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SHORT COMMUNICATION

Proposal of reclassification of *Mycobacterium celatum* type 2 as *Mycobacterium kyorinense*

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Abstract 16S rRNA, *rpoB*, and *sodA* genes of *Mycobacterium celatum* type 2 were re-examined to clarify its taxonomic position. Reevaluation of these sequences revealed that *M. celatum* type 2 strain ATCC 51130 was 100% identical to *Mycobacterium kyorinense* type strain, but differed substantially from *M. celatum* types 1 and 3 at these genes. A phylogenetic tree analysis placed *M. celatum* type 2 in proximity with *M. kyorinense*, with a clear-cut distinction from *M. celatum* types 1 and 3. We propose the reclassification of *M. celatum* type 2 as *M. kyorinense*, with both species maintaining their respective names and standing in the literature.

Keywords *hsp65* · *rpoB* · 16S rRNA · *sodA* · subspecies

Findings

Mycobacterium celatum was first described in 1993 as a new mycobacterial species responsible for human infections (Butler et al. 1993). That report described two subtypes (types 1 and 2) of *M. celatum* with slightly different genetic profiles and similar phenotypic features. A subsequent report described an additional subtype of *M. celatum* (type 3), whose genetic features were distinct from, but very similar to, those of the previously reported subtype 1 (Bull et al. 1995). In the same year, *Mycobacterium branderi*, which exhibited a close phylogenetic relationship to *M. celatum*, was described as a novel species (Koukila-Kahkola et al. 1995). More recently, we have identified *Mycobacterium kyorinense* and *Mycobacterium fragae* as new species closely related to *M. celatum* and *M. branderi* (Okazaki et al. 2009; Ramos et al. 2013).

In the original description of *M. celatum*, types 1 and 2 were reported to differ by 10 bp (out of 1479 nucleotides) in the 16S rRNA gene sequence (Butler et al. 1993). The same report indicated that types 1 and 2 differed in PRA (PCR restriction analysis) profiles of the *hsp65* gene. Given the phenotypic similarity of types 1 and 2, such genetic divergences were not considered sufficient to define the two types as separate species. Subsequently, Picardeau et al. (1997) investigated the three types of *M. celatum* by molecular assays, demonstrating that types 1 and 3 were indistinguishable on the basis of restriction fragment length polymorphism (RFLP) and pulsed field gel electrophoresis (PFGE) analyses. That work also reported that types 1 and 3 harbored multiple copies of a unique new insertion element, IS*1407*, in identical genomic positions. In contrast, type 2 presented a distinct RFLP pattern and lacked IS*1407* elements (Picardeau et al. 1997). More recently, on occasion of the description of *M. fragae* sp. nov., we found (using a multilocus phylogenetic tree incorporating 16S rRNA sequences deposited by Butler et al.) that *M. celatum* type 2 was most closely related to *M. kyorinense* and placed apart from the branch including *M. celatum* types 1 and 3 (Ramos et al. 2013).

The accumulation of these data has prompted us to further investigate the genetic features of *M. celatum* type 2. Direct

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sequencing of the 16S rRNA gene sequences (1,404 bp; accession number: AB824284) of *M. celatum* type 2 (strain ATCC 51130) revealed ambiguities at nine nucleotide positions (Fig. 1a). This result suggests the presence of two copies of the 16S rRNA gene in this strain, in accordance with a previous report that strain ATCC 51130 harbors two copies of the 16S rRNA gene (Stadthagen-Gomez et al. 2008). Interestingly, we have observed an identical pattern of heterogeneity in the 16S rRNA gene sequences of *M. kyorinense* strains, both in the type strain (accession number: AB370111.2) and in some clinical isolates (Ohnishi et al. 2013). Alignment of our ATCC 51130 16S rRNA gene sequence with that of the original *M. celatum* type 2 description (GenBank L08170) revealed mismatches at 12 positions, including four that occurred at positions with ambiguous nucleotides (Fig. 1b). If these mismatches are corrected as possible sequence errors, the similarity of the 16S rRNA sequence of *M. celatum* type 2 with the sequences of types 1 and 3 drops to 98.6 and 98.4%,

