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GENOME-BASED TAXONOMIC REVISION DETECTS A NUMBER OF SYNONYMOUS TAXA IN THE GENUS *MYCOBACTERIUM*

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Abstract

The aim of this study was to clarify the taxonomic status of named species within the genus *Mycobacterium*. The analysis of genomes belonging to 174 taxa (species or subspecies) of the genus *Mycobacterium* was conducted using both the Average Nucleotide Identity and the Genome to Genome Distance. A number of synonymous taxa were detected. The list of synonyms includes: two subspecies of *M. chelonae* (*M. chelonae* subsp. *bovis* and *M. chelonae* subsp. *gwanakae*), two subspecies of *M. fortuitum* (*M. fortuitum* subsp. *fortuitum* and *M. fortuitum* subsp. *acetamidolyticum*), four subspecies of *M. avium* (*M. avium* subsp. *avium*, *M. avium* subsp. *silvaticum*, *M. avium* subsp. *paratuberculosis* and “*M. avium* subsp. *hominissuis*”), two couples of subspecies of *M. intracellulare* (*M. intracellulare* subsp. *intracellulare*/*M. intracellulare* subsp. *paraintracellulare* and *M. intracellulare* subsp. *chimaera*/*M. intracellulare* subsp. *yongonense*), the species *M. austroafricanum* and *M. vanbaalenii*, the species *M. senegalense* and *M. conceptionense*, the species *M. talmoniae* and *M. eburneum* and the species *M. marinum*, *M. ulcerans* and *M. pseudoshottsii*. Furthermore one species were reclassified as subspecies of another mycobacterium: *M. lepraemurium* was reclassified as a subspecies of *M. avium* (*M. avium* subsp. *lepraemurium*). The updates to nomenclature are proposed basing on the priority of names according the Code of nomenclature of prokaryotes. For two species (*M. bouchedurhonense* and *M. marseillense*) the loss of standing in nomenclature is proposed because of unavailability of respective type strains in culture collections.

Keywords: Mycobacterium; taxonomy; synonymous taxa; Average Nucleotide Identity; Genome to Genome Distance

INTRODUCTION

The genus *Mycobacterium* includes, once the members of *Mycobacterium tuberculosis* complex are regarded as a single species (Riojas et al., 2018), 192 species of which five are split into subspecies. Recently the division of this genus into five genera has also been proposed (Gupta et al., 2018). Although we prefer to use here the classical nomenclature, a prerogative granted by taxonomic rules (Tindall et al., 2006; Tortoli et al., 2019), a conversion table with novel and classical names is given in Supplementary Table 1. The vast majority of the *Mycobacterium* species have had their genomes sequenced in the past years, allowing for genome-based taxonomic classification to occur.

The gold standard for species circumscription in *Archaea* and *Bacteria* is the DNA-DNA Hybridization (DDH), a laboratory test consisting of mixing the denatured DNA of two organisms and determining the proportion of hybrid DNA formed. A hybrid DNA percentage over 70% is considered to prove that the two organisms belong to the same species (Wayne et al., 1987). This test is cumbersome and, being error prone, requires multiple repetitions to obtain confident results.

Nowadays reliable and objective computational techniques are available for the comparison of genomes. The Average Nucleotide Identity (ANI) uses the average of identical nucleotides between two genomes (Lee et al., 2016a). A number of studies have demonstrated that strains with pairwise ANI>95-96% correspond to DDH>70% and therefore belong to the same species; in contrast strains with pairwise ANI<95% belong to distinct species (Goris et al., 2007). The Genome to Genome Distance (GGD) is the equivalent in silico of the wet-lab DDH and produces results convertible into DDH-equivalent percentages (Meier-Kolthoff et al., 2013).

In a previous genomic study we detected that several *Mycobacterium* species with standing in nomenclature are likely synonyms (Tortoli et al., 2017). We decided therefore to extend the analysis to all the *Mycobacterium* taxa with a genome available in GenBank to undertake a genus-wide review.

MATERIALS AND METHODS

We analyzed all the official mycobacterial taxa with an available genome in GenBank, including 164 species with five of them split into 15 subspecies for a total of 174 taxa. Out of the 174 genomes 156 were from type strains. The complete list of taxa investigated is available in Supplementary Table 2.

Each taxon was compared with all others for determining ANI and GGD pairwise values (15,051 comparisons per method). OrthoANI (ver. 0.93) (Lee et al., 2016a) was used for calculating ANI, while for GGD was resorted to the Genome to Genome Distance Calculator 2.1 available at <http://ggdc.dsmz.de/ggdc.php> (Meier-Kolthoff, 2013 66095 /id), employing the Formula 2 option.

