



## Research paper

# A novel DNA chromatography method to discriminate *Mycobacterium abscessus* subspecies and macrolide susceptibility

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## ARTICLE INFO

## Article History:

Received 5 November 2020

Revised 28 November 2020

Accepted 11 December 2020

Available online 11 January 2021

## Keywords:

DNA chromatography

Drug susceptibility

Subspecies discrimination

Non-tuberculosis mycobacteria

ATS/ERS/ESCMID/IDSA guideline

Whole genome sequencing

## ABSTRACT

**Background:** The clinical impact of infection with *Mycobacterium* (*M.*) *abscessus* complex (MABC), a group of emerging non-tuberculosis mycobacteria (NTM), is increasing. *M. abscessus* subsp. *abscessus/bolletii* frequently shows natural resistance to macrolide antibiotics, whereas *M. abscessus* subsp. *massiliense* is generally susceptible. Therefore, rapid and accurate discrimination of macrolide-susceptible MABC subgroups is required for effective clinical decisions about macrolide treatments for MABC infection. We aimed to develop a simple and rapid diagnostic that can identify MABC isolates showing macrolide susceptibility.

**Methods:** Whole genome sequencing (WGS) was performed for 148 clinical or environmental MABC isolates from Japan to identify genetic markers that can discriminate three MABC subspecies and the macrolide-susceptible *erm*(41) T28C sequevar. Using the identified genetic markers, we established PCR based- or DNA chromatography-based assays. Validation testing was performed using MABC isolates from Taiwan.

**Finding:** We identified unique sequence regions that could be used to differentiate the three subspecies. Our WGS-based phylogenetic analysis indicated that *M. abscessus* carrying the macrolide-susceptible *erm*(41) T28C sequevar were tightly clustered, and identified 11 genes that were significantly associated with the lineage for use as genetic markers. To detect these genetic markers and the *erm*(41) locus, we developed a DNA chromatography method that identified three subspecies, the *erm*(41) T28C sequevar and intact *erm*(41) for MABC in a single assay within one hour. The agreement rate between the DNA chromatography-based and WGS-based identification was 99.7%.

**Interpretation:** We developed a novel, rapid and simple DNA chromatography method for identification of MABC macrolide susceptibility with high accuracy.

**Funding:** AMED, JSPS KAKENHI

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## 1. Introduction

The *Mycobacterium* (*M.*) *abscessus* (heterotypic synonym; *Mycobacteroides abscessus*) [1,2] complex (MABC) is a group of rapid-

growing non-tuberculosis mycobacteria (NTM) that includes three subspecies: *M. abscessus* subsp. *abscessus* (*M. abscessus*), *M. abscessus* subsp. *massiliense* (*M. massiliense*), and *M. abscessus* subsp. *bolletii* (*M. bolletii*) [3,4]. MABC causes a range of clinical infections including chronic pulmonary disease even in immunocompetent persons, as well as postsurgical or traumatic infections and skin and soft tissue infections [4–8].

Among NTM infections, treatment outcomes for MABC infections are relatively worse and the in-hospital mortality rate can reach 16%

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## Research in context

### Evidence before this study

*Mycobacterium (M.) abscessus* complex (MABC) lung diseases is one of the most frequent and increasing non-tuberculosis mycobacteria (NTM) infections. Treatment outcomes for MABC infections are relatively worse due in part to the extensive antibiotic resistance of MABC. There are three main MABC subspecies: *M. abscessus* subsp. *abscessus* (*M. abscessus*), *M. abscessus* subsp. *bolletii* (*M. bolletii*), and *M. abscessus* subsp. *massiliense* (*M. massiliense*). Although some MABC isolates have multidrug resistance, particularly resistance to macrolides, a key drug used to treat NTM infections, some MABC are susceptible to macrolides. As such, methods to discriminate MABC subspecies and macrolide susceptibility in patient samples would be valuable to guide treatment decisions. Such methods should be rapid, user-friendly and not labor-intensive however, existing methods do not adequately meet those requirements.

On September 30, 2020, we searched PubMed for articles using the search terms (macrolide OR clarithromycin OR azithromycin OR erythromycin) AND (subspecies) AND ("*Mycobacterium abscessus*"), without any date or language restrictions. This search yielded 62 articles, of which four were relevant in that, they were original research articles describing the methods other than conventional sequencing and/or drug susceptibility testing to discriminate subspecies and macrolide susceptibility of MABC. Among these articles, one described subspecies-associated genomic regions to develop a PCR-based method to discriminate subspecies of MABC. However, genomic regions described in the previous study are completely different from those described in this study. There are three remaining studies in which the performance of a commercial kit has been tested using clinical isolates of MABC. We could not find any studies which propose a methodology to differentiate subspecies and macrolide susceptibility of this pathogen based on whole genome sequence analysis of a set of clinical isolates, and implement it in a user-friendly and rapid DNA chromatography assay.

### Added value of this study

We identified unique sequence regions that can be used to differentiate the three subspecies. In addition, we found that *M. abscessus* carrying the macrolide-susceptible erythromycin ribosomal methylase (*erm*)(41) T28C sequevar were tightly clustered, and identified 11 genes that were significantly associated with the lineage for use as genetic markers. By implementing these two findings about MABC genome architecture in a DNA chromatography, we developed a novel assay that identified three subspecies, the *erm*(41) T28C sequevar and intact *erm*(41) for MABC in a single assay within one hour. Overall agreement rate between the DNA chromatography-based and WGS-based identification was 99.7%. Considering the performance of our DNA chromatography, we believe that this assay could be introduced into clinical laboratory practice to facilitate selection of effective treatments.

### Implications of all available evidence

Although MABC mycobacteria grow rapidly, they nonetheless require several days of culture as compared to other bacteria that can be cultured in several hours. Previously-described assays to discriminate MABC subspecies require an initial culture. Upon further development, we anticipate that the sensitivity of the DNA chromatography method described in this

study will not require initial culture amplification as only 2000 bacterial copies are needed as the initial input. Our analysis indicated that the macrolide-susceptible *M. abscessus erm*(41) T28C sequevar is clustered and shares specific genetic loci. To best of our knowledge, this is the first report to describe the cluster and its associated genetic loci. Although the incidence of the *erm*(41) T28C substitution is unknown, a population of *M. abscessus* in our Japanese sample set carried it. Phylogenetic relationships between these mutants and global circulating clones of *M. abscessus* described in several studies should be addressed in the future. Further genomic epidemiology of international MABC clinical isolates is needed to uncover these upcoming research questions.

[5,9–13]. These poor outcomes are due in part to the extensive antibiotic resistance of MABC [14]. However, some MABC patients achieve good clinical outcomes with standard antibiotic regimens [15–17]. Out of the three subspecies, *M. massiliense* is susceptible to macrolide antibiotics whereas *M. abscessus* and *M. bolletii* are resistant [18,19]. In the presence of macrolide antibiotics *M. abscessus* and *M. bolletii* exhibit inducible expression of erythromycin ribosomal methylase (*erm*)(41), which produces the Erm protein that reduces macrolide affinity for the ribosome exit tunnel [20–22]. *M. massiliense* harbors a truncated *erm*(41) that produces inactive Erm(41). A T-to-C sequence variant (sequevar) at position 28 (T28C) of the *erm*(41) gene also results in production of an inactive enzyme, and does not result in inducible macrolide resistance of *M. abscessus* and *M. bolletii*, which are generally macrolide resistant. These observations indicate that determination of subspecies and detection of intact *erm*(41) and the *erm*(41) T28C sequevar of MABC can inform prediction of clinical course and treatment outcome. In fact, the 2020 ATS/ERS/ESCMID/IDSA Clinical Practice Guideline strongly recommends a macrolide-containing multidrug treatment regimen for patients with MABC respiratory disease caused by strains without inducible macrolide resistance [23]. Accordingly, discrimination of subspecies and identification of the *erm*(41) T28C sequevar is crucial.

Sequencing of single 16S rRNA or the RNA polymerase beta subunit (*rpoB*) cannot distinguish MABC subspecies because these loci are nearly identical [13,24]. Several studies have examined use of multi-locus sequencing typing (MLST) of housekeeping genes to separate subspecies [25–28]. Advances in whole-genome sequencing (WGS) technology allowed MABC clinical isolates to be phylogenetically divided into three subspecies even at the whole genome level [29,30]. However, detection of the *erm*(41) T28C sequevar still requires sequencing of the entire *erm*(41) gene. Although MLST and/or WGS analyses allow discrimination of *erm* genes, these analyses are time-consuming and labor intensive in clinical practice. Thus, novel assays that are simple and rapid yet retain discriminatory power required to distinguish subspecies and to identify the *erm*(41) T28C sequevar are needed.

