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Research Paper

Nontuberculous mycobacteria: Insights on taxonomy and evolution

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ABSTRACT

Seventy years have passed since Ernest H. Runyon presented a phenotypic classification approach for nontuberculous mycobacteria (NTM), primarily as a starting point in trying to understand their clinical relevance. From numerical taxonomy (biochemical testing) to 16S rRNA gene sequencing to whole genome sequencing (WGS), our understanding of NTM has also evolved. Novel species are described at a rapid pace, while taxonomical relationships are re-defined in large part due to the accessibility of WGS. The evolutionary course of clonal complexes within species is better known for some NTM and less for others. In contrast with *M. tuberculosis*, much is left to learn about NTM as a whole.

1. Introduction

Publications describing mycobacteria often begin with a statement that they are acid-fast aerobic organisms that includes the obligate pathogens *Mycobacterium tuberculosis* and *M. leprae*, as well as nontuberculous mycobacteria (NTM). The latter are soil and water-dwelling saprophytic species that occasionally cause opportunistic infections in humans and animals. Although true in a general sense, this is a simplistic view of a genus. As the label implies, nontuberculous mycobacteria excludes organisms of the *M. tuberculosis* complex (MTBC). In contrast to the pathogenic MTBC, which spread exclusively from host-to-host and have no environmental reservoir, NTMs are not transmitted from person-to-person. Acquisition from the environment is a hallmark of NTM infection. As such, NTM also excludes *M. leprae* and other host-restricted species that are not saprophytes and not known to inhabit any environmental niche.

The prevalence of disease due to NTM appears to be increasing in some countries (Adjemian et al., 2012; Marras et al., 2013) and there is growing appreciation for the economic burden posed by these organisms. Along with clinical presentation, identification to the species–level is an essential component of assessing the clinical significance of an NTM. Molecular and genomic approaches to taxonomy have revealed limitations to the traditional phenotype-based schemes for NTM taxonomy. Phenotypically-defined groups can be genomically diverse and there is increasing recognition that some disease-causing NTMs represent pathogenic clones of otherwise weakly virulent species.

In recent years, there has been an explosion in NTM species, and consequently essential periodic updates and perspectives on NTM taxonomy (Forbes, 2017; Tortoli, 2003, 2006, 2012, 2014; Tortoli et al., 2017). This review strives to complement previous discussions, address current issues in NTM taxonomy, and highlight the evolutionary complexities within key NTM species groups.

2. NTM classification: the early days

The earliest formal species description of mycobacterial organisms began in the early 1880s with the obligate pathogens *M. leprae* and *M. tuberculosis*. The earliest NTM described were the saprophytic species *M. smegmatis* (1889) and *M. phlei* (1899). The agent of avian tuberculosis was recognized in the 1890s, but the specific epithet, *M. avium*, wasn't introduced until the turn of the century (1901). Identification of *M. lepraemurium* (rat leprosy) and *M. paratuberculosis* (Johne's Disease) followed, although the notion that these represented clonal variants of *M. avium* was not suspected. Only a few other *Mycobacterium* species were described before 1950, including *M. chelonae*, *M. marinum* and *M. fortuitum*.

In the clinical setting, NTM – many with no names (often referred to as “anonymous atypical acid-fast bacteria”), were considered mere saprophytes since, unlike agents of tuberculosis, they were not virulent in guinea pigs. However, it became evident that some NTMs, primarily those isolated in the context of diagnosing TB, were associated with diseases distinct from tuberculosis and leprosy. Those most commonly recognized as potentially pathogenic in humans were *M. kansasii* and the “Battey bacillus” (known today as *M. intracellulare,* a member of the

Abbreviations: NTM, nontuberculous mycobacteria; RGM, rapidly growing mycobacteria; SGM, slowly growing mycobacteria; WGS, whole genome sequencing

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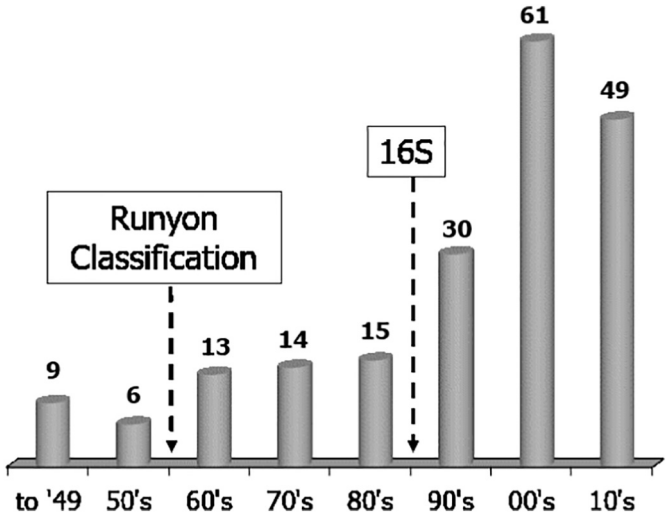

Fig. 1. Number of validated *Mycobacterium* species through the last 7 decades, highlighting the impact of 2 taxonomical milestones: Runyon classification and 16S rRNA gene sequencing.

M. avium complex [MAC]), in addition to other less frequent NTM with an uncertain propensity to cause disease (Runyon, 1959, 1965). The first adequate phenotypic classification system for NTMs is attributed to Ernest Runyon, who categorized NTM into 4 easily recognizable phenotypic groups I – IV (Runyon, 1959). This was followed by introduction of a numerical taxonomy approach that used a larger number of phenotypic characters to assist in identification, promote speciation of novel taxa, and better understand the pathogenic potential and epidemiology of NTM (Bojalil et al., 1962; Tsukamura, 1967; Wayne, 1966). As a result, the number of NTM species described nearly quadrupled between 1960 and 1990 (Fig. 1).

Most NTM correspond to one of two main groups, slowly growing mycobacteria (SGM) and rapidly growing mycobacteria (RGM). This phenotype is defined on the basis of growth (from a diluted inoculum) occurring either before or after 7 days. The SGM are further categorized as photochromogenic (Group I: pigmentation upon exposure to light), scotochromogenic (Group II: always pigmented) or non-photochromogenic (Group III: no, weak or late-pigmenting, irrespective of light); RGM belong to Group IV. The division of SGM and RGM into subgenera *Mycobacterium* and *Mycomycobacterium*, respectively, was proposed (Tsukamura, 1967) but aside from growth rate, far too many characteristics, including DNA-DNA hybridization, phage susceptibility, or lipid composition, were shared to justify a new subgenus (Magee and Ward, 2012). Furthermore, growth rate and pigmentation can be subjective and vary even within a single species. Though tremendously useful, the Runyon classification was not intended as absolute, but served as a starting point to help assess clinical significance of NTM since Groups I and III (e.g. *M. kansasii* and *M. avium* complex) contained the more pathogenic groups, while Groups II (e.g. *M. gordonae*) and IV (RGM) were deemed *usually* not clinically relevant (Runyon, 1959).