The couples of taxa presenting either an ANI>95% or a GGD>70% or both were said to belong to the same species. Any such couples that also had an ANI≤97% and a GGD≤80% were then classified as subspecies (Meier-Kolthoff et al., 2014). A phylogenetic tree including *Hoyosella altamirensis* as an outgroup was constructed using the UPGMA algorithm.

Initial comparisons of genomes resulted in exclusion of *M. hodleri* and *M. pyrenivorans* due to highly fragmented genomes producing ANI=0% when compared with a number of other species. Additionally, the analysis of *M. bouchedurhonense* and *M. timonense* demonstrated that the respective type strains available in the public culture collections are incompatible with their description (Ben Salah et al., 2009). Both are actually *M. avium* and both were excluded from the study.

RESULTS AND DISCUSSION

A number of comparisons resulted in the fusion of synonymous taxa and/or the creation of new subspecies. They are as follows:

The three *M. abscessus* subspecies (*M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii* and *M. abscessus* subsp. *massiliense*) all fulfilled the threshold of subspecies (Table 1), confirming their current taxonomic status (Tortoli et al., 2016).

The three *M. chelonae* subspecies (*M. chelonae* subsp. *chelonae*, *M. chelonae* subsp. *bovis* and *M. chelonae* subsp. *gwanakae* (Kim et al., 2017; Kim et al., 2018) all had ANI and GGD values very close to the thresholds. Both *M. chelonae* subsp. *bovis* and *M. chelonae* subsp. *gwanakae* were distant enough from *M. chelonae* subsp. *chelonae* to fulfill the criteria of subspecies. However they were so close to one another to

make it likely they represent synonym subspecies. Phenotypic characters reported in respective protologues do not appear significantly different. We propose therefore they are synonymous subspecies of *M. chelonae* with the name *M. chelonae* subsp. *bovis* having the priority.

M. fortuitum includes two subspecies *M. fortuitum* subsp. *fortuitum* and *M. fortuitum* subsp. *acetamidolyticum* (Tsukamura et al., 1986a). Both indicators (Table 1) suggested their fusion into a single taxon. Consequently we propose that the species *M. fortuitum* no longer includes any subspecies.

M. avium includes three subspecies, *M. avium* subsp. *avium*, *M. avium* subsp. *paratuberculosis*, *M. avium* subsp. *silvaticum* (Thorel et al., 1990). A fourth subspecies "*M. avium* subsp. *hominissuis*" has been described (Mijs et al., 2002) but has no official standing in nomenclature. All the subspecies above were characterized by very high values of ANI and GGD, well above the thresholds of subspecies (Table 1). This suggests they should be fused within a single taxon. Very recently the unculturable species *M. lepraemurium*, previously regarded as closely related to *M. leprae* because of the parallel massive gene decay, revealed surprising similarity with *M. avium* (Benjak et al., 2017). Both computational parameters suggested that *M. lepraemurium* should be reclassified as a subspecies of *M. avium*. We propose therefore *M. avium* is split in two subspecies *M. avium* subsp. *avium* and the new subspecies *M. avium* subsp. *lepraemurium* is created. Akin to what was recently proposed for the species of *M. tuberculosis* complex (Riojas et al., 2018), we recommend here that the previous subspecies of *M. avium*, in consideration of their adaptation to specific hosts, to retain the previous names at level of variants instead of subspecies. An exception could be made, in case, maintaining the status of subspecies for *M. avium* subsp. *paratuberculosis* which differs significantly from other variants in terms of epidemiology, pathogenicity, evolution and physiology.

M. intracellulare includes three subspecies *M. intracellulare* subsp. *intracellulare*, *M. intracellulare* subsp. *yongonense* (Castejon et al., 2018) and *M. intracellulare* subsp. *chimaera* (Nouioui et al., 2018). This division was also supported by our findings. *M. paraintracellulare* (Lee et al., 2016b) is also closely related to *M. intracellulare*; our data (Table 1) suggest that *M. paraintracellulare* is synonym of *M. intracellulare* subsp. *intracellulare* while *M. intracellulare* subsp. *chimaera* is synonym of *M. intracellulare* subs. *yongonense*.

Thus, within the group of taxa closely related to *M. intracellulare* we propose the novel taxonomy to recognize a single species, *M. intracellulare*, with two subspecies, *M. intracellulare* subsp. *intracellulare* (absorbing *M. paraintracellulare*), and *M. intracellulare* subsp. *chimaera* (comprising *M. intracellulare* subsp. *chimaera* and *M. intracellulare* subsp. *yongonense*, with the name *chimaera* having priority over *yongonense*).