We previously reported a PCR-based method to differentiate MABC subspecies, but the capacity of this test was limited [31]. In the present study, we analyzed WGS data for 148 MABC isolates from Japan to explore genetic markers associated with each subspecies and the *erm*(41) T28C sequevar. We propose a novel, rapid and easy-to-use DNA chromatography method that can identify all MABC subspecies as well as intact *erm*(41) and the *erm*(41) T28C sequevar in a single assay.

## 2. Methods

For DNA sequencing and genomic analyses including construction of primers, see the supplemental methods.

## 2.1. Bacterial isolates

A total of 147 MABC clinical isolates and one environmental isolate (strain MabLRBC1) obtained for differential diagnosis at 20 hospitals (listed in Acknowledgments) in Japan were considered. Of the clinical isolates, 138 originated in the respiratory system, 8 were isolated from skin lesions, and 1 strain was isolated from a blood sample (Table S1). Another 103 clinical isolates were obtained from Taiwan National University Hospital (Table S2). All strains were classified as *M. abscessus* complex using a DDH Mycobacteria kit (Kyokuto Pharmaceutical Industrial, Tokyo, Japan) or by MALDI-TOF MS (Bruker Daltonics, Billerica, MA, USA). *M. abscessus* JCM 13569 (=ATCC 19977), *M. massiliense* JCM 15300 and *M. bolletii* JCM 15297 (=BD) type strains were obtained from the Japan Collection of Microorganisms of the Riken Bio-Resource Center (BRC-JCM; Ibaraki, Japan). All bacterial strains/isolates were sub-cultured on 2% Ogawa egg slants or 7H10 agar plates supplemented with 10% OADC.

## 2.2. PCR assays for discriminating *M. abscessus* subspecies and *erm(41)* T28C sequevar

Single-PCR and multiplex PCR assays differentiating *M. abscessus*, *M. massiliense*, and *M. bolletii*, as well as the *erm(41)* T28C sequevar were conducted essentially as described previously [31] using newly constructed primers (see supplemental methods). Briefly, template DNA for PCR assays was isolated from one loopful of a mycobacterial colony grown on Middlebrook 7H10 agar that were resuspended in 300  $\mu$ l sterilized water, boiled at 95 °C for 15 min and frozen at -30 °C. PCR amplification was performed using a Mastercycler gradient (Eppendorf) with 95 °C for 10 min; 30 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 40 s; and extension at 72 °C for 10 min. Amplification to identify the *erm(41)* T28C sequevar was performed in the Mastercycler gradient using 95 °C for 10 min; 35 cycles of 95 °C for 1 min, 62 °C for 1 min, and 72 °C for 1 min; and extension at 72 °C for 4 min. The PCR products were separated by 2% agarose gel electrophoresis and stained with ethidium bromide. The analytical limit of detection of the multiplex PCR assay was estimated by applying serial dilutions of DNA from *M. abscessus* ATCC19977, *M. massiliense* JCM 15300, and *M. bolletii* BD, in addition to several clinical MABC isolates. To assess the multiplex PCR assay specificity, several laboratory strains and clinical isolates were used including the *M. avium* complex (strain 104 and 10 clinical isolates), *M. chelonae* JCM 6388 (one strain), *M. conceptionense* JCM 15299 (one strain), *M. fortuitum* (JCM 6387 and two clinical isolates), *M. gordonae* JCM 6382 (one strain), *M. houstonense* JCM 15656 (one strain), *M. kansasii* JCM 6379 (one strain), *M. leprae* (strain Thai-53 and 3 clinical isolates), *M. lentiflavum* JCM 13390 (one strain), *M. peregrinum* (two clinical isolates), *M. salmoniphilium* ATCC 13758 (one strain), *M. senegalense* JCM 15467 (one strain), *M. shimoidei* (one clinical isolate), *M. smegmatis* JCM 6386 (one strain), *M. szulgai* (two clinical isolates), *M. triplex* JCM 14744 (one strain), *M. tuberculosis* (strain H37Rv and 10 clinical isolates), and *M. xenopi* (JCM 15661 and two clinical isolates).

## 2.3. DNA chromatography assay for discriminating MABC subspecies, intact *erm(41)* and the *erm(41)* T28C sequevar

We applied a DNA chromatography method that was described elsewhere [32–34] to distinguish the MABC subspecies, intact *erm(41)* gene and the *erm(41)* T28C sequevar. This assay comprises PCR amplification and amplicon detection. All primers used in the assay are listed in Table 3. Briefly, primers with 5' tags, which have a domain that anneals to the target sequence and a tag domain that hybridizes to a single-stranded DNA probe on the chip or gold nanoparticle, were used for amplification. DNA extraction was performed essentially as described previously [6,35]. Total genomic DNA was

extracted from frozen samples (as described above) using a Kaneka easy DNA extraction kit for *Mycobacteria* (KANEKA, Osaka, Japan). PCR was performed using a 20  $\mu$ l mixture containing 10  $\mu$ l PCR Mix (KANEKA, Osaka, Japan), 5  $\mu$ l primer mix (5 primer sets, 0.5  $\mu$ M each, Table 3), 1  $\mu$ l template DNA. Amplification was performed in a Life ECO thermocycler (BIOER Co. Ltd., Hangzhou, China) with 25 °C for 5 min and 94 °C for 1 min, followed by 35 cycles of 94 °C for 5 s, 65 °C for 10 s, and 72 °C for 15 s. An aliquot of the amplicons supplemented with 70  $\mu$ l development buffer (KANEKA, Osaka, Japan) was applied to the detection strip sample pad (KANEKA, Osaka, Japan). After 10 min, blue lines were confirmed visually. Sensitivity and specificity tests for the DNA chromatography were performed as described above.

## 2.4. Statistical analysis

Statistical analyses were performed with R software ([www.r-project.org](http://www.r-project.org)). The R function `binom.test()` was used to assess 95% confidence intervals (95% CIs) of agreement rates between the multiplex PCR/DNA chromatography and WGS-based identification. Differences in accuracy between the multiplex PCR developed in this study and our previous multiplex PCR were statistically assessed using the R function `prop.test()`.

## 2.5. Ethics statement

This study was reviewed and approved by the medical research ethics committee of the National Institute of Infectious Diseases for inclusion of human subjects (#1004 and #1005 for the Japanese and Taiwanese, respectively).

## 2.6. Role of funders

Funders have no role in this study design, data collection, data analyses, interpretation or writing of the report.

## 3. Results

### 3.1. Subspecies identification based on core gene alignment of MABC isolates

In the context of genome-based taxonomy, phylogeny involving concatenated core gene alignments is frequently used instead of 16S rRNA or *rpoB* gene sequences. We first examined whether a concatenated sequence of core genes, which are defined as homologous genes present in all strains examined, could distinguish the three MABC subspecies. Using a concatenated sequence of 2957 core genes, the 148 MABC isolates could clearly be divided into three clades, with 92 (62.2%), 52 (35.1%), and 4 (2.7%) isolates identified as *M. abscessus*, *M. massiliense*, and *M. bolletii*, respectively (Fig. 1). This result is consistent with our previous report showing that multi-locus sequence typing (MLST) of *rpoB*, *hsp65*, and the ITS region could discriminate the three subspecies with 97.5% accuracy [31]. Moreover, most isolates could be phylogenetically categorized in agreement with the core-locus phylogeny, although 4 (2.7%) were inconsistently categorized (Fig. S1 a). Another group proposed a MLST scheme using seven housekeeping genes [28]. We confirmed that this scheme could reliably discriminate the three MABC subspecies (Fig. S1 b). We also confirmed the subspecies identification by calculating the average nucleotide identity (ANI) for all MABC isolates (Fig. S2). The minimum ANI within each of the three subspecies was 98.4 (within *M. abscessus*), 98.3 (within *M. massiliense*), and 99.1 (within *M. bolletii*), while the maximum ANI between subspecies was 97.5 (between *M. abscessus* and *M. massiliense*), 97.7 (between *M. abscessus* and *M. bolletii*), and 97.0 (between *M. massiliense* and *M. bolletii*). These results indicated that phylogenetic analysis based on core-locus alignment



indeed differentiated the three MABC subspecies and suggested that their subspecies boundaries were approximately 98% ANI.

