequevar code 16 (26/77, 33.8%), which is a rare sequevar in other countries (Ichikawa et al., 2009; Iwamoto et al., 2012; Turenne et al., 2006). The majority of Korean isolates were ancestral type (AA02), whereas Japanese isolates predominated both AA02 and AA03. Moreover, other modern AA types showed different distributions between samples from these two countries.

4. Discussion

The precise functions of the PE and PPE families are still unknown except for a certain small number of members (Karboul et al., 2008; Mishra et al., 2008; Sassetti and Rubin, 2003), but these families are highly suspected to play key roles in the interaction between pathogens and their host (habitat) (Brennan et al., 2001; Sampson, 2011). A recent comparative study (Mackenzie et al., 2009) of the whole genomes of different MAC organisms revealed that two PPE paralogs, MACPPE4 and MACPPE12, were specifically found in MAH strain 104. Therefore, it would be a reasonable assumption that the two PPE genes reflect bacteriological characteristics of MAH in comparison with other MAC species.

Table 2
Characterization of MACPPE12 gene sequevars according to *hsp65* gene sequence and source of the 334 isolates.

MAC PPE 12		*hsp65* Code Type											Source			
AA type	NA type	C1	C2	C3	C7	C9	C15	C16	C17	N1	N2	N3	Human (Japan)	Human (Korea)	Bath-room	Pig
AA01	NA01	26	4	1	–	–	–	–	–	–	–	–	3	–	–	28
AA02	NA02-08	9	68	2	4	3	21	32	2	1	3	1	72	57	12	5
AA03	NA09	1	21	–	–	1	59	2	5	–	–	–	57	10	22	–
AA04	NA10	–	3	–	–	–	3	–	1	–	–	–	5	–	2	–
AA05	NA11	–	–	–	–	–	1	–		–	–	–	1	–	–	–
AA06	NA12	–	–	–	–	–	–	–	1	–	–	–	1	–	–	–
AA07	NA13	13	5	–	–	–	–	–	1	–	–	–	2	–	–	17
AA08	NA14	11	5	–	–	1	1	1	–	–	–	–	4	–	–	15
AA09	NA15	4	2	–	–	–	–	–	1	3	–	–	–	–	–	10
AA10	NA16	–	1	–	–	–	2	–	–	–	–	–	–	2	1	–
AA11	NA17	–	–	–	–	–	–	1	–	–	–	–	–	1	–	–
AA12	NA18	–	–	–	–	–	–	–	–	–	1	–	–	1	–	–
AA13	NA19	–	–	6	–	–	–	–	–	–	–	–	–	6	–	–

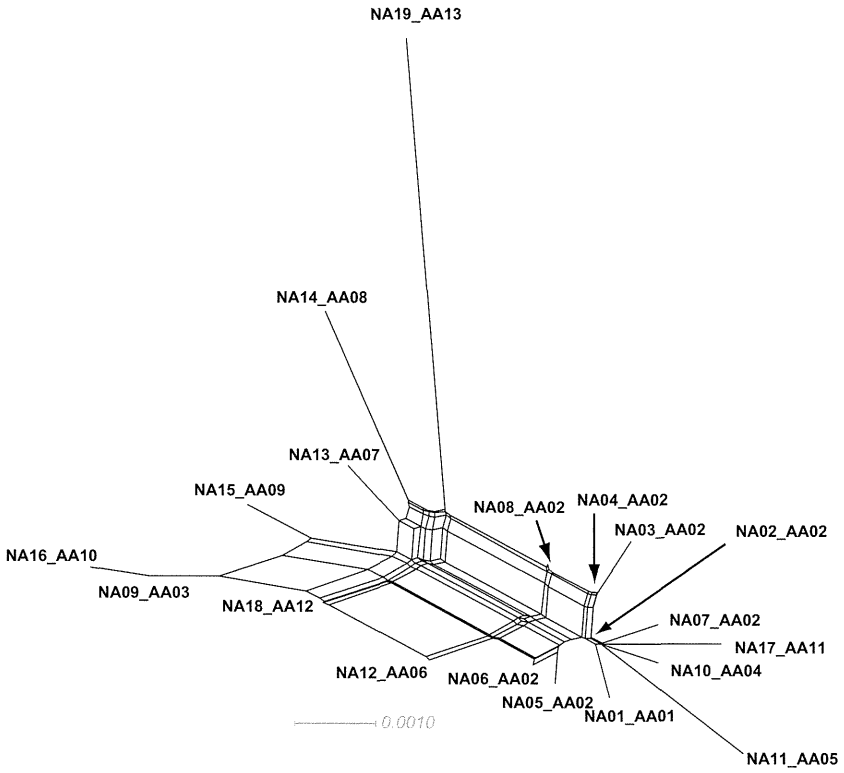

Fig. 1. Phylogenetic representation of each AA and NA type determined in this study and generated in SplitsTree4.

In this study, we evaluated the ubiquitous presence of these two PPE genes in MAH, and their genetic variability and association with the isolation sources and different genetic markers.

Our preliminary study using 16 MAH isolates suggested that MACPPE4 is not ubiquitous in MAH, although it is not certain that if this PPE gene is specific only for MAH strain104 or limited in subgroups of MAH. On the other hand, MACPPE12 was present in all of the 334 MAH isolates. Since our 334 isolates were a set of high heterogeneous isolates, the ubiquitous presence of the PPE gene in this sample set strongly supports the idea that this gene was present in the most recent common ancestor of MAH and universally retained in the subspecies. Because of the absence of the MACPPE12 gene in other members of the MAC and the ubiquitous presence in MAH isolates, MACPPE12 can be considered as a relatively new gene but ubiquitous in MAH.

By using the large number of isolates obtained from different sources, we demonstrated the variability of MACPPE12, which includes 19 different NA types and 13 AA types (Table 1). The web-like topology of the unrooted phylogeny (Fig. 1) and the estimated minimum number of recombination events suggest that genetic recombination plays a role in the divergence of this gene, although its mechanism is unknown. On the basis of the distribution of SNPs, isolation sources, and *hsp65* code types in the different MACPPE12 gene AA types, we classified the PPE gene into two groups; one is an ancestral type (AA02) and the other is a modern type. MAH is generally characterized as its ubiquitous host distribution and heterogeneous grouping (Turenne et al., 2008, 2007). However, when we look closely at the correlation between AA types and isolation sources, the distribution of the modern AA types well reflect their isolation sources, whereas the ancestral AA type (AA02) was observed in all of the different isolation sources. Thus, it can be hypothesized that emergence of the modern AA types somehow reflects an on-going evolution of MAH toward specialization (narrower range for host specificity and higher fitness to its habitat)

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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SHORT REPORT Open Access

Molecular identification of non-tuberculous mycobacteria isolated from clinical specimens in Zambia

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Abstract

Background: The emergence of Acquired Immunodeficiency Syndrome has highlighted the increased incidence and importance of the disease caused by Non-tuberculous Mycobacteria (NTM). While disease due to *M. avium-intracellulare* complex is apparently common throughout the world, other Non-tuberculous mycobacterial species have been isolated from both immunocompromised and immunocompetent individuals. The increasing number of infections caused by these organisms has made it clinically important to quickly identify mycobacterial species. The diagnosis of a pathogenic versus a non-pathogenic species not only has epidemiological implications but is also relevant to the demands of patient management. Since antibiotic treatment varies according to the species encountered, species identification would reduce the burden of some of these emerging opportunistic pathogens especially in immunocompromised patients and improve their quality of life.

Findings: A total of 91 NTM suspected isolates from four regions of Zambia were included in the study. These isolates were identified using the sequence analysis of the *16S-23S* rRNA intergenic transcribed spacer (ITS) region of Mycobacteria.
Fifty-four of the 91 (59%) isolates were identified as NTM and these included *M. intracellulare* (27.8%), *M. lentiflavum* (16.7%), *M. avium* (14.8%), *M. fortuitum* (7.4%), *M. gordonae* (7.4%), *M. kumamotonense* (3.7%), *M. indicus pranii* (3.7%), *M. peregrinum* (3.7%), *M. elephantis* (1.85%), *M. flavescens* (1.85%), *M. asiaticum* (1.85%), *M. bouchedurhonense* (1.85%), *M. chimaera* (1.85%), *M. europaeum* (1.85%), *M. neourum* (1.85%), *M. nonchromogenicum* (1.5%).

Conclusion: The study has shown that DNA sequencing of the ITS region may be useful in the preliminary identification of NTM species. All species identified in this study were potentially pathogenic.

Keywords: Non-tuberculous mycobacteria, Identification, Zambia

Findings

Members of the genus *Mycobacterium* are important causes of respiratory disease, thereby posing an important public health threat to people and animals worldwide. Recently, there has been increased cognisance of a variety of diseases that have been caused by Non-tuberculous Mycobacteria (NTM) [1]. The current unprecedented high level of interest in NTM infections is mainly the result of the association of NTM infection with immune-suppression [2] and the recognition that NTM pulmonary infections are encountered with increasing frequency in the immune-competent patients. Another major factor contributing to the increased awareness of the importance of NTM as human pathogens is the improvement in the mycobacteriology laboratory techniques, resulting in enhanced isolation and more rapid and accurate identification of NTM from clinical specimens [3]. Consistent with advances in mycobacteriological laboratory techniques is the emphasis on the identification of individual NTM species and the clinical disease-specific syndromes they produce [4]. The number of NTM species has been steadily

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increasing [5] and currently there are more than 160 NTM species [6].

