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ORIGINAL ARTICLE

Careful use of 16S rRNA gene sequence similarity values for the identification of *Mycobacterium* species

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Abstract

In order to evaluate the suitability of 16S rRNA nucleotide sequence similarity for the classification of new *Mycobacterium* isolates at the species level, we systematically studied the pairwise identity values of this gene for 131 *Mycobacterium* species with standing in nomenclature. Only one of the studied species, *M. poriferae* (0.76%), strictly respected the 95% and 98.65% threshold values currently recommended to determine the affiliation of bacterial isolates to an existing or new genus or species, respectively. All other species exhibited at least an identity value >98.65% and/or <95% with another *Mycobacterium* species. Therefore, we suggest that interpretation of interspecies 16S rRNA identity values should be made cautiously when classifying a new mycobacterial isolate at the species level.
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Introduction

Taxonomy provides scientists with essential information, enabling them to understand the relationships between living organisms and their different ecosystems [1]. For prokaryotes, taxonomy allows the reliable identification of microbial strains from clinical or environmental samples [2]. Bacterial taxonomy was initiated in the late 19th century, when phenotypic characteristics were incorporated into bacterial description, including motility, growth requirements, morphology, staining properties, colony size and colour, and chemical reactions [3]. Between the mid-1950s and the 1980s, new parameters were progressively added, notably chemotaxonomy [4], numerical taxonomy, genomic DNA-DNA hybridization and G+C content [5]. In the 1980s, the advent of DNA amplification and sequencing techniques, in particular of the 16S rRNA gene, constituted a major step forwards by facilitating bacterial classification [6,7]. The 16S rRNA gene is a highly conserved gene that is made of nine hypervariable domains separated by more preserved fragments in which universal primers can be designed. More than three million 16S rRNA gene sequences are currently available in public databases [8]. In 1996, Vandamme et al. [9] suggested that polyphasic taxonomy, which takes into account all available phenotypic and genotypic data and integrates them into a consensus classification, should include 16S rRNA gene sequence identity. In 2010, Tindall et al. [10], in a reevaluation of the various available methods, proposed a combination of phenotypic and genotypic criteria within which 16S rRNA gene sequence similarity, and phylogeny was included as a first-line tool.

In 1994, scientists considered two strains as belonging to different species if they shared 16S rRNA gene sequence similarity values <97% and to a distinct genus if this value was <95% [11]. The cutoff value at the species level was later reevaluated at 98.7% [12] and then 98.65% [13]. However, several authors have shown that these thresholds, originally designed to standardize the use of sequences of 16S rRNA genes in taxonomy, are not applicable to multiple genera. In 2015, we demonstrated that many of the current bacterial species with validly published names do not respect the 95% and 98.7% thresholds [14].

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TABLE 1. List of species with standing in nomenclature used in our study