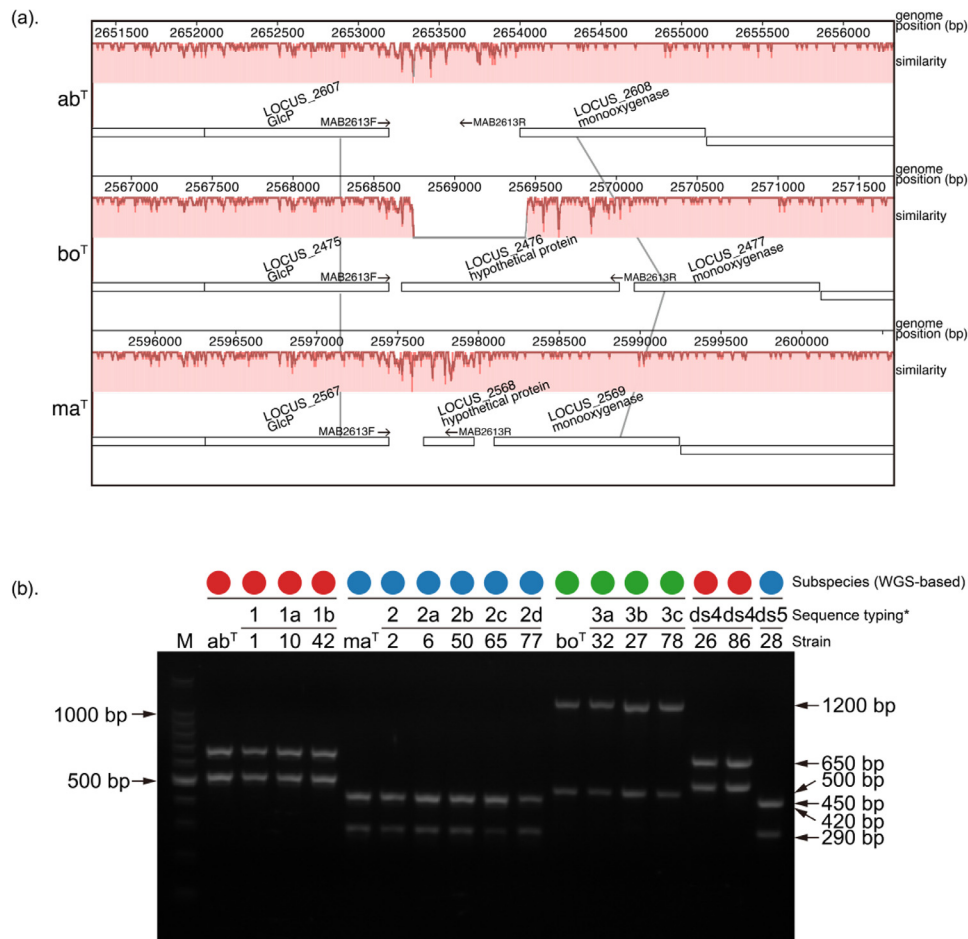
### 3.2. Multiplex PCR assay for discriminating the three MABC subspecies

Since WGS and/or MLST are so far not feasible for routine diagnostics at clinical settings [36,37], we sought to develop an alternative method to distinguish the three subspecies of MABC. We focused on “genetic markers” specific to each subspecies. We first aligned the complete genome sequences of type strains (ATCC 19977, JCM 15300, and BD) and draft genome sequences of 14 representative clinical isolates that covered the phylogeny (arrowheads in Fig. 1) with progressiveMauve [38] and visually identified unique insertion/deletion (indel) regions in each of the three subspecies. We then designed three primer sets specific for sequences around the indel regions for size-based differentiation of PCR amplicons (Fig. 2a, Table 1). All primer sets could sharply discriminate reference strains and clinical isolates as evidenced by a single band on an agarose gel (Fig. 2b and Fig. S3). A sensitivity test showed the limit of detection was 100 pg DNA (Fig. S4). All other laboratory and clinical isolates of NTM and *M. tuberculosis* tested were negative in this PCR assay (Fig. S5). We subsequently examined the primer set accuracy using the 148 MABC isolates, and confirmed that 91/92 *M. abscessus* (98.9%), 52/52 *M.*

*massiliense* (100%), and 4/4 *M. bolletii* (100%) isolates were in agreement with the WGS-based subspecies identification, and the overall agreement rate between the PCR assay and WGS-based subspecies identification was 99.3% (95% CI: 96.3% to 100%). Results of our previous MLST [31] showed several sequence variants in the isolates, but the PCR assay could still differentiate between strains (Fig. 2b, Fig. S3). Notably, discordant sequencing type 4 (MabLRC28 and MabLRC86) and ds type 5 (MabLRC28) were distinguished as *M. abscessus* and *M. massiliense*, respectively, in accordance with WGS-based subspecies identification (Fig. 2). Although our previous multiplex PCR assay [31] could not distinguish all *M. bolletii* from others (Table S1), the present PCR assay could distinguish them. Moreover, the agreement rate with WGS-based subspecies identification was significantly higher ( $P < 0.01$ , two-proportion Z test) than that of the previous multiplex PCR assay (92.6%, 95% CI: 87.1% to 96.2%).

### 3.3. Multiplex PCR assay for discriminating macrolide susceptibility of MABC

MABC have an Erm(41)-mediated inducible mechanism for resistance to macrolide antibiotics. Earlier studies demonstrated that a T-to-C substitution at position 28 in the *erm* gene results in macrolide antibiotic susceptibility [20,39]. Thus, discriminating isolates that



**Fig. 2. Example of indels among MABC subspecies.** (a) progressiveMauve alignment of the three reference strains of MABC is shown. Each genome is laid out in a horizontal track and white boxes indicate coding sequences annotated by dfast\_core [55]. A colored similarity plot is shown for each genome; the height is proportional to the sequence identity in that region. F and R indicate primer position of MAB2613F and MAB2613R (listed in Table 1), respectively, in each reference strain genome. (b) Representative multiplex PCR results for reference strains and clinical isolates amplified with primer pair MAB2613F and MAB2613R and primer pair MAB\_1655F and MAB\_1655R. Types 1, 1a, 1b, 2, 2a, 2b, 2c, 2d, 3, 3a, 3b, 3c, ds4 and ds5 are the sub-groupings (sequevars) of the clinical isolates based on their sequences [Table 3 of the previous article [31]]. Numerals below the sequevars correspond to reference strains (*ab*<sup>T</sup>, *ma*<sup>T</sup>, or *bo*<sup>T</sup>) or the strain numbers of clinical isolates described in the previous article [31]. Colored circles correspond to each member of MABC determined by WGS-based analysis. Lanes: M, DNA marker (100 bp ladder); *ab*<sup>T</sup>, *M. abscessus* (ATCC19977); *ma*<sup>T</sup>, *M. massiliense* (JCM15300); *bo*<sup>T</sup>, *M. bolletii* (BD). The PCR reaction products were electrophoresed on 2% agarose gels.