Although the reservoir of infection in most cases remains unclear, there is a general notion that NTM infections are derived mainly from the environment. NTM are widely distributed in nature and have been isolated from water and soil with water being the major reservoir [7]. There are a variety of situations where human and mycobacterial geographical and environmental distributions can overlap leading to exposure of humans. A major overlap occurs with water where humans are exposed to mycobacteria in water through drinking, swimming and bathing [8]. Aerosols generated during some of these activities can also lead to human exposure [9]. The presence of NTM in water, coupled with their disinfectant resistance, leads to their presence in hot tubs, solutions used in medical treatments and water–oil emulsions used to cool metal working tools [10]. It is however, generally believed that the majority of human-mycobacterial interactions are transient, self-curing colonisations [11,12]. These subclinical human-mycobacterial interactions may give a transient stimulation of certain pathways that may set the stage for manifestation of other diseases [4].

Non-tuberculous Mycobacteria are often involved in nosocomial outbreaks [13], although there is little or no evidence for person-to-person transmission of these organisms [3]. However, the significance of isolation of these organisms in clinical samples remains unclear since the number of diseases they cause is difficult to assess and no system for notification exists as in the case of *M. tuberculosis*. In addition, treatment and infection control measures vary according to the aetiological species [3]. Therefore, rapid and accurate identification of mycobacteria to the species level is essential to facilitate early treatment of mycobacterioses.

Zambia is a high burden country for tuberculosis and patients with chronic pneumonia, lymphadenitis, pyrexia of unknown origin and other chronic infections are evaluated for tuberculosis through microbiological cultures of various clinical specimens. In the process of isolating *M. tuberculosis*, NTM are also isolated from these specimens, without any attempt to identify them to species level. Therefore this study was initiated to identify NTM to species level for ease of managing such suspect conditions.

Materials and methods

This was a retrospective study of 91 isolates stored over a period of three and half years from January 2009 to June 2012 from four regions of Zambia (Eastern, Lusaka, Southern and Western). The stored isolates were revived using Lowestein Jensen (LJ) and Mycobacterium Growth Indicator Tube (MGIT) by standard microbiological procedures [14]. The cultures were then subjected to PCR identification and DNA sequencing of the 23S rRNA (ITS) region with primers Sp1 (5′-ACC TCC TTT CTA AGG AGC ACC-3′) and Sp2 (5′-GAT GCT CGC AAC CAC TAT CCA-3′) [15]. The obtained sequences were compared with those available in GenBank by BLAST searches. Sequences that displayed at least 98% sequence identity when compared to those in the GenBank were preliminary considered as identified species [16].

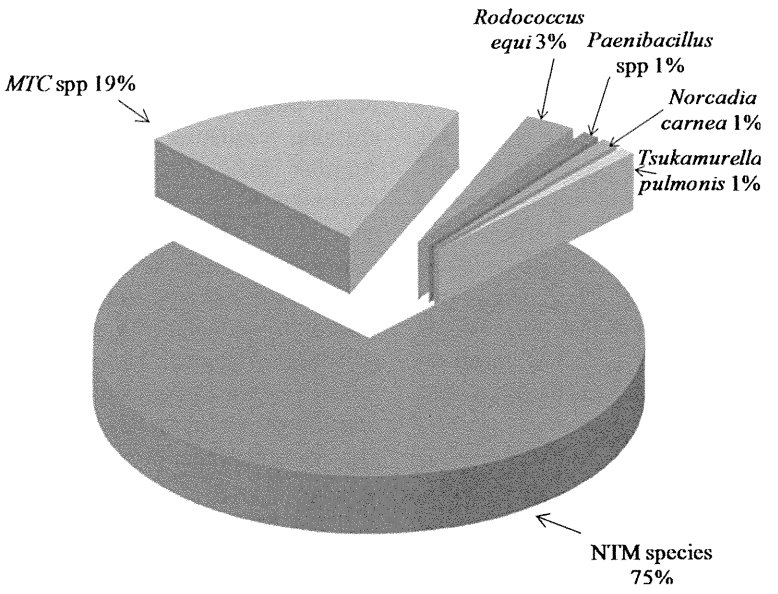

Figure 1 Organisms identified by sequence analysis of the ITS positive PCR amplicons.

Table 1 Spectrum and Identity of NTM species

NTM species	No.	Frequency (%)
M. intracellulare	15	27.8
M. lentiflavum	9	16.7
M. avium	8	14.8
M. fortuitum	4	7.41
M. gordonae	4	7.41
M. kumamotonense	2	3.70
M. indicus pranii	2	3.70
M. peregrinum	2	3.70
M. elephantis	1	1.85
M. flavescens	1	1.85
M. asiaticum	1	1.85
M. bouchedurhonense	1	1.85
M. chimaera	1	1.85
M. europaeum	1	1.85
M. neoaurum	1	1.85
M. nonchromogenicum	1	1.85
Total	**54**	**100**

Results

Sequence analysis and identification of the ITS region of the 91 strains showed: NTM species (68), *Mycobacterium tuberculosis* complex (17), *Rodococcus equi* (3), *Tsukamurella pulmonis* (1), *Norcadia carnea* (1) and *Paenibacillus* species (1) as shown in Figure 1 and Additional file 1: Table S1. Of the 68 NTM isolates, 54 were identified to species level as shown in Table 1, while 14 could not be identified. The 54 NTM species identified belonged to 16 different species with *M. intracellulare* exhibiting the highest frequency of identity (Additional file 2). Furthermore, *M. intracellulare* was the only NTM specie identified in the four regions of Zambia under study, with Lusaka region having a higher frequency (10), Southern (3), Western (1) and Eastern (1). *M. fortuitum* was identified in the Eastern and Lusaka regions, with one and three isolates respectively. All the other 14 species identified were from the region of Lusaka. A map of Zambia showing regions of distribution of various NTM identified in this study is shown in Figure 2.

Discussion

Non-tuberculous Mycobacteria have gained a lot of clinical significance in the last couple of decades in immunocompromised and immunocompetent individuals or patients [2]. Their ubiquitous distribution in nature put them at an advantage of having hosts close to ecological niches compounded by human activities. This might be the first study in Zambia to identify NTM species using PCR and DNA sequencing of the ITS region. This study has provided a range of NTM species which are potentially pathogenic. A total of 64 isolates were initially identified as NTM species. On sequencing and GenBank comparison, only 54 were identified to species level using the preliminary identification strategy which

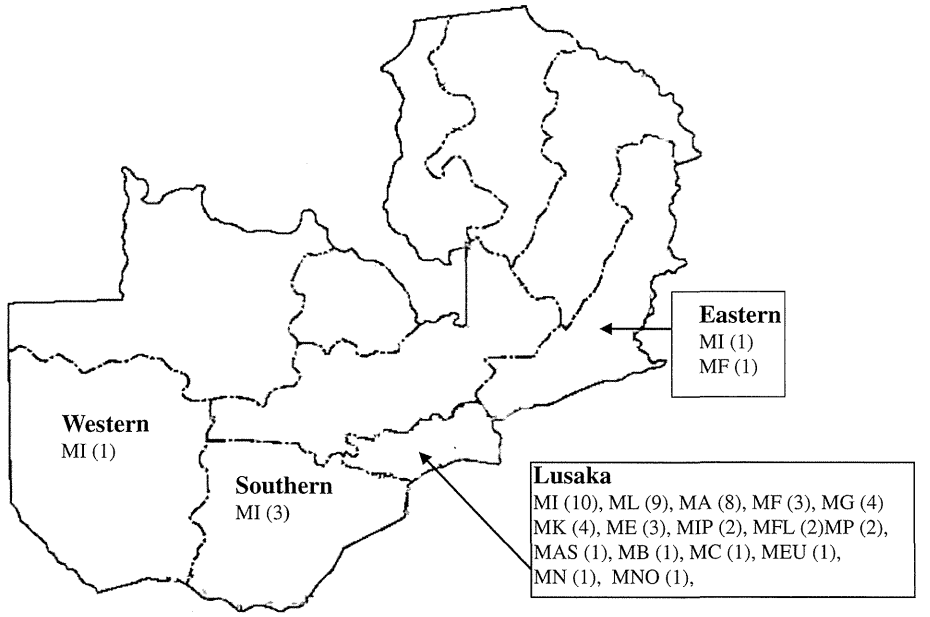

Figure 2 Map of Zambia showing regions of various NTM identified. The regions are indicated in bold with the identified NTM. MI (*M. intracellulare*), ML (*M. lentiflavum*), MA (*M. avium*), MF (*M. fortuitum*), MG (*M. gordonae*), MK (*M. kumamotonense*), ME (*M. elephantis*), MIP (*M. indicus pranii*), MFL (*M. flavescens*), MP (*M. peregrinum*), MAS (*M. asiaticum*), MB (*M. bouchedurhonense*), MC (*M. chimaera*), MEU (*M. europaeum*), MN (*M. neoaurum*), MNO (*M. nonchromogenicum*).

has been previously described [16]. The most prevalent species was *M. intracellulare* followed by *M. lentiflavum* and *M. avium*. This was in partial agreement with the findings of the study conducted by Buijtels and others [17] in the Eastern region of Zambia where sputum Mycobacterial culture isolates were identified by 16S rRNA gene sequencing. In this study *M. fortuitum*, was isolated from a clinical case. The other studies conducted in the Western and Northern regions of Zambia [18] and other parts of the world [19,20] were in contrast with these findings. The reason for this difference is that NTM species distribution differs from one geographical region to another [21].

