Development of a multilocus sequence typing scheme for *Mycobacterium abscessus* complex strains and their association with the clinical characteristics and the antibiotic resistance patterns

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ABSTRACT

Development of a multilocus sequence typing scheme for *Mycobacterium abscessus* complex strains and their association with the clinical characteristics and the antibiotic resistance patterns

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(Directed by Professor Young Ae Kang)

Mycobacterium abscessus (*M. abscessus*) is a rapid growing mycobacteria which causes a wide spectrum of disease in humans. Recent studies revealed that *M. abscessus* (hereafter referred to *M. abscessus* complex) was shown to comprise three closely related species: *M. abscessus*, *Mycobacterium massiliense (M. massiliense)*, and *Mycobacterium bolletii (M. bolletii).* It was reported that *M. abscessus*, *M. massiliense* and *M. bolletii* had differences in antibiotic susceptibility, in treatment response and in clinical outcome. However, those clinical characteristics could be various even in the same species when the results of the previous studies were reviewd. These findings suggested that there could be different clinical characteristics and prognosis in different strains within the same species of *M. abscessus* complex.

Multilocus sequence typing (MLST) is a PCR-based technique to identify sequence types (STs) of strains by compounding of allelic types of several housekeeping genes. The method is now available and widely used in many different bacterial species, because it provides reproducible and reliable results in studying infection epidemiology.

Therefore, identifying STs of strains of *M. abscessus* complex through the MLST can be clinically important. However, there was no report about the MLST of *M. abscessus* complex and the association of *M. abscessus* complex strains with the clinical characteristics and the antibiotic resistance patterns. The purpose of this study is to develop an MLST scheme for *M. abscessus* complex for the typing of stains of these species and to determine the association of *M. abscessus* complex strains with the clinical characteristics and antibiotic resistance patterns.

A total of 89 clinical isolates of *M. abscessus* complex from 71 patients of 2 tertiary care hospitals in South Korea were included. Forty-two isolates were identified as *M. abscessus*, and 29, as *M. massiliense* through sequencing of 8 housekeeping genes (*argH, cya, glpK, gnd, murC, pgm, pta and purH*) and *rpoB*. We excluded two genes that showed the lowest frequency of polymorphic sites before developing MLST scheme.

The MLST scheme identified 26 different sequence types (STs) and 13 different clonal complexes (CCs) in *M. abscessus* and 12 different STs and 6 different CCs in *M. massiliense*. The MLST data showed high concordance with the XbaI-macrorestriction patterns of pulsed-field gel electrophoresis in the duplicated isolates.

The association of *M. abscessus* strains/ CCs with disease progression was not distinct, but CC5 and CC8 had tendency to stable disease. And, the isolates

which were not included in the clusters within CCs showed the trends of stable disease (strain number 2, 3 and 21) in terms of correlation of *M. massiliense* strains/ CCs with disease progression.

The correlation of *M. abscessus* strains/ CCs with the antibiotics resistance patterns was not clear, nevertheless, susceptibility to many antibiotics was observed in CC2. And the correlation of *M. massiliense* strains/ CCs and antibiotic resistance patterns was not remarkable.

In summary, MLST scheme developed in this study could identify different strains, and similar strains can be classified as CCs. The MLST also showed adequate reproducibility compared with the studies of PFGE using duplicated isolates. Although the strains/ CCs determined by MLST scheme were not clearly associated with clinical characteristics and antibiotic resistance patterns, some trends were observed. Therefore, this MLST scheme may be useful for studying the epidemiology of *M. abscessus* complex infections and for managing the patients with *M. abscessus* complex.

Key words : *Mycobacterium abscessus*, *Mycobacterium massiliense*, multilocus sequence analysis, *rpoB*, multilocus sequence typing, drug susceptibility test

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I. INTRODUCTION

Mycobacterium abscessus (*M. abscessus*) is a rapid growing mycobacteria (RGM) which causes a wide spectrum of disease in humans.[1,2] It accounts for 65-80 % of RGM lung disease,[3,4] and is the second most common RGM species presented in extrapulmonary disease.[2,3,5]

M. abscessus is known to be resistant to many antibiotics in vitro and be difficult to treat, especially in patients with pulmonary disease.[1,2] American Thoracic Society/Infectious Diseases Society of America and many other experts has recommended intravenous amikacin with imipenem or cefoxitin and an oral macrolide as a regimen of *M. abscessus* complex.[1,2,5,6] However, treatment reasponse rates are unsatisfactory and optimal treatment duration or

regimen are not well studied yet.[7]

Recent studies revealed that *M. abscessus* (now *M. abscessus* complex or *M. abscessus* sensu lato) was shown to comprise three closely related species: *M. abscessus* (sensu stricto) (hereafter referred to as *M. abscessus*), *Mycobacterium massiliense* (*M. massiliense*), and *Mycobacterium bolletii* (*M. bolletii*).[8,9] In addition, because previous studies reported that *M. abscessus*, *M. massiliense* and *M. bolletii* had differences in antibiotic susceptibility, in treatment response and in clinical outcome,[10,11] importance of accurate identification of *M. abscessus* complex to the species level has been suggested in order to manage patients and to predict prognosis. Partial *rpoB* gene sequencing,[12-16] PCR restriction enzyme analysis of the *hsp*65 gene (PRA-*hsp*65), restriction fragment length polymorphims (RFLP) analysis of the 16S rRNA gene, DNA-DNA hybridization,[17] and multilocus sequence analysis (MLSA) have been used for the species identification of *M. abscessus* complex. [13,18,19] However, antibiotic susceptibility, treatment response and clincal outcome could be various within species level in the previous studies.[11] Although there was limited data about these findings in *M. abscessus* complex, it has been reported that different strains can result in different clinical characteristics and prognosis in other bacterial species, including *Propionibacterium acnes*, *Acinetobacter baumannii*, *Stenotrophomonas maltophilia*, and *Klebsiella pneumoniae*.[20-23] These findings suggested that there could be different clinical characteristics and prognosis in different strains within same species of *M. abscessus* complex.

Previously described methods are limited in their ability to determine *M. abscessus* complex strain types. First, methods such as partial *rpoB* gene

sequencing, PRA-*hsp*65 RFLP analysis of the 16S rRNA gene and DNA-DNA hybridization are unable to differentiate some closely related species like *M. abscessus* complex.[18,24] Second, MLSA can be used only in species identification and not strain typing. Third, pulsed-field gel electrophoresis (PFGE), which has been regarded as the gold standard technique for epidemiologic studies of NTM, shows poor inter-laboratory reproducibility.[25]

Multilocus sequence typing (MLST) is a PCR-based technique to identify sequence types (STs) of strains by compounding of allelic types of several housekeeping genes.[26] The method is now available and widely used in many different bacterial species, including *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*, because it provides reproducible and reliable results in studying infection epidemiology.

Therefore, identifying STs of strains of *M. abscessus* complex through the MLST can be clinically important. However, there was no report about the MLST of *M. abscessus* complex and the association of *M. abscessus* complex strains with the clinical characteristics and the antibiotic resistance patterns. In the present study, we are to develop an MLST scheme to type the strains within each species of the *M. abscessus* complex and to determine whether there was any association of *M. abscessus* complex strains with the clinical characteristics and the antibiotic resistance patterns.

II. MATERIALS AND METHODS

1. Setting and bacterial strains

This study included a total of 89 clinical *M. abscessus* complex isolates from 71 patients of two tertiary care hospitals (Severance Hospital and Seoul National University Hospital) in Seoul, Republic of Korea between January 2011 and August 2011. Duplicate isolates were obtained from 16 patients (interval: mean, 2.5 months; range, 0–9.6 months). All isolates were recovered from respiratory specimens of patients with *M. abscessus* complex lung diseases. All patients met the American Thoracic Society guidelines[1] diagnostic criteria for NTM lung disease. The type strains of *M. abscessus* [CIP 104536^T (ATCC19977^T)] and *M. massiliense* (CIP 108297^T) were also included in this study.[18]

2. DNA extraction

Cryopreservation beads were used when bacterial strains were stored at -70℃, and bacterial strains were grown on Middlebrook 7H10 media (BD, Franklin Lakes, NJ, USA) at 37℃ for 4 days before being used. Genomic DNA was extracted using Tris-EDTA, lysozyme, and proteinase K, as described previously.[18,27]

3. MLSA and rpoB sequencing

Fragments of *rpoB* and eight housekeeping genes, which have been used previously in the MLSA of *M. abscessus* complex,[27] were amplified using the primer sets listed in Table 1. Amplification experiments were performed using 25 μL of ReddyMix PCR master mix (Thermo Fisher Scientific Inc., Epsom,

UK) and 1 µL of each primer (10 pmol). The deoxynucleotide sequencing of the amplified gene fragments was carried out on both strands with the Big Dye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) using the same primers as those for amplification. Sequencing products were purified and analysed with an ABI 3700 DNA analyser (International Equipment Trading Ltd., IL, USA). Contiguous sequences were formed and trimmed using SeqMan II software system (DNASTAR, Inc., Madison, WI, USA). Phylogenetic trees were obtained using concatenated sequences of *rpoB* and eight housekeeping genes of MLSA.

Table 1. Primers used for PCR and sequencing

Gene	Primer	Primer sequences (5' to 3')	Amplicon size (bp)	Tm (℃)
argH	argH-F	GACGAGGGCGACAGCTTC	629	60
(argininosuccinate lyase)	argH-R	GTGCGCGAGCAGATGATG		58
cya	cya-F	TAAGGGTGATGACGTGCTGT	807	59
(adenylate cyclase)	cya-R	GTGAACGGCAACGCCTAC		60
glpK	glpK-F	AATCTCACCGGCGGTGTC	609	58
(glycerol kinase)	glpK-R	GGACAGACCCACGATGGC		60
gnd	gnd-F	GTGACGTCGGAGTGGTTGG	634	62
(6-phosphogluconate deshydrogenase)	gnd-R	CTTCGCCTCAGGTCAGCTC		62
murC	murC-F	TCAATGAAGCCGGTACCAAT	730	60
(UDP N-acetylmuramate-1-Ala ligase)	murC-R	GCGAATTCTGTAGCGAAAGC		60
pgm	pgm-F	CCATTTGAACCCGACCGG	596	60
(phosphoglucomutase),	pgm-R	GTGCCAACGAGATCCTGCG		66
pta	pta-F	GATCGGGCGTCATGCCCT	720	60
(phosphate acetyltransferase)	pta-R	ACGAGGCACTGCTCTCCC		66
purH	purH-F	CGGAGGCTTCACCCTGGA	634	64
(phoshoribosylaminoimiazolcarboxylase ATPase subunit)	purH-R	CAGGCCACCGCTGATCTG		60
rpoB	rpoB-F	TCCGATGAGGTGCTGGCAGA	940	68
(RNA polymerase, beta subunit)	rpoB-R	ACTTGATGGTCAACAGCTCC		68

4. Development of MLST scheme

After MLSA and *rpoB* sequencing, we compared the polymorphic sites for each gene fragment. We excluded two genes that showed the lowest frequency of polymorphic sites. Sequence data were aligned by CLUSTALW (http://www.genome.jp/tools/clustalw/). For each locus, arbitrary allele numbers were assigned to distinct allele sequences. Each isolate was identified by a pattern of allelic numbers, defined as ST. We defined clonal complex (CC) as strains sharing at least five of seven alleles. To assess the genetic relatedness of various *M. abscessus* complex strains, phylogenetic trees were obtained from concatenated sequences using the MEGA 5 software (http://www.megasoftware.net/) by UPGMA (unweighted pair-group method with arithmetic averages) analysis based on the nucleotide differences. Allele profiles and STs were set at a website (http://pubmlst.org/mabscessus/) in which database software was used.[28]

5. PFGE

PFGE experiments were performed as described previously[29] to evaluate the reproducibility of our MLST scheme when duplicate clinical isolates were obtained from the same patients. *Xba*I-macrorestriction experiments were performed as described previously.[30] PFGE was performed using a CHEF-DR II System (Bio-Rad, Hercules, CA, USA) for 20 h at 6 V/cm at 11℃ with initial and final pulse times of 0.5 s and 30 s, respectively. Gels were stained with ethidium bromide for visualisation of DNA bands and photographed.