3. The molecular era of taxonomy

In 1987, an International Committee for Systematic Bacteriology convened to resolve aspects of bacterial systematics, among them renowned mycobacteriologists. It was agreed that use of the complete genome served best to determine phylogeny, and consequently, taxonomy. The definition of a species included strains with approximately 70% (or more) DNA-DNA relatedness (by DNA re-association techniques) and 5 °C or less ΔT_m (a measure of thermal stability comparing homoduplexes from a single strain vs heteroduplexes from a mix of two strains). It was also recommended that unless different species could be identified by some phenotypic property, that they not yet be named (Wayne et al., 1987).

Around the same time, the use of 16S rRNA gene sequencing started to dominate the field of bacterial systematics. Carl Woese's seminal work provided the first phylogenetic trees that spanned all prokayotes, revealing for the first time their genetic relationships (Woese, 1987). 16S was, and continued to be, a powerful tool providing a phylogenetic framework for the classification of organisms. This was revolutionary and offered an important criterion to complement phenotypic classification systems used in the definition of novel taxa (Stackebrandt et al., 2002). The RGM and SGM division is maintained in molecular phylogeny. In most cases, SGM contain only a single copy of the ribosomal operon, whereas RGM contain two. Therefore, it has been hypothesized that number of ribosomal operons in mycobacteria correlates with growth rate, though this has not been demonstrated to be true, nor is the correlation consistent. Some of the slowest growers, e.g. *M. celatum* and *M. xenopi*, contain two copies of the rRNA operon, as do members of the *M. terrae* complex (SGM with an intermediate growth rate). In contrast to other RGM, members of the *M. abscessus-M. chelonae* group contain only a single copy.

Mycobacteria species can be identified to the species level by sequencing the complete 16S rRNA gene, with some exceptions (Tortoli, 2014; Tortoli et al., 2017). While many can also be identified using only the first 500 bp (containing two hypervariable regions), clinically significant RGM that belong to the *M. fortuitum* or *M. abscessus-M. chelonae* clades require the full 16S rRNA gene, and/or alternate target (see below) for species resolution (CLSI, 2018). For mycobacteria, a 100% sequence identity in the 16S sequence against the type strain of a species is generally expected for species identification, therefore proper sequence editing is essential (Tortoli, 2010). A few species that contain 2 copies depict inter-operon variability, usually in 1 to 3 positions, which may result in either “false” differences or ambiguities. This is common in several members of the *M. terrae* complex, as well as *M. xenopi*. Some strains of *M. celatum* contain a T insert at position 214 in only 1 of its 2 16S copies in the genome, causing total sequence ambiguity downstream of the indel (in either directions) by direct sequencing (Reischl et al., 1998). While RGM contain two copies, very few present with ambiguous bases, presumably because in most species, the two 16S copies are identical.

Secondary genes targets, e.g. housekeeping genes, evolve more rapidly than ribosomal genes and therefore have higher discriminatory power. This is practical in order to distinguish between very closely related species or complexes that cannot be identified by 16S. A 441-bp region of the *hsp*65 gene, often referred to as the Telenti fragment (Telenti et al., 1993) and region V of the *rpoB* (Adekambi et al., 2003) are the most commonly used for mycobacteria and are used routinely in some laboratories (de Zwaan et al., 2014; McNabb et al., 2004). There are limitations to consider. With high sequence identity values, e.g. > 99%, identification is clear when matched to a single species, and this is the case for most isolates (de Zwaan et al., 2014; McNabb et al., 2004). At levels below that, depending on the species, it may become difficult to determine closest relatives due to equal distance to several unrelated species. Secondly, horizontal gene transfer events may result in incongruity and therefore, it is recommended to use more than one target, especially in the context of novel species description (Macheras et al., 2014; Tortoli et al., 2013). The analysis of multiple strains and multilocus sequencing for species characterization does help to overcome some of these limitations. And finally, these targets are underrepresented in publicly available sequence databases (de Zwaan et al., 2014), though this limitation becomes less of an issue as we move into the era of whole genome sequencing (WGS).

The use of sequencing for NTM identification in routine practice, starting with 16S, quickly replaced time-consuming biochemical testing and eventually high-pressure liquid chromatography of mycolic acids due to overlap of profiles as new species were described (Tortoli, 2014). To this day, no other gene is depicted with such comprehensiveness, in that every known species is represented in public sequence databases.

As a result, for the next 3 decades and into the present, NTM taxonomy expanded > 3-fold (Fig. 1).

4. Current NTM taxonomy

Mycobacteria are aerobic to microaerophilic, non-motile straight or slightly curved acid-fast bacilli with a high G + C content (57–73%). They contain complex long chain mycolic acids (60–90 carbons), comparable only to *Tsukamurella* (62–78) and some *Gordonia* (46–77) (Magee and Ward, 2012), the latter two being partially acid fast. *Mycobacterium* remains a well-demarcated and distinct monophyletic group of the family *Mycobacteriaceae* (Magee and Ward, 2012; Nouioui et al., 2018), within the Order *Actinomycetales*. Nearly 200 *Mycobacterium* species have been validated (http://www.bacterio.net/mycobacterium.html) with approximately half considered RGM and half SGM. Despite the large number of species in the genus, 16S identity values of all species are above 94.9% ± 0.4 relative to the type species (i.e. *M. tuberculosis*), identified as the minimum value for belonging to a single genus (Yarza et al., 2008). The last published recommendations specific for mycobacteria were provided for SGM by Lévy-Frebault et al. > 25 years ago (Levy-Frebault and Portaels, 1992), before the routine use of sequencing-based identification that facilitates obvious classification into the genus.

Phylogenetic taxonomy by 16S rRNA gene sequencing reveals several main groups or clades, all of which contain genus-specific 16S nucleotide signatures and some that are clade-specific (Magee and Ward, 2012; Tortoli, 2003). These groups are interspersed with unique single-membered clades that are distant enough from their closest relative to be considered their own branch (Magee and Ward, 2012). Among the RGM, 3 main clades are represented by species clusters that contain *M. fortuitum*, *M. smegmatis*, and the *M. abscessus-chelonae* group. The *M. terrae* complex is situated in an intermediary position between SGM and RGM, along with *M. triviale* and *M. talmoniae*, though these three complexes/species are relatively distant from each other. Clades within SGM include MAC, MTBC, and groups that cluster with *M. kansasii*, *M. celatum*, *M. marinum-M. ulcerans*, or *M. simiae*.