M. intracellulare has been identified as the important species of the *Mycobacterium avium* complex. It has been identified together with *M. avium* as a complex because of their close similarities. *M. intracellulare* has been found to be more pathogenic than *M. avium* [22] and have been reported to cause disease not only in immunocompromised but also in immunocompetent subjects [23]. Other NTM species such as *M. lentiflavum* and *M. avium* have been implicated in clinical disease of immunocompromised as well as immunocompetent individuals [24,25]. *M. lentiflavum* has been isolated from various human specimens including pleural effusions, ascites and lung tissue [26,27] and have mainly been associated with causing an array of infections in immunocompromised patients [28]. Unlike *M. intracellulare,* most *M. avium* species do not multiply in monocytes of healthy individuals [29]. *M. fortuitum* infrequently cause a variety of diseases including bone and soft tissue infections, lymphadenitis and post-surgical infections and lung disease [30]. *M. kumamotonense, M. indicus pranii, M. flavescens, M. bouchedurhonense, M. chimaera, M. europaeum* and *M. nonchromogenicum* were identified and reported for the first time in Zambia. Some of these NTM have been associated with clinical disease [31,32] while *M. indicus pranii* is an atypical saprophytic bacterium that has raised a lot of research interest in leprosy immunotherapeutic [33]. *M. flavescens* has been isolated from the synovial fluid of an AIDS patient [34], whereas *M. bouchedurhonense* and *M. chimaera* have been documented in some respiratory tract infections [35]. *M. europaeum* was isolated from the sputum samples of an Iranian human immunodeficiency virus-infected patient and a cystic fibrosis patient with chronic pulmonary disease [36] while *M. nonchromogenicum* has been associated with sarcoidosis [37].

Other organisms which are not NTM that were identified include *Mycobacterium tuberculosis* complex species, *Rodococcus equi, Nocardia carnea, Tsukamura pulmonis* and *Paenibacillus* species. Of significance is the identification of *Rodococcus equi* from a clinical specimen in Zambia. This is the second time *Rodococcus equi* has been reported in Zambia [38]. The organisms: *Rodococcus equi, Nocardia carnea, Tsukamura pulmonis* and *Paenibacillus* species have been known to cause pulmonary diseases that are similar to tuberculosis [39-41]. Management of infections by these agents is different from that of tuberculosis. Therefore species identification of NTM remains of great importance as it provides an opportunity to develop a database that may help increase the scope of mycobacterioses.

Availability of supporting data

The data supporting the results of this study are included within this article.

Additional files

Additional file 1: Table S1. Species with the highest degree of nucleotide sequence identity to isolates from Zambia.

Additional file 2: Nucleotide sequences of Zambian isolates.

Abbreviations

NTM: Non-tuberculous mycobacteria; MGIT: Mycobacteria growth indicator tube; PCR: Polymerase chain reaction; LJ: Lowestein Jensen; ITS: Intergenic transcribed spacer.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MG and KG conceived and designed the experiments. MG, HBM and SE performed the experiments. MG, KG, HBM, SE and SS analysed and interpreted the data. KT, NS and SY helped in study design, coordinated the study and reviewed the manuscript. MG and HBM wrote the manuscript. All authors have read and approved the final manuscript.

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Genetic Diversity and Dynamic Distribution of Mycobacterium tuberculosis Isolates Causing Pulmonary and Extrapulmonary Tuberculosis in Thailand

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Genetic Diversity and Dynamic Distribution of *Mycobacterium tuberculosis* Isolates Causing Pulmonary and Extrapulmonary Tuberculosis in Thailand

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This study examined the genetic diversity and dynamicity of circulating *Mycobacterium tuberculosis* strains in Thailand using nearly neutral molecular markers. The single nucleotide polymorphism (SNP)-based genotypes of 1,414 culture-positive *M. tuberculosis* isolates from 1,282 pulmonary tuberculosis (PTB) and 132 extrapulmonary TB (EPTB) patients collected from 1995 to 2011 were characterized. Among the eight SNP cluster groups (SCG), SCG2 (44.1%), which included the Beijing (BJ) genotype, and SCG1 (39.4%), an East African Indian genotype, were dominant. Comparisons between the genotypes of *M. tuberculosis* isolates causing PTB and EPTB in HIV-negative cases revealed similar prevalence trends although genetic diversity was higher in the PTB patients. The identification of 10 reported sequence types (STs) and three novel STs was hypothesized to indicate preferential expansion of the SCG2 genotype, especially the modern BJ ST10 (15.6%) and ancestral BJ ST19 (13.1%). An association between SCG2 and SCG1 genotypes and particular patient age groups implies the existence of different genetic advantages among the bacterial populations. The results revealed that increasing numbers of young patients were infected with *M. tuberculosis* SCGs 2 and 5, which contrasts with the reduction of the SCG1 genotype. Our results indicate the selection and dissemination of potent *M. tuberculosis* genotypes in this population. The determination of heterogeneity and dynamic population changes of circulating *M. tuberculosis* strains in countries using the *Mycobacterium bovis* BCG (bacillus Calmette-Guérin) vaccine are beneficial for vaccine development and control strategies.

Tuberculosis (TB) remains one of the most infectious and deadly diseases worldwide and has significant medical, social, and economic impacts. The *Mycobacterium bovis* BCG (bacillus Calmette-Guérin) vaccine was first used in humans in 1921, while the first effective anti-TB drug, isoniazid, was developed in 1952 (1). The incidence of TB has declined with the worldwide distribution of the BCG vaccine and improving living conditions (2); however, the protective effect of the vaccine remains controversial. The likely factors influencing the degree of disease variation, including host and environmental factors, and/or mycobacterial traits are largely unknown (3). There are several lines of evidence that indicate that genetic variation in *Mycobacterium tuberculosis* contributes to the ambiguities concerning disease presentation, frequency of transmission, BCG vaccine response, and treatment outcome (4–6). Host factors, immune status, nutrition, and genetic polymorphisms are known to affect BCG vaccine efficacy (7, 8). The protective effect of the Glaxo freeze-dried BCG vaccine was more than 75% in the United Kingdom but displayed low efficiency in Malawi (8). Genetic evolution trees of humans and *M. tuberculosis* strongly indicate an adaptation of TB bacteria to the changing human population (3). Improving TB control requires a better understanding of the impact of the environment on the interaction between the pathogen and its host. Like other high-TB-burden countries, Thailand has officially included the BCG vaccine in the national vaccination program since 1977. However, the production of BCG vaccine was started and held in a small-scale setting in 1953 by the Queen Saovabha Memorial Institute (QSMI). In 1987, the BCG vaccine strain in Thailand was changed from a Danish strain to a Tokyo strain, and the dose was also changed from two doses to one dose at birth; 100% coverage of BCG vaccination in Thailand was achieved in 1990 (9, 10). Although the BCG vaccine appears to provide a high level of protection against tuberculous meningitis and disseminated TB in children, Beijing (BJ) strains have been reported as the most common genotype causing both pulmonary tuberculosis (PTB) and tuberculous meningitis in Thailand (6, 11, 12). Therefore, BJ strains might be postulated to represent one of the most common Thai-adapted genotypes in the mass vaccination environment.

Several different molecular typing methods have been used to study genetic diversity within *M. tuberculosis* complex (MTBC)

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This work is dedicated to Her Royal Highness Princess Galyanivadhana KromLaung Narathiwas Rajnakarindth on her 90th birthday.

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TABLE 1 Incidence of PTB and EPTB cases in Thailand from 1995 to 2011 by age group and HIV status

Age group (yr)	No. of PTB patients (%)			No. of EPTB patients (%)			Total no. of patients (%)
	HIV^{-a}	HIV^{+a}	Unknown	HIV^{-}	HIV^{+}	Unknown	
0–10	4 (0.4)	0 (0.0)	0 (0.0)	0 (0.0)	1 (2.8)	11 (11.8)	16 (1.1)
11–20	61 (5.7)	1 (0.5)	1 (5.6)	1 (33.3)	1 (2.8)	4 (4.3)	69 (4.9)
21–30	142 (13.4)	54 (26.6)	2 (11.1)	0 (0.0)	14 (38.9)	22 (23.7)	234 (16.5)
31–40	171 (16.1)	98 (48.3)	0 (0.0)	1 (33.3)	13 (36.1)	31 (33.3)	314 (22.2)
41–50	227 (21.4)	35 (17.2)	7 (38.9)	1 (33.3)	4 (11.1)	12 (12.9)	286 (20.2)
51–60	182 (17.2)	14 (6.9)	1 (5.6)	0 (0.0)	2 (5.6)	7 (7.5)	206 (14.6)
61–70	146 (13.8)	1 (0.5)	2 (11.1)	0 (0.0)	0 (0.0)	3 (3.2)	152 (10.7)
71–80	105 (9.9)	0 (0.0)	4 (22.2)	0 (0.0)	1 (2.8)	2 (2.2)	112 (7.9)
>80	23 (2.2)	0 (0.0)	1 (5.6)	0 (0.0)	0 (0.0)	1 (1.1)	25 (1.8)
Total	1,061 (82.8)	203 (15.8)	18 (1.4)	3 (2.3)	36 (27.3)	93 (70.5)	1,414 (100.0)