6. Nucleotide sequence accession numbers

The GenBank accession numbers for the unique sequences generated in this study are JX041946–JX042025.

7. Clinical information

We classified the imaging findings as upper lobe cavitary type or nodular bronchiectatic disease based on chest radiography (CXR) and high resolution computed tomography (HRCT) findings which is performed at the time of diagnosis.[11,31,32]

We defined disease progression as the case in which the disease was progressive (treatment within 24 months of diagnosis) or imaging findings (CXR or HRCT) was progressive after diagnosis. Those criteria were modified from the other study. [33]

8. Antibiotic susceptibility testing

Minimal inhibitory concentrations (MICs) of antibiotics (amikacin, cefoxitin, ciprofloxacin, clarithromycin, doxycyclin, moxifloxacin, linezolid, clofazimine, tigecycline, and azithromycin) were determined by the broth microdilution method.[34] The MIC was read on the 4^{th} and 5^{th} day. The inocula were prepared from actively growing bacterial in 10 mL of cation-adjusted Mueller-Hinton broth. The strains was then adjusted with cation-adjusted Mueller-Hinton broth to a bacterial cell density of 10^6 colony forming units/mL (cfu/mL), and diluted to a final inoculum of approximately $5 * 10^4$ cfu/well. The MIC breakpoints was interpreted according to the recommendations of the Clinical and Laboratory

Standards Institute (CLSI)[34] and the modified values for tigecycline by Petrini[35] and for clofazimine by Shen.[36] Because the MIC of azithromycin for the *M. abscessus* complex has not been described by the CLSI yet, recommendation for *Mycobacterium avium* in CLSI M24-A of 2003 was used (Table 2).[37] To test for inducible resistance to clarithromycin or azithromycin, clarithromycin or azithromycin susceptibility tests were conducted by a broth microdilution method with preincubation with clarithromycin or azithromycin. The MICs were determined on Days 3 and 14 after incubation.[38]

Table 2. Antibiotics and MIC breakpoints

Drug	MIC (μg/mL)		
	S	I	R
Amikacin	≤16	32	≥64
Cefoxitin	≤16	32-64	≥128
Ciprofloxacin	≤1	2	≥4
Clarithromycin	≤2	4	≥8
Doxycycline (Minocycline)	≤1	2-4	≥8
Moxifloxacin	≤1	2	≥4
Linezolid	≤8	16	≥32
Clofazimine	≤1	2	≥4
Tigecycline	≤4		>4
Azithromycin	≤128		>256

MIC, minimal inhibitory concentration.

9. Statistical Analysis

Pearson's chi-squared test or Fisher's exact test was used to analyze categorical variables, and the Mann–Whitney *U*-test was used to analyze continuous variables. A two-tailed *p* value of <0.05 was considered to be statistically significant. The SPSS software (ver. 18.0; SPSS Inc., Chicago, IL, USA) was used for statistical analysis.

III. RESULTS

We identified the species of *M. abscessus* complex through multilocus sequence analysis (MLSA) and *rpoB* sequencing, and developed an MLST scheme for *M. abscessus* complex for the typing of strains within species. Then, we checked that there was any association of *M. abscessus* complex strains with the clinical characteristics and the antibiotic resistance patterns.

1. Species identification of and MLST scheme for *M. abscessus* complex

A. Identification of the M. abscessus complex by rpoB sequencing and MLSA

Phylogenetic trees were obtained from the partial sequences of eight housekeeping genes of *rpoB* and MLSA (Figures 1A and 1B). The trees showed that our collection of isolates comprised two principal groups (*rpoB* sequencing: group A′, n = 43; group B′, n = 28; MLSA: group A, n = 42; group B, n = 29), and each group included type strain *M. abscessus* CIP104536T and *M. massiliense* CIP108297T, respectively. Therefore, the groups were assigned as *M. abscessus* and *M. massiliense*, respectively. The identification of strains according to MLSA and partial *rpoB* sequences showed identical results in all isolates, except one. Strain 6 was identified as *M. abscessus* by partial *rpoB* sequencing, but as *M. massiliense* by MLSA. *M. bollettii* was not detected in our collection.

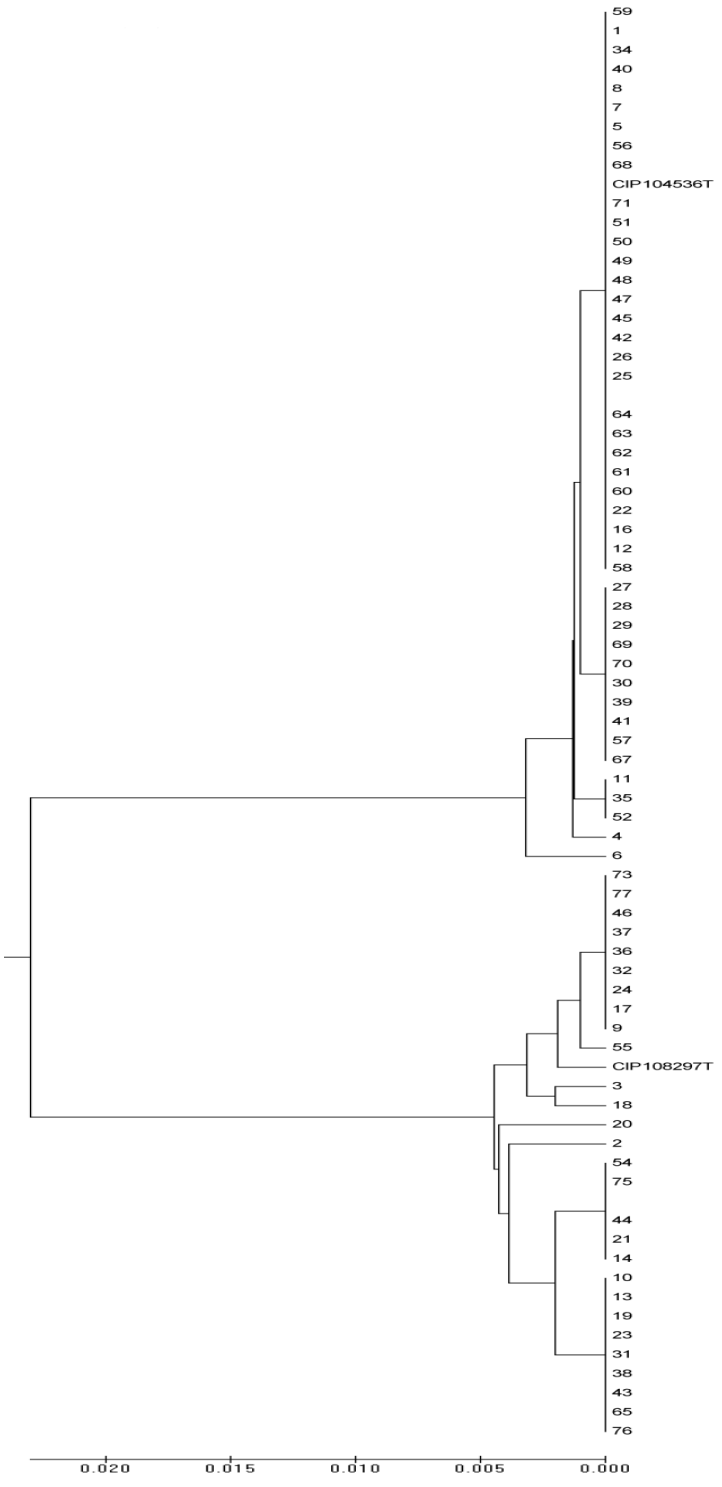

Figure 1A. Tree constructed using *rpoB* gene sequences.

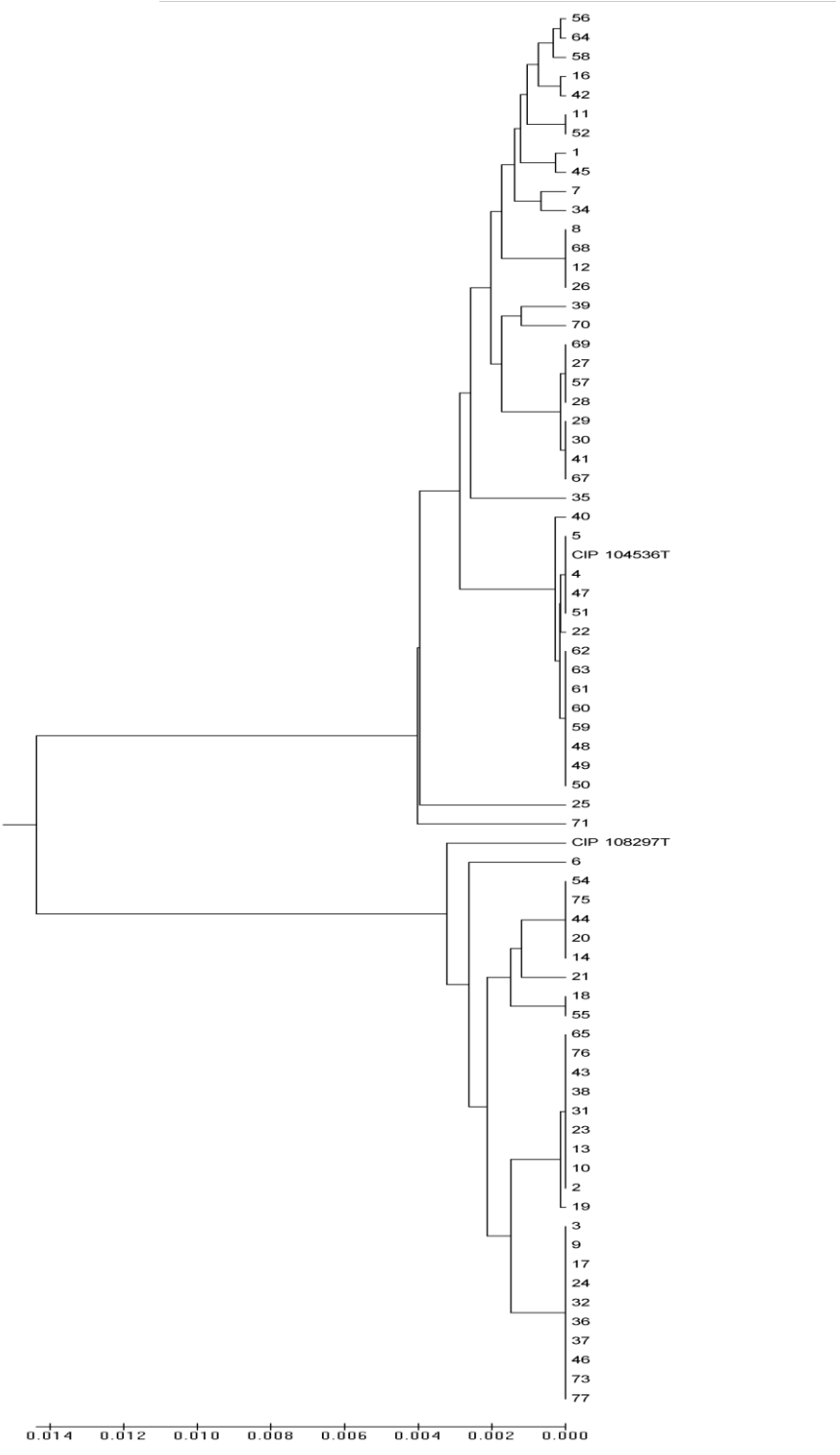

Figure 1B. Tree constructed using concatenated MLSA sequences.