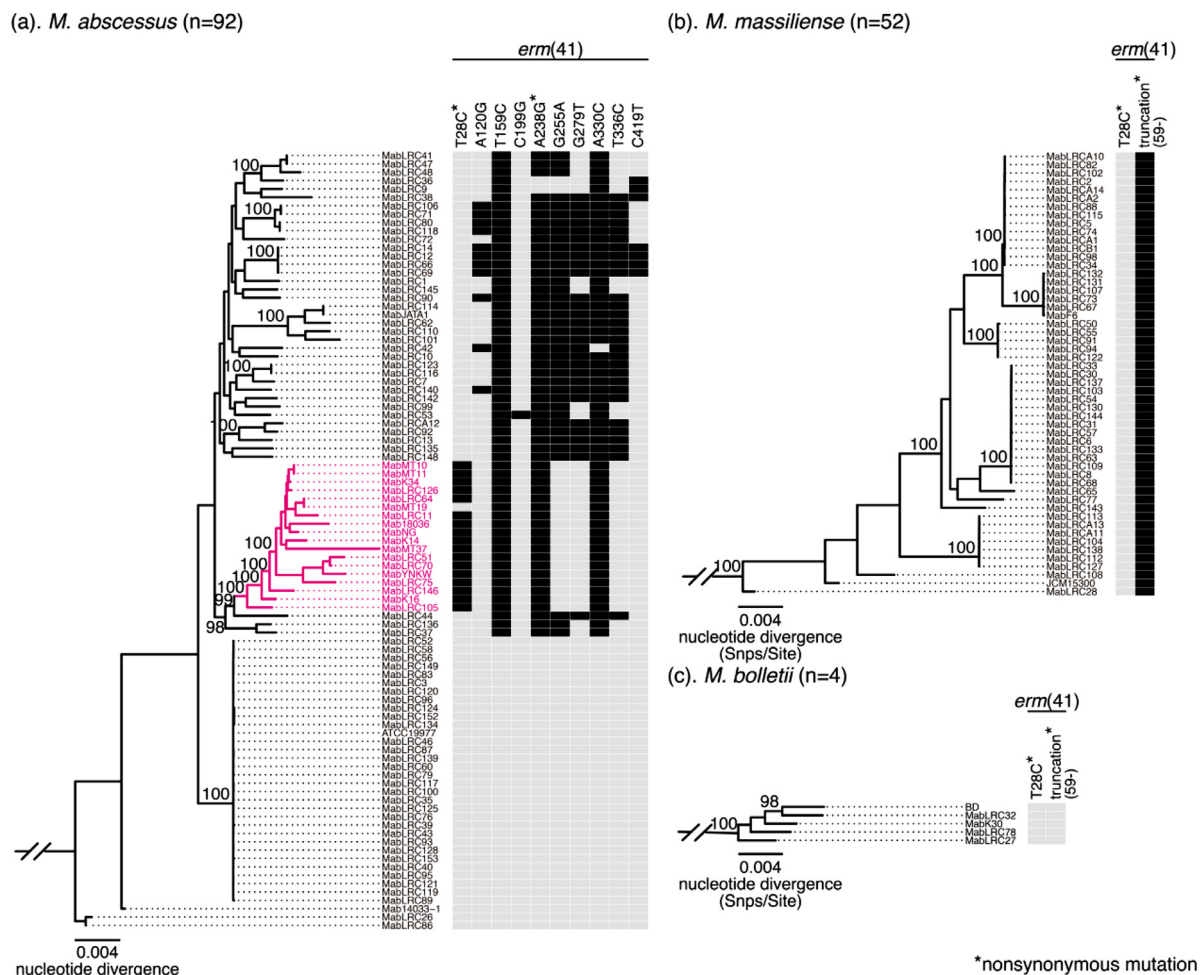
**Table 1**  
Primers for discrimination of MABC subspecies.

Set	Primer name	Sequence (5'–3')	Expected product size (bp)			Note
			<i>M. abscessus</i>	<i>M. massiliense</i>	<i>M. bolletii</i>	
1	MAB2613F	gttcggatcgcatggcgtgtgtctg	503	422	1204	ATCC 19977 (2,653,095 to 2,653,597), C-term of MAB_2613 to downstream of MAB_2613
	MAB2613R	Gggatgctgtgatcgaggtcggc				
2	MAB_1655F	gagggcacgggagagaccaccggag	652	291	452	ATCC 19977 (1,685,112 to 1,685,763), C-term of MAB_1655 to downstream of MAB_1655
	MAB_1655R	ccatttcYctatcYcgcccg				
3	MAB_4665F	gatcccgttactagcgtgctttac	332	538	666	ATCC 19977 (4,747,125 to 4,747,435), intergenic region, downstream of MAB_4665c
	MAB_4665R	Tcccgttcgactggcggccgga				

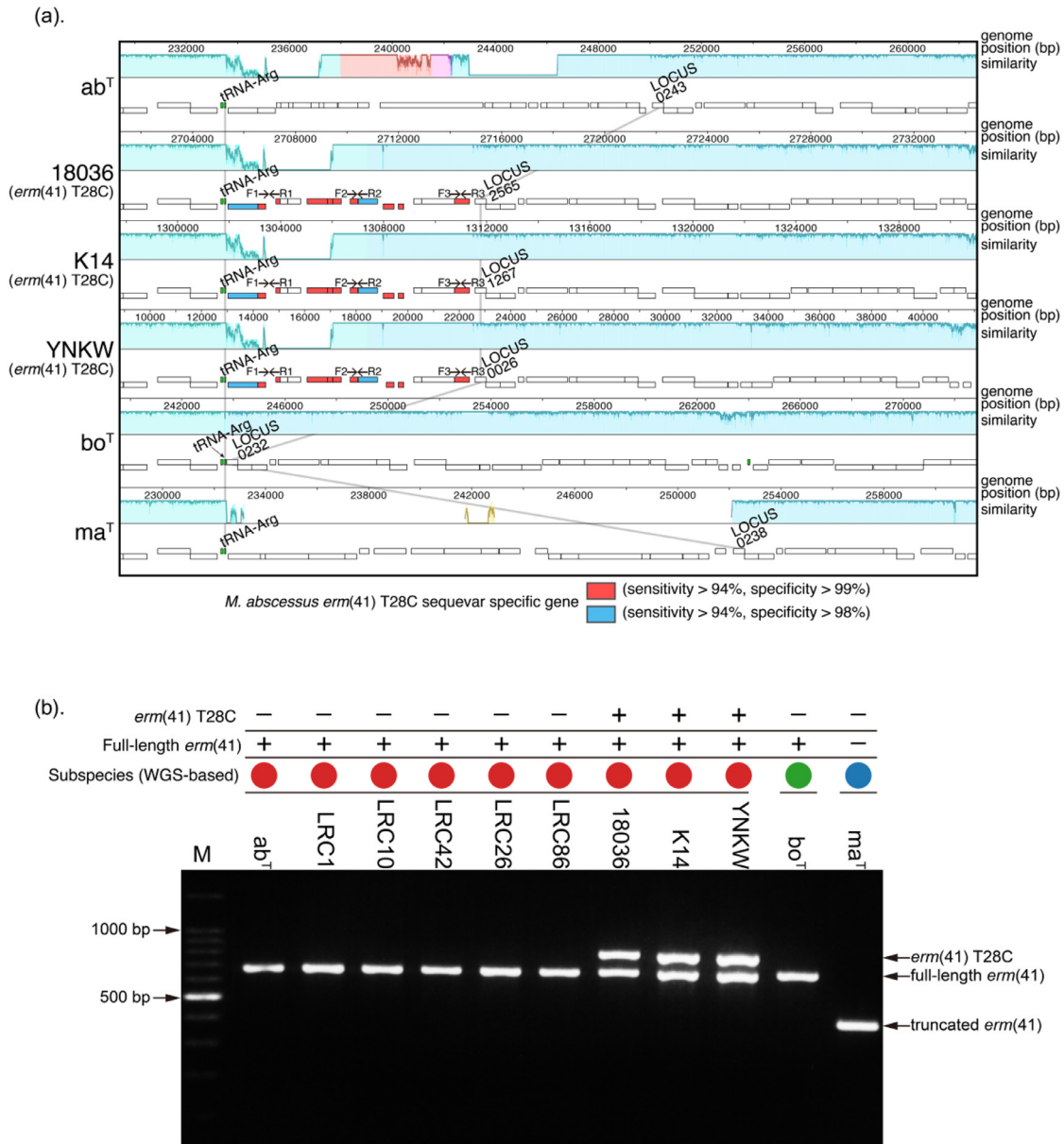
Y; c or t

carry the *erm(41)* T28C sequevar could guide antibiotic selection. To identify genetic markers associated with the *erm(41)* T28C sequevar, we investigated the *erm(41)* genotype of the 148 MABC isolates and their phylogenetic relationship (Fig. 3). Of the 92 *M. abscessus*, 17 (18.5%) had the *erm(41)* T28C mutation and all but MablRC70 were susceptible to clarithromycin (CAM) (Table S1). Notably, our phylogenetic analysis showed that these clinical isolates were tightly clustered (Fig. 3). Scoary analysis of lineage-associated genes indicated

that 68 genes were significantly associated with the lineage to which all *M. abscessus* with the *erm(41)* T28C sequevar belonged (Bonferroni corrected  $P$ -value <  $1E-8$ , Fisher's exact test, sensitivity >80% and specificity > 80%, Table S4). In a whole-genome alignment, 11/68 lineage-associated genes having the highest sensitivity and specificity were on a lineage-specific genetic locus (Fig. 4a). To determine whether these findings applied to other sample sets, we used public WGS data for MABC clinical isolates from two European countries



**Fig. 3.** Macrolide susceptibility-associated genotypes of 148 MABC isolates. Maximum likelihood core-gene phylogeny of (a) *M. abscessus*, (b) *M. massiliense*, (c) *M. bolletii* correspond to those depicted in Fig. 1. The presence (black) and absence (gray) of macrolide resistance-associated mutations is indicated. The presence of a T-to-C substitution in position 28 or a truncation of the *erm(41)* gene, which are both associated with inducible resistance to macrolides, was detected. Substitutions or truncations with asterisks indicate nonsynonymous mutations. The lineage to which all *M. abscessus* *erm(41)* T28C mutants belong is highlighted in magenta. The maximum likelihood trees, bootstrap values and scale bars correspond to those depicted Fig. 1.