Species	Accession no.	Size (bp)
Mycobacterium abscessus subsp. *bolletii*	AY859681	1481
Mycobacterium africanum	AF480605	1433
Mycobacterium agri	AJ429045	1456
Mycobacterium aichiense	X55598	1456
Mycobacterium alvei	AF023664	1465
Mycobacterium anyangense	KJ855063	1420
Mycobacterium arosiense	EF054881	1493
Mycobacterium arupense	DQ157760	1487
Mycobacterium asiaticum	AF480595	1466
Mycobacterium aubagnense	AY859683	1482
Mycobacterium aurum	X55595	1458
Mycobacterium austroafricanum	X93182	1462
Mycobacterium avium subsp. *avium*	AJ536037	1472
Mycobacterium avium subsp. *silvaticum*	EF521891	1442
Mycobacterium bouchedurhonense	EF591053	1498
Mycobacterium branderi	AF480574	1469
Mycobacterium brisbanense	AY012577	1499
Mycobacterium brumae	AF480576	1449
Mycobacterium canariasense	AY255478	1433
Mycobacterium caprae	AJ131120	1524
Mycobacterium celatum	L08169	1460
Mycobacterium celeriflavum	KJ607136	1442
Mycobacterium chelonae	AY457072	1481
Mycobacterium chitae	X55603	1457
Mycobacterium chlorophenolicum	X79292	1466
Mycobacterium chubuense	AF480597	1472
Mycobacterium conceptionense	AY859684	1483
Mycobacterium confluentis	AJ634379	1504
Mycobacterium conspicuum	X88922	1433
Mycobacterium cosmeticum	AY449728	1507
Mycobacterium crocinum	DQ534008	1398
Mycobacterium diernhoferi	AF480599	1458
Mycobacterium doricum	AF264700	1450
Mycobacterium duvalii	U94745	1502
Mycobacterium elephantis	AJ010747	1517
Mycobacterium fallax	AF480600	1470
Mycobacterium farcinogenes	AY457084	1483
Mycobacterium flavescens	X52932	1454
Mycobacterium fluoranthenivorans	AJ617741	1494
Mycobacterium fortuitum subsp. *acetamidolyticum*	FR733720	1505
Mycobacterium fortuitum subsp. *fortuitum*	AY457066	1483
Mycobacterium fragae	JQ898451	1452
Mycobacterium frederiksbergense	AJ276274	1474
Mycobacterium gadium	X55594	1456
Mycobacterium gastri	AF480602	1469
Mycobacterium genavense	X60070	1449
Mycobacterium goodii	Y12872	1417
Mycobacterium gordonae	X52923	1461
Mycobacterium hassiacum	U49401	1491
Mycobacterium heidelbergense	AJ000684	1445
Mycobacterium hodleri	X93184	1459
Mycobacterium holsaticum	AJ310467	1526
Mycobacterium houstonense	AY457067	1483
Mycobacterium interjectum	HM037998	1431
Mycobacterium intermedium	X67847	1441
Mycobacterium intracellulare	AJ536036	1440
Mycobacterium iranicum	HQ009482	1450
Mycobacterium kansasii	AJ536035	1470
Mycobacterium komossense	X55591	1462
Mycobacterium koreense	JF271826	1474
Mycobacterium kubicae	AF133902	1321
Mycobacterium kyorinense	AB370111	1470
Mycobacterium lacus	AF406783	1470
Mycobacterium lentiflavum	AF480583	1452