B. Characteristics of patients

Table 3 shows the baseline characteristics of patients with infections caused by *M. abscessus* complex. The median age was 62 yrs (range 20 – 83 yrs) and the proportion of female was 63.4% (n= 45). Thirty six patients (50.7%) had history of active tuberculosis and 10 patients (14.1%) had diabetes mellitus. Radiologic studies revealed that the nodular bronchiectatic form was the dominant pattern (n = 47, 66.2%), followed by the upper lobe cavitary form (n = 14, 19.7%). And radiologic patterns in 10 patients (14.1%) were not classifiable.

C. *Development of MLST scheme*

Partial sequences of six housekeeping genes of MLSA and *rpoB*, ranging from 445 bp (*murC*) to 540 bp (*cya*) in *M. abscessus* and from 422 bp (*glpK*) to 530 bp (*argH*) in *M. massiliense*, were used in the MLST. We excluded *pgm* and *glpK* housekeeping genes from the MLST because of the low frequency of polymorphic sites in these gene fragments, as shown in Table 4. The number of allelic types of housekeeping genes identified in the 96 clinical isolates ranged from 2 (*glpK*) to 11 (*pta*) in *M. abscessus* and from 1 (*pgm*) to 10 (*rpoB*) in *M. massiliense*. Nucleotide variations were identified at 1 to 18 sites of each housekeeping gene in *M. abscessus* and at 0 to 25 sites in *M. massiliense*. The percentages of nucleotide variability ranged from 0.19% (*glpK*) to 3.46% (*pta*) in *M. abscessus* and from 0.00% (*pgm*) to 4.99% (*rpoB*) in *M. massiliense* (Table 3). The maximum sequence divergence was identified in *cya* (2.96 %) of *M. abscessus* and in *rpoB* (4.59 %) of *M. massiliense*, and the minimum sequence divergence was identified in *glpK* (0.19 %) of *M. abscessus* and in *pgm* (0.00%) of *M. massiliense*.

Table 3. Baseline characteristics of 71 patients with *M. abscessus* complex lung disease

	Total (n = 71)
Age, yrs (median, range)	62 (20 - 83)
Sex, female	45 (63.4)
BMI, Kg/m2 (median, IQR)	20.5 (18.7 - 22.9)
Institution	
SEV[a]	46 (64.8)
SNU[b]	25 (35.2)
Smoking	
Non-smoker	53 (74.6)
Ex-smoker	16 (22.5)
Current smoker	2 (2.8)
Underlying disease	
Previous TB	36 (50.7)
COPD	3 (4.2)
DM	10 (14.1)
Lung cancer	1 (1.4)
Other malignancy	12 (16.9)
Symptoms	
Cough	39 (54.9)
Sputum	47 (66.2)
Hemoptysis	33 (46.5)
Other symptoms	10 (14.1)
Positive AFB smear	11 (15.5)
Type of disease	
NB type	47 (66.2)
UC type	14 (19.7)
Unclassifiable	10 (14.1)

BMI, body mass index; IQR, interquartile range; TB, tuberculosis; COPD, chronic obstructive pulmonary disease; DM, diabetes mellitus; AFB, acid-fast bacilli; NB, nodular bronchiectasis; UC, upper lobe cavity.

[a] SEV, Severance hospital.

[b] SNU, Seoul National University Hospital.

Table 4. Variation in loci of *M. abscessus* and *M. massiliense*

Locus	Species	Fragment size (bp)	No. of alleles	No. of polymorphic nucleotide sites	% Variable sites	% Nucleotide divergence between alleles	
						Maximum	Average
argH	*M.abscessus*	510	6	14	2.75	1.76	1.19
	M.massiliense	530	3	6	1.13	0.94	0.75
cya	*M.abscessus*	540	5	16	2.96	2.96	1.30
	M.massiliense	454	4	14	3.08	2.64	1.58
gnd	*M.abscessus*	506	7	14	2.77	2.37	0.98
	M.massiliense	476	3	3	0.63	0.63	0.42
murC	*M.abscessus*	445	8	9	2.02	1.35	0.68
	M.massiliense	459	6	14	3.05	2.18	1.16
pta	*M.abscessus*	520	11	18	3.46	2.50	1.39
	M.massiliense	486	3	2	0.41	0.41	0.27
purH	*M.abscessus*	497	7	10	2.01	1.21	0.73
	M.massiliense	502	3	16	3.19	2.79	1.96
rpoB	*M.abscessus*	503	4	3	0.60	0.40	0.30
	M.massiliense	501	10	25	4.99	4.59	1.49
glpK	*M.abscessus*	519	2	1	0.19	0.19	0.10
	M.massiliense	422	2	1	0.24	0.24	0.12
pgm	*M.abscessus*	406	5	9	2.22	1.72	0.25
	M.massiliense	515	1	0	0.00	0.00	0.00

D. *The MLST scheme for M. abscessus*

MLST identified 26 STs and 13 CCs in 42 clinical isolates of *M. abscessus* (Table 5). CC1 included 14 clinical isolates and the reference strain CIP 104536^{T} belonging to ST5, 6, 7, 8 and 13; and CC2 included eight clinical isolates belonging to ST11, 17, 18, 19 and 20. Figure 2A, which was obtained with concatenated sequences of six housekeeping genes and *rpoB* gene using MLST, shows that clinical *M. abscessus* isolates were grouped into three clusters using a bootstrap cut-off value of 0.0035. The distances between clonal complexes were < 0.001.

E. *The MLST scheme for M.massiliense*

MLST identified 12 STs and 6 CCs in 29 clinical isolates of *M. massiliense* (Table 6). CC1 harboured 10 clinical isolates belonging to three STs (ST1, 2 and 8) and CC2 harboured 10 clinical isolates belonging to two STs (ST9 and 10). The phylogenetic tree generated using the MLST results of *M. massiliense* is shown in Figure 2B. Three clusters were observed using a bootstrap cut-off value of 0.003, and two of the three clusters comprised only one isolate. The solitary strains were the reference strain and strain 6, which showed discordant results between MLSA and *rpoB* sequencing in terms of identifying the species of the *M. abscessus* complex. CCs of *M. massiliense* were accurately distinguishable when 0.0015 was used as the bootstrap cut-off value.

Table 5. Epidemiologic characteristics and MLST scheme of *M. abscessus* used in this study

Strain number	Institution	Year of isolation	Source	ST (number)	CC	Allelic type (number)						
						cya	*gnd*	*murC*	*pta*	*purH*	*argH*	*rpoB*
1	SEV[a]	2011	respiratory specimen	22	6	4	3	2	11	7	4	1
4	SEV	2011	respiratory specimen	7	1	1	4	3	1	2	3	3
5	SEV	2011	respiratory specimen	5	1	1	4	3	1	2	3	1
7	SEV	2011	respiratory specimen	21	10	4	3	2	8	2	1	1
8	SEV	2011	respiratory specimen	1	3	1	1	2	2	3	2	1
11	SEV	2011	respiratory specimen	2	8	1	1	2	7	4	4	4
12	SEV	2011	respiratory specimen	1	3	1	1	2	2	3	2	1
16	SEV	2011	respiratory specimen	12	7	2	1	2	2	5	1	1
22	SEV	2011	respiratory specimen	13	1	2	4	3	1	2	3	1
25	SEV	2011	respiratory specimen	3	4	1	1	4	5	6	5	1
26	SEV	2011	respiratory specimen	1	3	1	1	2	2	3	2	1
27	SEV	2011	respiratory specimen	11	2	2	3	1	3	1	1	2
28	SEV	2011	respiratory specimen	19	2	3	3	1	3	1	1	2
29	SEV	2011	respiratory specimen	17	2	3	2	1	3	1	1	2
30	SEV	2011	respiratory specimen	17	2	3	2	1	3	1	1	2
34	SEV	2011	respiratory specimen	9	11	2	1	2	4	4	1	1
35	SEV	2011	respiratory specimen	4	12	1	7	2	9	2	2	4
39	SEV	2011	respiratory specimen	14	9	2	5	1	2	1	1	2

40	SEV	2011	respiratory specimen	8	1	1	4	5	1	2	3	1
41	SEV	2011	respiratory specimen	19	2	3	3	1	3	1	1	2
42	SEV	2011	respiratory specimen	16	7	3	1	2	6	5	1	1
45	SNU[b]	2011	respiratory specimen	23	6	4	3	2	11	7	1	1
47	SNU	2011	respiratory specimen	5	1	1	4	3	1	2	3	1
48	SNU	2011	respiratory specimen	6	1	1	4	8	1	2	3	1
49	SNU	2011	respiratory specimen	6	1	1	4	8	1	2	3	1
50	SNU	2011	respiratory specimen	6	1	1	4	8	1	2	3	1
51	SNU	2011	respiratory specimen	5	1	1	4	3	1	2	3	1
52	SNU	2011	respiratory specimen	2	8	1	1	2	7	4	4	4
56	SNU	2011	respiratory specimen	24	5	4	3	2	2	4	1	1
57	SNU	2011	respiratory specimen	20	2	3	3	1	2	1	1	2
58	SNU	2011	respiratory specimen	26	5	4	3	2	2	7	1	1
59	SNU	2011	respiratory specimen	6	1	1	4	8	1	2	3	1
60	SNU	2011	respiratory specimen	6	1	1	4	8	1	2	3	1
61	SNU	2011	respiratory specimen	6	1	1	4	8	1	2	3	1
62	SNU	2011	respiratory specimen	6	1	1	4	8	1	2	3	1
63	SNU	2011	respiratory specimen	6	1	1	4	8	1	2	3	1
64	SNU	2011	respiratory specimen	25	5	4	3	2	2	4	6	1
67	SNU	2011	respiratory specimen	18	2	3	2	1	2	1	1	2
68	SNU	2011	respiratory specimen	1	3	1	1	2	2	3	2	1

69	SNU	2011	respiratory specimen	20	2	3	3	1	2	1	1	2
70	SNU	2011	respiratory specimen	15	9	2	6	6	2	1	1	2
71	SNU	2011	respiratory specimen	10	13	2	1	7	10	2	4	1
reference strain				5	1	1	4	3	1	2	3	1

ST, sequence types; CC, clonal complex.

[a] SEV, Severance hospital.

[b] SNU, Seoul National University Hospital.