In addition to valid species, many NTM described in the literature are not currently validated. Many of these are likely true species but haven't yet fulfilled validation criteria as per the Prokaryotic Code (Oren et al., 2018). New journals have surfaced in which novel taxa are being described at a rapid pace, but have a poor record of completing the validation process (Oren et al., 2018). With regards to mycobacteria, some of these are often cited in the current literature and/or appear to be generally (albeit unofficially) accepted in the mycobacterial community, e.g. "*M. avium* subsp. *hominissuis*", "*M. canettii*", "*M. lepromatosis*". It is without a doubt that novel taxa currently remain in laboratory freezers or have been discovered but have yet to make it to the publication stage as a novel species (de Zwaan et al., 2014; Pauls et al., 2003; Tortoli et al., 2001). Depositing unique 16S rRNA sequence data in a public database provides a foundation for future analyses. Ideally, if multiple laboratories find strains with identical sequences, collaborative efforts could lead to a better description of a novel species (both phenotypic and genotypic) and ultimately, validation. Public databases do contain mycobacterial sequences that have not been assigned any names, and hints at the greater diversity that exists for mycobacteria.

At the same time, the validation of a novel species name does not necessarily indicate that it is truly novel or distinct from its closest relative. In order for a novel taxon to be considered validly published, it must comply with the rules set out by the International Code of Nomenclature of Prokaryotes (Parker et al., 2019). However, The Prokaryotic Code only regulates the nomenclature aspect, not the features or uniqueness of taxa. In summary, the minimum requirements for validating a species name include 1) choosing a name and providing the etymology, 2) designation of a type strain, deposit into two separate international collections, and available without restriction and 3) a description of the species. Finally, it must be published in the International Journal of Systematic and Evolutionary Microbiology (IJSEM), the previous International Journal of Systematic Bacteriology (IJSB) or included in the Validations list published by IJSB/IJSEM (if published elsewhere and submitted for validation in IJSB/IJSEM). Approval (or rejection) of novel taxon descriptions occur only as part of the review process upon submission, and the reasons for suggesting that a taxon is novel are not bound by any rules. What is often not appreciated is the fact that once valid, a name remains valid unless rejected according to strict criteria. The burden then lies on the scientific community to make a judgment on proposed taxonomy and decide whether or not to adopt it in common usage. However, novel taxa or the latest taxonomical reclassifications that are supported by strong evidence are generally accepted, even in the clinical setting where the impact on results reporting and patient management is the greatest. When describing novel species, multiple strains should be included to offset intra-species variation. Unfortunately, the description of a novel species on the basis of a single strain does not preclude their validation and contributes to 90% of taxa validly published (Oren and Garrity, 2014).

5. WGS as a phylogenetic tool

For many years, DNA-DNA hybridization, a cumbersome technique for assessing genome similarity between strains, served as a gold standard for species delineation in support of phenotypic classification. The advent of WGS has brought on a new revolution, clarifying phylogenetic relationships to a scale not previously possible, and only recently has this been undertaken for the type strains of most NTM (Fedrizzi et al., 2017; Tortoli et al., 2017).

Overall, WGS largely confirms the phylogenetic relationships and positions observed for 16S and other single-gene targets. The main clades remain unchanged, with a segregation of RGM and SGM and several branches in between (Fedrizzi et al., 2017; Tortoli et al., 2017). RGM appear ancestral, with the *M. abscessus* group being putatively closest to a common ancestor. Generalizations have been made for genome size, where SGM tend to be smaller and RGM larger. However, being first and foremost environmental organisms, genome content varies significantly, such that some of the largest (*M. gordonae* at 7.6 Mb) and smallest (*M. triviale* at 3.6 Mb) genomes are both SGM (Fedrizzi et al., 2017). Consistent with 16S phylogenies, SGM species *M. doricum* and *M. tusciae* remain clustered among the RGM.

Several NTM species share identical 16S sequences with another valid species. The reasons for this varies, but have been rationalized a) by using methods not well validated such as dot-blot DNA-DNA hybridization, b) by differences observed in techniques normally employed as a typing tool (e.g. PFGE and MLST), c) on the basis of genetic variation in alternate genes (e.g. *rpoB*) and/or d) by subjective interpretation of characteristics such as pigmentation, or phenotypic differences based on single strains (where intra-species variation cannot be ascertained). Once WGS became available, it became clear that some of these do in fact belong to a same species. As a result, it is proposed that one of two ensue: fusion with the earliest valid name (e.g. *M. vanbaalenii* becomes *M. austroafricanum*) or subspeciation (e.g. *M. engbaekii* could become *M. hiberniae* subsp. *engbaekii*) (Tortoli et al., 2017). Additional species combinations proposed as a result of WGS findings are described below for MAC, *M. marinum-M. ulcerans* complex, and *M. senegalense-M. conceptionense*.

An early definition of subspecies was provided by Wayne et al.: "genetically close organisms that diverge in phenotype", and admittedly, further guidelines were needed (Wayne et al., 1987). Whereas average nucleotide identity (ANI) values of > 95% are commonly used as a measure to delineate species (Goris et al., 2007; Richter and Rosselló-Móra, 2009), values between 95 and 98% reasonably exemplify subspecies if supported by epidemiological, phenotypic, or other relevant trait. While there are no current genomic standards for genus delineation, and ANI values cannot be used to do so, > 2/3 of

interspecies ANI values within a genus are around 68–72% (Qin et al., 2014).

Recently, it was proposed to formalize some of the known divisions in the genus by demarcating them into 5 distinct genera consisting of two large groups containing the majority of SGM (*Mycobacterium*) and RGM (*Mycolicibacterium*), and three smaller groups represented by the *M. terrae* complex (*Mycolicibacter*), *M. abscessus-M. chelonae* complex (*Mycobacteroides*), and a genus (*Mycolicibacillus*) with only *M. triviale* and two rare related species (Gupta et al., 2018). The basis for this is the identification of molecular signatures, specifically the presence or absence of proteins or amino acid insertions and deletions (indels) shared within a group of related taxa. This approach is not widely practiced for demarcation at the genus level and is not without controversy (Margos et al., 2017). It becomes further complicated by the discovery of novel species or by the analysis of genomes that were not included in such studies. For example, *M. talmoniae* shares biomarkers specific for SGM, specific for RGM, and some specific for the *M. terrae-M. triviale* clade. Phylogenetically, it is in an intermediate position between RGM and SGM and is potentially the same species as *M. eburneum* (WGS not yet available) (Vasireddy et al., 2018), which was described almost at the same time. Using the approach by Gupta et al., *M. talmoniae* and *M. eburneum* may warrant re-assignment to a 6th novel genus, while reducing further the number of markers deemed specific for each genus. Several other NTM species exist that form phylogenetically unique branches, relatively distant from other species, where one could easily identify unique proteins or indels, e.g. *M. fallax*, *M. insubricum M. botniense*, *M. chitae*, *M. heckeshornense* or *M. xenopi*. There are more questions than answers in implementing such taxonomical changes, though the methodology can certainly be used to study evolutionary relationships between taxa (Patel and Gupta, 2018) and as a tool in the development of diagnostics and therapeutics.