Mycobacterium litorale	GU997640	1380
Mycobacterium llatzerense	AJ746070	1397
Mycobacterium madagascariense	AB537170	1470
Mycobacterium mageritense	AJ699399	1497
Mycobacterium malmoense	X52930	1457
Mycobacterium mantenii	FJ042897	1471
Mycobacterium marinum	AF456240	1522
Mycobacterium marseillense	EU266632	1440
Mycobacterium microti	AF480584	1484
Mycobacterium monacense	AF107039	1473
Mycobacterium moriokaense	AJ429044	1493
Mycobacterium mucogenicum	AY457074	1482
Mycobacterium murale	AB537171	1459
Mycobacterium nebraskense	AY368456	1506
Mycobacterium neoaurum	AF480593	1470
Mycobacterium neworleansense	AY457068	1483
Mycobacterium noviomagense	EU239955	1478
Mycobacterium novocastrense	U96747	1513
Mycobacterium obuense	X55597	1458
Mycobacterium pallens	DQ370008	1435
Mycobacterium paraense	KJ948996	1480
Mycobacterium paraffinicum	GQ153270	1492
Mycobacterium parafortuitum	X93183	1460
Mycobacterium paragordonae	KC525204	1393
Mycobacterium parakoreense	JF271823	1465
Mycobacterium parascrofulaceum	AY337273	1468
Mycobacterium paraseoulense	DQ536404	1522
Mycobacterium paratuberculosis	X52934	1458
Mycobacterium parmense	AF466821	1529
Mycobacterium peregrinum	AY457069	1483
Mycobacterium phlei	AF480603	1461
Mycobactérie phocaicum	AY859682	1482
Mycobacterium porcinum	AY457077	1483
Mycobacterium poriferae	AF480589	1449
Mycobacterium pseudoshottsii	AY570988	1453
Mycobacterium psychrotolerans	AJ534886	1516
Mycobacterium pulveris	AJ429046	1492
Mycobacterium pyrenivorans	AJ431371	1481
Mycobacterium rhodesiae	AJ429047	1485
Mycobacterium riyadhense	EU274642	1475
Mycobacterium rufum	AY943385	1322
Mycobacterium rutilum	DQ370011	1417
Mycobacterium scrofulaceum	AF480604	1466
Mycobacterium sediminis	KC010490	1515
Mycobacterium senegalense	AY457081	1483
Mycobacterium senuense	DQ536408	1526
Mycobacterium seoulense	DQ536403	1522
Mycobacterium septicum	AY457070	1483
Mycobacterium setense	EF138818	1336
Mycobacterium sherrisii	AY353699	1510
Mycobacterium shinjukuense	AB268503	1505
Mycobacterium shottsii	AY005147	1491
Mycobacterium simiae	X52931	1479
Mycobacterium smegmatis	AJ131761	1482
Mycobacterium sphagni	FR733719	1505
Mycobacterium stomatepiae	AM884331	1471
Mycobacterium szulgai	X52926	1454
Mycobacterium thermoresistibile	X55602	1464
Mycobacterium tokaiense	AF480590	1451
Mycobacterium triplex	U57632	1474
Mycobacterium triviale	DQ058405	1362
Mycobacterium tusciae	AF058299	1409
Mycobacterium ulcerans	AB548725	1475
Mycobacterium vaccae	AF480591	1439
Mycobacterium vulneris	EU834055	1471
Mycobacterium wolinskyi	AY457083	1485
Mycobacterium yongonense	JF738056	1395