Between *M. chubuense* (Tsukamura et al., 1981) and *M. kyogaense* (Nouioui et al., 2018) the ANI was slightly over, and the GGD slightly below, the cutoff of species (Table 1). In consideration of this inconsistency we think preferable not making recommendations for these taxa.

ANI and GGD values (Table 1) supported the synonymy of *M. vanbaalenii* (Khan et al., 2002) and *M. austroafricanum* (Tsukamura et al., 1983) with *M. austroafricanum* having the priority over *M. vanbaalenii*. *M. conceptionense*, *M. farcinogenes* and *M. senegalense* also appeared to be synonyms of each other (Table 1). It has however been pointed out that the genome available in GenBank, and investigated in this study, as *M. farcinogenes*^T (CCAY010000000), has been obtained from a strain of *M. senegalense* (Turenne, 2019). We propose therefore only that *M. conceptionense* is a synonym of *M. senegalense* with the last name having the priority.

The cluster including *M. crocinum*, *M. pallens* (Hennessee et al., 2009) and *M. aromaticivorans* (Kwak et al., 2014) was characterized by very inconsistent outputs. While *M. crocinum* and *M. pallens* seemed to be synonym species and *M. pallens* and *M. aromaticivorans* seemed synonym subspecies as well, *M. crocinum* and *M. aromaticivorans* appeared to be unrelated species (Table 1). This may be due to genome quality and requires further investigation. We suggest that the status of the species *M. crocinum*, *M. pallens* and *M. aromaticivorans* remains unsettled until genome of better quality (less fragmented) is available at least for *M. crocinum*.

Two recently described species, *M. talmoniae* (Davidson et al., 2017) and *M. eburneum* (Nouioui et al., 2017) are, in fact, practically identical (Table 1). Interestingly *M. eburneum* had been described as a rapid grower (growth rate 7 days) and *M. talmoniae* as a slow grower (7-10 days). They actually present in the

16S rRNA the genetic signature of slow growers and accordingly cluster in the phylogenetic tree (Fig. 1). Therefore *M. talmoniae* overlaps *M. eburneum* and has the first has the priority.

Although *M. hiberniae* and *M. engbaekii* had a GGD=60.6, supporting the status of distinct species, their ANI value fell within the uncertainty interval 95-96% (Table 1). Thus we avoid making any recommendation in this case.

The last group of taxa included *M. marinum*, *M. ulcerans* and *M. pseudoshottsii*. Both computational values agree about their synonymy (Table 1). However the species *M. marinum* and *M. ulcerans*, which are very common worldwide, are responsible for very different clinical pictures; the fusion of these taxa in a single species could be a cause of great confusion for clinicians and even of harm for patients. Although we are certain that our decision is not taxonomically sound, we prefer not to recommend any change in this case.

Conclusions

Overall, the analysis of available genomes of *Mycobacterium* species (type strains whenever available) revealed a number of species and subspecies which were indeed synonym. Additionally, we propose that *M. bouchedurhonense* and *M. timonense*, because of the lack of type strains available in public culture collections, lose, according to the Code of nomenclature of prokaryotes (2019), the status of being validly published.

EMENDED DESCRIPTION OF MYCOBACTERIUM CHELONAE SUBSP. BOVIS.

M. chelonae subsp. *bovis* (bo'vis. L. gen. n. *bovis* of the ox). The description emerges by the combinations of those given for *M. chelonae* subsp. *bovis* (Kim et al., 2017) and *M. chelonae* subsp. *gwanakae* (Kim et al., 2018). The type strain is QIA-37^T=KCTC 39630^T=JCM 30986^T.

EMENDED DESCRIPTION OF MYCOBACTERIUM FORTUITUM.

M. fortuitum (for.tu'it.um. L. neut. adj. *fortuitum*, casual). The description combines the ones given for *Mycobacterium fortuitum* (da Costa Cruz, 1938; Skerman et al., 1980) and for *M. fortuitum* subsp. *acetamidolyticum* (Tsukamura et al., 1986b). The type strain is ATCC 6841^T=CCUG 20994^T=CIP 104534^T=DSM 46621^T=JCM 6387^T=CTC 10394^T.

EMENDED DESCRIPTION OF MYCOBACTERIUM AVIUM SUBSP. AVIUM.