Mislabeling of taxa from WGS data is another problem (Tortoli, 2018; Tortoli et al., 2017) but can be overcome by limiting analysis to the type strain of a species or ensuring adequate intra-species similarity values against the type strain (Ciufo et al., 2018). In the least, phylogenies that appear erroneous must be investigated further and identification confirmation by 16S and concurrence with a species' original description should be carried out. However, even type strains are not exempt from mislabeling and care must be taken verify that these represent in fact the strain they are supposed to be. For example, the WGS of the type strain of *M. vulneris* (DSM 45247^T; accession CCBG00000000), represents instead a strain of the RGM *M. porcinum* rather than a member of the *M. avium* complex (Tortoli, 2018), whereas investigations using the type strains of *M. bouchedurhonense* and *M. timonense* revealed they represent strains of *M. avium*, inconsistent with their original description (van Ingen et al., 2018). This may lead researchers less familiar with NTM taxonomy off course.

With the help of WGS, we continue to dissect and learn more about the propensity of some species or lineages to be more often associated with infection. However, compared to the wealth of data available on evolution and virulence determinants of *M. tuberculosis*, similar investigations in NTM has been hampered by the extremely limited availability of WGS representing NTM species in public databases. As a result, the assessment on the distribution of putative virulence factors among NTM, particularly those known to have a propensity to cause disease, has been mostly speculative, with a few obvious exceptions (e.g. mycolactone in *M. ulcerans*). It has only been until very recently that researchers have assumed the task of generating WGS sequence data for the type strains of most NTM species (Fedrizzi et al., 2017; Tortoli et al., 2017), providing an important foundation to study phylogenetic relationships, the impact of horizontal gene transfer (Panda et al., 2018), and virulence.

6. Evolution and heterogeneity within complexes and species

Studies on the evolution of *M. tuberculosis* are understandably the most advanced. As a result, evolutionary aspects of MTBC are sometimes erroneously generalized for all of mycobacteria. The environmental niche and whether or not the organism is host-associated will have significant impact on the evolution of a species and the various lineages within. For this reason, obligate intracellular pathogens that reside in a sterile environment (e.g. *M. tuberculosis*) will have limited opportunity for gene exchange and hence, behave as a highly clonal species. They may also evolve through gene loss or decay, as certain functions that are no longer essential in the host-adapted species begin to disappear. Reductive evolution occurred on a massive scale in *M. leprae* (Cole et al., 2001). In contrast, NTM as a whole demonstrate greater genetic variability, at both the intra- and interspecies level. Remarkably, some species do contain within them pathogenic lineages that appear clonal in contrast with the environmental species from which they derive. These are primarily animal pathogens, e.g. birds, bovine, while humans tend to develop opportunistic infections with strains free-living in the environment. Phenotypic and genotypic differences that may be observed between animal and humans strains of a species are one example where designation at the subspecies level is warranted.

6.1. *M. avium* complex (MAC)

The most frequently isolated NTM from clinical specimens are members of the *M. avium* complex (MAC). Prior to antigen-based or genetic approaches, the relatedness of MAC organisms was not necessarily obvious. Different lineages of *M. avium* cause distinct disease manifestations: avian tuberculosis, Johne's Disease/paratuberculosis, pulmonary human disease (“Battey disease” prior to the 1960s), and feline leprosy. In addition, growth of MAC organisms vary in temperature, rate and in the requirement for mycobactin. In contrast, clinically or phenotypically distinguishing human strains of *M. avium* from *M. intracellulare* proved challenging (Wayne, 1966), but genomic studies by means of DNA-DNA hybridization confirmed that human *M. avium* and *M. intracellulare* were distinct taxa (Baess, 1983). As well, studies demonstrated that the agents of paratuberculosis, avian tuberculosis and the “wood-pigeon mycobacteria” belonged to the same species (Saxegaard and Baess, 1988), subsequently formalized by the validation of 3 subspecies consisting of *M. avium*: *M. avium* subsp. *avium* (MAA), *M. avium* subsp. *paratuberculosis* (MAP), and *M. avium* subsp. *silvaticum* (MAS), respectively (Thorel et al., 1990). RFLP and 16S-23S rDNA internally transcribed spacer (ITS1) sequencing characteristics highlighted that human and porcine strains of *M. avium* were distinct from MAA and “*M. avium* subsp. *hominissuis*” (MAH) was proposed (Mijs et al., 2002). While not validly published according to Rule 27 of the Bacteriological Code, MAH is universally accepted.

The MAC includes 12 validly published species: *M. avium*, *M. intracellulare*, *M. chimaera, M. colombiense*, *M. arosiense*, *M. vulneris*, *M. marseillense*, *M. timonense*, *M. bouchedurhonense*, *M. yongonense, M. paraintracellulare, and M. lepraemurium* (van Ingen et al., 2018). Species belonging to MAC share high sequence identity in 16S and other commonly used targets, as well as > 85% ANI values (van Ingen et al., 2018). By 16S, the following are 100% identical: *M. intracellulare* and *M. paraintracellulare*, *M. marseillense* and *M. yongonense*, while *M. chimaera* can be distinguished from *M. intracellulare* by a single bp within the first 500 bp of the 16S. WGS analyses of their type strains suggests that *M. paraintracellulare* is a strain of *M. intracellulare*, whereas subspecies status was proposed for *M. chimaera*, *M. intracellulare* and *M. yongonense* (Tortoli et al., 2017). Subsequently, the formal description of *M. intracellulare* subsp. *intracellulare* and *M. intracellulare* subsp. *yongonense* was published (Castejon et al., 2018), as was the formal validation of *M. intracellulare* subsp. *chimaera* (Oren and Garrity, 2018). By and large, the species *M. avium* and *M. intracellulare* remain the most commonly isolated from clinical specimens, followed by *M. chimaera*, responsible for what could essentially be described as the first global outbreak due an NTM species (van Ingen et al., 2017). The remaining

species are infrequently isolated to date.