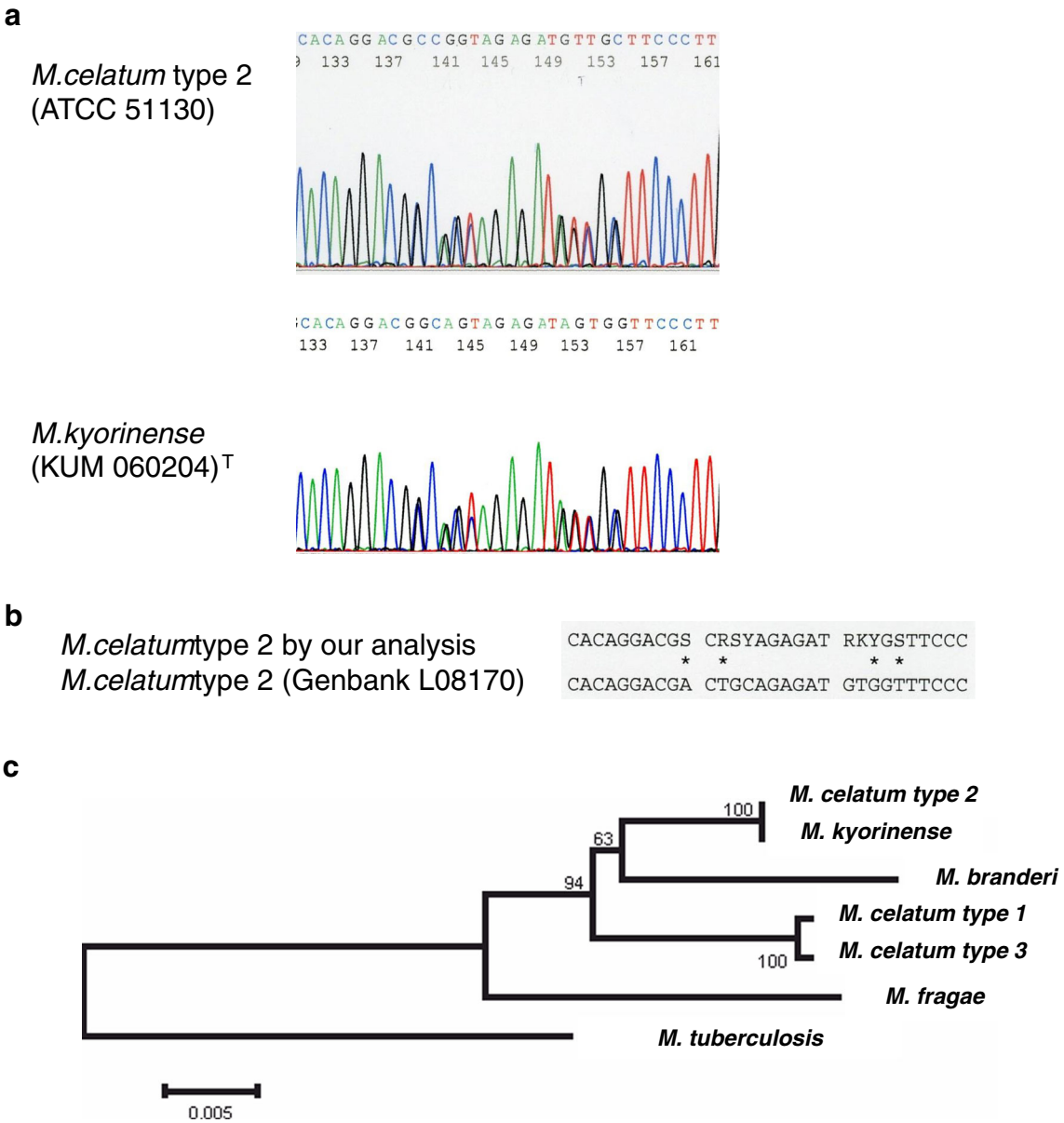

Fig. 1 (**a**). Graphic plot of direct 16S rRNA sequences at nucleotide positions (corresponding to the numbering system of *M. tuberculosis*) 993-1021 of *M. celatum* type 2 and *M. kyorinense*, showing identical ambiguities at eight nucleotide positions. (**b**). The discrepancy of 16S rRNA sequences of ATCC 51130 at nucleotide positions 993-1021 between our analysis and the original work deposited by Butler et al. (Genbank L08170). Note that the four positions of sequence discrepancy belong to those with ambiguous nucleotides. (**c**) Phylogenetic tree constructed by the neighbor-joining method, using the concatenated sequences of 16S rRNA, *hsp65*, and *rpoB* genes. The robustness of branches is indicated by bootstrap values calculated on 1000 replicates. *M. tuberculosis* was used as the outgroup

respectively, while the similarity with *M. kyorinense* rises to 100%.

Direct sequencing of an almost complete *rpoB* gene (accession number: AB824285) and a partial *sodA* gene (accession number: AB824286) from *M. celatum* type 2 (ATCC 51130) revealed 100% identity with the respective sequences of *M. kyorinense*. In contrast, the *rpoB* sequence of *M. celatum* type 1 differed from that of *M. kyorinense*, exhibiting more than 150 mismatches across 3,489 bp (95.4% identity); a similar comparison between *sodA* gene sequences revealed 12 mismatches across 490 bp (97.6% identity). As we have previously reported, *M. celatum* type 2 and *M. kyorinense* exhibited identity in a partial *hsp65* sequence (Okazaki et al. 2009), in contrast to mismatches at 8 of 402 bp (98.0% identity) upon comparison to the equivalent sequence from *M. celatum* type 1.