**Fig. 4. Visualization of lineage-specific genomic loci.** (a) A progressiveMauve alignment of three clinical isolates carrying the *erm(41)* T28C mutation and the reference strains is shown. Boxes indicate coding sequences annotated by dfast\_core [55]. Red and blue boxes indicate genes that are significantly associated with the lineage to which all *M. abscessus* *erm(41)* T28C mutants belong (see Methods and Table S3). F1, R1, F2, R2, F3, and R3 indicate primer position of MAB18036\_2551F, MAB18036\_2551R, MAB18036\_2558F, MAB18036\_2558R, MAB18036\_2560F, and MAB18036\_2560R (listed in Table 2), respectively, in each genome of the presented clinical isolates. A similarity plot for each genome is colored as described for Fig. 2a. (b) Representative multiplex PCR results to identify inducible macrolide resistance in MABC. PCR was performed for the reference strains and clinical isolates were amplified with primer pairs MAB18036\_2558F and MAB18036\_2558R (listed in Table 2) and primer pair *ermF* (gaccggggccttctctgtgatc) and *ermR* (agcttccccgcacc-gattcca) [56]. Colored circles correspond to each member of MABC determined by WGS-based analyses: red, *M. abscessus*; blue, *M. massiliense*; green, *M. bolletii*. The presence (+) or absence (-) of genotypes associated with the inducible macrolide resistance are shown. Lanes: M, DNA marker (100 bp ladder); *ab<sup>T</sup>*, *M. abscessus* (ATCC19977); *ma<sup>T</sup>*, *M. massiliense* (JCM15300); *bo<sup>T</sup>*, *M. bolletii* (BD). The PCR reaction products were electrophoresed on 2% agarose gels. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

[40,41]. A phylogenetic analysis based on the core-gene alignment indicated that the *M. abscessus* *erm(41)* T28C sequevar was also clustered and had the abovementioned genetic locus (Fig. S6). Among primer sets designed to amplify part of the genetic locus (Table 2, Fig. 4a), one set detected only the *M. abscessus* *erm(41)* T28C sequevar with a single band (Fig. 4b). Among the 148 MABC isolates, all 17 *M. abscessus* *erm(41)* T28C sequevars were positive in the PCR assay, whereas all other clinical isolates examined, except for MabMT19 and MabLRC77, were negative (Table S1). The agreement between the multiplex PCR assay results with WGS-based discrimination of the *erm(41)* T28C sequevar was 98.6% (95% CI: 95.2% to 99.8%).

### 3.4. DNA chromatography to discriminate subspecies and macrolide susceptibility of MABC

Based on the results for the two PCR-based assays to discriminate MABC subspecies and the *erm(41)* T28C sequevar, we developed a simple DNA chromatography-based assay to discriminate MABC subspecies, intact *erm(41)*, and the *erm(41)* T28C sequevar. We could discriminate subspecies and macrolide resistance in a single assay that in a sensitivity test had a detection limit of 10 pg DNA (Fig. 5), which was more sensitive than that of the multiplex PCR assay (Fig. S4, S7). All other laboratory and clinical isolates of NTM as well as *M. leprae* and *M. tuberculosis* that were tested were negative in the assay

**Table 2**  
Primers for discrimination of the *erm(41)* T28C sequevar.

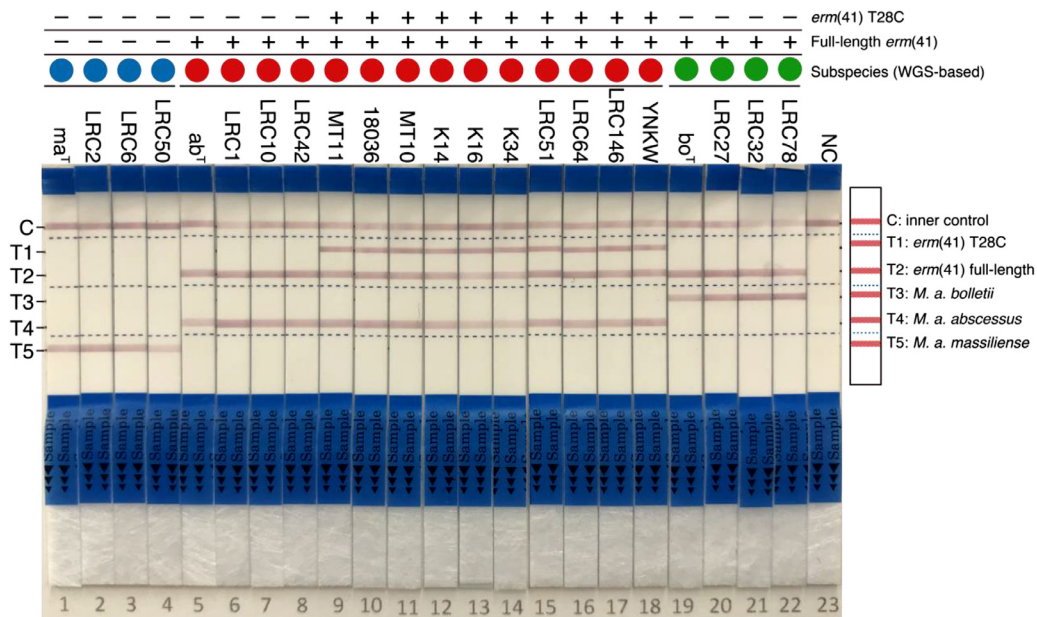
Set	Primer name	Sequence (5'–3')	Expected product size of <i>M. abscessus</i>	Note
1	MAB18036_2551F	ccgaatcggatacgggcccgggtaca	601	Mab18036 (contig11: 13,301 to 13,902), C-term of Mab18036_2551 to N-term of Mab18036_2553
	MAB18036_2551R	cgcctcgatactcagcgcgccttca		
2	MAB18036_2558F	caagaaccacatggataaacccgactg	730	Mab_18,036 (contig11: 16,597 to 17,327), Upstream of Mab18036_2558 to Mab18036_2559
	MAB18036_2558R	catcgctcgggatcattcagcggcag		
3	MAB18036_2560F	caggagcatcgtgcagatccgctgctg	820	Mab18036 (contig11: 18,219 to 19,039), middle of Mab18036_2560 to downstream of Mab18036_2561
	MAB18036_2560R	accctgtttgccagcagcctaact		

(Fig. S8 and data not shown). Using the 148 MABC isolates, we also examined the agreement between the DNA chromatography assay and WGS-based discrimination. All *M. abscessus* ( $n = 92$ ), *M. massiliense* ( $n = 52$ ), and *M. bolletii* ( $n = 4$ ) isolates were positive with T4, T5, and T3 bands respectively, while all other isolates identified as the remaining two subspecies were negative for these bands (Table 4, Table S1). All *M. abscessus* carrying the *erm(41)* T28C sequevar were positive, whereas other strains carrying wild-type T28 were negative for the T1 band, except for MabMT19 and MabLRC77 clinical isolates. All *M. abscessus* ( $n = 92$ ) and *M. bolletii* ( $n = 4$ ) strains carrying a full-length *erm(41)* gene were positive for the T2 band but all *M. massiliense* ( $n = 53$ ) carrying a truncated *erm(41)* gene were negative, which is consistent with WGS-based analyses (Table 4, Fig. 3). Overall agreement between the DNA chromatography results with WGS-based discrimination was 99.7% (95% CI: 99.0 to 100%). We also used the DNA chromatography method to analyze another sample set comprising 103 MABC clinical isolates from Taiwan for validation (Table S2). Using this method, within only a few hours we could determine that 49, 2, and 50 clinical isolates were *M. abscessus*, *M. bolletii* and *M. massiliense*, respectively; subspecies of TJMA-002 and TJMA-104 (1.9%) were not determined because these isolates showed multiple bands in subspecies identification (Table S2). Of 47 clinical isolates showing the T4 band, 12 also showed the T1 and T2 band, which corresponded to the *M. abscessus erm(41)* T28C sequevar, while 37 isolates showed only the T2 band corresponding to *M. abscessus* with an intact *erm(41)* gene. Two clinical isolates showing

the T3 band also showed the T2 band, indicating that all *M. bolletii* had an intact *erm(41)* gene. Of 50 isolates showing the T5 band, 47 did not show the T2 band, indicating an *erm(41)* gene truncation. However, the remaining three isolates (TJMA-024, TJMA-041, TJMA-046) unexpectedly showed the T2 band. Using PCR amplification, TJMA-024 and TJMA-041 isolates had intact *erm(41)*, whereas TJMA-046 had both an intact and truncated *erm(41)* gene (data not shown). These results suggested that TJMA-024 and TJMA-041 were *M. massiliense* with an intact *erm(41)* gene and TJMA-041 was probably a mixed isolate of *M. abscessus* or *M. bolletii* having an intact *erm(41)* gene and *M. massiliense* with a truncated *erm(41)* gene.