MLST approaches have uncovered that the species *M. avium* is highly heterogenous and contains independently evolved host-specific pathogenic clones derived from a common ancestor, presumably MAH (Turenne et al., 2008). MAP consists of two host-associated lineages that have evolved by acquisition of genomic elements not present in other *M. avium* strains, followed by reductive genomic events characterizing cattle type (MAP-C) and sheep type (MAP-S) clones (Alexander et al., 2008). It is hypothesized that an alternate iron-uptake system may have been acquired in MAP, allowing for the impairment or loss of mycobactin synthesis and iron acquisition pathways functional in other *M. avium* (Wang et al., 2015). WGS investigations confirm the existence of two lineages and reveal exceptionally high genetic stability consistent with monomorphic pathogens (Bryant et al., 2016).

MAA and MAS exemplify two phenotypically distinct clones, where MAS is slower, rough, and mycobactin-dependent upon primary isolation, in contrast to MAA. However, they are so close genetically that the development of a reliable NAAT assay to distinguish between the two lineages was only recently achieved (Ronai et al., 2015).

Although host-associated, strains of MAA, MAS, MAP-C and MAP-S are not restricted to a single host, and may cause disease in other animals that represent true or accidental hosts. Similarly, the natural hosts of pathogenic *M. avium* clones may also be infected by the generalist MAH or other NTM species, with variable ability to cause disease. In a recent investigation of mycobacteriosis in birds, almost all harbored either *M. avium* or *M. genavense*. WGS revealed that half of *M. avium* were the clonal MAA, while the rest were a heterogeneous assortment of MAH (Pfeiffer et al., 2017). Humans are predominantly infected by MAH acquired from the environment, with rare isolation of MAA. Not surprisingly, WGS analyses of clinical MAH demonstrates extensive diversity, with geographically-associated lineages and clusters potentially associated with progressive pulmonary disease (Uchiya et al., 2017; Yano et al., 2017).

Finally, the species *M. avium* also likely generated the murine pathogen *M. lepraemurium*, which presents with ANI values of > 97% vs other *M. avium* subspecies (van Ingen et al., 2018). *M. lepraemurium* evolved through massive gene decay and reductive evolution akin to *M. leprae* (Benjak et al., 2017). Causing leprosy in cats and rodents, and unculturable by standard methods, the assumption was that the organism was closely related to *M. leprae*.

6.2. M. marinum and M. ulcerans

M. marinum and *M. ulcerans* are very distinct from each other in the diseases that they cause, their epidemiology and their phenotype. For this reason, maintaining their distinct species status is warranted, despite ANI values ~98% from each other (Doig et al., 2012; Stinear et al., 2007; Tortoli et al., 2017). *M. ulcerans* causes Buruli ulcer, a tropical disease limited to western and central Africa and pockets of Australia, with an uncertain environmental reservoir. *M. marinum* cutaneous mycobacterioses occur worldwide.

M. ulcerans and closely related strains are unique in the genus in that they are toxin-producing NTM due to acquisition of a plasmid that produces the immunosuppressive polyketide mycolactone. *M. ulcerans* has undergone extensive reductive genomics, driven by expansion of IS*2404* (not present in *M. marinum*), resulting in ~770 pseudogenes and subsequent loss of ~1 Mb in genetic content (Stinear et al., 2007). *M. ulcerans*, a nonphotochromogen, can be cultured in the laboratory, but growth from clinical specimens is excessively slow, requiring additional incubation (e.g. 12 weeks) if suspected. *M. ulcerans* forms a phylogenetically tight cluster with other mycolactone-producing mycobacteria (MPM): *M. pseudoshottsii*, *M. shinshuense*, "*M. liflandii*" and some strains of *M. marinum*. These do not necessarily cause Buruli ulcer, but similarly to *M. ulcerans*, share a common ancestor with *M. marinum* and are distinguished from it by DNA-DNA hybridization experiments, mycolactone production, and high-copy IS*2404*. This strongly supports that these should in fact be considered a single species: *M. ulcerans* (Pidot et al., 2010).

M. marinum is an aquatic photochromogenic NTM that causes disease in fish and cutaneous infections in the humans that handle them. It is both a successful pathogen of humans, and is ubiquitous in aquatic environments. It is categorized as a SGM, but may grow relatively rapidly, possibly within 7 days when subcultured from a heavy inoculum. WGS of *M. marinum* strains from various sources reveals two main clusters, I and II associated with the M strain and the Aronson (type) strain, respectively (Das et al., 2018). Cluster I isolates were found to be more closely related to *M. ulcerans* and "*M. liflandii*" (ANI values > 98.6) than to cluster II. Interestingly, a duplication event in cluster II gave rise to two ribosomal operons, while cluster I contains only one, similar to *M. ulcerans* and most SGM. However, this difference does not contribute to variations in growth rate. Classifying each cluster as subspecies *M. marinum* subp. *marinum* and *M. marinum* subsp. *moffat* (for the M strain cluster) was proposed, and could be important to consider in comparative genomics studies for members of the *M. marinum-ulcerans* complex. It could also be argued that the M cluster itself consists of two lineages, where the *M. marinum* strains that cluster with *M. ulcerans* happen to contain a mycolactone-producing plasmid. This would be consistent with the previously proposed re-classification of these organisms as *M. ulcerans* (Pidot et al., 2010).

6.3. M. kansasii

M. kansasii has historically been easily identifiable by its photochomogenicity, quite useful to distinguish it from its closest neighbor (100% by 16S), the non-pathogenic *M. gastri*. Early on, however, it was hypothesized that *M. kansasii* contained both pathogenic and non-pathogenic strains, and that *M. gastri* bared close resemblance to the lesser pathogenic, low-catalase group of *M. kansasii* (Wayne, 1962). Being one of the more important NTM pulmonary pathogens, a commercially available DNA probe assay specific for *M. kansasii* was developed, but the existence of probe-negative *M. kansasii* was also reported (Ross et al., 1992). Various typing and sequence-based methods using 16S, ITS1, or *hsp65*, have consistently shown heterogeneity within *M. kansasii*, with up to 7 subtypes identified (Alcaide et al., 1997; Picardeau et al., 1997; Richter et al., 1999; Taillard et al., 2003). Remarkably, some studies have observed that strains associated with clinical disease almost all belong to a single clonal entity known as Type I (Bakula et al., 2018; Santin et al., 2004; Zhang et al., 2004), which includes the type strain (ATCC 12478^T). Others have shown a predominance of Type I in clinical disease, though a subset were also caused by Type II strains, found in water sources, in the context of immunosuppression or HIV infection (Alcaide et al., 1997; Taillard et al., 2003). Other types (III-VII) are uncommonly associated with disease and are infrequently isolated from clinical specimens (Taillard et al., 2003); strains from the environment, primarily obtained from tap water, feature all genotypes (Alcaide et al., 1997; Picardeau et al., 1997), demonstrating significant variation.