In 2000, Woo et al. [15] proposed that 16S rRNA gene sequencing was the reference standard for the identification of *Mycobacterium* species. Genotypic investigations based on the sequencing of the 16S rRNA gene have played a significant role in the taxonomic classification of members of the genus *Mycobacterium* [16]. However, to date, no systematic study of the degree of 16S rRNA divergence among *Mycobacterium* species has been conducted.

Here we evaluate the value of current 16S rRNA cutoff values at the species and genus levels by systematically calculating the pairwise degree of 16S rRNA similarity between all *Mycobacterium* species with standing in nomenclature.

Methods

Collection of 16S rRNA gene sequences from members of the genus *Mycobacterium*

Within the List of Prokaryotic Names with Standing in Nomenclature website (http://www.bacterio.net/mycobacterium.html),

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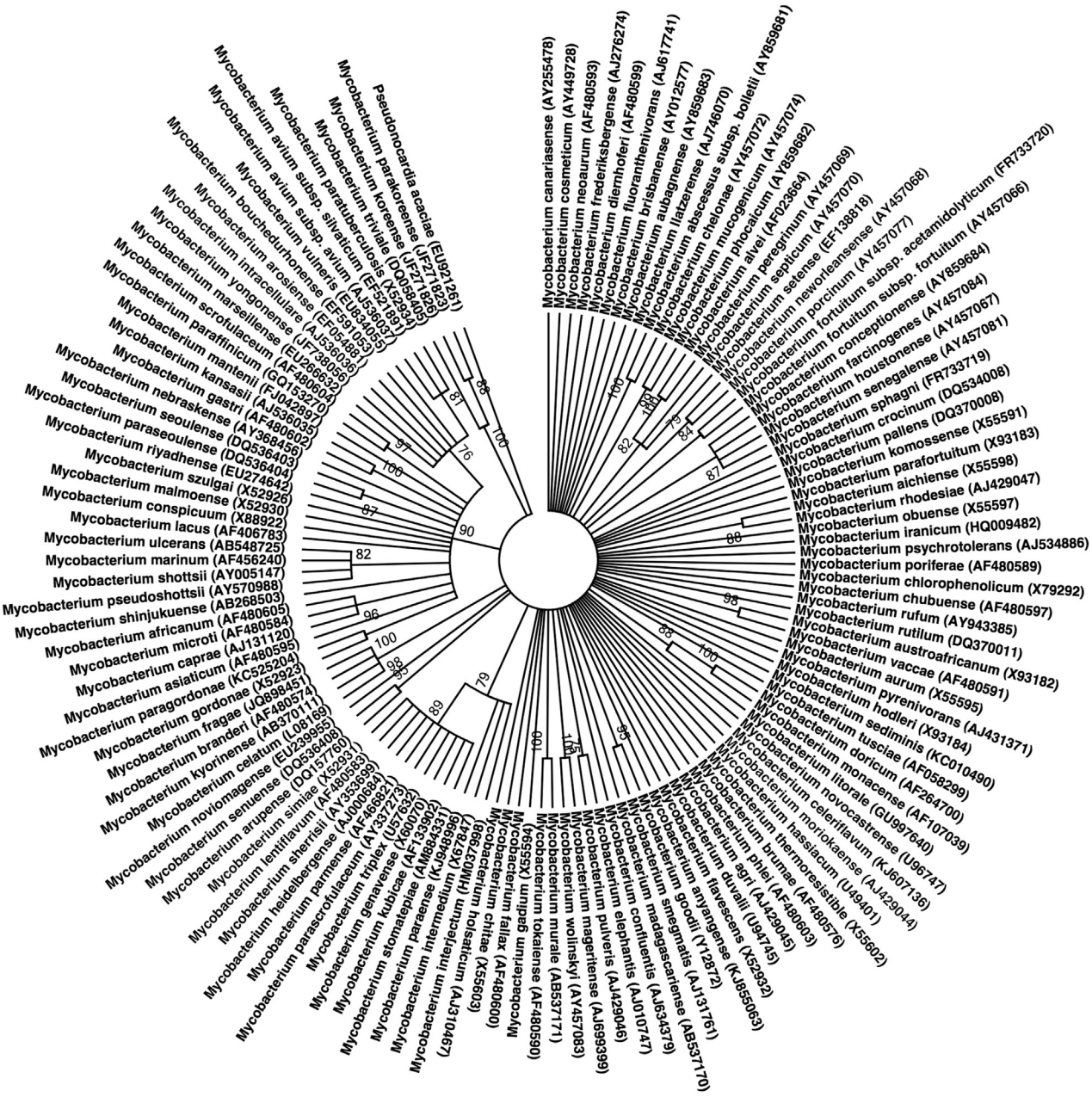


FIG. 1. Phylogenetic distribution of *Mycobacterium* species used in present study based on comparison of 16S rRNA sequences. Sequences were aligned by MUSCLE [14], and phylogenetic inferences were obtained by maximum likelihood method and Kimura two-parameter model in MEGA software. Numbers at nodes are percentages of bootstrap values obtained by repeating analysis 1000 times to generate majority consensus tree. *Pseudonocardia acaciae* (EU921261) was used as outgroup.

we selected all *Mycobacterium* species with a validly published name as of 25 March 2016, and we collected the 16S rRNA gene accession numbers from type strains.

As a result of the wide heterogeneity in length and quality of the 16S rRNA gene sequences of type strains, we did not use sequences shorter than 1320 nt. We created a FASTA format file containing all selected sequences.