#### 4. Discussion

MABC is the most frequent clinical isolate of rapidly growing mycobacteria and an increased emergence has recently been observed in Japan and other developed countries [42–44]. Commercially available DNA-DNA hybridization kits or MALDI-TOF MS are available in clinical laboratories in Japan and Taiwan to identify mycobacterium isolates [45,46], but they cannot discriminate between either subspecies or the macrolide-susceptible *erm(41)* truncation and T28C polymorphism [47]. Since macrolide susceptibility is crucial for effective treatment of MABC infection, here we developed a novel multiplex PCR and DNA chromatography method to identify subspecies and macrolide susceptibility. This assay allows rapid and accurate identification of inducible-macrolide resistance



**Fig. 5.** DNA chromatography to differentiate subspecies and macrolide susceptibility of MABC. DNA chromatography results for reference strains ( $ab^T$ ,  $ma^T$ , or  $bo^T$ ) or representative clinical isolates are shown. Colored circles above the strain names correspond to each member of MABC determined by WGS-based analyses: red, *M. abscessus*; blue, *M. massiliense*; green, *M. bolletii*, respectively. The presence (+) or absence (-) of genotypes associated with inducible macrolide resistance are shown. Bands: C, inner (negative) control; T1, *erm(41)* T28C polymorphism; T2, intact *erm(41)* genes; T3, *M. bolletii*; T4, *M. abscessus*; T5, *M. massiliense*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)



**Table 3**  
DNA chromatography primers.

Set	Primer name	Sequence (5'–3')	Target
T1	C28–1f	tcctcggaatcggcactgtccgttg	<i>erm(41)</i> T28C sequevar
	C28–1r	tacagcagctcaacagtgacaccgaag	
T2	erm–4f	cgctgccgaatccggtgttcgc	intact <i>erm(41)</i>
	erm–4r	ctcggcaaacctggaacgaaggtgtc	
T3	MBO–22f	cggtacgtcttacagctcagattgt	<i>M. bolletii</i>
	MBO–22r	acgaggtggataccgcgatcatt	
T4	MAB–25f	atgttggaccgcaaggggttcgacac	<i>M. abscessus</i>
	MAB–23r	gtcaatacagatgaagccgacctgg	
T5	MMA–20f	tgctcgagagggatgtatccaccac	<i>M. massiliense</i>
	MMA–20r	atatcacatcagcgaagccgaag	

**Table 4**  
Accuracy of DNA chromatography test using MABC isolates from Japan.

	<i>erm(41)</i> C28	<i>erm(41)</i> T28	Agreement rate with WGS-based identification
T1 positive	17	2	0.986 (95% CI: 0.952 to 0.998)
T1 negative	0	129	
	<i>erm(41)</i> full-length	<i>erm(41)</i> truncated	Agreement rate with WGS-based identification
T2 positive	96	0	1.000 (95% CI: 0.975 to 1.000)
T2 negative	0	52	
	<i>M. bolletii</i>	not <i>M. bolletii</i>	Agreement rate with WGS-based identification
T3 positive	4	0	1.000 (95% CI: 0.975 to 1.000)
T3 negative	0	144	
	<i>M. abscessus</i>	not <i>M. abscessus</i>	Agreement rate with WGS-based identification
T4 positive	92	0	1.000 (95% CI: 0.975 to 1.000)
T4 negative	0	56	
	<i>M. massiliense</i>	not <i>M. massiliense</i>	Agreement rate with WGS-based identification
T5 positive	53	0	1.000 (95% CI: 0.975 to 1.000)
T5 negative	0	51	

without need for sequencing of the *erm(41)* gene and/or 14-day drug susceptibility testing as recommended in the recent ATS/ERS/ESCMID/IDSA Clinical Practice Guideline [23]. Our methodology is based on two findings about MABC genome architecture: (i) indel regions are robustly conserved at the subspecies level, and (ii) the macrolide-susceptible *M. abscessus* T28C sequevar is phylogenetically clustered and shares specific genetic loci. We thus used subspecies-associated and *erm(41)* T28C sequevar-associated genomic sequences as genetic markers to predict macrolide susceptibility. By combining detection of these genetic markers with simple DNA chromatography without DNA degeneration processes, our assay could discriminate both subspecies and their macrolide susceptibility more quickly and easily than previously described MLST or WGS methods [45].

DNA chromatography produces clear visual results using only a thermocycler rather than more expensive and complex genome sequencers or MALDI-TOF MS instruments. The one hour turnaround time between DNA extraction to availability of results would substantially reduce the need for MLST or WGS to differentiate MABC subspecies and inducible macrolide susceptibility. Compared with the commercially available GenoType NTM-DR kit (Hain Lifesciences GmbH, Bruker Corporation, Nehren, Germany), the present DNA chromatography method is simpler (DNA chromatography vs. Southern blotting) and faster (1 hour vs. >4 h) but the accuracy of subspecies identification and the ability to discriminate the *erm(41)* T28C sequevar are comparable [48–50], although the GenoType NTM-DR kit can detect additional acquired macrolide resistance and amikacin resistance.

Our DNA chromatography method had an analytical limit of detection of 10 pg DNA, which is substantially more sensitive than the GenoType NTM-DR kit (2 ng) [50]. This amount of DNA theoretically corresponds to approximately  $2.2 \times 10^3$  MABC cells (DNA content/cell = genome size(bp)/0.978  $\times 10^9 \approx 5.11 \times 10^{-3}$ ) [51]. There was no cross-reactivity with *M. leprae*, *M. tuberculosis*, MAC, or other representative NTM. These observations suggest that the DNA

chromatography assay can identify MABC isolates directly from liquid MGIT or solid Löwenstein-Jensen (LJ) or Ogawa cultures at early time points after decontamination of non-mycobacterial organisms. However, we have not yet tested this DNA chromatography method with highly contaminated nucleotide samples extracted directly from sputum or skin specimens. Future improvements to further increase the speed of this method will focus on direct detection from tissue specimens without culturing.

In the present work, we sought to detect associations between indel regions and the subspecies, and between genetic loci and the *erm(41)* T28C mutation. Our method successfully discriminated the subspecies and inducible macrolide susceptibility of almost all of the clinical isolates, but the few exceptions represent a limitation of the method that should be considered. MabMT37 yielded a 1200 bp and 450 bp product for locus MAB\_2613 and MAB\_1655 (similar to *M. bolletii* strains) and a 330 bp product for MAB\_4665 (similar to *M. abscessus* strains). However, WGS-based phylogenetic and ANI analyses unambiguously categorized MabMT37 as *M. abscessus* (Table S1). We mapped raw sequence reads of MabMT37 to the *M. abscessus* ATCC 19977 genome and confirmed that there was no heterogeneity among the MAB2613, MAB\_1655, and MAB\_4665 loci (data not shown), suggesting that MabMT37 was likely a mono-clonal isolate. This result also suggested that, with respect to these genetic markers, MabMT37 had a chimeric genome structure between *M. abscessus* and *M. bolletii*. MabMT19 showed inducible clarithromycin resistance and had no mutation at *erm(41)* position 28, but it did cluster with other *erm(41)* T28C mutants and was positive for the primer set MAB18036\_2558F and MAB18036\_2558R (Table S1), suggesting that MabMT19 had a chimeric genome between *M. abscessus* clades. These observations are supported by a previous genomic study describing an asymmetrical gene flow between MABC subspecies that resulted in a highly mosaic genome architecture [52]. Since our assays do not directly analyze housekeeping genes or the *erm(41)* gene itself, our approach could be affected by genome architecture mosaicism arising

via horizontal gene transfer. To address this limitation, accumulation of genomic information for MABC clinical isolates from across the world and successive adjustments of target genomic sequences will be important.