[a] HIV status is indicated as negative (−) or positive (+).

clinical isolates. Because MTBC isolates contain less genetic diversity than many other bacteria, molecular markers that reveal individual and group differences of the strains under investigation have to be selected according to the study purpose. The unique rate of change of each marker is an important factor in marker selection. Repetitive sequences evolve more quickly than single nucleotide polymorphisms (SNPs) and large-sequence polymorphisms. Therefore, evolutionary studies do not commonly use repetitive sequences. The unique polymorphic properties of SNPs are a valuable measurement for categorizing *M. tuberculosis* clinical isolates into genetically related groups. Recently, we developed a high-throughput DNA chip that provides genotyping results congruent with corresponding phylogenies inferred from large-sequence polymorphisms, spoligotyping, mycobacterial interspersed repetitive-unit–variable-number tandem repeat analysis based on 12 loci, principal genetic groups based on *katG463-gyrA*95 polymorphisms, and another SNP set (13, 14). In particular, *M. tuberculosis* snip cluster group 1 (SCG1) and SCG2 strains were completely comparable to East African-Indian (EAI)/Indo-Oceanic and BJ/East Asia genotypes, respectively (14). Interestingly, neutral genetic variations across modern *M. tuberculosis* populations are categorized into different groups based on spoligotype, IS*6110* sequence, or mycobacterial interspersed repetitive-unit–variable-number tandem repeat typing, in which outlier genetic diversity is commonly caused by genetic convergence.

In Asian countries such as China (15), Myanmar (16), Japan (17), South Korea (18), Taiwan (19), Thailand (6, 11, 12), and Vietnam (20, 21), *M. tuberculosis* strains belonging to the BJ and EAI genotypes are predominant. In Europe and America, *M. tuberculosis* Haarlem (H), S, X, T, and Latin American and Mediterranean (LAM) genotypes, alternatively known as the Euro-American lineage, have been predominantly isolated from TB-positive patients. In addition, evolution and phylogeography studies indicate that EAI strains represent the ancestral *M. tuberculosis* genotypes and are linked to ancestral African predecessors (3). For BJ genotype strains, several human-mycobacterial coevolution studies have provided evidence of BCG vaccine impact on the selection of *M. tuberculosis* isolates (22). Differential protein expression of the BJ genotype contributes to heterogeneous immune responses (23). In countries where BCG vaccination is uncommon, such as Ethiopia, the genetic diversity of *M. tuberculosis* strains is higher than in countries where it is common, such as Tunisia. It is therefore likely that selection of *M. tuberculosis* strains resistant to BCG vaccine-induced immunity has occurred (24).

This study aimed to assess the genetic diversity and dynamicity of the *M. tuberculosis* population in different age groups of patients in Thailand using the aforementioned high-throughput DNA chip, which is based on the DigiTag2 platform (13). The results provide useful, up-to-date epidemiological data on tuberculosis and may be able to help predict future trends in the prevalence of certain genotypes.

MATERIALS AND METHODS

Study population and patient data. A total of 1,414 culture-positive *M. tuberculosis* isolates from 1,282 PTB and 132 extrapulmonary TB (EPTB) patients in Thailand were characterized in this study (Table 1). PTB is defined as a case of TB in which the patient has disease only in the lungs, whereas in EPTB cases, the disease involves at least one nonpulmonary site. All isolates causing PTB and two causing EPTB (one from a patient with lymph node infection and one from a patient with TB pleurisy) were obtained from 13 hospitals covering 13 of 18 districts in Chiang Rai Province, Thailand, from 1998 to 2011. To increase the number of isolates associated with cases of EPTB, which are extremely rare, an additional 130 previously collected *M. tuberculosis* isolates from cases of meningitis, collected from 1995 to 2008, were retrieved from the Molecular Mycology and Mycobacteriology Laboratory (Drug-Resistant Tuberculosis Research Fund), Department of Microbiology, Faculty of Medicine, Siriraj Hospital, Mahidol University, Thailand, and included in this study (6, 11). Demographic data, including age and human immunodeficiency virus (HIV) status, were collected by physicians and trained medical staff. For determining the changing prevalence of *M. tuberculosis* genotypes across the patients from the pre- and post-BCG vaccine era, the genotype of 396 *M. tuberculosis* isolates from PTB patients who were born prior to 1953 and of 873 isolates from PTB patients born postintroduction of the BCG vaccine were recruited. Because approximately 70% of EPTB patients were of unknown HIV status, only the genotypes of the 1,269 isolates from PTB patients with HIV-negative status who were 15 to 65 years old were analyzed. Isolates from patients in this group who were born before and after 1953 were analyzed in parallel. The ethical and scientific committees of the Faculty of Medicine, Siriraj Hospital, Mahidol University, approved the study protocols (EC no. 759/2551 and EC no. 604/2555).

SNP genotyping. The sequence types (STs) and SCGs of all *M. tuberculosis* isolates were determined using the MTBC-targeting DigiTag2 assay, based on 51 highly informative SNPs (13, 14). The first four SNPs were used to screen MTBC species. The next 47 SNPs were chosen for genotyping *M. tuberculosis* isolates, with 2 SNP positions for determina-

tion of principal genetic groups and 45 SNPs for characterizing *M. tuberculosis* isolates into six SCGs (14, 25). Based on the DNA sequence of *M. tuberculosis* H37Rv (GenBank accession no. NC_000962), SNPs at either position 43943 or 3440542 were able to discriminate between ancestral (TbD1[+]) and modern (TbD1[−]) *M. tuberculosis* genotypes. In addition, the polymorphisms at nucleotide 4280708 are the same as the absence or presence of RD105 and were used to define the BJ genotype (13). The SNP at position 1477598 is the same as the *ogt12* polymorphism and was used to classify the ancestral and modern BJ genotypes (26). The perfect concordance of the evolutionary events between either SNP 797736 or SNP 2825581 and the RD181 marker was also used to subdivide the ancestral BJ genotype into early and late genotypes (27).

Genomic DNA from all *M. tuberculosis* isolates was extracted using the cetyltrimethylammonium bromide (CTAB) method (28). PCRs (10 μl) contained 20 to 40 ng of bacterial DNA, 25 nM each primer, and 1× KAPA2G Robust HotStart Ready Mix with dye (Kapa Biosystems, Woburn, MA, USA). Two-step multiplex PCR for increasing target SNPs was performed for 15 min at 95°C, followed by 40 cycles of 95°C for 90 s and 68°C for 2 min using a TGradient thermal cycler (Biometra, Göttingen, Germany). In the ligation reaction, the perfect-match 3′ query-cD1s probes were first phosphorylated at 37°C for 30 min and then incubated at 95°C for 3 min in a phosphorylation mixture containing 1× protruding-end kinase buffer, 30 mM ATP, 40 U of polynucleotide kinase, and 1 μM each 3′ query probe (Kination kit, Toyobo, Osaka, Japan) using a TGradient thermal cycler (Biometra). Next, 100 nM mismatch-induced 5′ query cEDs probes were mixed with 100 nM phosphorylated perfect-match 3′ query cD1s probes for the encoding reaction. The encoding mixture, containing 1 μl of multiplex PCR product, 50 nM each 5′ query cED and 3′ query cD1s probe mixture, 1× *Taq* DNA ligase reaction buffer, and 10 U of T4 DNA ligase (New England BioLabs, Beverly, MA, USA), was incubated at 95°C for 5 min and at 59°C for 15 min and then held at 10°C using a TGradient thermal cycler (Biometra). For the labeling reaction, the mixture (12-μl total volume) contained 6 μl of ligation product, 3 nM each D1s primer, 0.5 μM (each)Alexa 647-ED-2 and Alexa 555-ED-1 primer, 1.5× KAPA2G Fast buffer, 4.5 mM Mg^{2+}, 0.2 mM (each) deoxynucleoside triphosphate (dNTP), and 0.4 U of KAPA2G Fast HotStart DNA polymerase (Kapa Biosystems). The reaction mixture was cycled for 15 min at 95°C for the initial denaturation step, followed by 30 cycles of 95°C for 1 min, 55°C for 6 min, and 72°C for 30 s, before being held at 10°C using a TGradient thermal cycler (Biometra) (13). To define the SNP sequence type of each position on a DNA microarray, fluorescent signal intensities were evaluated using SNPStar software (version 0.0.0.8; Olympus, Tokyo, Japan). The SNP profiles indicating either the ST or SCG were compared with previous data (14, 26, 29). When necessary, the novel STs were confirmed by direct sequencing along with previous spoligotyping results (N. Smittipat, personal communication).

Statistical analysis. Descriptive statistics of patient clinical and demographic data, including patient age, HIV status, and disease phenotypes, are presented as the number, percentage, or median, as appropriate. The association between bacterial genotypes among the PTB and EPTB groups was evaluated using a chi-square test. A P value of ≤ 0.05 was considered statistically significant. Associations between the bacterial genotypes and pre- and post-BCG vaccination eras among all TB cases were determined by calculating the odds ratio with 95% confidence intervals (CI).

