Reinstating *Mycobacterium massiliense* and *Mycobacterium bolletii* as species of the *Mycobacterium abscessus* complex

Toidi Adekambi,1,* Mohamed Sassi,2 Jakko van Ingen3 and Michel Drancourt4,*

**Abstract**

The *Mycobacterium abscessus* complex is a group of rapidly growing, multiresistant mycobacteria previously divided into three species. Proposal for the union of *Mycobacterium bolletii* and *Mycobacterium massiliense* into one subspecies, so-called *M. abscessus* subsp. *massiliense*, created much confusion about the routine identification and reporting of *M. abscessus* clinical isolates for clinicians. Results derived from multigene sequencing unambiguously supported the reinstatement of *M. massiliense* and *M. bolletii* as species, culminating in the presence of *erm(41)*-encoded macrolide resistance in *M. bolletii*. Present genome-based analysis unambiguously supports the reinstatement of *M. massiliense* and *M. bolletii* as species after the average nucleotide identity values of 96.7% for *M. abscessus* versus *M. bolletii*, and 96.4% for *M. abscessus* versus *M. massiliense*, and the 96.6% identity between *M. bolletii* and *M. massiliense* was put into the perspective of a larger, 28-species analysis. Accordingly, DNA–DNA hybridization values predicted by the complete rpoB gene sequencing analysis were between 68.7 and 72.3% in this complex. These genomic data as well as the phenotypic characteristics prompted us to propose to reinstate the previously known *M. massiliense* and *M. bolletii* into two distinct species among the *M. abscessus* complex.

The *Mycobacterium abscessus* complex is a group of rapidly growing, multiresistant mycobacteria [1–5]. Isolates of this complex have become one of the most frequent nontuberculous mycobacteria (NTM) recovered from clinical samples [6–11]. They are responsible for chronic, recurrent infections that are difficult to treat because of their resistance to many of the usual medications for NTM infections [8, 12–14]. This complex was previously divided into three species: *M. abscessus* [15], *Mycobacterium massiliense* [16, 17] and *Mycobacterium bolletii* [18]. In earlier publications, *M. massiliense* [19, 17] and *M. bolletii* [18] have been described as two different species in the *M. abscessus* complex. In 2009, Leao et al. proposed the union of *M. bolletii* and *M. massiliense* into one subspecies, so-called *M. abscessus* subsp. *massiliense* based on the fact that phenotypic tests, DNA–DNA hybridization (DDH) and 16S rRNA gene sequencing could not separate *M. bolletii* and *M. massiliense* [20]. However, *M. abscessus* subsp. *massiliense* cannot be validly published by citation in a validation list since the authors did not provide protologues for the novel subspecies [21] (Rule 27 of the Bacteriological code, 1990 Revision). In 2011, Leao et al. further proposed that *M. massiliense* and *M. bolletii* had to be united and reclassified as *M. abscessus* subsp. *bolletii* [21].

This proposal created much confusion regarding the routine identification and reporting of *M. abscessus* clinical isolates for clinicians [11, 22, 23]. This proposal has also undoubtedly led to inconsistencies in the medical literature about the appropriate name of *M. massiliense* and *M. bolletii* [13, 24–26].

Results derived from multigene sequencing [7, 16, 18, 27, 28–33], postgenomic analyses [14, 34–40], presence of the inducible *erm(41)* gene [10, 28, 41–44] and *in vitro* antibiotic susceptibility tests [4, 5, 8, 12, 28, 32, 41–43, 45, 46] unambiguously supported the reinstatement of *M. massiliense* and *M. bolletii* as species (Fig. 1). *M. massiliense* with truncated, non-functional *erm(41)* exhibits macrolide susceptibility whereas *M. bolletii* with a full length and functional *erm(41)* exhibits macrolide resistance [10, 28, 35, 41, 42].

At the genomic level, a previous study found a mean DDH between *M. massiliense* and *M. bolletii* of 73.4±8.5%, close to the speciation threshold of 70% [20]. We have previously shown that the complete rpoB gene sequence provides an efficient supplement to DDH to delineate bacterial species [34]. We found that the mean DDH value reported by Leao et al. [20] is close to the 72.3% DDH value predicted by the complete rpoB gene sequencing analysis (Table 1).
[34]. We also found that the DDH values between *M. abscessus*, *M. massiliense* and *M. bolletii* were 68.7–69.7% (Table 1). These values contradict the ones found by Leao et al. [20], where the mean DDH values between *M. abscessus*, *M. massiliense* and *M. bolletii* were >92.02±13.4% (Table 1), confirming that DDH is a dependable technique [47]. It is also well known that some species have higher DDH threshold values [34, 47]. Therefore, using a DDH value with a large margin of error creates confusion in the literature about the species delineation in this complex.