16S rRNA gene sequence analysis: calculation of pairwise 16S rRNA gene sequence similarities

Sequences were aligned using Muscle software with default settings [17]. In this study, pairwise 16S rRNA gene sequence similarities between all species of the genus *Mycobacterium* were first estimated by MEGA 5 phylogeny software [18]. Then the highest and lowest values computed by this software were

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TABLE 2. Species that do not respect pairwise similarity thresholds of <95% and >98.65%

Species (accession no.)	No. for <95% threshold	No. for >98.65% threshold
Mycobacterium abscessus subsp. *bolletii* (AY859681)	41	2
Mycobacterium africanum (AF480605)	23	7
Mycobacterium agri (AJ429045)	32	0
Mycbacterium aichiense (X55598)	18	1
Mycobacterium alvei (AF023664)	17	13
Mycobacterium anyangense (KJ855063)	3	5
Mycobacterium arosiense (EF054881)	16	11
Mycobacterium arupense (DQ157760)	12	0
Mycobacterium asiaticum (AF480595)	6	4
Mycobacterium aubagnense (AY859683)	6	3
Mycobacterium aurum (X55595)	23	0
Mycobacterium austroafricanum (X93182)	17	1
Mycobacterium avium subsp. *avium* (AJ536037)	30	9
Mycobacterium avium subsp. *silvaticum* (EF521891)	23	13
Mycobacterium bouchedurhonense (EF591053)	20	11
Mycobacterium branderi (AF480574)	66	0
Mycobacterium brisbanense (AY012577)	1	1
Mycobacterium brumae (AF480576)	32	0
Mycobacterium canariasense (AY255478)	20	4
Mycobacterium caprae (AJ131120)	17	7
Mycobacterium celatum (L08169)	23	0
Mycobacterium celeriflavum (KJ607136)	0	1
Mycobacterium chelonae (AY457072)	47	2
Mycobacterium chitae (X55603)	17	0
Mycobacterium chlorophenolicum (X79292)	0	3
Mycobacterium chubuense (AF480597)	0	1
Mycobacterium conceptionense (AY859684)	2	17
Mycobacterium confluentis (AJ634379)	1	0
Mycobacterium conspicuum (X88922)	8	3
Mycobacterium cosmeticum (AY449728)	6	6
Mycobacterium crocinum (DQ534008)	5	6
Mycobacterium diernhoferi (AF480599)	23	5
Mycobacterium doricum (AF264700)	42	1
Mycobacterium duvalii (U94745)	3	2
Mycobacterium elephantis (AJ010747)	6	0
Mycobacterium fallax (AF480600)	2	0
Mycobacterium farcinogenes (AY457084)	2	21
Mycobacterium flavescens (X52932)	36	0
Mycobacterium fluoranthenivorans (AJ617741)	10	5
Mycobacterium fortuitum subsp. *fortuitum* (AY457066)	6	16
Mycobacterium fortuitum subsp. *acetamidolyticum* (FR733720)	6	16
Mycobacterium fragae (JQ898451)	17	0
Mycobacterium frederiksbergense (AJ276274)	11	3
Mycobacterium gadium (X55594)	6	0
Mycobacterium gastri (AF480602)	26	7
Mycobacterium genavense (X60070)	11	4
Mycobacterium goodii (Y12872)	11	3
Mycobacterium gordonae (X52923)	27	1
Mycobacterium hassiacum (U49401)	52	0
Mycobacterium heidelbergense (AJ000684)	5	5
Mycobacterium hodleri (X93184)	5	0
Mycobacterium holsaticum (AJ310467)	1	0
Mycobacterium houstonense (AY457067)	2	21
Mycobacterium interjectum (HM037998)	1	1
Mycobacterium intermedium (X67847)	2	0
Mycobacterium intracellulare (AJ536036)	29	12
Mycobacterium iranicum (HQ009482)	13	0
Mycobacterium kansasii (AJ536035)	26	7
Mycobacterium komossense (X55591)	10	0
Mycobacterium koreense (JF271826)	6	1
Mycobacterium kubicae (AF133902)	1	1
Mycobacterium kyorinense (AB370111)	70	0
Mycobacterium lacus (AF406783)	17	14