RESULTS

Genetic diversity of the studied *M. tuberculosis* PTB- and EPTB-causing strains based on SNP typing. The age of the PTB and EPTB patients ranged from 9 to 104 years (median, 45 years) and from 3 months to 83 years (median, 33 years), respectively. The HIV status was known in most of the PTB cases (n = 1,264). Unfortunately, for approximately 70% of the EPTB cases (n = 93), the HIV status was unknown (Table 1). Based on comparison with the global collection of *M. tuberculosis* strains, the 1,414 clinical *M. tuberculosis* isolates were classified into eight SCGs: including SCG1 (39.4%), -2 (44.1%), -3a (1.6%), -3b (1.6%), -3c (0.1%), -4 (0.1%), -5 (11.8%), and -6a (0.6%). Among this collection, we found 28 STs, including nine novel STs (named STTh1 to STTh9) (Table 2). Interestingly, the incidence of SCG3a (1.7%) was higher than that of SCG3b (1.5%) among PTB patients while SCG3a was not found among the EPTB patients, and SCG3b was detected in a higher percentage of samples (3.0%). The population size may affect this finding, as well as the greater variation observed in *M. tuberculosis* genotypes of the PTB patients (27 STs) than in the EPTB patients (13 STs). STF was not identified in PTB patients but was found in EPTB patients. Conversely, STTh1 to STTh9 and STs 3, 9, 11, 21, 25, and 41 were detected only in PTB patients. However, the prevalence trend of *M. tuberculosis* infections causing PTB and EPTB in this study displayed no significant association between the site of disease and the SCG of the infecting bacterial isolate (P = 0.07).

Interestingly, our results revealed that in Thailand, *M. tuberculosis* SCG2 accounted for a large proportion of the strains and also displayed the highest genetic diversity (13 STs). There was a preferential expansion of modern ST10 (15.6%) and ancestral ST19 (13.1%) strains of the BJ genotype, which indicates either good adaptation or high efficiency of transmission. The few predominant *M. tuberculosis* subtypes in this population indicate that the Thai-adapted *M. tuberculosis* genotypes have a selective advantage. Moreover, 13 isolates from PTB patients were heterozygous mixed-strain *M. tuberculosis* infections that included ST15 plus ST3 (1 isolate), ST15 plus ST10 (2 isolates), ST15 plus ST19 (1 isolate), ST15 plus ST18 (1 isolate), ST15 plus ST24 (2 isolates), ST10 plus ST24 (1 isolate), ST19 plus ST24 (1 isolate), ST25 plus ST24 (2 isolates), and ST18 plus ST19 (2 isolates). These findings were very similar to the spoligotyping results (data not shown).

Dynamic change in *M. tuberculosis* genotypes between different patient age groups. To clarify whether *M. tuberculosis* co-adapted with its host to cause disease, the distribution of bacterial genotypes within the different patient age groups was determined. Patient ages were obtained from hospital registration records. We divided the subjects into eight age groups in 10-year intervals to determine the dominant *M. tuberculosis* genotype for each age group. Mixed-genotype strains were excluded because of ambiguity between true mixed infection and DNA contamination during processing. The results revealed that all *M. tuberculosis* genotypes were distributed evenly within both patient groups (PTB and EPTB) but that the predominant strains within these populations differed between the age groups (Fig. 1). Among the 1,269 PTB patients, *M. tuberculosis* SCG1 was predominant in elderly patients, including patients aged 51 to 60 (46.4%), 61 to 70 (56.1%), 71 to 80 (53.7%), and >80 years (78.3%). Conversely, the most common *M. tuberculosis* genotype from samples collected from younger patients, including patients aged <10 years (75.0%), 11 to 20 (65.1%), 21 to 30 (57.1%), 31 to 40 (43.9%), and 41 to 50 years (42.7%), was SCG2. In addition, *M. tuberculosis* SCG5 was significantly more common in young patients than in elderly patients. Although there were fewer cases of EPTB (n = 132), the prevalence still clearly displayed an infection trend of *M. tuberculosis* SCG1 in elderly patients and SCG2 in younger patient groups.

There is some evidence of disruption of sympatric host-pathogen coevolution by HIV infection (30). The dynamic distribution of *M. tuberculosis* genotypes among different age groups of the HIV-negative PTB patients indicated the presence of clonal selection among patient age groups (Fig. 1b). HIV infection has fre-

TABLE 2 Diversity of SNP genotyping patterns between *M. tuberculosis* strains causing PTB and EPTB

SCG	ST	No. of PTB cases (%)	No. of EPTB cases (%)	SNP profile[a]
1	15	506 (39.9)	47 (35.6)	GGGCTGCCTTCCCTCCGACGTCGGAAGATTCAGGGCCTGCCCGGG
	Th1	1 (0.1)	0 (0.0)	...T.....
	Th2	3 (0.2)	0 (0.0)	.....G.....
2	3	20 (1.6)	0 (0.0)	.A....T.....GG.....A.....T.T.A
	8	4 (0.3)	2 (1.5)	.A....TT.....T.G..C...G.AGG.....A.....TTT.A
	10	179 (14.1)	42 (31.8)	.A....TT.....T....C.....GG.....A.....TTT.A
	11	1 (0.1)	0 (0.0)	.A.....G.....A.....T.T.A
	19	176 (13.9)	9 (6.8)	.A....TT.....C.....GG.....A.....TTT.A
	22	70 (5.5)	14 (10.6)	.A....TT.....T....C...G.AGG.....A.....TTT.A
	25	39 (3.1)	0 (0.0)	.A....T.....C.....GG.....A.....TTT.A
	26	10 (0.8)	2 (1.5)	.A.....T.....G.....A.....T.T.A
	F	0 (0.0)	1 (0.8)	.A....T.....T....C.....GG.....A.....TTT.A
	K	50 (3.9)	1 (0.8)	.A....TT.....GG.....A.....T.T.A
	Th3	1 (0.1)	0 (0.0)	.A....TT.....GG.....A.....TTT.A
	Th4	1 (0.1)	0 (0.0)	.A....TT.....C.....C.....GG.....A.....TTT.A
	Th5	2 (0.2)	0 (0.0)	.A....TT.....T....C.....AGG.....A.....TTT.A
3a	21	22 (1.7)	0 (0.0)	.A.....G.....A.....T.T..
3b	20	12 (0.9)	2 (1.5)	.A.....A.....T....
	34	7 (0.6)	2 (1.5)	.A.T.....A.....A..CA.T....
3c	Th6	1 (0.1)	0 (0.0)	.A.T....CA.....TA.....A..CA.T....
4	9	1 (0.1)	0 (0.0)	.A.T....CA.....A.TA.....A..CA.T....
5	7	47 (3.7)	3 (2.3)	.A.....ATG...T....
	24	103 (8.1)	6 (4.5)	.A.....G.....ATG...T....
	41	1 (0.1)	0 (0.0)	.A.....C.....G.....ATG...T....
	Th7	1 (0.1)	0 (0.0)	.A.....T.....ATG...T....
	Th8	1 (0.1)	0 (0.0)	.A.....G.G.....ATG...T....
	Th9	3 (0.2)	0 (0.0)	.A.....G.....ATG.....
6a	18	7 (0.6)	1 (0.8)	CAT.....G.....A.G..TT..A.

[a] The dot indicates that the nucleotide is identical to that in the reference sequence.

quently been reported to be a risk factor for manifestation of EPTB (31). Our data strongly agreed with these previous findings, with 36/39 EPTB patients being HIV positive (Table 1). Unfortunately, the bacterial genotype data across different age groups of HIV-negative EPTB patients could not be determined in this study (Fig. 1b).

Frequency distribution of circulating *M. tuberculosis* strains in Thailand. The most prevalent genotypes were SCG1, -2, and -5 among PTB cases (Table 3). While the rare *M. tuberculosis* genotypes SCG3a, -3b, -3c, -4, and -6a were found among isolates examined in the current study, they were statistically analyzed as "other" genotypes for the main trait. The frequency of distribution of bacterial genotypes of strains isolated from different age groups was significantly different between these populations ($P < 0.01$). Interestingly, a decrease in the prevalence of SCG1 was significantly associated with patients who were born in the post-BCG vaccination environment. On the other hand, statistical analysis revealed an increasing number of patients harboring *M. tuberculosis* SCG2 and -5 in the postvaccination population.

We attempted to reduce the possible risk factors of human hosts by adjusting the results of *M. tuberculosis* genotyping by the selective force of BCG vaccination for adult PTB patients who were 15 to 65 years old and HIV negative. Within the PTB patient group, there were changing dynamics in the prevalence of *M. tuberculosis* genotypes SCG1, -2, and -5 between the two groups (pre-BCG vaccine, $n = 181$ isolates; and post-BCG vaccine, $n = 662$ isolates) (Table 3). There was a negative correlation between patients who were born in the post-BCG vaccine era and *M. tuberculosis* SCG1 though there was a positive association with SCG2 and SCG5.