A phylogenetic tree was constructed, using MEGA software version 5.2 (Tamura et al. 2011), based on the concatenated sequences of the 16S rRNA, *rpoB*, and *hsp65* genes (Fig. 1c), according to the neighbor-joining method (Saitou and Nei 1987) under total gap removal and Kimura's two-parameter substitution model (Kimura 1980). The tree revealed a clear-cut distinction of *M. celatum* type 2 from types 1 and 3. Identical topology was obtained according to the maximum likelihood method (Felsenstein 1981).

The sequence analysis of the 16S rRNA, *rpoB*, *hsp65*, and *sodA* genes in the present study and our previous report (Ramos et al. 2013) clearly shows that genetic profiles of *M. celatum* type 2 and *M. kyorinense* are identical in these genes. In addition, *M. celatum* type 2/*M. kyorinense* showed similar levels of differences from both *M. celatum* types 1 and 3 and *M. branderi* in these genes (Table 1), supporting the hypothesis that *M. celatum* type 2/*M. kyorinense* is a species distinct from *M. celatum* types 1 and 3 or *M. branderi*. Phenotypic features of these species are also similar to, but distinct from, each other (Okazaki et al. 2009).

Two major factors contributed to the original classification of strain ATCC 51130 within the species *M. celatum*. First, the sequencing of the 16S rRNA apparently was incorrect in the original report. Second, the phenotypic characteristics of types 1 and 2 were similar, with both subtypes being non-chromogenic slow growers with overlapping biochemical profiles. Regarding the second issue, it should be noted that the taxonomic weight of classical biochemical tests has been greatly re-evaluated in recent years. Approximately 20 tests generally used for description of mycobacteria lack discriminative power among over 150 species of the genus *Mycobacterium*, and reproducibility of these assays is poor (Springer et al. 1996). According to the respective original descriptions, *M. celatum* (including type 2) and *M. kyorinense* appeared to differ based on growth temperature range and on the results of 3-day arylsulfatase, pyrazinamidase, and tellurite reduction tests. However, the features of *M. celatum* in the original description are not consistent with the properties described in several subsequent papers (Tortoli et al. 1995; Piersimoni et al. 1997; Gholizadeh et al. 1998). For example, Butler et al. (1993) reported that *M. celatum* was unable to grow at temperatures higher and lower than 37°C. It is noteworthy, however, that that observation was limited to three weeks, and it seems likely that strains whose colonies were defined as "barely visible after three weeks" required longer incubation times in order to develop at suboptimal incubation temperatures. In fact, several strains of *M. celatum* type 1 reportedly can grow at 25°C and 45°C (Tortoli et al. 1995; Piersimoni et al. 1997).

We conclude that *M. celatum* type 2 and *M. kyorinense* are genetically indistinguishable from each other by multiple analyses generally used for distinction of mycobacterial species. Nonetheless, the legitimacy of the status of *M. kyorinense* as a species distinct from *M. celatum* (type 1) is demonstrated by the percentage of DNA-DNA hybridization, which was clearly below the recommended threshold for species identity (Stackebrandt et al. 2002; Okazaki et al. 2009). We therefore propose the reclassification of *M. celatum* type 2 as *M. kyorinense*, with both species maintaining their respective names and standing in the literature.

Table 1 Percent similarity of gene sequence between *Mycobacterium celatum* subtypes, *Mycobacterium kyorinense*, and *Mycobacterium branderi*

	16S rRNA	*rpoB*	*hsp65*	*sodA*
M. celatum type 1 vs *M. celatum* type 2	98.6	96.8	98	97.6
M. celatum type 1 vs *M. celatum* type 3	99.7	100	100	N/A
M. celatum type 2 vs *M. celatum* type 3	98.4	96.8	98	N/A
M. branderi vs *M. celatum* type 1	97.8	94.8	98.3	89.8
M. branderi vs *M. celatum* type 2	97.8	95.7	98.7	90.1
M. branderi vs *M. celatum* type 3	97.7	94.8	98.3	N/A
M. celatum type 2 vs *M. kyorinense*	100	100	100	100

Strains ATCC 51131^T, ATCC 51130, NCTC 12882, CIP 104592, and KUM 060204^T were used as *M. celatum* type 1, 2, 3, *M. branderi*, and *M. kyorinense*, respectively. The values for *rpoB*, *hsp65*, and *sodA* are based on their partial sequences. N/A: not available.

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