M. avium subsp. *avium* (a'vi.um. L. gen. pl. n. *avium*, of birds) and *M. avium* subsp. *silvaticum* are reclassified as later heterotypic synonyms of *M. avium* subsp. *avium*. The subspecies described with the effectively, but not validly, published name "*M. avium* subsp. *hominissuis*" is reclassified as belonging to the subspecies *M. avium* subsp. *avium*. The previously named subspecies should be reclassified as variants. The characteristics, including host adaptations, described for the previously named subspecies (Mijs et al., 2002; Thorel et al., 1990) are now combined in the description of *M. avium* subsp. *avium*. The differential features remain therefore unchanged but are now applied at lower taxonomic level (variant instead of subspecies). Although variants are not governed by the rules of prokaryotic nomenclature, it is recommended that they retain the previous subspecies epithet (e.g. *M. avium* subsp. *avium* var. *avium*). The type strain of *M. avium* subsp. *avium* is ATCC 25291^T=CCUG 20992^T=CIP 104244^T=DSM 44156^T=NCTC 13034^T.

DESCRIPTION OF CANDIDATUS TAXON MYCOBACTERIUM AVIUM SUBSP. LEPRAEMURIUM SUBSP. NOV.

M. avium subsp. *lepraemurium* (le.prae.mu'ri.um. L. fem. n. *lepra*, leprosy; L. masc. n. *mus*, of mice; N.L. gen. pl. n. *lepraemurium*, of leprosy of mice). The description of this subspecies is as given for *M. lepraemurium* (Marchoux & Sorel, 1912). The sequence of 16S rRNA differs by only 4 bp from the one of *M. avium* subsp. *avium*. The type strain is not available due to difficulty in cultivation.

EMENDED DESCRIPTION OF *M. INTRACELLULARE* SUBSP. *CHIMAERA*.

M. intracellulare subsp. *chimaera* (chi.mae'ra. L. fem. n. *chimaera* the chimaera, the mythological being made up of parts of three different animals, referring to the apparent mix of genetic features characterizing the strains). The description of *M. intracellulare* subsp. *chimaera* combines the ones given for *M. chimaera* (Tortoli et al., 2004) and for *M. intracellulare* subsp. *yongonense* (Castejon et al, 2018). The type strain of *M. intracellulare* subsp. *chimaera* is CCUG 50989^T=CIP 107892^T=DSM 44623^T=JCM 14737^T.

EMENDED DESCRIPTION OF *MYCOBACTERIUM AUSTROAFRICANUM*.

The species *M. austroafricanum* (au.stra.lis. L. adj. *australis* southern; a.fri.ca.nus. L. adj. *africanus* pertaining to Africa; N.L. neut. adj. *austroafricanum*, of or pertaining to South Africa, the source of the isolates) has the priority on the synonymous species *M. vanbaalenii*. The description is a combination of the

one given in 1983 (Tsukamura et al., 1983) and that given for *M. vanbaalenii* (Khan et al., 2002). The type strain of *M. austroafricanum* is ATCC 33464^T=CCUG 37667^T=CIP 105395^T=DSM 44191^T= HAMB1 2271^T= JCM 6369^T.

EMENDED DESCRIPTION OF *MYCOBACTERIUM SENEGALENSE*.

The species *M. senegalense* (*se.ne.gal.en'se*. L. adj. *senegalensis* pertaining to Senegal) has the priority on the synonymous species *M. conceptionense*. The description is as given in 1979 (Chamoisseau, 1979) integrated by the one of *M. conceptionense* (Adékambi et al., 2006). The type strain of *M. senegalense* is IEMVT 378^T=ATCC 35796^T=CCUG 21001^T=CIP 104941^T=DSM 43656^T=JCM 15467^T=NCTC 10956^T.

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Table 1. Pairwise comparative ANI and GGD data for the taxa presenting at least one of such values contradicting the status of diverse species/subspecies.