DISCUSSION

Our results provide up-to-date information on the genotypes of circulating *M. tuberculosis* strains after the first introduction of the BCG vaccine in Thailand in 1953. Several previous studies indicated the possible association between genotype and clinical expression; however, the small sample sizes of these studies meant that the results were unreliable (12, 32, 33). In this study, SNP-based genetic variation of circulating *M. tuberculosis* isolates was investigated by examining a large number of *M. tuberculosis* isolates from patients with PTB and EPTB in Thailand. All isolates were collected from individual patients, and probable outbreak cases were ruled out. A previous study on TB meningitis by our group provided data similar to the data from the current study in

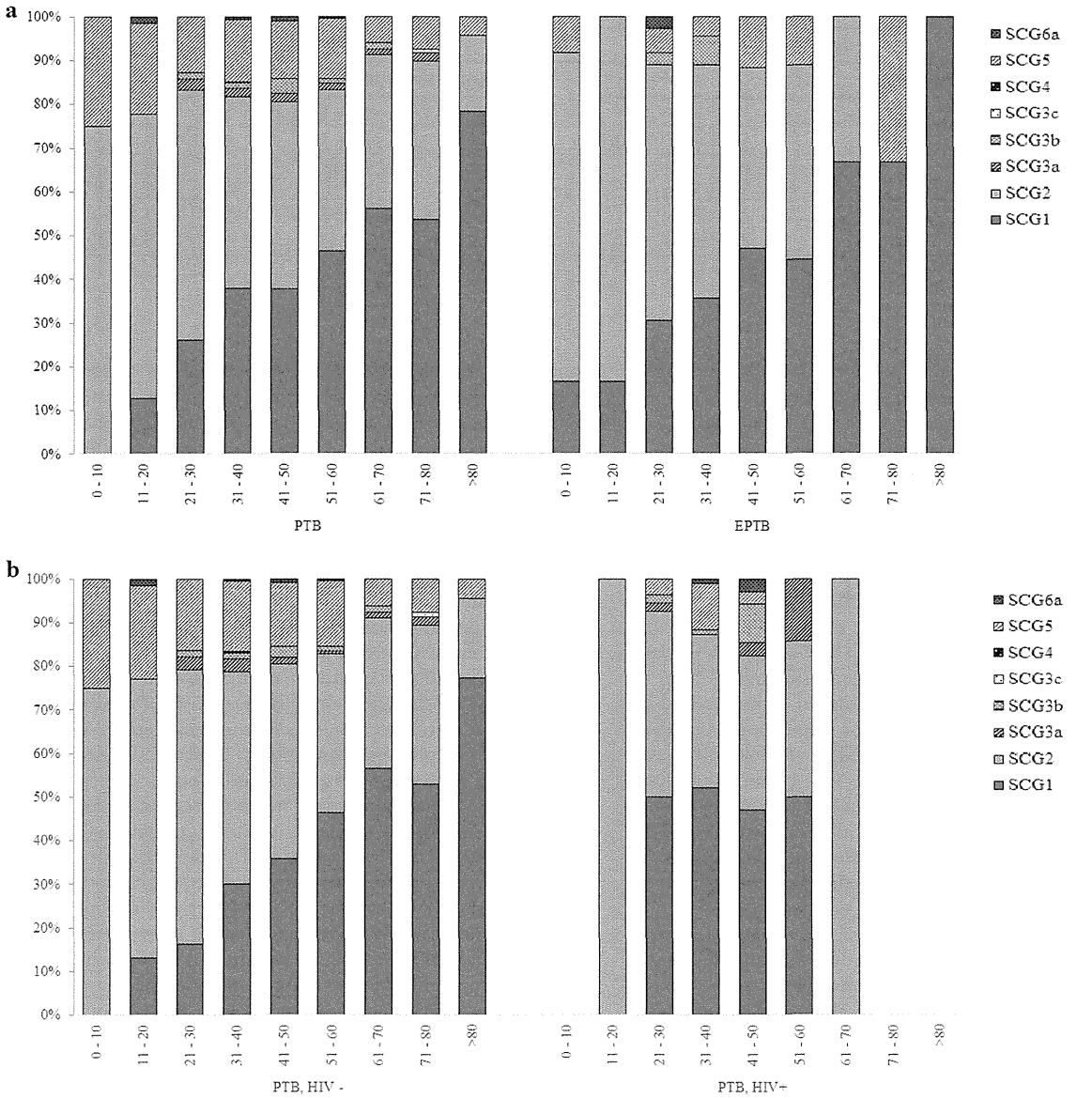

FIG 1 Trend of *M. tuberculosis* genotypes for the different patient age groups. The SCG genotypes in the PTB and EPTB groups (a) and HIV-negative and -positive PTB groups (b) are shown as percentages. Age groups (years) are indicated along the *x* axes.

regard to the most prevalent *M. tuberculosis* genotypes (BJ strains, 56%; EAI, 31%; T, 10%; and H, 3%) (6, 11). Even with the addition of the PTB cases, both the BJ and EAI genotypes, which are classified as SCG2 and SCG1, respectively, were still found to be the endemic genotypes causing TB in Thailand (6, 11, 12, 34–36). The prevalences of each genotype did not differ significantly between the PTB and EPTB cases in our study. Studies from Madagascar (37) and Brazil (38) using both PTB and EPTB cases also implied that host or environmental factors, rather than bacterial lineages, have an impact on disease phenotypes. In our study, there was no significant difference in the association between the genotypes of *M. tuberculosis* strains and the locations from which the strains were collected. However, the high prevalence of the EAI genotype in PTB patients (48.4%, $n = 150$) might be caused by the migratory habits of people living in Chiang Rai Province, where there is a border between Thailand and Myanmar (16). The presence of the largest cluster, *M. tuberculosis* ST15 belonging to SCG1, could be caused by the low discriminatory power of the selected markers or by special characteristics of this ST when it is the cause of PTB in Thailand. As described previously by spoligotyping analysis, SCG1 strains are subdivided into many different genotypes (14). To better classify SCG1 strains, other highly informative SNPs or markers should be developed. In addition, in this study, it was not clear if the mixed *M. tuberculosis* infections evident from DNA samples were caused by contamination, mixed infection, or coevolution in the host. Our results based on the

TABLE 3 Prevalence comparison of SCGs between *M. tuberculosis* strains causing PTB

SCG	Prevalence by patient age and group (no. of patients [%])[a]					
	PTB patients (n = 1,269)			HIV-negative adult PTB patients (n = 843)		
	Born after 1953 (n = 873)	Born before 1953 (n = 396)	Odds ratio (95% CI)	Born after 1953 (n = 662)	Born before 1953 (n = 181)	Odds ratio (95% CI)
1	292 (33.4)	218 (55.1)	0.4 (0.3–0.5)	191 (28.9)	89 (49.2)	0.4 (0.3–0.6)
2	415 (47.5)	138 (34.8)	1.7 (1.3–2.2)	331 (50.0)	70 (38.7)	1.6 (1.1–2.2)
5	128 (14.7)	28 (7.1)	2.3 (1.5–3.5)	112 (16.9)	16 (8.8)	2.1 (1.2–3.6)
Other[b]	38 (4.4)	12 (3.0)	1.5 (0.8–2.8)	28 (4.2)	6 (3.3)	1.3 (0.5–3.2)

[a] Prevalence was determined by the number of patients (%) positive for the SCG.
[b] Other, SCGs 3a, 3b, 3c, 4, and 6a.

developed DNA chip revealed evidence of mixed DNA in an individual sample by displaying heterogeneous results.

The predominant genotype in the current populations was SCG2, followed by SCG1 and SCG5, which frequently display higher transmissibility and virulence than other genotypes (22, 29). In other high-BJ-burden countries, such as China, a high prevalence of modern BJ strains has also been reported; however, in other countries, such as Japan and South Korea, more strains from ancestral BJ genotypes (ST3 and ST19 in Japan and ST11 or ST26 in South Korea) have been found. Modern BJ genotypes (ST10 and ST22) are also predominant in Taiwan and Peru (17, 18, 29). The close historical relationship between China (39) and Japan (17) might be responsible for the BJ sublineages in Thailand. The most frequently identified strains in Taiwan were similar to predominant BJ subtypes in our populations, including ST10 (35.42%), ST19 (29.65%), and ST22 (13.46%) (40). Advanced means of global travel could affect the spread of infectious strains among countries, and bacterial adaptation raises concern over transnational transmission. BCG mass vaccination and the high prevalence of BJ genotypes in Southeast Asia support the hypothesis of BCG vaccine subtype selection being responsible for limiting the dynamic distribution of BJ genotypes (41).

Differences in the distribution of *M. tuberculosis* genotypes across patient generations were observed in our study. Using different molecular markers and population age groupings, previous observations from countries where vaccination is required, including Thailand (12, 35), Vietnam (20, 21, 42), Taiwan (19), and China (15), have also revealed a higher frequency of *M. tuberculosis* genotype SCG2 strains causing TB in younger patients than in elderly patients. The association trend between EAI lineage strains (SCG1) and older patient age has also been reported in neighboring countries, including Vietnam (42). These results imply that BJ strains take the place of the EAI genotype in the current population. The differences in the percentages of particular bacterial genotypes across patient generations make it difficult to specify the roles of adaptation factors in this distribution. It is becoming increasingly accepted that the differential protein expression of bacterial genotypes could contribute to heterogeneous immune responses. The unique protein expression profile of the BJ genotype that affects immune suppression or modulation of the host may result in differences in BCG vaccine protection ability (23). Age of the patient and level of immunity at the time of bacterial reactivation and the degree of transmissibility could be emphasized. Our study was inconclusive in regard to the periods of infected individuals. Age at onset of disease was also not significantly associated with the duration of TB infection. The impacts of genotype-to-genotype variation, host immune response, and host susceptibility on the onset of the disease are largely unknown. However, based on our findings in PTB patients, it can be presumed that HIV coinfection probably interferes with this sympatric relationship (Fig. 1b). Further studies using a larger sample size and including data on host social behavior and demographic factors are needed to confirm the results of this pilot study.