M. persicum, a novel species recently described as closely related to *M. kansasii* and *M. gastri* (Shahraki et al., 2017), corresponds to *M. kansasii* Type II by ITS1 and 16S rRNA gene sequencing. By WGS, the species exhibits 92.7 and 92.2 ANI values with the type strains of *M. kansasii* and *M. gastri*, respectively, substantiating its status as a distinct species. It is likely that the remaining subtypes represent either subspecies of *M. kansasii* or possibly novel species. Genotype-level discrimination by MALDI-ToF was successfully demonstrated for six subtypes (Murugaiyan et al., 2018). As evident from the number of genome sequences now deposited in public databases, including different subtypes (Borowka et al., 2017), efforts are underway to characterize what may eventually become known as *M. kansasii* complex.

M. kansasii is phylogenetically in close proximity to *M. tuberculosis*. Its ability to cause TB-like disease, yet isn't known to be transmitted person-to-person, make it an ideal candidate for comparative and

functional genomic analyses. Such investigations have uncovered genes in *M. tuberculosis* that have no homology in other NTM, which suggests horizontal gene transfer in a phase of genomic expansion prior to a phase of host-adaptation and reductive evolution in *M. tuberculosis* (Veyrier et al., 2009; Wang et al., 2015). Considering including less virulent strains of an *M kansasii* complex in future analyses may provide further insight on the pathogenicity of both *M. kansasii* and *M. tuberculosis*.

6.4. M. chelonae and M. abscessus

The *M. abscessus-M. chelonae* group currently contains 8 species: *M. abscessus*, *M. chelonae*, *M. immunogenum*, *M. salmoniphilum*, *M. franklinii*, *M. saopaulense* and *M. stephanolepidis*. The 2 most common species, *M. abscessus* and *M. chelonae* are further delineated into 3 subspecies each. Species level identification by 16S is challenging for this group, since they are very similar, with 0 to 11 bp differences between them; *M. abscessus* subspecies share identical 16S sequences and only differ by a single bp from the type strain of *M. chelonae* and *M. franklinii* (which are identical to each other).

The type strain of *M. chelonae* was isolated from a tortoise tubercle. Human isolates of *M. chelonae* consistently present with a 3 bp difference in the 3′ end of the 16S rRNA gene from the type strain (unpublished data). *M. chelonae* "chemovar niacinogenes" ATCC 19237, a reference strain from a gastric lavage, harbors those same differences. In fact, this 16S sequence is far more common than that of the type strain. In *M. chelonae*, intraspecies variability in the 16S alone is depicted by at least 4 sequevars, three of which present with 1–3 bp from the type strain (Simmon et al., 2011). None of the "classic" human *M. chelonae* strains match the type strain, with the exception of a group of isolates from Pennsylvania with an atypical susceptibility pattern for *M. chelonae*. This prompted further investigation and the subsequent description of *M. franklinii* (Lourenco Nogueira et al., 2015; Simmon et al., 2011), despite showing 100% by 16S sequencing, exhibiting significant genetic divergence from *M. chelonae* by MLST, DNA-DNA hybridization and deep sequencing.

Recently, *M. chelonae* has been divided into 3 subspecies: 1) *M. chelonae* subsp. *chelonae* (Mchc), the tortoise isolate deposited as NCTC 946 in 1921, for which the true distribution remains unknown; 2) *M. chelonae* subsp. *bovis* (Mchb) representing strains isolated from the lymph nodes of cattle (Kim et al., 2017) and 3) *M. chelonae* subsp. *gwanakae* (Mchg) from sputa of patients from Korea (Kim et al., 2018). Demarcation at the subspecies level was proposed on the basis of ~95% ANI values from the Type strain and other subspecies, phenotypic characteristics (such as the ability to grow at 37C or colony morphology) and MLST. Mchb shares the 3 bp 16S differences observed in ATCC 19237, but demonstrate divergence by MLST, whereas Mchg presents with 2 unique bp differences in the 16S gene. *M. saopaulense* also presents with the same 16S sequence as ATCC 19237, but is otherwise distinct from other members of the *M. chelonae-abscessus* complex (Nogueira et al., 2015). Since it is not a type strain, ATCC 19237 is not always included in analyses or novel species descriptions, therefore its taxonomic position within the complex remains uncertain. Currently, > 40 genome projects for *M. chelonae* isolates can be found in NCBI. The majority belong to 2 Bioprojects: 1) PRJNA347845, an ongoing study of *M. chelonae* from biofilms, fish, reptiles, mammals and humans and 2) PRJNA323571 is a collection of clinical (human) and water isolates. These projects will no doubt provide much needed insight on the taxonomy and epidemiology of *M. chelonae* organisms. It will be of interest to determine if the Type strain of *M. chelonae* is in fact an outlier within the species, as preliminary data suggests.

Studies on the *M. abscessus* complex far outnumber those on *M. chelonae.* In many parts of the world, *M. abscessus* is second only to MAC as a commonly isolated and clinically important NTM. After several taxonomical proposals, debates and re-classifications, *M. abscessus* currently consists of 3 subspecies: *M. abscessus* subsp. *abscessus* (Mab), *M. abscessus* subsp. *massiliense* (Mma), and *M. abscessus* subsp. *bolletii* (Mbo) (Tortoli et al., 2016). The majority of clinical isolates consist of Mab and Mma; Mbo is rare in the US and East Asia, but appears more frequent in Europe (Koh et al., 2014). Subspecies distinction is clinically relevant in that the presence of a functional erythromycin ribosomal methylase gene, *erm*(41), is associated with delayed or failed response to macrolide combination therapy (Koh et al., 2011), despite in vitro susceptibility to clarithromycin at 3 days. Nash et al. were first to demonstrate that *erm*(41), present in *M. abscessus* (but not *M. chelonae*), conferred inducible resistance (through methylation of 23S ribosomal RNA) in a majority of Mab isolates but was mutated in a smaller number of susceptible strains (Nash et al., 2009). The gene is also functional in most Mbo, conferring inducible resistance unless mutated, while deletions/truncation present in Mma renders it susceptible (Nash et al., 2009). High-level resistance (not inducible) can also occur in all 3 subspecies due to single point mutations in the 23S rRNA gene. To detect inducible macrolide resistance, MIC determination requires incubation for up to 14 days (CLSI, 2011) and can be predicted by *erm*(41) gene sequencing (Brown-Elliott et al., 2015; Kim et al., 2010).