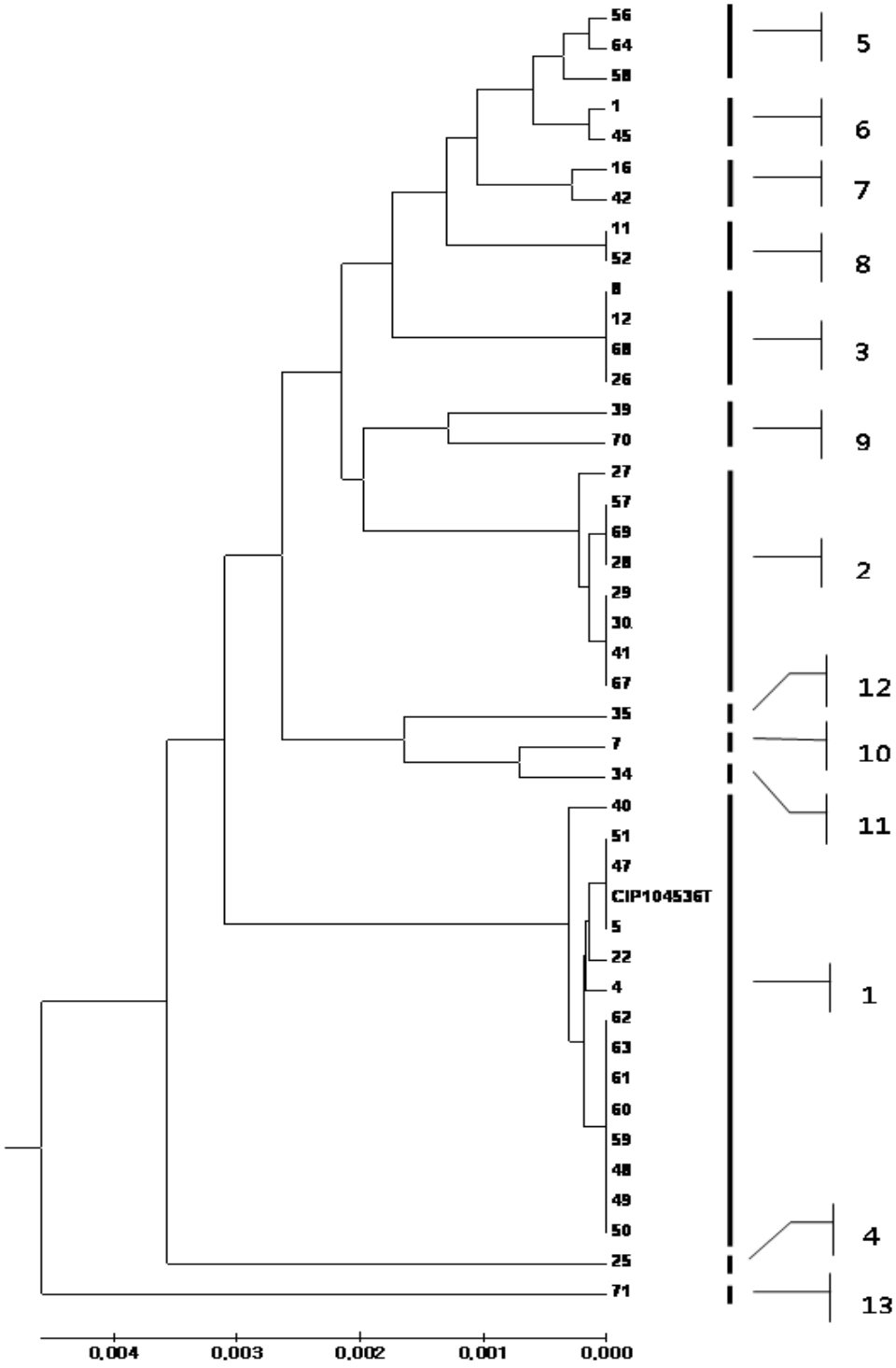
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Figure 2A. Tree constructed by UPGMA cluster analysis obtained by sequencing *cya, gnd, murC, pta, purH, argH* and *rpoB* gene fragments in *M. abscessus*. Strain numbers and clonal complex numbers are shown from left to right.

Table 6. Epidemiologic characteristics and MLST scheme of *M. massiliense* used in this study

Strain number	Institution	Year of isolation	Source	ST (number)	CC	Allelic type (number)						
						cya	*gnd*	*murC*	*pta*	*purH*	*argH*	*rpoB*
2	SEV	2011	respiratory specimen	1	1	1	1	1	1	1	2	2
3	SEV	2011	respiratory specimen	9	2	2	2	1	2	1	1	5
6	SEV	2011	respiratory specimen	7	6	3	1	5	3	2	1	4
9	SEV	2011	respiratory specimen	10	2	2	2	1	2	1	1	1
10	SEV	2011	respiratory specimen	2	1	1	1	1	1	1	2	8
13	SEV	2011	respiratory specimen	2	1	1	1	1	1	1	2	8
14	SEV	2011	respiratory specimen	4	4	1	1	2	3	2	1	3
17	SEV	2011	respiratory specimen	10	2	2	2	1	2	1	1	1
18	SEV	2011	respiratory specimen	11	3	4	3	4	2	2	3	6
19	SEV	2011	respiratory specimen	8	1	1	1	6	1	1	2	8
20	SEV	2011	respiratory specimen	5	4	1	1	2	3	2	1	9
21	SEV	2011	respiratory specimen	6	4	1	1	3	3	2	1	3
23	SEV	2011	respiratory specimen	2	1	1	1	1	1	1	2	8
24	SEV	2011	respiratory specimen	10	2	2	2	1	2	1	1	1
31	SEV	2011	respiratory specimen	2	1	1	1	1	1	1	2	8
32	SEV	2011	respiratory specimen	10	2	2	2	1	2	1	1	1
36	SEV	2011	respiratory specimen	10	2	2	2	1	2	1	1	1
37	SEV	2011	respiratory specimen	10	2	2	2	1	2	1	1	1
38	SEV	2011	respiratory specimen	2	1	1	1	1	1	1	2	8

43	SEV	2011	respiratory specimen	2	1	1	1	1	1	1	2	8
44	SEV	2011	respiratory specimen	4	4	1	1	2	3	2	1	3
46	SNU	2011	respiratory specimen	10	2	2	2	1	2	1	1	1
54	SNU	2011	respiratory specimen	4	4	1	1	2	3	2	1	3
55	SNU	2011	respiratory specimen	12	3	4	3	4	2	2	3	10
65	SNU	2011	respiratory specimen	2	1	1	1	1	1	1	2	8
73	SEV	2011	respiratory specimen	10	2	2	2	1	2	1	1	1
75	SEV	2011	respiratory specimen	4	4	1	1	2	3	2	1	3
76	SEV	2011	respiratory specimen	2	1	1	1	1	1	1	2	8
77	SEV	2011	respiratory specimen	10	2	2	2	1	2	1	1	1
reference strain				3	5	1	1	1	1	3	1	7

ST, sequence types; CC, clonal complex.

[a] SEV, Severance hospital.

[b] SNU, Seoul National University Hospital.

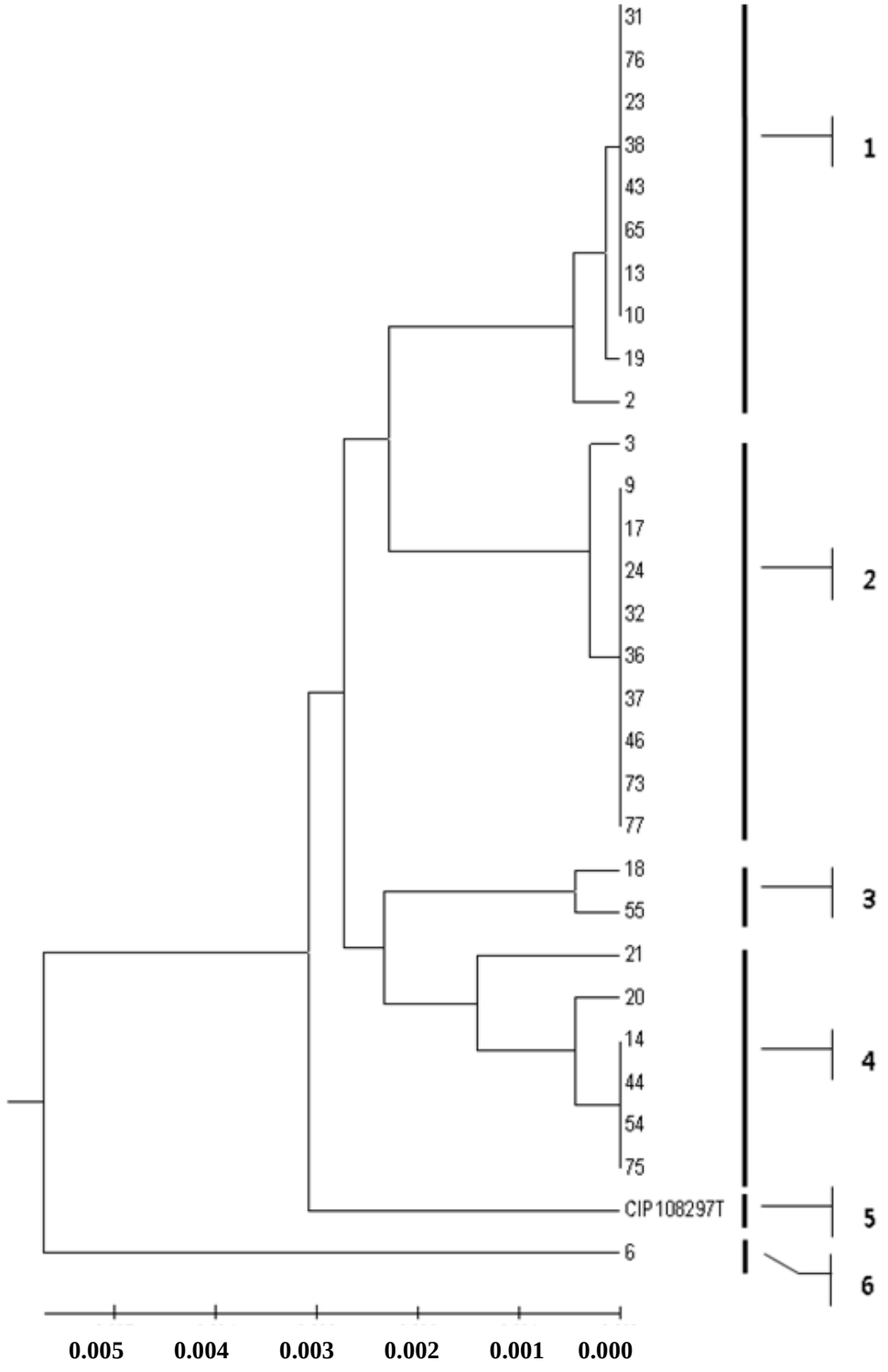
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Figure 2B. Tree constructed by UPGMA cluster analysis obtained by sequencing *cya, gnd, murC, pta, purH, argH* and *rpoB* gene fragments in *M. massiliense*. Strain numbers and clonal complex numbers are shown from left to right.

F. ***Comparison of MLST and PFGE***

We compared the MLST results with *Xba*I-macrorestriction patterns in the 34 duplicate isolates from 16 patients to evaluate the reproducibility of our MLST. All duplicate clinical isolates from the same patient belonged to the same STs, except two pairs of isolates, which shared six identical allelic types of housekeeping genes but carried a single different allele. All duplicate clinical isolates from the same patient showed similar *Xba*I-macrorestriction patterns with > 85% similarity (Figure 3A and 3B).