According to several human-mycobacterial coevolution studies, there is evidence of the vaccination having an impact on the selection of *M. tuberculosis* genotypes (22). Most of the elderly patients included in the current study were born prior to the introduction of the BCG vaccine in Thailand in 1953. The particular *M. tuberculosis* genotypes of strains isolated from the younger patients were dynamic in terms of dominance. We hypothesized that BCG vaccine distribution accidentally introduced selective pressure into the *M. tuberculosis* population in Thailand. We identified an association between *M. tuberculosis* SCG and the clinical phenotype in all patients, regardless of whether they had exposure to the prevaccine environment. The results of the current study highlight the genetic diversity of cultured *M. tuberculosis* strains in Thailand. The data revealed that the majority of isolates were classified as genotypes SCG2 and SCG1, belonging to the BJ and EAI genotypes. An increase in the prevalence of *M. tuberculosis* SCG2 and SCG5 isolates was observed in patients who were born post-BCG vaccine implementation. These results indicate an ongoing transmission of these genotypes in the younger generation. Conversely, SCG1 genotype strains tended to cause disease in patients who were born prior to 1953. The presence of transmission dynamics among patients who were born during the BCG vaccination era indicates that mass BCG vaccination might be one of the selective factors that have driven the spread of the *M. tuberculosis* BJ genotype in Thailand. These epidemiological data on the currently circulating strains of *M. tuberculosis* could assist in the selection of appropriate vaccine strains in the future. Whether these strains are associated with BCG vaccination requires further study. In addition, further extended studies on successful BJ strains in vaccination countries are needed.

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We declare that we have no conflicts of interest.

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Mycobacteriology

Rapid identification of strains belonging to the *Mycobacterium abscessus* group through *erm*(41) gene pyrosequencing

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ABSTRACT

Mycobacterium abscessus and *Mycobacterium massiliense* lung infections have different clarithromycin susceptibilities, making proper identification important; however, standard multi-gene sequencing in clinical laboratories is laborious and time consuming. We developed a pyrosequencing-based method for rapid identification of strains belonging to the *M. abscessus* group by targeting *erm*(41). We examined 55 isolates from new pulmonary *M. abscessus* infections and identified 28 *M. abscessus*, 25 *M. massiliense*, and 2 *Mycobacterium bolletii* isolates. Multi-gene sequencing of 16S rRNA, *hsp65*, *rpoB*, and the 16S–23S ITS region was concordant with the results of *erm*(41) pyrosequencing; thus, the *M. abscessus* group can be identified by single-nucleotide polymorphisms in *erm*(41). The method also enables rapid identification of polymorphic, inducible clarithromycin-resistant sequevars (T28 or C28). Pyrosequencing of *erm*(41) is a rapid, reliable, high-throughput alternative method for identifying and characterizing *M. abscessus* species. Further testing of a diverse collection of isolates is necessary to demonstrate the discriminatory power of *erm*(41) sequencing to differentiating species with this highly divergent group.

1. Introduction

Genotypic analysis using single-target sequencing of the 16S rRNA gene cannot distinguish *Mycobacterium massiliense* from other mycobacteria in the *Mycobacterium abscessus* group (Leao et al., 2011; Zelazny et al., 2009). The *M. abscessus* group has been subclassified into 3 new species—*M. abscessus* (sensu stricto), *M. massiliense*, and *Mycobacterium bolletii* (Macheras et al., 2011; Nakanaga et al., 2011)—based on the sequences of *rpoB* and other housekeeping genes. The differentiation of these species is well founded: even long-read targets by multiple gene sequencing approached simultaneous detection (Nakanaga et al., 2011). However, these approaches are not rapid, and interpretation of the lengthy sequences produced by conventional methods is time consuming.

Pyrosequencing is a semi-automated method based on real-time monitoring of DNA synthesis (Heller et al., 2008; Jureen et al., 2006). Pyrosequencing is broadly applicable to analysis of single-nucleotide polymorphisms (SNPs), as well as identification and quantification of short DNA sequences for bacterial and viral typing (pyrosequencing generates short DNA sequences—typically 20–70 bp in length [Heller et al., 2008]). Unlike conventional Sanger sequencing, which lays a reading gap of roughly 20–30 bases from the sequencing primer, pyrosequencing can generate sequence signals immediately downstream of the primer. As sequencing starts with the first base next to the annealed primer, pyrosequencing primer design is quite flexible. Although pyrosequencing presents several advantages in comparison to standard sequencing, it is insufficient for identification of nontuberculous mycobacteria because not all species can be identified by the complete 16S rRNA gene sequence; additional phenotypic characterization is required (Cristia-Fernström et al., 2007; Tuohy et al., 2005). We selected an alternative target, the *erm*(41) gene, because of its uneven distribution of hypervariable and conserved regions. Methylase function is dependent on nucleotide 28 in *erm*(41) (Bastian et al., 2011; Nash et al., 2009). Importantly, the *erm*(41) sequence differentiates species within the *M. abscessus* group (Bastian et al., 2011). Specific features, such as the presence of deletions and the nature of the amino acid at position 10, are predictive of clarithromycin susceptibility or resistance (Bastian et al., 2011). We hypothesize that the short segment of the *erm*(41) gene may provide the information necessary for identification of these species.

Clarithromycin and other macrolides are common treatments for *M. abscessus* group infections. Bacteria gain resistance to macrolides through post-transcriptional methylation of the 23S bacterial ribosomal RNA, which inhibits drug attachment. *M. abscessus* infections tend

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to respond poorly to macrolide-based chemotherapy due to acquired and inducible resistance mechanisms. The expression of an inducible *erm*(41) gene confers high-level clarithromycin resistance in clinical isolates of *M. abscessus*. Species of the *M. abscessus* group have different inducible susceptibilities to clarithromycin (Bastian et al., 2011; Kim et al., 2010; Nessar et al., 2012), and their *erm*(41) gene features and inducible clarithromycin susceptibility patterns vary. *M. massiliense* harbors a truncated *erm*(41) gene and is intrinsically susceptible to clarithromycin, whereas *M. abscessus* has a complete *erm*(41) gene and variable clarithromycin susceptibility. The C28 polymorphism in *M. abscessus* (C28 sequevar) is associated with clarithromycin susceptibility; the T28 polymorphism (T28 sequevar) is associated with resistance. *M. bolletii*, which contains the T28 polymorphic *erm*(41) gene, is resistant to clarithromycin (Bastian et al., 2011; Kim et al., 2010; Koh et al., 2011). These susceptibility and resistance genotypes are especially important for differentiating species within *M. abscessus*.

Our objectives were to i) develop an efficient method for identification within the *M. abscessus* group and ii) evaluate the method for use in identifying clarithromycin resistance-associated mutations in clinical isolates of *M. abscessus*.

2. Materials and methods

2.1. Clinical isolates

We studied a sample of 55 rapidly growing mycobacteria that belonged to the *M. abscessus* group using a commercial DNA-DNA hybridization kit (DDH Mycobacteria; Kyokuto Pharmaceutical Industrial, Tokyo, Japan). Forty-nine of these isolates overlapped with a set of 143 isolates previously used to analyze genetic diversity (Yoshida et al., 2013). These 49 isolates were provided by the NHO Kinki-Chuo Chest Medical Center in Japan and collected between 2007 and 2010. We added 6 isolates obtained from patients in 2010 and 2011. All strains were stored in Middlebrook 7H9 broth with 15% glycerol at −80 °C. Each isolate was subcultured in Mycobacterium Growth Indicator Tubes (MGIT; Beckton Dickinson & Company, Fukushima, Japan) and Ogawa slant medium.

2.2. Sequence analysis

We evaluated the sensitivity of pyrosequencing using genomic DNA prepared from clinical isolates and reference strains (*M. abscessus* JCM13569T, *M. massiliense* JCM15300T, and *M. bolletii* JCM15297T). For DNA preparations suitable for PCR, culture samples were obtained by removing 1 mL from the bottom of the MGIT to a 1.5-mL screw cap tube. Tubes were centrifuged at 10,000*g* for 15 min, the supernatant was removed, and 0.1 mL Tris-EDTA buffer (TE; pH8.0) buffer was added. Tubes were heated at 95–100 °C for 30 min and centrifuged at 10,000*g* for 10 s. The supernatants were transferred to new tubes for pyrosequencing and multi-gene sequencing. Conventional sequencing methods were used to target the 16S rRNA gene, *hsp65*, *rpoB*, and the internal transcribed spacer (ITS) region between the 16S and 23S rRNA genes (Adékambi and Drancourt, 2009; Kim et al., 2008). To compare the genetic diversity of the 6 new isolates and the previous 49 isolates, we analyzed the same genetic markers used in the previous study (Yoshida et al., 2013) [the multiple gene sequence, *erm*(41) gene sequences, and 23S rRNA sequence (*rrl*)]. PCR products were sequenced using the Big Dye Terminator cycle sequencing kit v3.1 (Applied Biosystems, Tokyo, Japan) and the ABI Prism 310 genetic analyzer (Applied Biosystems). Species identification of *M. abscessus* group isolates and reference strains was accomplished using the Basic Local Alignment Tool (BLAST; http://www.ncbi.nlm.nih.gov/BLAST) to measure similarities.

2.3. Phenotypic characterization and drug susceptibility testing

BLAST searching yielded sequence identities; phenotypic characterization was performed by measuring growth rates and pigment production.