An extensive multinational WGS study of > 1000 *M. abscessus* strains from > 500 cystic fibrosis (CF) patients revealed that recent spread of 3 dominant circulating clones of *M. abscessus* (2 Mab and 1 Mma) has occurred within the CF community between the US, Europe and Australia (Bryant et al., 2016). It was suggested that *M. abscessus* is likely spreading indirectly between patients through fomites or possibly by long-lived (desiccation resistant) aerosols. Furthermore, clustered isolates tended to correlate with chronic infection, worse clinical outcomes and high rates of amikacin and/or macrolide resistance. Clustered isolates also demonstrated increased phagocytic uptake and intracellulare survival in macrophages compared to non-clustered isolates, indicating increased pathogenic potential. It is possible that *M. abscessus* is evolving to become a true pulmonary pathogen (Bryant et al., 2016).

6.5. M. senegalense, M. farcinogenes and their human counterparts

The *M. fortuitum* group includes > 15 RGM species clustered together on a phylogenetic branch. The earliest validated species include (in order of validation) *M. fortuitum, M. farcinogenes, M. senegalense, M. porcinum* and *M. peregrinum*. Genetically, most species have been recognized as distinct based on earlier biochemical profiles, or later by 16S, *hsp65* or *rpoB*-based characterization. Recent WGS-based phylogenies continue to support the close proximity of these species within a branch containing *M. fortuitum* (Tortoli et al., 2017). Taxonomical changes within this group has for the most part remained unchanged, though there may be host-associated lineages that are not often considered, as well as misperceptions derived from inconsistencies in sequencing data in the type strains from various international collections.

As early as 1985, it was observed that while clinical *M. peregrinum* isolates appeared phenotypically indistinguishable, resistance and molecular patterns divided them into two groups. These became known as *M. peregrinum* types I and II. Type I included the type strain ATCC 14467^T, while type II included ATCC 35755, a sputum isolate. It wasn't until 2005 that 16S sequencing revealed *M. peregrinum* type II isolates to be human strains of *M. senegalense*, an agent of bovine farcy in Africa (Wallace et al., 2005). Supporting this further, 4 additional ATCC and DSMZ reference strains (not type) of *M. senegalense* derived from cases of bovine farcy were sequenced, and all matched 100% by 16S. Consistent with intra-species divergence using alternate targets, the human strains of *M. senegalense* presented with 2–5 bp differences (98.6–99.5% identity) in the 441 bp (Telenti region) of the *hsp65* and 1–7 bp differences (99.0–99.9% identity) in *rpoB*-V. Interestingly, all animal strains shared the same *hsp65*, *rpoB* and ITS1 sequevar, while human strains exhibited variability, albeit distinct from the animal strains. Shortly thereafter, *M. conceptionense* was validated partly on the basis a unique 16S and *rpoB*-V sequence deemed closest to *M. porcinum* and *M.*

fortuitum (Adekambi et al., 2006) and 4 bp from *M. senegalense* CIP 104941^T by 16S. However, the 16S sequence of the type strain from CIP is not consistent with that of *M. senegalense* NCTC 10956^T, ATCC 35796^T and other reference strains of *M. senegalense* from cases of bovine farcy. Instead, the designated type strain of *M. conceptionense* is 100% identical by 16S to *M. senegalense* NCTC 10956 ^{T} and ATCC 35796^T. The use of the CIP strain possibly contributed to proposing *M. conceptionense* as a novel species. The type strain of *M. senegalense* was deposited in CIP at a later time than NCTC or ATCC, but appears to be no longer available. WGS also supports that *M. conceptionense* is the same species as *M. senegalense* (Tortoli et al., 2017). It is not unreasonable to consider that human and bovine strains may represent two sub-species, one being an opportunistic environmental species that is most commonly isolated from humans, and the other representing an animal host-adapted lineage. One could propose *"M. senegalense* subsp. *senegalense"* and *"M. senegalense* subsp. *conceptionense"*.

The other agent of bovine farcy, *M. farcinogenes*, shares 100% identity by 16S with *M. houstonense*. What is often not appreciated, since *M. farcinogenes* per se does not appear to be common, is that it is in fact a slow grower (15–20 days)(Chamoiseau, 1979), despite having genetic characteristics of RGM. It is possible that this species has evolved along with its animal host, as observed in other species of mycobacteria, resulting in slower growth in vitro. There is no data to support this yet, but it would worth investigating.

At the time of writing, an accurate representative WGS of the type strains of both *M. senegalense* and *M. farcinogenes* is currently lacking. Three genomes are available for *M. senegalense*; clinical strains CK1 and CK2 and NCTC 4524, none of which represent the type strain. All 3 correspond taxonomically to *M. senegalense* combining 16S, *hsp65* and *rpoB*-V. Unfortunately, the taxonomic affiliation of the only available *M. farcinogenes* genome (Genbank accession no. HG964481-HG964485) is uncertain. Designated as a type strain (DSM 43637^T), it is nevertheless inconsistent with type strains obtained from ATCC or NCTC, as determined using a multigene approach (Beaz-Hidalgo et al., 2015). Instead, it is identical to *M. senegalense* ATCC 35796^T by *hsp65* and *rpoB*-V, as well as 100% identical over the entire *rpoB* gene (> 3900 bp) compared to *M. senegalense* NCTC 4524 (NZ_UGQQ01000002.1) and with an ANI value of > 99%. Interestingly, both DSM 43637^T and NCTC 4524 share a unique bp difference in the 16S compared to *M. senegalense*. *M. farcinogenes* ATCC 35753^T does in fact grow slowly, as originally described. If the growth rate of the strain DSM 43637^T used for WGS is rapid, it further supports that it does not represent the true type strain.

Since *M. senegalense* and *M. farcinogenes* were described long before 16S sequencing arrived, it is of no use to refer to the original species description for sequencing information. However, transmission history reveals that the earliest collections to have received the strains described by Chamoiseau were the NCTC and TMC (later transferred to ATCC) in 1974. WGS of the type strains obtained directly from these collections, where the least number of transfers occurred, should be sought in such cases where inconsistencies are observed. *M. syngnathidarum*, the most recently described member of the *M. fortuitum* group, is a fish pathogen closely related to *M. senegalense/M. conceptionense* group (Fogelson et al., 2018).

6.6. Known diversity in other NTM species

Genetic heterogeneity has been identified in other NTM species not discussed above, primarily on the basis of 16S and other targets such as *hsp65* and *rpoB*. Commonly, this is known to occur in *M. gordonae*, a very common contaminant in the clinical setting, where up to 5 genetic variants have been identified (de Zwaan et al., 2014; Itoh et al., 2003; Kirschner and Bottger, 1992; McNabb et al., 2004; Telenti et al., 1993). Some of these may consist of different species or subspecies, but pursuing this further is of low priority due to their common inability to cause disease. Other examples of NTM that exhibit intra-species variability beyond expected levels includes *M. interjectum* (de Zwaan et al., 2014; Lumb et al., 1997) and *M. simiae* (de Zwaan et al., 2014). In addition, there is significant divergence between clinical isolates and their type strain for *M. simiae* and *M. haemophilum*. Such as appears to be the case for *M. chelonae*, these type strains may not be the most suitable strain for the species that they represent.