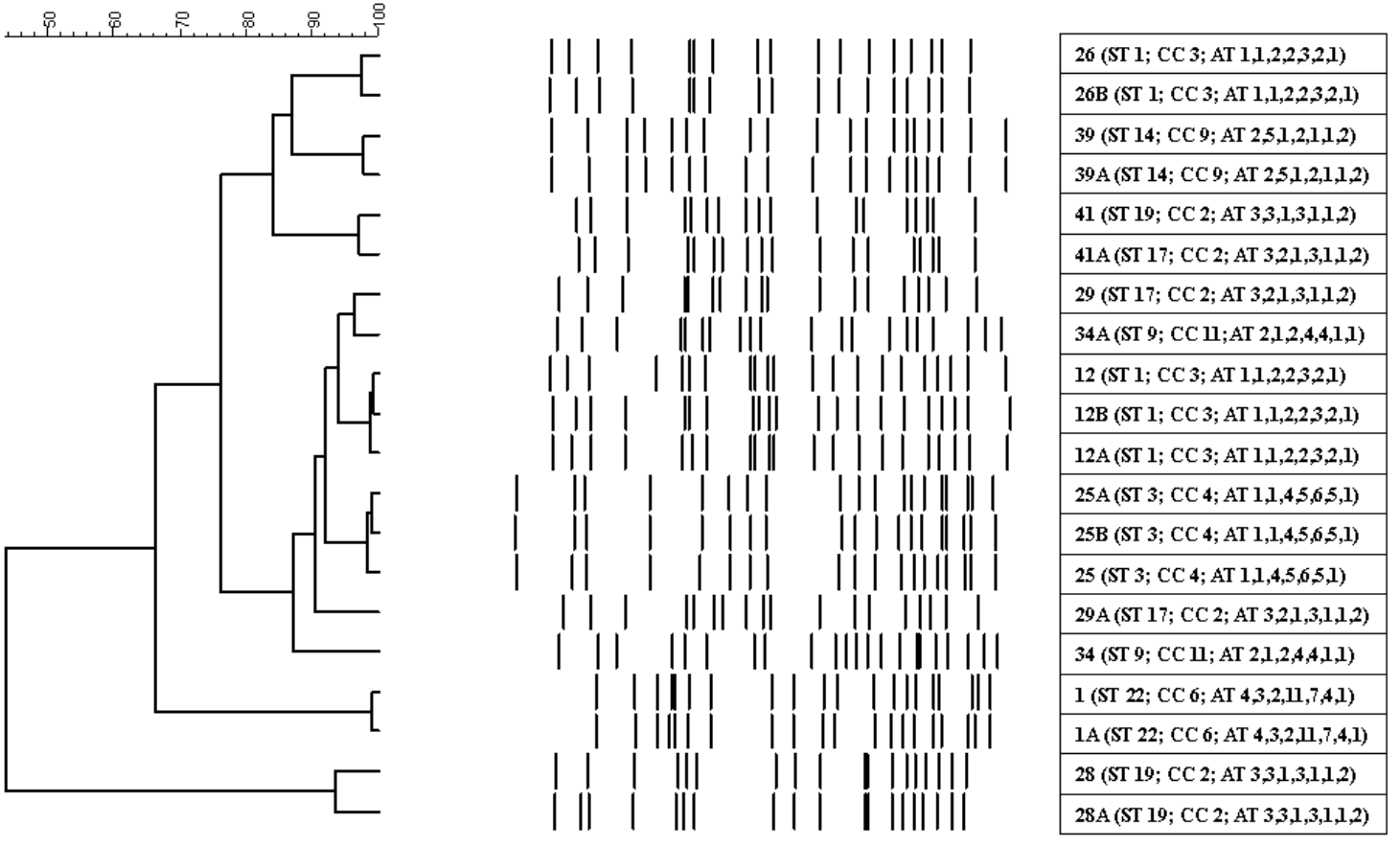

Figure 3A. Comparison of MLST and PFGE among duplicate *M. abscessus* isolates. Dendrogram constructed using the PFGE pattern. Dendrograms, PFGE patterns, isolate numbers, ST numbers, CC numbers and allelic type numbers are indicated from left to right. *Cya, gnd, murC, pta, purH, argH* and *rpoB* allelic types are expressed in order.

ST, sequence type; CC, clonal complex; AT, allelic type; MLST, multilocus sequence typing; PFGE, pulsed field gel electrophoresis.

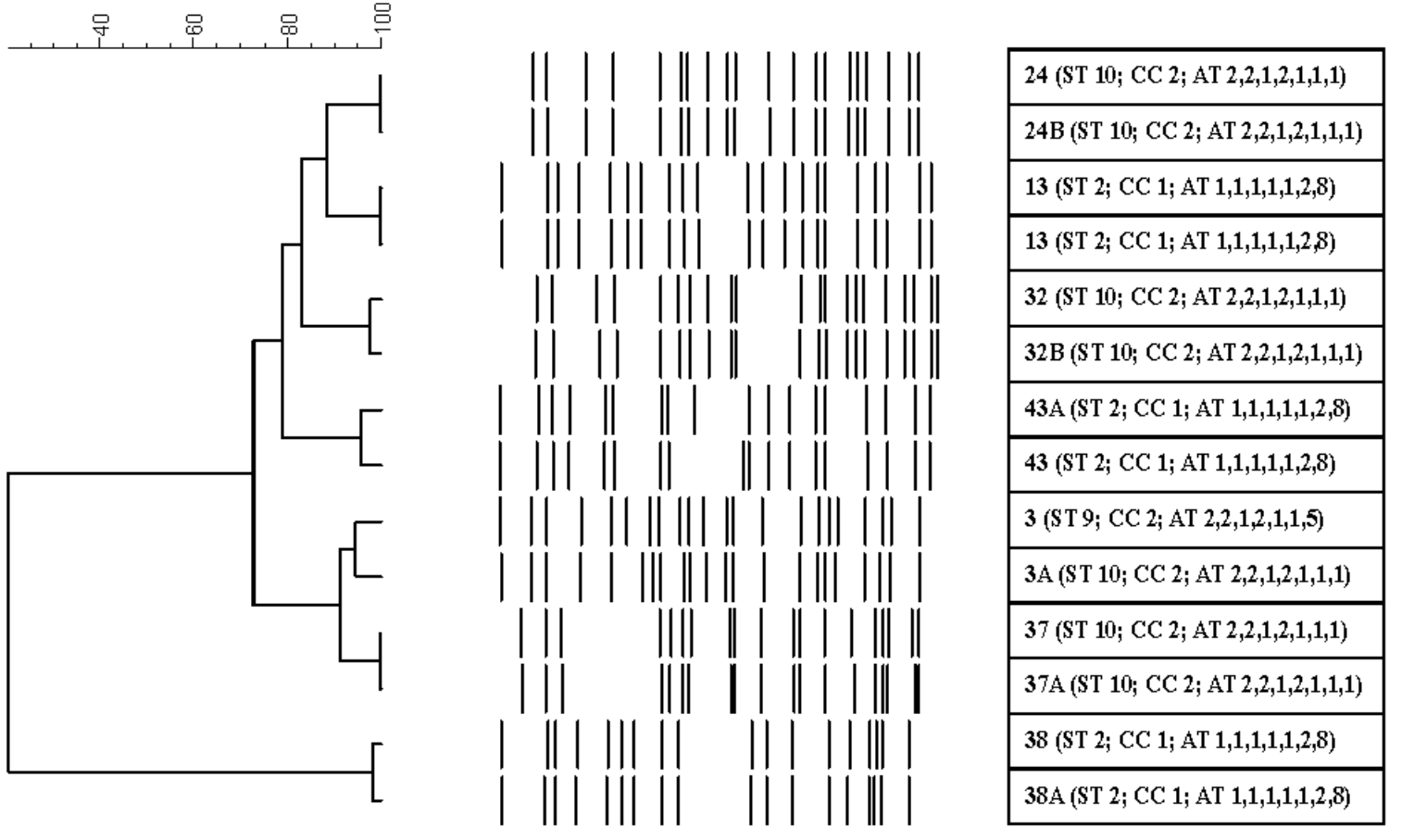

Figure 3B. Comparison of MLST and PFGE among duplicate *M. massiliense* isolates. Dendrogram constructed using the PFGE pattern. Dendrograms, PFGE patterns, isolate numbers, ST numbers, CC numbers and allelic type numbers are indicated from left to right. *Cya*, *gnd*, *murC*, *pta*, *purH*, *argH* and *rpoB* allelic types are expressed in order.

ST, sequence type; CC, clonal complex; AT, allelic type; MLST, multilocus sequence typing; PFGE, pulsed field gel electrophoresis.

2. The associatioin of *M. abscessus* complex strains with the clinical characteristics

A. Clinical characteristics of patients with *M. abscessus* and *M. massiliense* lung disease

Table 7 shows the clinical characteristics of patients with infections caused by *M. abscessus* or *M. massiliense*. No significant differences were found between patients with *M. abscessus* and *M. massiliense*. Median age was 66 yrs (range 20 – 83 yrs) in patients with *M. abscessus* and 59 yrs (range 42 – 78 yrs) in *M. massiliense*. Twenty (47.6%) patients with *M. abscessus* infection and 16 (55.2%) with *M. massiliense* infection had a history of active tuberculosis. Radiologic studies revealed that the nodular bronchiectatic form was the dominant pattern in patients with *M. abscessus* infection (n = 25, 59.5%) and *M. massiliense* infection (n = 22, 75.9%), followed by the upper lobe cavitary form in *M. abscessus* infection (n = 11, 26.2%) and *M. massiliense* infection (n = 3, 10.3%). The overall proportion of patients who experienced disease progression was not different between *M. abscessus* and *M. massiliense* (n=22, 52.4% in *M. abscessus vs*. n=18, 62.1% in *M. massiliense*; *P*= 0.292).

B. Association of *M. abscessus* strains/ CCs with the clinical characteristics

Figure 4A shows an association of *M. abscessus* strains/ CCs with disease progression. (Disease progression was presented with box.) Although the association of strains/ CCs with disease progression was not distinct, CC5 and CC8 had tendency to stable disease.

C. Association of *M. massiliense* strains/CCs with the clinical characteristics

An association of *M. massiliense* strains/ CCs with disease progression was shown in Figure 4B. (Disease progression was presented with box.) The isolates which were not included in the cluster within CCs showed the trends of stable disease. (strain number 2, 3 and 21)

Table 7. Clinical characteristics of patients with *M. abscessus* and *M. massiliense* lung disease

	M. abscessus (n=42)	*M. massiliense* (n=29)	*P*
Age, yrs (median, range)	66 (20 - 83)	59 (42 - 78)	0.096
Sex, female	23 (54.8)	22 (73.3)	0.109
BMI, Kg/m2 (median, IQR)	19.7 (18.1 - 22. 6)	21.1 (19.5-23.5)	0.173
Institution			0.002
SEV[a]	21 (50.0)	25 (86.2)	
SNU[b]	21 (50.0)	4 (13.8)	
Smoking			
Non-smoker	29 (69.0)	24 (82.8)	
Ex-smoker	11 (26.2)	5 (17.2)	
Current smoker	2 (4.8)	0 (0)	0.298
Underlying disease			
Previous TB	20 (47.6)	16 (55.2)	0.631
COPD	1 (2.4)	2 (6.9)	0.563
DM	5 (11.9)	5 (17.2)	0.730
Lung cancer	1 (2.4)	0 (0)	1.000
Other malignancy	7 (16.7)	5 (17.2)	1.000
Symptoms			
Cough	23 (54.8)	16 (55.2)	1.000
Sputum	27 (64.3)	20 (69.0)	0.8
Hemoptysis	19 (45.2)	14 (48.3)	0.814
Other symptoms	6 (14.3)	4 (13.8)	1.000
Positive AFB smear	8 (19.0)	3 (10.3)	0.506
Type of disease			
NB type	25 (59.5)	22 (75.9)	
UC type	11 (26.2)	3 (10.3)	
Unclassifiable	6 (14.3)	4 (13.8)	0.237
Disease progression	22 (52.4)	18 (62.1)	0.472
Treatment[c]	10 (24.4)	11 (37.9)	0.292
Imaging progression	17 (44.7)	13 (44.8)	1.000

BMI, body mass index; IQR, interquartile range; TB, tuberculosis; COPD, chronic obstructive pulmonary disease; DM, diabetes mellitus; AFB, acid-fast bacilli; NB, nodular bronchiectasis; UC, upper lobe cavity

[a] SEV, Severance hospital; [b] SNU, Seoul National University Hospital;

[c]Treatment was defined as the case in which the disease was progressive (treatment within 24 months of diagnosis).