The MIC of clarithromycin was determined by the broth microdilution method for the previous 49 isolates (Yoshida et al., 2013) and the 6 new isolates. To determine reproducibility, we tested each isolate 3 times on 3 separate days. The broth microdilution method was performed with serial double dilutions of clarithromycin (0.015–64 mg/L), according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2011). Inoculated plates were placed in plastic bags, incubated at 35 °C in ambient air, and read at 72 h and 14 days. The MIC was defined as the lowest concentration of drug that inhibited visible growth.

2.4. Pyrosequencing

We performed PCR with a PyroMark PCR Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. The PCR product was converted to a single-strand DNA template and purified with the PyroMark Vacuum Workstation. We prepared 2 sets of primers for *erm* (41) detection: the forward sequencing PCR primer set are ERM F1 (5′-CCTAAGCGCAGTTCTGAC-3′) and ERM R1 (5′-(Biotin)-CCGCCCAGTCATCAGTGAG-3′), as well as the reverse sequencing PCR primer set are ERM F3 (5′-(Biotin)-GCCTAAGCGCACGTTCTGA-3′) and ERM R3 (5′-CTCCCCTGAGCGAACACC-3′). Each sequencing primer (0.3 μmol/L) for *M. abscessus* group identification (Sequence Analysis: SQA) and *erm*(41) genotyping (Allele Quantification: AQ) was selected to capture differences in *erm*(41). The SQA target was the −35 promoter region, which contains SNPs specific to *M. abscessus*, *M. massiliense*, and *M. bolletii*. The AQ target was the *erm*(41) coding sequence that includes the T/C 28 polymorphism associated with inducible clarithromycin resistance. Sequencing primers SQA1 (SQA: 5′-ACCGGGGCCTTCTTCGT-3′) and AQ1 (AQ: 5′-AACGGTCGCGACGC-3′) were used with the ERM F1R1 PCR products, and sequencing primers SQA2 (SQA: 5′-GGGCATGCAACTGGT-3′) and AQ2 (AQ: 5′-AGCGGATACCAGCC-3′) were used with the ERM F3R3 PCR products (Fig. 1). We followed the methods of Haanpera et al. (2005) to detect acquired macrolide resistance mutations at positions 2058 and 2059 (*Escherichia coli* numbering) of the 23S rRNA gene. Pyrosequencing was performed and analyzed in a PyroMark Q24 (QIAGEN) with PyroMark Gold Q24 Reagents and software (QIAGEN).

2.5. Statistical analysis

Using χ^2 tests, we analyzed the susceptibility of each subspecies based on their genetic patterns. All *P*-values were 2 sided, and values of $P < 0.05$ were defined as statistically significant. Statistical analyses were performed in Excel 2011 (Microsoft, Redmond, WA, USA).

3. Results

3.1. Genotypic identification

We performed multi-gene sequencing for 6 clinical isolates and 3 reference strains (*M. abscessus*, *M. massiliense*, and *M. bolletii*). The 6 clinical isolates, previously identified as *M. abscessus*, were successfully identified to the subspecies level. The pyrosequencing results of *erm*(41) for these 6 clinical isolates and the previously characterized 49 isolates led to the identification of sequevars, that is, 28 of *M. abscessus* (50.9%), 25 of *M. massiliense* (45.5%), and 2 of *M. bolletii* (3.6%), which was in 100% agreement with the multi-gene sequencing results (Table 1).

Our pyrosequencing approach yielded up to 24 short-reads in parallel sequences in less than 1 h. The pyrosequencing software accurately interpreted high-quality sequences, as assessed by matching accuracy. The *erm*(41) analysis with 2 primers yielded sequences (F1R1 and F3R3). Fig. 2 presents the SQA and AQ target pyrograms for each subspecies using the F1R1 primer set. We found full agreement between the F1R1 and F3R3 pyrosequencing results of 53 of 55 isolates (96.3%); however, F1R1 could not resolve 2 *M. abscessus* strains. Therefore, pyrosequencing with the F3R3 reverse primer set was necessary for complete identification of the mycobacterial species.

Mycobacterium abscessus HQ127365(T28), HQ127366(C28) (360 bp)

```
121 ggctcgctcg ccaacgacga gcagctcgcc gcactgcgcg agaagctggc aggcaacgcc
181 taagcgcacg ttctgacgaa agaaggcccc ggtcatggcg accggggcct tcttcgtgat
241 ctatcgaaac cagttgcatg ccccgatatc tttggagcat gggcatattc atgatggtgc
301 tgcgtcgtgt ccggccaacg gtcgcgacgc cagc/tggggct ggtatccgct cactgatgac
361 tgggcggcgc ggatcgtcgc cgaatccggt gttcgctcag gggagttcgt tgtggatctg
```

Mycobacterium massiliense HQ127368 (282 bp)

```
121 ggctcgctcg ccaacgacga gcagctcgcc gcactgcgcg agaagctggc aggcaacgcc
181 taagcgcacg ttctgacgaa agaaggcccc ggtcatggcg accggggcct tcttcgtgat
241 ttgtcgagac cagttgcatg ccccgatatc tttggagcgt gggcatattc atgatggtgc
301 tgtgtcgtgt ccggccaacg gtcgcgacgc cagtggggct ggtatcagct cgctgatgac
361 tgggcggcgg atcgtcgccg aatccggttt tcgttcaggg gagttcgttg tgaatctggg
```

Mycobacterium bolletii HQ127367 (360 bp)

```
121 ggctcgctcg ccaacgacga gcagctcgcc gcactgcgcg agaagctggc aggcaacgcc
181 taagcgcacg ttctgacgaa agaaggcccc ggtcatggcg accggggcct tcttcgtgat
241 ctgtcgaaac cagttgcatg ccccgatatc tttggagcat gggcatattc atgatggtgc
301 tgcgtcgtgt ccggccaacg gtcgcgacgc cagtggggct ggtatccgct cactgatgac
361 tgggcggcgc ggatcgtcgc cgaatccggt gttcgctcag gggagttcgt tgtggatctg
```

Primer	Id	Sequence	Nt	Tm, °C	%GC
PCR	ERM F1	CCTAAGCGCACGTTCTGAC	19	68.6	57.9
PCR(biotin)	ERM R1	CCGCCCAGTCATCAGTGAG	19	71.9	63.2
Sequencing	SQA 1	ACCGGGGCCTTCTTCGT	17	64.9	64.7
Target sequence (*M. abscessus*)	GATCTATCGAAACCAGTTGCATGCCCCGATA				
Sequencing	AQ 1	CAACGGTCGCGACGC	15	64.6	73.3
Target sequence (*M. abscessus*)	CAGC/TGGGGCTGGTATCCG				

Fig. 1. The forward ERM F1R1 primer set design for pyrosequencing. Gray characters: *erm*(41) promoter target(SQA), c/t:28 SNP target(AQ).

3.2. Susceptibility testing for clarithromycin and erm(41) genotyping

Table 2 presents the MIC_{50} (MIC at which 50% of the isolates tested are inhibited), MIC_{90} (MIC at which 90% are inhibited), and full MIC ranges for susceptibility to clarithromycin. Clarithromycin susceptibility was significantly greater for *M. massiliense* than for *M. abscessus* ($P < 0.05$). Of the 55 isolates that were tested using the proportion method, 51 were susceptible to clarithromycin at day 3. At 14 days, all 25 *M. abscessus* strains in the T28 sequevar gained resistance to clarithromycin (Table 2). In contrast, no strains of the *M. massiliense* or *M. abscessus* C28 sequevar appeared to be clarithromycin resistant.

erm(41) gene sequences were compared to published sequences (HQ127366, HQ127365, HQ127368, and HQ127367) (Bastian et al., 2011). Our results and the published sequences were in complete agreement on the following discriminable features of the *erm*(41) gene sequences that appeared to be species-specific: deletions, promoter sequences, and the 28th nucleotide belonging to the 10th codon (Bastian et al., 2011). *erm* (41) genotyping by pyrosequencing was completely consistent with the published *erm*(41) gene sequences. Assessment of the T28/C28 genotype of the *M. abscessus* and *M. bolletii* strains revealed that 89% (25/28) of the *M. abscessus* and 100% (2/2) of the *M. bolletii* strains belonged to the T28 sequevar, while 11% (3/28) of *M. abscessus* strains belonged to the C28 sequevar (Table 1). Fig. 2B presents the pyrograms for each of these sequevars.

Of the 55 studied isolates, 4 acquired resistance to clarithromycin: 7.1% (2/28) of *M. abscessus*, 100% (2/2) of *M. bolletii*, and 0% (0/26) of *M. massiliense* strains. Of these isolates, 1 *M. bolletii* strain contained a mutation (A2058G) in the 23S RNA sequence, while no mutations were observed in the remaining 3 strains.

Table 1
M. abscessus group identification by multiple gene sequencing and pyrosequencing.

Results of multiple gene sequencing (16S rRNA gene, *hsp65*, *rpoB*, and 16S–23S ITS region)	n	Results of pyrosequencing of *erm*(41)	n
M. abscessus	28	*M. abscessus*	28[a]
		T28 sequevar	25[a]
		C28 sequevar	3
M. massiliense	25	*M. massiliense*	25
		T28 sequevar	25
		C28 sequevar	0
M. bolletii	2	*M. bolletii*	2
		T28 sequevar	2
		C28 sequevar	0

[a] Two strains were required for pyrosequencing with the F3R3 primer set because pyrograms with the F1R1 primer set resolved the small peaks.