In conclusion, we developed a rapid, easy-to-use, and accurate assay to identify subspecies and macrolide susceptibility of MABC by analyzing WGS data of clinical isolates. This assay could be introduced into clinical laboratory practice to facilitate selection of effective treatments, development of assays having improved discrimination and diagnostic capacity, and acquisition of precise, nation-wide epidemiological information for MABC. Although the incidence of the macrolide-susceptible *erm*(41) T28C mutation is unknown, a population of *M. abscessus* in our Japanese sample set (18.5%) carried this mutation. Phylogenetic relationships between these mutants and global circulating clones of *M. abscessus* [30] should be addressed in future studies. Additional international corroboration studies based on epidemiological and population genomic approaches will be required to address these fundamental research questions.

#### Data sharing statement

All raw data and materials are available by request to the corresponding authors. All raw read data for the newly sequenced isolates in this study are available at the National Center for Biotechnology Information (NCBI) under BioProject accession number PRJDB10333.

#### Declaration of Competing Interest

Mitsunori Yoshida, Sotaro Sano, Shigehiko Miyamoto and Yoshiko Hoshino are listed on a pending patent in Japan for the DNA chromatography methodology to distinguish MABC and identify macrolide susceptibility (JP2020–066277 and JP2020–066306). Dr. Morimoto and Dr Kurashima report personal fees as Consultant of INSMED, outside the submitted work. The other authors have nothing to disclose under this manuscript.

#### Acknowledgments

This work was supported in part by grants from the Japan Agency for Medical Research and Development/Japan International Cooperation Agency (AMED) to Y.H. (jp19fk0108043, jp19fk0108064, jp19fk0108075, and jp19jm0510004) and to M.A. (jp19fk0108043 and jp19fk0108049); by grants-in-aid from the Japan Society for Fostering Joint International Research (B) to Y.H. and M.Y. (jp19KK0217), for Early-Career Scientists to M.Y. (jp20K17205) and H.F. (jp18K15966), and for Scientific Research (C) to Y.H. (jp18K08312). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The clinical isolates used in this study were sent from the hospitals and universities listed below. We appreciate the work of all of the clinicians at the following institutions who cared for patients infected with these mycobacteria: Hokkaido Social Insurance Hospital, Fukujiji Hospital Japan Anti-Tuberculosis Association (JATA), Saitama Medical University Hospital, National Hospital Organization (NHO) Tokyo Hospital, Showa University Fujigaoka Hospital, Keio University Hospital, National Defense Medical College Hospital, Kyorin University Hospital, Nagaya City University Hospital, NHO Minami-Kyoto Hospital, Kyoto Prefectural University of Medicine Hospital, Osaka City Northern-City Hospital, Osaka Hospital JATA, NHO Kinki-Chuo Chest Medical Center, NHO Matsue Medical Center, Kawasaki Medical School, NHO Higashi-Hiroshima Medical Center, Kyosai-Yoshijima Hospital, Kyushu University Hospital, and NHO Omuta Hospital. Computations were performed in part on the NIG supercomputer at the ROIS National Institute of Genetics. We thank Ms. Maki Okuda, Sayaka Kashiwagi, and Ginko Kaneda for their assistance.

#### Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ebiom.2020.103187.

#### References

- Oren A, Garrity GM. List of new names and new combinations previously effectively, but not validly, published. *Int J Syst Evol Microbiol* 2016;66:2463–6.
- Gupta RS, Lo B, Son J. Phylogenomics and comparative genomic studies robustly support division of the genus *Mycobacterium* into an emended genus *Mycobacterium* and four novel genera. *Front Microbiol* 2018;9:67.
- Adekambi T, Sassi M, van Ingen J, Drancourt M. Reinstating *Mycobacterium massiliense* and *Mycobacterium bolletii* as species of the *Mycobacterium abscessus* complex. *Int J Syst Evol Microbiol* 2017;67:2726–30.
- Tortoli E, Kohl TA, Brown-Elliott BA, Trovato A, Cardoso-Leão S, Garcia MJ, et al. *Mycobacterium abscessus*, a taxonomic puzzle. *Int J Syst Evol Microbiol* 2018;68:467–9.
- Griffith DE, Aksamit T, Brown-Elliott BA, Catanzaro A, Daley C, Gordin F, et al. An official ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. *Am J Respir Crit Care Med* 2007;175:367–416.
- Nakanaga K, Hoshino Y, Era Y, Matsumoto K, Kanazawa Y, Tomita A, et al. Multiple cases of cutaneous *Mycobacterium massiliense* infection in a “hot spa” in Japan. *J Clin Microbiol* 2011;49:613–7.
- Shields RK, Clancy CJ, Minces LR, Shigemura N, Kwak EJ, Silveira FP, et al. Epidemiology and outcomes of deep surgical site infections following lung transplantation. *Am J Transplant* 2013;13:2137–45.
- Lee MR, Cheng A, Lee YC, Yang CY, Lai CC, Huang YT, et al. CNS infections caused by *Mycobacterium abscessus* complex: clinical features and antimicrobial susceptibilities of isolates. *J Antimicrob Chemother* 2012;67:222–5.
- Choi H, Kim SY, Kim DH, Huh HJ, Ki CS, Lee NY, et al. Clinical characteristics and treatment outcomes of patients with acquired macrolide-resistant *Mycobacterium abscessus* lung disease. *Antimicrob Agents Chemother* 2017;61:e01146–17.
- Diel R, Ringshausen F, Richter E, Welker L, Schmitz J, Nienhaus A. microbiological and clinical outcomes of treating non-*Mycobacterium avium* complex nontuberculous mycobacterial pulmonary disease: a systematic review and meta-analysis. *Chest* 2017;152:120–42.
- Griffith DE, Girard WM, Wallace RJ. Clinical features of pulmonary disease caused by rapidly growing mycobacteria. An analysis of 154 patients. *Am Rev Respir Dis* 1993;147:1271–8.
- Sfeir M, Walsh M, Rosa R, Aragon L, Liu SY, Cleary T, et al. *Mycobacterium abscessus* complex infections: a retrospective cohort study. *Open Forum Infect Dis* 2018;5:ofy022.
- Huang YC, Liu MF, Shen GH, Lin CF, Kao CC, Liu PY, et al. Clinical outcome of *Mycobacterium abscessus* infection and antimicrobial susceptibility testing. *J Microbiol Immunol Infect* 2010;43:401–6.
- Nessar R, Cambau E, Reyrat JM, Murray A, Gicquel B. *Mycobacterium abscessus*: a new antibiotic nightmare. *J Antimicrob Chemother* 2012;67:810–8.
- Jeon K, Kwon OJ, Nam YL, Kim BJ, Kook YH, Lee SH, et al. Antibiotic treatment of *Mycobacterium abscessus* lung disease: a retrospective analysis of 65 patients. *Am J Respir Crit Care Med* 2009;180:896–902.
- Lyu J, Jang HJ, Song JW, Choi CM, Oh YM, Do Lee S, et al. Outcomes in patients with *Mycobacterium abscessus* pulmonary disease treated with long-term injectable drugs. *Respir Med* 2011;105:781–7.
- Morimoto K, Nakagawa T, Asami T, Morino E, Fujiwara H, Hase I, et al. Clinicomicrobiological analysis of 121 patients with pulmonary *Mycobacteroides abscessus* complex disease in Japan – An NTM-JRC study with RIT. *Respir Med* 2018;145:14–20.
- Koh WJ, Jeon K, Lee NY, Kim BJ, Kook YH, Lee SH, et al. Clinical significance of differentiation of *Mycobacterium massiliense* from *Mycobacterium abscessus*. *Am J Respir Crit Care Med* 2011;183:405–10.
- Park J, Cho J, Lee C-H, Koo S, Yim J-J. Progression and treatment outcomes of lung disease caused by *Mycobacterium abscessus* and *Mycobacterium massiliense*. *Clin Infect Dis* 2017;64:301–8.
- Nash KA, Brown-Elliott AB, Wallace RJ. A Novel gene, *erm*(41), confers inducible macrolide resistance to clinical isolates of *Mycobacterium abscessus* but is absent from *Mycobacterium chelonae*. *Antimicrob Agents Chemother* 2009;53:1367–76.
- Luthra S, Rominski A, Sander P. The role of antibiotic-target-modifying and antibiotic-modifying enzymes in *Mycobacterium abscessus* drug resistance. *Front Microbiol* 2018;9:2179.
- Choi GE, Shin SJ, Won CJ, Min KN, Oh T, Hahn MY, et al. Macrolide treatment for *Mycobacterium abscessus* and *Mycobacterium massiliense* infection and inducible resistance. *Am J Respir Crit Care Med* 2012;186:917–25.
- Daley CL, Iaccarino Jr JM, Lange C, Cambau E, Wallace RJ, Andrejak C, et al. Treatment of nontuberculous mycobacterial pulmonary disease: an official ATS/ERS/ESCMID/IDSA clinical practice guideline: executive summary. *Clin Infect Dis* 2020;71:e1–e36.
- Macheras E, Roux AL, Ripoll F, Sivadon-Tardy V, Gutierrez C, Gaillard JL, et al. Inaccuracy of single-target sequencing for discriminating species of the *Mycobacterium abscessus* group. *J Clin Microbiol* 2009;47:2596–600.
- Macheras E, Roux A-L, Bastian S, Leão SC, Palaci M, Sivadon-Tardy V, et al. Multilocus sequence analysis and *rpoB* sequencing of *Mycobacterium abscessus* (*sensu lato*) strains. *J Clin Microbiol* 2011;49:491–9.