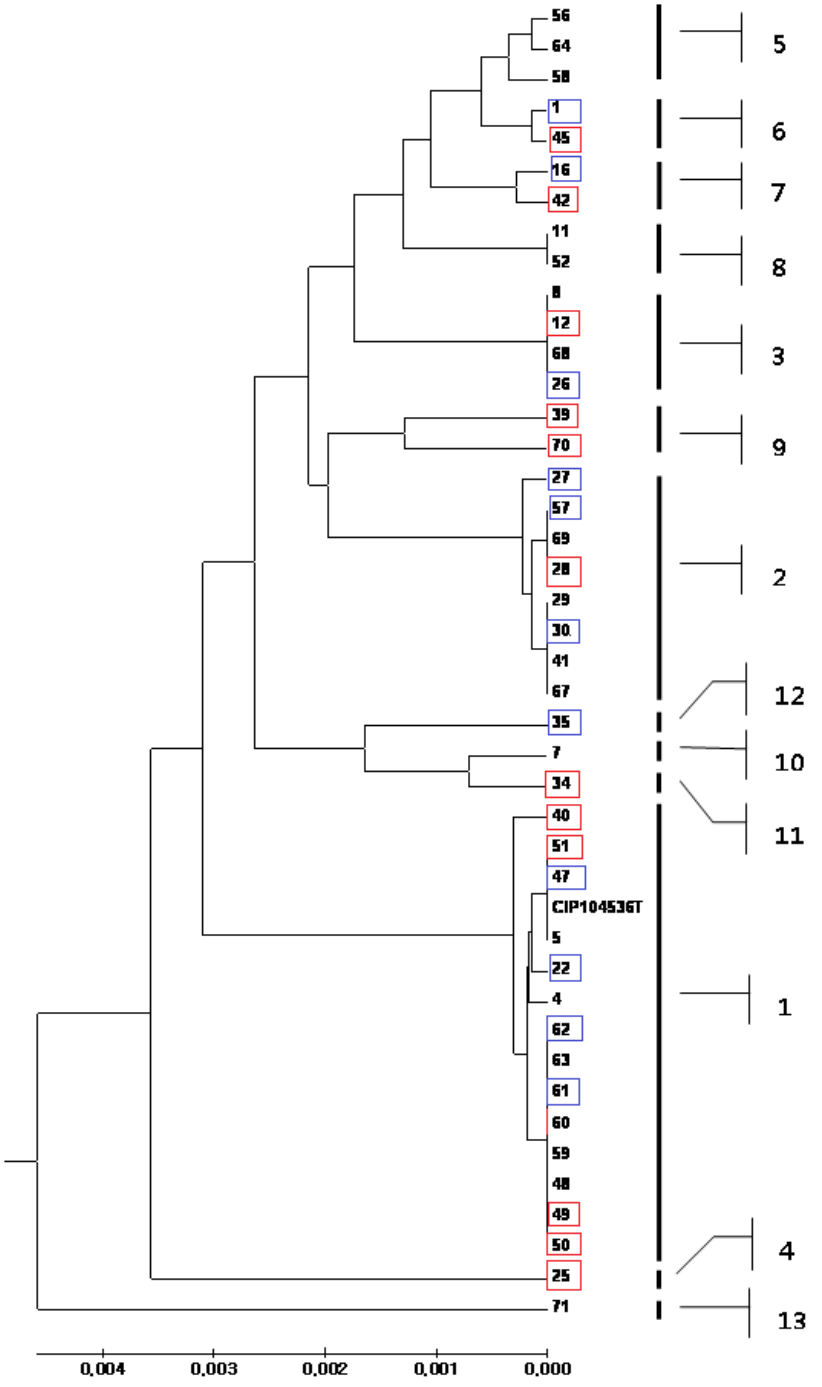

Figure 4A. Association of *M. abscessus* strains/ CCs with the clinical characteristics. CC5 and CC8 had tendency to stable disease. Disease progression was presented with box.

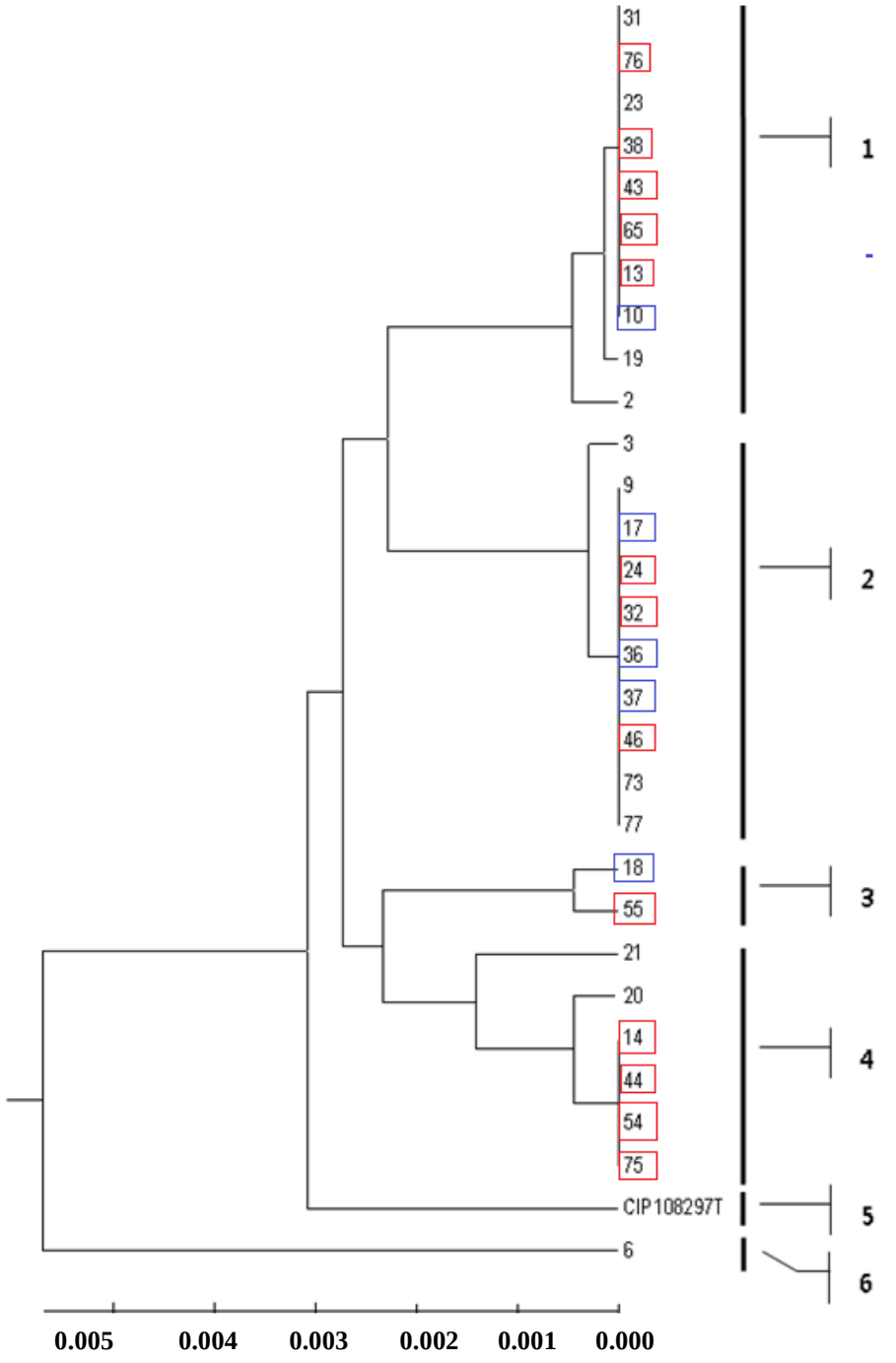

Figure 4B. Association of *M. massiliense* strains/CCs with the clinical characteristics. The isolates which were not included in the cluster within CCs showed the trends of stable disease. Disease progression was presented with box.

3. The association of *M. abscessus* complex strains with the antibiotic resistance patterns

A. Antibiotic resistance patterns of *M. abscessus* and *M. massiliense* from patients with lung disease

Antibiotic susceptibility testing was performed on *M. abscessus* from 35 patients and on *M. massiliense* from 27 patients. The results of antibiotic resistance patterns are presented in Table 8. The difference of antibiotic resistance patterns between *M. abscessus* and *M. massiliense* was observed in cefoxitin. The proportion of susceptible isolates was higher in *M. abscessus* than in *M. massiliense*. (60.6% *vs.* 25.0%; $P = 0.015$). Amikacin (91.2% in *M. abscessus vs*. 100% in *M. massiliense*), moxifloxacin (72.7% *vs*. 66.7%), linezolid (97.0% *vs*. 87.5%), clofazimine (87.9% *vs*. 95.8%) and tigecycline (100% *vs*. 100%) were active against most *M. abscessus* and *M. massiliense*. Cefoxitin (60.6% *vs*. 25.0%) and ciprofloxacin (14.3% *vs*. 7.7%) had activity against a moderate number of isolates of *M. abscessus* and *M. massliense*. Regarding to clarithromycin and azithromycin, *M. abscessus* became further highly resistant to clarithromycin or azithromycin on Day 14 after incubation. (the proportion of resistant to clarithromycin on Day 3, 2.9% *vs*. on Day 14, 88.6%; azithromycin on Day 3, 0% *vs*. on Day 14, 85.7%) In contrast, the susceptibility to clarithromycin or azithromycin was relatively remained in *M. massiliense* on Day 14 after incubation. (the proportion of susceptible to clarithromycin on Day 3, 92.3 % *vs*. on Day 14, 92.3%; azithromycin on Day 3, 100% *vs*. on Day 14, 100%) Inducible resistance to clarithromycin or azithromycin was significantly different between *M. abscessus* and *M. massiliense* (clarithromycin, $P < 0.001$; azithromycin, $P < 0.001$).