Mycobacterium lentiflavum (AF480583)	4	6
Mycobacterium litorale (GU997640)	20	0
Mycobacterium llatzerense (AJ746070)	14	1
Mycobacterium madagascariense (AB537170)	3	1
Mycobacterium mageritense (AJ699399)	0	1
Mycobacterium malmoense (X52930)	57	5
Mycobacterium mantenii (FJ042897)	5	7
Mycobacterium marinum (AF456240)	12	7
Mycobacterium marseillense (EU266632)	22	14
Mycobacterium microti (AF480584)	23	6
Mycobacterium monacense (AF107039)	35	1
Mycobacterium moriokaense (AJ429044)	0	3
Mycobacterium mucogenicum (AY457074)	4	11
Mycobacterium murale (AB537171)	1	0
Mycobacterium nebraskense (AY368456)	20	12
Mycobacterium neoaurum (AF480593)	5	4
Mycobacterium neworleansense (AY457068)	2	17
Mycobacterium noviomagense (EU239955)	48	0
Mycobacterium novocastrense (U96747)	44	0
Mycobacterium obuense (X55597)	21	0
Mycobacterium pallens (DQ370008)	5	6
Mycobacterium paraense (KJ948996)	0	1
Mycobacterium paraffinicum (GQ153270)	3	6
Mycobacterium parafortuitum (X93183)	3	6
Mycobacterium paragordonae (KC525204)	17	2
Mycobacterium parakoreense (JF271823)	2	0
Mycobacterium parascrofulaceum (AY337273)	6	1
Mycobacterium paraseoulense (DQ536404)	10	16
Mycobacterium paratuberculosis (X52934)	45	8
Mycobacterium parmense (AF466821)	46	0
Mycobacterium peregrinum (AY457069)	9	15
Mycobacterium phlei (AF480603)	4	0
Mycobacterium phocaicum (AY859682)	4	10
Mycobacterium porcinum (AY457077)	4	16
Mycobacterium poriferae (AF480589)	0	0
Mycobacterium pseudoshottsii (AY570988)	22	6
Mycobacterium psychrotolerans (AJ534886)	6	1
Mycobacterium pulveris (AJ429046)	2	1
Mycobacterium pyrenivorans (AJ431371)	4	0
Mycobacterium rhodesiae (AJ429047)	6	12
Mycobacterium riyadhense (EU274642)	10	18
Mycobacterium rufum (AY943385)	37	0
Mycobacterium rutilum (DQ370011)	17	6
Mycobacterium scrofulaceum (AF480604)	7	8
Mycobacterium sediminis (KC010490)	37	0
Mycobacterium senegalense (AY457081)	2	21
Mycobacterium senuense (DQ536408)	30	0
Mycobacterium seoulense (DQ536403)	10	16
Mycobacterium septicum (AY457070)	8	13
Mycobacterium setense (EF138818)	14	16
Mycobacterium sherrisii (AY353699)	3	7
Mycobacterium shinjukuense (AB268503)	42	0
Mycobacterium shottsii (AY005147)	16	7
Mycobacterium simiae (X52931)	6	6
Mycobacterium smegmatis (AJ131761)	15	7
Mycobacterium sphagni (FR733719)	3	10
Mycobacterium stomatepiae (AM884331)	2	4
Mycobacterium szulgai (X52926)	33	2
Mycobacterium thermoresistible (X55602)	48	1
Mycobacterium tokaiense (AF480590)	0	1
Mycobacterium triplex (U57632)	3	6
Mycobacterium triviale (DQ058405)	2	0
Mycobacterium tusciae (AF058299)	25	0
Mycobacterium ulcerans (AB548725)	16	7
Mycobacterium vaccae (AF480591)	31	1
Mycobacterium vulneris (EU834055)	26	12
Mycobacterium wolinskyi (AY457083)	2	0
Mycobacterium yongonense (JF738056)	15	14

For each studied species, we indicate for each threshold numbers of pairwise comparisons for which abnormal values were observed.

more accurately determined by pairwise BLASTN. We defined as expected values of interspecies 16S rRNA gene sequence similarity percentages that were between 95% and 98.65% or intraspecies percentages that were greater than 98.65% [13], and as abnormal values interspecies percentages that were >98.65% or <95% [14] or intraspecies percentages of <98.65%.