Taxon A	Taxon B	ANI%^a	GGD%^b
<i>M. abscessus</i> subsp. <i>abscessus</i>	<i>M. abscessus</i> subsp. <i>bolletii</i>	97.40	77.30
<i>M. abscessus</i> subsp. <i>bolletii</i>	<i>M. abscessus</i> subsp. <i>massiliense</i>	97.23	75.40
<i>M. abscessus</i> subsp. <i>massiliense</i>	<i>M. abscessus</i> subsp. <i>abscessus</i>	97.16	75.50
<i>M. chelonae</i> subsp. <i>chelonae</i>	<i>M. chelonae</i> subsp. <i>bovis</i>	95.63	64.40
<i>M. chelonae</i> subsp. <i>bovis</i>	<i>M. chelonae</i> subsp. <i>gwanakae</i>	98.39	87.10
<i>M. chelonae</i> subsp. <i>gwanakae</i>	<i>M. chelonae</i> subsp. <i>chelonae</i>	95.94	66.30
<i>M. fortuitum</i> subsp. <i>fortuitum</i>	<i>M. fortuitum</i> subsp. <i>acetamidolyticum</i>	98.99	88.40
<i>M. avium</i> subsp. <i>avium</i>	" <i>M. avium</i> subsp. <i>hominissuis</i> "	99.13	93.70
<i>M. avium</i> subsp. <i>paratuberculosis</i>	" <i>M. avium</i> subsp. <i>hominissuis</i> "	98.79	89.20
<i>M. avium</i> subsp. <i>paratuberculosis</i>	<i>M. avium</i> subsp. <i>avium</i>	98.67	87.30
<i>M. avium</i> subsp. <i>silvaticum</i>	" <i>M. avium</i> subsp. <i>hominissuis</i> "	99.19	93.40
<i>M. lepraemurium</i>	" <i>M. avium</i> subsp. <i>hominissuis</i> "	97.27	74.60
<i>M. lepraemurium</i>	<i>M. avium</i> subsp. <i>avium</i>	97.08	72.80
<i>M. lepraemurium</i>	<i>M. avium</i> subsp. <i>silvaticum</i>	97.08	73.20
<i>M. lepraemurium</i>	<i>M. avium</i> subsp. <i>paratuberculosis</i>	97.03	73.00
<i>M. avium</i> subsp. <i>paratuberculosis</i>	<i>M. avium</i> subsp. <i>silvaticum</i>	98.67	87.80
<i>M. avium</i> subsp. <i>silvaticum</i>	<i>M. avium</i> subsp. <i>avium</i>	99.92	99.30
<i>M. kyogaense</i>	<i>M. obuense</i>	96.19	68.10
<i>M. intracellulare</i> subsp. <i>intracellulare</i>	<i>M. paraintracellulare</i>	98.71	89.90
<i>M. intracellulare</i> subsp. <i>chimaera</i>	<i>M. intracellulare</i> subsp. <i>yongonense</i>	97.81	81.90
<i>M. paraintracellulare</i>	<i>M. intracellulare</i> subsp. <i>chimaera</i>	97.61	79.20
<i>M. intracellulare</i> subsp. <i>yongonense</i>	<i>M. intracellulare</i> subsp. <i>intracellulare</i>	97.57	78.10
<i>M. intracellulare</i> subsp. <i>intracellulare</i>	<i>M. intracellulare</i> subsp. <i>chimaera</i>	97.57	78.70
<i>M. paraintracellulare</i>	<i>M. intracellulare</i> subsp. <i>yongonense</i>	97.63	78.60
<i>M. austroafricanum</i>	<i>M. vanbaalenii</i>	98.71	88.80
<i>M. conceptionense</i>	<i>M. senegalense</i>	99.36	90.70
<i>M. senegalense</i>	<i>M. farcinogenes</i>	98.34	85.10
<i>M. farcinogenes</i>	<i>M. conceptionense</i>	98.35	85.40
<i>M. crocinum</i>	<i>M. pallens</i>	98.36	81.90
<i>M. crocinum</i>	<i>M. aromaticivorans</i>	86.00	32.00
<i>M. pallens</i>	<i>M. aromaticivorans</i>	96.54	78.00
<i>M. eburneum</i>	<i>M. talmoniae</i>	99.73	100
<i>M. hiberniae</i>	<i>M. engbaekii</i>	95.12	60.60
<i>M. marinum</i>	<i>M. pseudoshottsii</i>	98.21	81.90
<i>M. pseudoshottsii</i>	<i>M. ulcerans</i>	99.07	89.90
<i>M. ulcerans</i>	<i>M. marinum</i>	98.03	80.70

^a ANI <95% diverse species; ≥96% synonymous species; ≥98% synonymous subspecies

^b GGD <70% diverse species; ≥70%: synonymous species; ≥80% synonymous subspecies

Figure 1. Phylogenetic tree of the genus *Mycobacterium* constructed using the UPGMA algorithm, from the distance matrix of ANI-divergence scores. Continuous circle, species threshold (ANI=96%). Broken circle, subspecies threshold (ANI=98%). Black dots, confirmed species/subspecies; gray dots, synonymous subspecies; white dots, synonymous species.

ACCEPTED MANUSCRIPT

- All the Mycobacterium species with available genome have been investigated
- Average Nucleotide Identity and Genome to genome distance were used for comparison
- A number of synonymous species were detected and a revision of the taxonomy was proposed

ACCEPTED MANUSCRIPT