Table 8. Antibiotic resistance patterns of patients with *M. abscessus* and *M. massiliense* lung disease

			No. (%) of isolates			
Drug	Species	Total No.	Susceptible	Intermediate	Resistant	*P*
Amikacin	*M. abscessus*	35	31 (91.2)	3 (8.8)	0 (0)	0.255
	M. massiliense	26	25 (100)	0 (0)	0 (0)	
Cefoxitin	*M. abscessus*	34	20 (60.6)	13 (41.2)	0 (0)	0.015
	M. massiliense	24	6 (25.0)	18 (75.0)	0 (0)	
Ciprofloxacin	*M. abscessus*	36	5 (14.3)	16 (45.7)	14 (40.0)	0.305
	M. massiliense	27	2 (7.7)	17 (65.4	7 (26.9)	
Clarithromycin 3d	*M. abscessus*	34	33 (94.3)	1 (2.9)	1 (2.9)	0.482
	M. massiliense	25	24 (92.3)	2 (7.7)	0 (0)	
Clarithromycin 14d	*M. abscessus*	34	4 (11.4)	0 (0)	31 (88.6)	<0.001
	M. massiliense	25	24 (92.3)	2 (7.7)	0 (0)	
Doxycycline	*M. abscessus*	34	0 (0)	0 (0)	33 (100.0)	0.103
	M. massiliense	24	1 (4.3)	2 (8.7)	20 (87.0)	
Moxifloxacin	*M. abscessus*	34	24 (72.7)	7 (21.2)	2 (6.1)	0.694
	M. massiliense	25	16 (66.7)	5 (20.8)	3 (12.5)	
Linezolid	*M. abscessus*	34	32 (97.0)	1 (3.0)	0 (0)	0.324
	M. massiliense	25	21 (87.5)	2 (8.3)	1 (4.2)	
Clofazimine	*M. abscessus*	34	29 (87.9)	2 (6.1)	2 (6.1)	0.439
	M. massiliense	25	23 (95.8)	0 (0)	1 (4.2)	
Tigecycline	*M. abscessus*	34	33 (100.0)	0 (0)	0 (0)	

	M. massiliense	25	24 (100.0)	0 (0)	0 (0)	
Azithromycin_3d	*M. abscessus*	36	35 (100.0)	0 (0)	0 (0)	
	M. massiliense	28	27 (100.0)	0 (0)	0 (0)	
Azithromycin_14d	*M. abscessus*	36	4 (11.4)	1 (2.9)	30 (85.7)	<0.001
	M. massiliense	28	27 (100.0)	0 (0)	0 (0)	

B. Association of *M. abscessus* strains/ CCs with the antibiotic resistance patterns

Figure 5A shows an association of *M. abscessus* strains/ CCs with the antibiotic resistance patterns. The correlation was not clear, nevertheless, susceptibility to many antibiotics was observed in CC2.

C. Association of *M. massiliense* strains/ CCs with the antibiotic resistance patterns

M. massiliense strains/CCs and antibiotic resistance pattern was presented in figure 5B. The association was not remarkable.

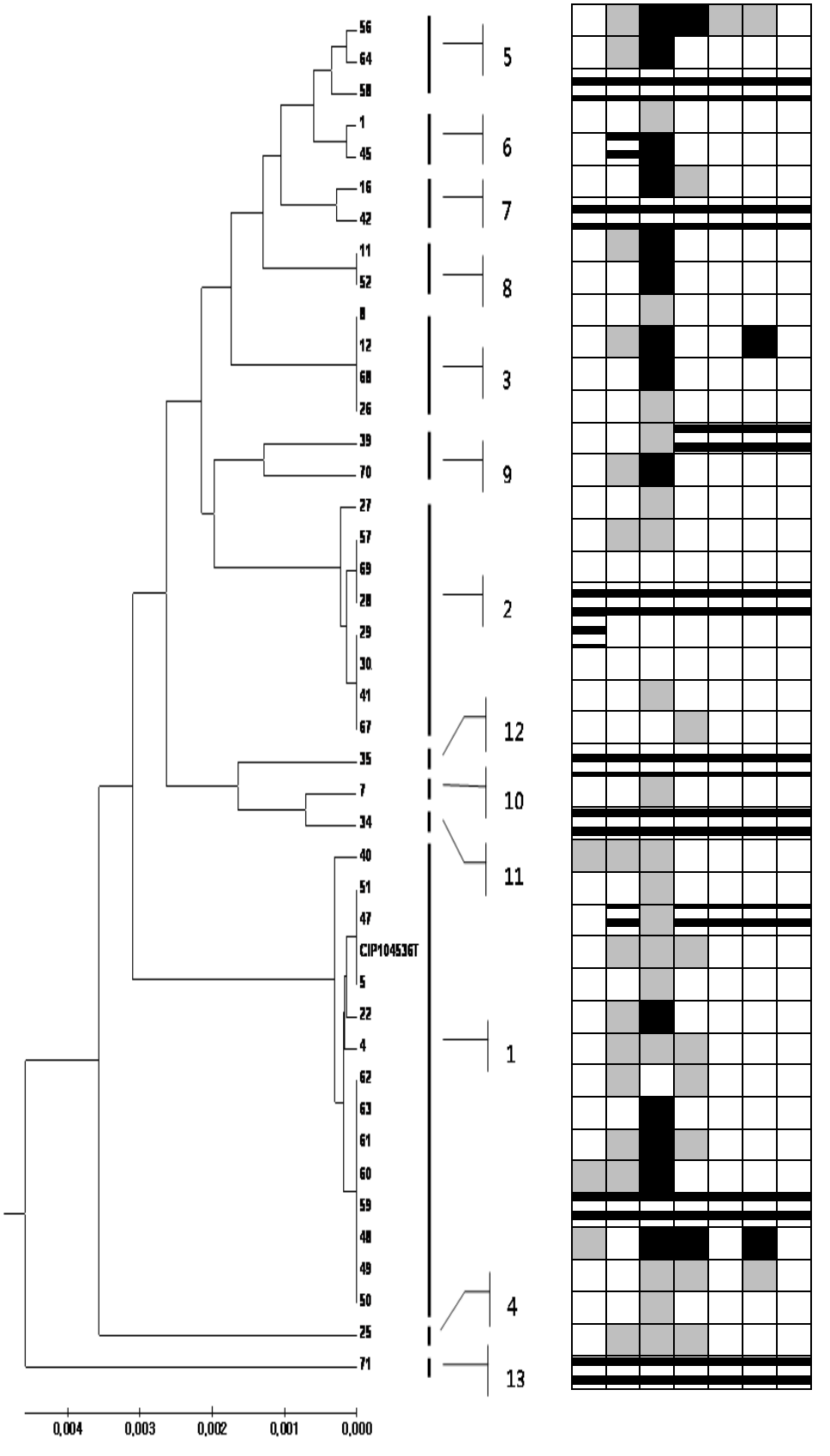
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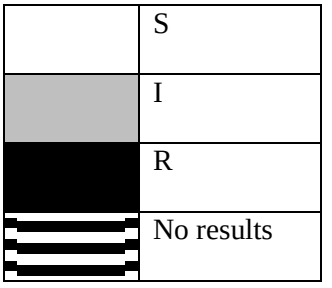
Figure 5A. Association of *M. abscessus* strains/ CCs with the antibiotic resistance patterns. The correlation was not clear, nevertheless, susceptibility to many antibiotics was observed in CC2. The results of antibiotic susceptibility test are presented and Amikacin- Cefoxitin- Ciprofloxacin- Moxifloxacin - Linezolid- Clofazimine- Tigecycline are presented in order.



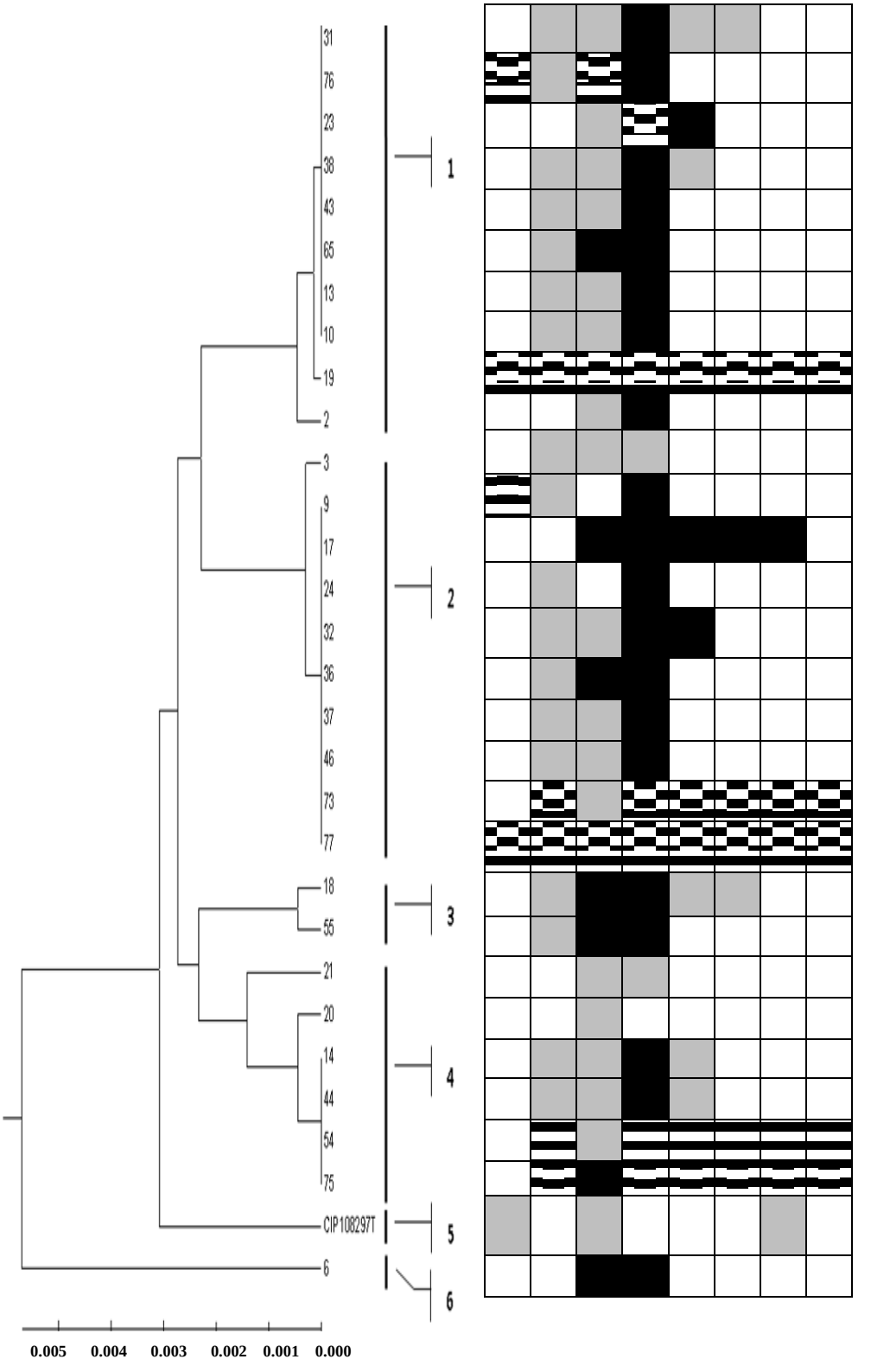

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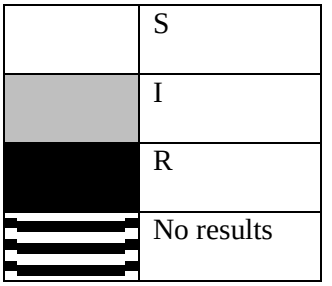
Figure 5B. Association of *M. massiliense* strains/ CCs with the antibiotic resistance patterns. The association was not remarkable. The results of antibiotic susceptibility test are presented and Amikacin- Cefoxitin- Ciprofloxacin- Doxycycline- Moxifloxacin – Linezolid- Clofazimine- Tigecycline are presented in order.