Results

Of the 182 *Mycobacterium* species and subspecies with a validly published name at the time of our study and for which a 16S rRNA sequence was available, we included 131 species with 16S rRNA sequences longer than 1320 nt (Table 1). For two of those species, *M. avium* and *M. fortuitum*, we included two subspecies (Table 1). The phylogenetic distribution of the

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studied *Mycobacterium* species is presented in Fig. 1. Among the 131 studied species, the pairwise 16S rRNA gene sequence similarity values ranged from 93.00% between *M. chelonae* and *M. kyorinense* to 100% between *M. fortuitum* subsp. *acetamidolyticum* and *M. fortuitum* subsp. *fortuitum, M. africanum* and *M. caprae, M. farcinogenes, M. houstonense* and *M. senegalense, M. gastri* and *M. kansasii, M. mucogenicum* and *M. phocaicum, M. murale* and *M. tokaiense,* and *M. paraseoulense* and *M. seoulense,* respectively (Supplementary Table S1).

Of the 131 studied *Mycobacterium* species, 90 (68.7%) exhibited at least one 16S rRNA gene sequence similarity value greater than 98.65% with another species in this genus (Table 2, Supplementary Table S1). Among 131 studied species, 123 (93.9%) exhibited at least one 16S rRNA gene sequence similarity value lower than 95% with another species in the genus (Table 2, Supplementary Table S1). Only one (0.76%) of the 131 studied species, i.e. *M. poriferae,* exhibited only expected values (Table 2, Supplementary Table S1). At the intraspecies level, only expected values were observed.

Discussion

Over the past decade, several authors suggested that the inter- and intraspecies discriminatory power of 16S rRNA gene sequences was insufficient for some bacterial genera [19,20]. As examples, *Streptococcus pneumoniae* and *S. mitis* exhibit only a 3 nt difference (99.7% identity), which would classify them in the same species. In contrast, major interspecies differences may be observed, as is the case in the genus *Clostridium,* with *C. tetani* and *C. innocuum* exhibiting a 104 nt divergence (93.7% identity). The strict application of the 95% threshold would justify their classification in distinct genera [19]. In addition, in 2010, Pei et al. [21] identified an intragenomic sequence divergence greater than 1.3% among 16S rRNA genes copies in 11 bacterial species. Among these, *Borrelia afzelii,* an agent of Lyme disease in humans, exhibits a similarity of only 79.62% between its two 16S rRNA gene copies [21]. Thus, a strict application of the 98.65% threshold would classify these bacteria in different species depending on the 16S rRNA gene copy analysed [21,22]. According to Rossi-Tamisier et al. [14], among 158 studied bacterial genera, only members of 17 genera strictly respected the 95% and 98.65% thresholds. Among other studied genera, the percentage of species that respected strictly both thresholds varied from 0 (*Brucella*) to 93.9% (*Nocardia*) [14].

In the present report, we observed that the currently used 16S rRNA gene sequence similarity thresholds for delineating bacterial species are valid for only 0.76% of 131 studied *Mycobacterium* species with standing in nomenclature. Because our study covers 71.97% of the currently validly published *Mycobacterium* species names, we believe that the 95% and 98.65% thresholds are not suitable for this genus and should at the maximum be used as indicators, not as a reference standard, for classifying new *Mycobacterium* species.

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Conflict of interest

None declared.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.nmni.2017.12.009.

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