![Phylogenomic tree](image)

**Table 1.** Differential phenotypic, biochemical and genetic characteristics of the type strains *M. abscessus* CIP 104536^T, *M. massiliense* CIP 108297^T and *M. bolletii* CIP 108541^T

<table>
<thead>
<tr>
<th>Characteristic</th>
<th><em>M. abscessus</em></th>
<th><em>M. massiliense</em></th>
<th><em>M. bolletii</em></th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony morphology on 7H11 agar cultured at 37°C</td>
<td>Rough</td>
<td>Smooth</td>
<td>Rough</td>
<td>[55, 56]</td>
</tr>
<tr>
<td>β-Glucosidase</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>[20]</td>
</tr>
<tr>
<td>Catalase over 45 mm</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>[20]</td>
</tr>
<tr>
<td>Hydroxylamine tolerance</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>[20]</td>
</tr>
<tr>
<td>Inducible clarithromycin resistance after 14 days of culture erm(41)</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>[28]</td>
</tr>
<tr>
<td>Mycobacteriophage</td>
<td>Full length, functional</td>
<td>276 bp deletion, non-functional</td>
<td>Full length, functional</td>
<td>[44]</td>
</tr>
<tr>
<td>Mycobacteriophage</td>
<td>Prophage (80 545 bp)</td>
<td>Prophage (94 200 bp)</td>
<td>Araucaria (Dori-like, 64 129 bp)</td>
<td>[33, 57]</td>
</tr>
</tbody>
</table>

Fig. 1. Phylogenomic tree based on 28 *Mycobacterium* and one *Rhodococcus* genomes using the neighbour-joining algorithm in the package SplitsTree4. The percentage values represent the ANI within groups. Bar, 1 change per nucleotide position.
Percentage values represent the rpoB similarity by pairwise alignment of sequences and the ANI by pairwise alignment of genome stretches based on BLAST.

<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td></td>
<td>M.abs</td>
<td>M.mass</td>
<td>M.bol</td>
<td>M.abs</td>
</tr>
<tr>
<td>M. abscessus</td>
<td>96.4%</td>
<td>96.7%</td>
<td>96.0%</td>
<td>95.6%</td>
</tr>
<tr>
<td>M. massiliense</td>
<td>96.4%</td>
<td>96.6%</td>
<td>96.0%</td>
<td>97.0%</td>
</tr>
<tr>
<td>M. bolletii</td>
<td>96.7%</td>
<td>96.6%</td>
<td>95.6%</td>
<td>97.0%</td>
</tr>
</tbody>
</table>

M.abs, M. abscessus; M.mass, M. massiliense; M.bol, M. bolletii.

Further, the average nucleotide identity (ANI) values derived from pairwise alignment of the whole-genome stretches using BLAST yielded 96.7% for M. abscessus versus M. bolletii and 96.4% for M. abscessus versus M. massiliense; and M. bolletii and M. massiliense shared 96.6% similarity [36, 37]. There was an apparent distinction in the overall ANI distribution between intra- and interspecies relationships at around 95–96% [48] and, although the values are slightly higher than the cut-off value, the three taxa can be considered separate species in combination with the results of low values of DDH and distance based on rpoB (Table 2). In order to test this hypothesis, we next investigated the ANI values in 28 mycobacterial species (Fig. 1). The genomic-sequence-derived phylogenetic tree clearly shows closely related, albeit distinct, species exhibiting ANI values (>97.8%) higher than the ones of the M. abscessus-bolleti-massiliense complex (96.4–96.7%). This is the case of the Mycobacterium intracellularare-yongonense complex exhibiting ANI values of 98.1–98.6%; of the Mycobacterium tuberculosis complex (M. tuberculosis, M. africanum, ‘M. canetti’ and M. bovis) exhibiting ANI values of 98.9–99.7%; and the Mycobacterium marinum-ulcerans complex exhibiting an ANI value of 97.8%. In none of these Mycobacterium complexes, the speciation is a question of debate [49, 50].

At the subspecies level, Mycobacterium avium subsp. avium and M. avium subsp. paratuberculosis exhibited an ANI of 98.3%. This was in agreement with data reported by Tortoli et al. who showed that variability for M. abscessus, M. bolletii and M. massiliense isolates was higher than 98.4% [51]. Therefore, a subspecies status within the genus Mycobacterium should be considered when the ANI>98.3%.

Further in-depth analysis of genomes showed a total of 36 genes uniquely present in M. abscessus, 15 in M. massiliense and 15 in M. bolletii that could be used to identify these three species (Fig. 2) [38]. We further showed that speciation was partly driven by mycobacteriophage [37, 52] including the 64 129 bp phage Araucaria for M. bolletii, the 9200 bp prophage for M. massiliense and the 80 545 bp prophage for M. abscessus [53] as well as the pRAW-like extrachromosomal genetic elements [33, 54]. In contrast to M. bolletii, M. massiliense is characterized by the prevalence of homologous gene recombination [33]. Sapriel et al. [33] also showed that the three species do not behave the same way since M. bolletii is clearly less introgressed than the two other species, resulting in a relatively homogenous gene pool that might result from a distinct or isolated ecological niche.

All these data prompted us to reinstate the previously known M. massiliense and M. bolletii into two species. This specific identification of these two species which show different antibiotic susceptibilities will enable the clinician to manage the patient appropriately (Table 2) [9, 12]. The names of the two species, M. massiliense and M. bolletii, have already been validly published as M. massiliense [16, 17] and M. bolletii [18].

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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