IV. DISCUSSION

We identified species of *M. abscessus* complex using MLSA and *rpoB* before developing the MLST scheme. There was a discrepancy in the identification of *M. abscessus* complex between MLSA and *rpoB* sequencing in only a single isolate (strain 6). This isolate was identified as *M. massiliense* by MLSA but as *M. abscessus* by *rpoB* sequencing. The isolate was grouped as a different cluster from other clinical *M. massiliense* isolates in the phylogenetic tree generated using the concatenated sequences of seven MLST housekeeping genes. Macheras *et al*. reported that almost 10% of the isolates showed discordant results between MLSA and *rpoB* sequences and speculated that those findings resulted from the horizontal transfer of the *rpoB* gene.[27] Zelazny *et al*. also reported discordant identifications according to the method used. They suggested that the discordance may be due to the presence of distinct taxa at the subspecies level.[29]

We developed the MLST for the *M. abscessus* complex using six housekeeping genes and *rpoB* gene. The allelic diversities (*M. abscessus*: 0.60–3.46%, *M. massiliense*: 0.41–4.99%) of our samples were relatively low compared with other bacteria, although we excluded two genes that exhibited low frequencies of polymorphic sites. The allelic diversities of the MLST were 2.1% to 18% in *Acinetobacter baumannii,*[39] from 5.6% to 33.9% in *Neisseria meningitidis,*[40] and from 5.1% to 10.3% in *Corynebacterium diphtheriae.*[41] And, relatively low allelic diversity was reported in the MLST scheme of *Yersinia Ruckeri* (0.39% - 1.21%).[42] Nevertheless, the numbers of ST in our isolates were sufficient when compared to other MLST schemes.[39,42] Therefore, the selected genes are likely

suitable for population genetic studies of *M. abscessus* and *M. massiliense*, despite the low allelic diversities. However, because the housekeeping gene was selected for MLSA of *M. abscessus* complex previously, the used housekeeping genes could be inappropriate for MLST of *M. abscessus* complex. The screening of loci selection for MLST of *M. abscessus* is needed in the future study.

While various STs and CCs were identified in our MLST, identical STs or CCs were observed frequently, even among different patients and institutions. Among 21 clinical *M. abscessus* isolates from Seoul National University Hospital, 13 were identified as having an identical CC, CC1 (ST6, n = 8; ST5, n = 5). These results suggest that an outbreak occurred at the hospital during the study period. Although human-to-human transmission of NTM has not been documented,[43-47] a recent report stated that a high mycobacterial load in the index case could contaminate the clinical environment and might result in patient-to-patient transmission of *M. massiliense*.[48]

Currently, PFGE is considered as the gold standard among band-based typing techniques and epidemiological studies of NTM.[25,49] Therefore, the fact that all duplicated isolates from the same patients shared the same CCs by MLST and similar PFGE patterns showed that our MLST scheme was reliable and reproducible. In contrast, two pairs of duplicate isolates shared six identical allelic types of housekeeping genes but carried one different allele. Therefore they were identified as having different STs, but identical CCs. A similar phenomenon was observed in a study of *Pseudomonas aeruginosa*,[50] and the authors suggested that mutation of a housekeeping gene might have resulted

from frequent antibiotic therapy.[50]

Duplicate isolates presented at intervals of months. Interestingly, most duplicate clinical isolates from the same patient were identified as having some specific CCs (CC2 and CC3 in *M. abscessus*; CC1 and CC2 in *M. massiliense*). The results indicate that some *M. abscessus* and *M. massiliense* strains might be related to chronic infection. This is consistent with another report that some *M. abscessus* complex strains identified using VNTR profiles and rep-PCR cluster were discovered repeatedly in individual patients.[51] Further investigation using a larger sample group is needed due to the small number of duplicate isolates in this study.

Antibiotic treatment is not always required in NTM lung disease.[2] Because patients may not have severe or progressive disease, and the efficacy of treatment is low and drug toxicity is high, physicians may "wait and see" before treatment.[2, 52, 53] Therefore, it is important to find the factors related with the disease progression.

The clinical manifestation of patients was not different between *M. abscessus* and *M. massiliense*. These findings are similar to the results of previous study,[11] which showed that the clinical manifestation was not different between two species but treatment response was better in *M. massiliense* than in *M. abscessus*. However, the treatment response could not be evaluated in this study due to short follow-up duration. These findings suggest that the species-identification of *M. abscessus* complex cannot be the evidence about the initiation of treatment.

The progression of NTM lung disease was influenced by mycobacterial

virulence and host susceptibility.[2] There was report that the virulence of *M. avium* complex could be strain-specific.[54] *M. avium* complex strain from patients with progressive NTM lung disease showed strong virulence.[55] And, recently published study suggested that specific genotypes were associated with progression of disease in *M. avium*[56] and *M. abscessus* complex.[33] Taken together, progression of NTM lung disease could be associated with the specific strains. In this study, stable disease was observed in some CCs, however, validation is needed in larger cohort.

Antibiotic susceptibility test is recommended in NTM isolates, although the correlation between *in vivo* and *in vitro* susceptibility test has not been determined.[2,5] Antibiotic resistance patterns were similar between *M. abscessus* and *M. massiliense* except inducible resistance of clarithromycin and azithromycin. The results were consistent with previous studies.[11,38] Nash *et al.* reported that *erm* (41) gene confers inducible macrolide resistance to isolates of *M. abscessus.* Mutation or characteristics of *erm* (41) gene may be associated with the inducible resistance of macrolide and treatment response according to the recent studies.[11,38] Validation of this correlation in our isolates will be meaningful.

The association between antibiotic resistance patterns and specific strains or CCs was not distinct. However, CC2 in *M. abscessus* could be related with the susceptibility to many antibiotics, especially ciprofloxacin and moxifloxacin. These results suggest that ciprofloxacin or moxifloxacine can be useful in some strains of *M. abscessus*. The more clinical isolates and clinically follow-up is required to verify.

The MLST scheme provides appropriate resolution and reproducibility, nevertheless, there some limitations. This MLST scheme could be used for epidemiologic study like MLST scheme of other bacteria. However, defining MLST pattern of *M. abscessus* complex from environment is needed for epidemiologic study, because only *M. abscessus* complex from respiratory specimens were used in this study. And, MLST scheme did not show clear association with clinical characteristics and antibiotics resistance patterns, and there were some possible reasons. First, high-resolution power of MLST scheme which was similar to that of PFGE could be the reason although the high-resolution power is the strength of our MLST scheme. Second, because housekeeping genes used in this study was not selected based on the whole genome, the selected genes might not reflect the characteristics of strains. This is different finding compared to other study which showed the correlation of some CCs of *Propionibacterium acnes* with healthy skin or opportunistic infection.[57] Third, the clustering of clinical characteristics could be indefinite due to the short follow-up duration. Finally, the relatively small number of clinical isolates could be the reason.

V. CONCLULSIONS

An MLST scheme to type *M. abscessus* and *M. massiliense* was developed. The MLST scheme provides high-level resolution for identification of strains, and similar strains can be classified as CCs. The MLST also showed adequate reproducibility compared with studies of PFGE using duplicated isolates. Although the strains/ CCs determined by MLST scheme were not clearly associated with clinical characteristics and antibiotics resistance patterns, some trends were observed. Therefore, this MLST scheme may be useful for studying the epidemiology of *M. abscessus* complex infections and for managing the patients with *M. abscessus* complex.

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ABSTRACT(IN KOREAN)

Mycobacterium abscessus complex 의 multilocus sequence typing 을 통한 계통분석 및 계통과 임상 특성•항생제 내성 유형과의 관련

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Mycobacterium abscessus (*M. abscessus*) 는 인간에게 다양한 질병을 유발하는 신속성장 마이코박테리움의 한종류이다. 최근 연구에서는 *M. abscessus* (추후에는 라 칭한다.) 가 실제로는 *M. abscessus, Mycobacterium massiliense (M. massiliense), Mycobacterium bolletii* 세 균주로 분리된다는 보고들이 있다. 이 세 균주들은 항생제 감수성 결과나 치료 반응 및 임상 경과에 있어서 차이가 있다고 보고 되고 있다. 그리고 각각 *M. abscessus* complex 의 균주들 내에서도 임상 경과들이 다양하다고 알려져 있다. 이는 각각의 균주들 내에서도 다양한 계통(strain)으로 나뉘고, 이 내에서 다양한 임상양상과 예후가 나타날 수 있음을 시사한다.

Multilocus sequence typing (MLST) 는 PCR 기반의 테크닉으로 다양한 종류의 housekeeping 유전자들의 allelic type 을 조합하여 균주 계통의 sequence type (ST) 을 규명할 수 있는 방법이다. MLST 는 감염성 역학조사하는 데 있어서 적절한 재현성과 신뢰감 높은 방법으로, 현재 다양한 균주들에서 시작되어 사용되고 있다.

그러므로, MLST 를 사용하여 *M. abscessus* complex 균주의 ST 를 규명하는 것은 임상적으로 중요할 수 있다. 그러나 현재까지는 *M. abscessus* complex 의 MLST 구축에 관한 연구나, MLST 와 임상양상 및 항생제 감수성 결과와의 연관성에 관한 연구는 없다. 본 연구의 목적은 *M. abscessus* complex 의 MLST 를 구축하고, MLST 를 통해 확인한 균주들의 계통과 임상양상과 항생제 감수성 결과와의 연관성에 대해 규명하는 것이었다.

대한민국의 2개의 3차 의료기관에서 71명의 환자로부터 89개의 균주가 본 연구에서 이용되었다. 8 개의 housekeeping 유전자(*argH, cya, glpK, gnd, murC, pgm, pta and purH*) 의 다좌위서열분석(multilocus sequence analysis) 과 *rpoB* 유전자 sequencing 을 통하여 *M. abscessus* 는 42개, *M. massiliense* 는 29개로 분류되었다. MLST 를 구축하기 전에 polymorphic site 의 빈도가 가장 낮은 유전자 두개는 제외하였다.

MLST 구축 결과상, *M. abscessus* 는 26개의 ST 와 13개의 clonal complex (CC) 로 분류되었고, *M. massiliense* 는 12개의 ST 와 6개의 CC 로 분류되었다. MLST date 는 한 환자에서 배출된 반복균주를 이용하여 시행한 pulsed-field gel electrophoresis 의 *XbaI*-macrorestriction 패턴과 일치도가 높았다.

M. abscessus 의 계통/ CC 와 질병 경과와의 연관관계는 명확하지는 않았지만, CC5 와 CC8 에서는 질병 경과가 안정적인 경향성이 있었다. *M. massiliense* 는 CC 내에서 고립된 CC (균주 번호 2,3,21) 의 경우 질병 경과가 안정적인 경향이 있었다.

M. abscessus 의 계통/ CC 와 항생제 감수성 결과와의 관련성은 확실하지는 않으나, CC2 에 속하는 균주들은 비교적 여러 항생제에 감수성이 있었다. *M. massiliense* 의 계통/ CC 와 항생제 감수성 결과와의 관련성은 없었다.

요약하면, 본 연구에서 구축된 MLST 결과상 *M. abscessus* complex 균주를 다양한 계통을 구분할수 있었고, 비슷한 계통의 균주들은 CC 형태로 분류될 수 있었다. 반복균주에서 시행한 PFGE 결과와 MLST 결과를 비교했을 때, *M. abscessus* complex MLST 의 재현성은 적절하였다. MLST 로 결정된 계통/ CC 와 임상 양상 및 항생제 감수성 결과와의 연관관계가 명확하게 존재하지는 않았지만, 부분적인 경향성은 관찰되었다. 그러므로, 본 연구에서 구축된 *M. abscessus* complex 의 MLST 는 향후에 *M. abscessus* complex 의 감염역학 연구에 있어서, 그리고 *M. abscessus* complex 관련 질환에 이환된 환자들을 치료하는 데 있어서 유용할 것이다.

핵심되는 말 : *Mycobacterium abscessus, Mycobacterium massiliense*, 다좌위서열분석(multilocus sequence analysis), *rpoB*, 다좌위서열타이핑(multilocus sequence typing), 항생제 감수성 검사

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