6.7. Unculturable pathogenic mycobacteria

An underappreciated area of evolution concerns the growing number of unculturable pathogenic mycobacteria species that cause leprosy-like symptoms. Since these are hard to detect without direct molecular methods, and are also difficult to validate as novel species due to the lack of cultivable organism, some remained nameless until very recently. Their existence, however, is undeniable. Similar to *M. lepraemurium* described above, these likely evolved from NTM ancestors.

Diffuse lepromatous leprosy (DLL), also known as Lucio's phenomenon, was thought to be a severe manifestation of leprosy caused exclusively by *M. leprae*. In the last decade, *"M. lepromatosis"* was isolated from 2 patients who died of DLL and was identified as a novel species distinct from *M. leprae* based on multi target sequencing, including 16S (Han et al., 2008). WGS revealed that the two species diverged from each other 13.9 million years ago and incurred subsequent gene loss in different regions of their genomes (Singh et al., 2015). PCR-based survey of a large collection of leprosy strains showed that while DLL may also be caused by *M. leprae*, it is a rare manifestation that is more often caused by "*M. lepromatosis*" and appears to be restricted to Mexico (Singh et al., 2015). "*M. visibile*" was described as a novel species causing multisystemic granulomatous mycobacteriosis in cats (Appleyard and Clark, 2002). It contains a 9 bp insert in the V1 region of the 16S rRNA gene, a feature limited to *M. leprae* (12 bp) and "*M. lepromatosis"* (15 bp), while remaining unique in sequence with a ~97% identity against other species that include *M. leprae, M. hemophilum,* and members of MAC. "*M. lepraefelis*", a cause of feline leprosy, also clusters with this group (O'Brien et al., 2017a). "*M. uberis*", a cause of nodular thelitis and scrotitis in bovine and ovine, comprises the smallest mycobacterial genome to date due to massive genome decay akin to *M. leprae* (Benjak et al., 2018). Three distinct lineages of unculturable mycobacteria have also been described that cluster with *M. sherrisii* and *M. stomatopiae* (by 16S): "*M. tarwinense*", the most common cause of feline leprosy in Australia (O'Brien et al., 2017b); a yet unnamed *Mycobacterium* causing canine leproid granuloma syndrome (Dedola et al., 2014; Foley et al., 2002; Hughes et al., 2000) and "*M. tilburgii*" detected by molecular methods in tissue specimens from adults and children with disseminated disease, some with a documented immunodeficiency (Hartwig et al., 2011; Temmerman et al., 2014; Wagner et al., 2006). These "unculturable" organisms may grow to some extent in a liquid media culture but subculture onto various standard media and conditions is largely unsuccessful. It is fascinating that these mycobacteria have all been shown by genetic methods to belong to independent lineages among the NTM.

7. Final thoughts

NTM are characterized by various pigmentation, growth rates, epidemiology and pathogenic potential, often occurring within a species. In some ways, what was known in the early 1960's about NTMs is not that much different than what is known today. *M. kansasii* and MAC organisms are still some of the most common opportunistic pathogens in humans, and their prevalence varies by geographical distribution (Spaulding et al., 2017). *M. gordonae* remains a frequent contaminant of no clinical relevance, with very rare exceptions. Animal pathogens were well-known prior to our familiarization with NTM opportunistic infections in humans, but we have since recognized that some of these pathogens represent clones within more heterogenous species or complex

of environmental and opportunistic organisms.

The complementation of WGS with the robustness of the 16S phylogeny brings us into a new era of NTM taxonomy. WGS representation in public databases of the type strain of every valid species of mycobacteria is nearly complete. For those who use single gene targets for identification, be it 16S, *hsp65*, *rpoB* or any other, the data is immediately retrievable from WGS data. Some NTM are also represented by multiple WGS projects, essential for an in-depth understanding of a species and the variations within. While WGS offers the highest resolution, there remains an important role for 16S in the context of phylogenetic positions, but more importantly, to confirm species identification of strains which may only have been studied on a molecular level.

The recently proposed division of the mycobacteria into 2 large and 3 small genera offers questionable benefit in a monophyletic group of organisms that present with typical intragenus-level divergence by single gene, multi-locus sequencing and/or WGS analyses. Furthermore, considerations for diagnostics, clinical relevance and treatment decisions are generally species-specific and not so much between RGM vs SGM. A greater number of SGM species may be recognized pathogens, but SGM also harbor many saprophytic species of limited/no clinical relevance as the RGM group. Characteristic susceptibility profiles for RGM or SGM as a whole do not exist. When developing a diagnostic test for *M. tuberculosis*, it may be important to consider cross-reactivity against other NTM, including RGM. Other complications include the reclassification of *M. vulneris* on the basis of analyses with a mislabeled genome and the validation of other RGM species that are not re-classified to a new genus. If a novel taxon does not obviously cluster with a well-defined clade, it may be challenging to determine its phylogenetic placement in one of 5 taxa. That said, the inclusion of 4 novel genera in a validation list does not preclude the genus *Mycobacterium* from remaining validly published for *M. abscessus*, other RGM, *M. terrae* complex or *M. triviale* and therefore, *Mycobacterium* may continue to be used for these organisms (Tindall, 1999).

Species-level criteria have been the most studied and as a result, are easier to understand and apply. This may not be the case as much for genus demarcation. Similarly, the consistency at which sub-species are described varies, but the option serves to emphasize strains that genetically belong to a same species, but that may have a relevant characteristic (e.g. phenotypic, pathogenic, and epidemiologic) that warrants distinction. Knowing the exact species or subspecies within a complex, for example, can impact clinical management, e.g. *M. abscessus* complex, or have consequences for diagnostics and research. The question remains: should each branch become a novel species or subspecies? For example, it is probable that environmental NTM species such as MAH contain many “subspecies” within based on their diversity. Genomic analysis suggests geographic associated lineages, which is reminiscent of MTBC. These may vary in virulence potential or other relevant characteristic, but this is either not known or difficult to ascertain. In reality, many, if not most NTM species likely consist of several subspecies, but validating each and every one for the sake of being able to do so is not a high priority. In the clinical microbiology setting, identification to the subspecies level may not be possible and only undertaken where it is clinically relevant.

Novel mycobacterial taxa do still exist and will continue to be described in the foreseeable future. Genomic methods, both traditional and current, are essential in recognizing and solidifying strain relationships within NTM. In contrast to *M. tuberculosis*, we have only just begun to understand the genetic diversity that is present in the ~200 or more NTM species.

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Declarations of interest

None.

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