- [26] Adékambi T, Colson P, Drancourt M. *rpoB*-based identification of nonpigmented and late-pigmenting rapidly growing mycobacteria. *J Clin Microbiol* 2003;41:5699–708.
- [27] Wuzinski M, Bak AK, Petkau A, Demczuk WHB, Soualhine H, Sharma MK. A multi-locus sequence typing scheme for *Mycobacterium abscessus* complex (MAB-multi-locus sequence typing) using whole-genome sequencing data. *Int J Mycobacteriol* 2019;8:273–80.
- [28] Macheras E, Konjek J, Roux AL, Thiberge JM, Bastian S, Leão SC, et al. Multilocus sequence typing scheme for the *Mycobacterium abscessus* complex. *Res Microbiol* 2014;165:82–90.
- [29] Bryant JM, Grogono DM, Greaves D, Foweraker J, Roddick I, Inns T, et al. Whole-genome sequencing to identify transmission of *Mycobacterium abscessus* between patients with cystic fibrosis: a retrospective cohort study. *Lancet* 2013;381:1551–60.
- [30] Bryant JM, Grogono DM, Rodriguez-Rincon D, Everall I, Brown KP, Moreno P, et al. Emergence and spread of a human-transmissible multidrug-resistant nontuberculous mycobacterium. *Science* 2016;354:751–7.
- [31] Nakanaga K, Sekizuka T, Fukano H, Sakakibara Y, Takeuchi F, Wada S, et al. Discrimination of *Mycobacterium abscessus* subsp. *massiliense* from *Mycobacterium abscessus* subsp. *abscessus* in clinical isolates by multiplex PCR. *J Clin Microbiol* 2014;52:251–9.
- [32] Nagai S, Miyamoto S, Ino K, Tajimi S, Nishi H, Tomono J. Easy detection of multiple *Alexandrium* species using DNA chromatography chip. *Harmful Algae* 2016;51:97–106.
- [33] Sano S, Miyamoto S, Kawamoto S. Rapid multiplex nucleic acid amplification test developed using paper chromatography chip and azobenzene-modified oligonucleotides. *J Biosci Bioeng* 2018;126:397–403.
- [34] Yamamuro T, Miyamoto S, Kitamura M, Muro T, Iwata YT, Segawa H, et al. Development of simple and accurate detection systems for *Cannabis sativa* using DNA chromatography. *Forensic Sci Int* 2018;291:68–75.
- [35] Tortoli E, Nanetti A, Piersimoni C, Cichero P, Farina C, Mucignat G, et al. Performance assessment of new multiplex probe assay for identification of mycobacteria. *J Clin Microbiol* 2001;39:1079–84.
- [36] Motro Y, Moran-Gilad J. Next-generation sequencing applications in clinical bacteriology. *Biomol Detect Quantif* 2017;14:1–6.
- [37] MacCannell D. Next generation sequencing in clinical and public health microbiology. *Clin Microbiol News* 2016;38:169–76.
- [38] Darling AE, Mau B, Blattner FR, Perna NT. Mauve: multiple alignment of conserved genomic sequence with rearrangements. *Genome Res* 2004;14:1394–403.
- [39] Brown-Elliott BA, Vasireddy S, Vasireddy R, Iakhsiaeva E, Howard ST, Nash K, et al. Utility of sequencing the *erm(41)* gene in isolates of *Mycobacterium abscessus* subsp. *abscessus* with low and intermediate clarithromycin MICs. *J Clin Microbiol* 2015;53:1211–5.
- [40] Tortoli E, Kohl TA, Brown-Elliott BA, Trovato A, Leão SC, Garcia MJ, et al. Emended description of *Mycobacterium abscessus*, *Mycobacterium abscessus* subsp. *abscessus* and *Mycobacterium abscessus* subsp. *bolletii* and designation of *Mycobacterium abscessus* subsp. *massiliense* comb. nov. *Int J Syst Evol Microbiol* 2016;66:4471–9.
- [41] Lipworth S, Hough N, Leach L, Morgan M, Jeffery K, Andersson M, et al. Whole-genome sequencing for predicting clarithromycin resistance in *Mycobacterium abscessus*. *Antimicrob Agents Chemother* 2019;63:e01204–18.
- [42] Namkoong H, Kurashima A, Morimoto K, Hoshino Y, Hasegawa N, Ato M, et al. Epidemiology of pulmonary nontuberculous mycobacterial disease, Japan<sup>1</sup>. *Emerg Infect Dis* 2016;22:1116–7.
- [43] Wagner D, Van Ingen J, Van Der Laan R, Obradovic M. Non-tuberculous mycobacterial lung disease in patients with bronchiectasis: perceived risk, severity and guideline adherence in a European physician survey. *BMJ Open Respir Res* 2020;7:e000498.
- [44] Zhang ZX, Cherng BPZ, Sng LH, Tan YE. Clinical and microbiological characteristics of non-tuberculous mycobacteria diseases in Singapore with a focus on pulmonary disease, 2012–2016. *BMC Infect Dis* 2019;19:436.
- [45] Hoshino Y, Suzuki K. Differential diagnostic assays for discriminating mycobacteria, especially for nontuberculous mycobacteria: what does the future hold? *Future Microbiol* 2015;10:205–16.
- [46] Carbonnelle E, Mesquita C, Bille E, Day N, Dauphin B, Beretti J-L, et al. MALDI-TOF mass spectrometry tools for bacterial identification in clinical microbiology laboratory. *Clin Biochem* 2011;44:104–9.
- [47] Mase A, Yamaguchi F, Funaki T, Yamazaki Y, Shikama Y, Fukuchi K. PCR amplification of the *erm(41)* gene can be used to predict the sensitivity of *Mycobacterium abscessus* complex strains to clarithromycin. *Exp Ther Med* 2019;19:945–55.
- [48] Kehrmann J, Kurt N, Rueger K, Bange FC, Buer J. GenoType NTM-DR for identifying *Mycobacterium abscessus* subspecies and determining molecular resistance. *J Clin Microbiol* 2016;54:1653–5.
- [49] Huh HJ, Kim SY, Shim HJ, Kim DH, Yoo IY, Kang OK, et al. GenoType NTM-DR performance evaluation for identification of *Mycobacterium avium* complex and *Mycobacterium abscessus* and determination of clarithromycin and amikacin resistance. *J Clin Microbiol* 2019;57 e00516–19.
- [50] Mougari F, Loiseau J, Veziris N, Bernard C, Bercot B, Sougakoff W, et al. Evaluation of the new GenoType NTM-DR kit for the molecular detection of antimicrobial resistance in non-tuberculous mycobacteria. *J Antimicrob Chemother* 2017;72:1669–77.
- [51] Dolezel J, Bartos J, Voglmayr H, Johann G. Nuclear DNA content and genome size of trout and human. *Cytometry* 2003;51A:127–8.
- [52] Sapriel G, Konjek J, Orgeur M, Bouri L, Frézal L, Roux A-L, et al. Genome-wide mosaicism within *Mycobacterium abscessus*: evolutionary and epidemiological implications. *BMC Genomics* 2016;17:118.
- [53] Page AJ, Cummins CA, Hunt M, Wong VK, Reuter S, Holden MTC, et al. Roary: rapid large-scale prokaryote pan genome analysis. *Bioinformatics* 2015;31:3691–3.
- [54] Stamatakis A. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* 2006;22:2688–90.
- [55] Tanizawa Y, Fujisawa T, Nakamura Y. DFAST: a flexible prokaryotic genome annotation pipeline for faster genome publication. *Bioinformatics* 2018;34:1037–9.
- [56] Kim B-J, Kim G-N, Kim B-R, Shim T-S, Kook Y-H, Kim B-J. Phylogenetic analysis of *Mycobacterium massiliense* strains having recombinant *rpoB* gene laterally transferred from *Mycobacterium abscessus*. *PLoS ONE* 2017;12:e0179237.