Mycobacterium malmesburyense sp. nov: A novel non-tuberculous Mycobacterium species revealed by multiple gene sequence characterization

*Nomakorinte Gcebe[1, 2], Victor Rutten [2, 3], Nicolaas Gey van Pittius [4] , Brendon Naicker [5], Anita Michel[2]

[1]Tuberculosis Laboratory, Agricultural Research Council - Onderstepoort Veterinary Institute, Onderstepoort, South Africa; [2]Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa; [3]Division of Immunology, Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands; [4]Centre of Excellence for Biomedical Tuberculosis Research, Division of Molecular Biology and Human Genetics, Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, Stellenbosch University, Tygerberg, South Africa; [5]Polymers and Composites, Materials Science and Manufacturing, Council for Scientific and Industrial Research, Brummeria, South Africa

*Author for correspondence- Email: gceben@arc.agric.za , Tel: +2712 5299138

The Genbank accession numbers for the 16S rRNA, *hsp65*, *rpoB* and *sodA* genes of WCM 7299^T are KJ873241; KJ873243; KJ873245 and KJ873247 respectively.

ABSTRACT

Non-tuberculous mycobacteria (NTM) are ubiquitous in the environment and an increasing number of NTM species have been isolated and characterized from both humans and animals, highlighting the zoonotic potential of these bacteria. Host exposure to NTM may impact on cross-reactive immune responsiveness which may affect diagnosis of bovine tuberculosis and may also play a role in the variability of the efficacy of *Mycobacterium bovis* BCG vaccination against tuberculosis. In this study we characterized 10 NTM isolates originating from water, soil, nasal swabs of cattle and African buffalo as well as bovine tissue samples. These isolates were previously identified during an NTM survey and were all found, using 16S rDNA sequence analysis to be closely-related to *Mycobacterium moriokaense*. A polyphasic approach that includes phenotypic characterization, antibiotic susceptibility profiling, mycolic acid profiling and phylogenetic analysis of four gene loci, *viz* 16S rDNA, *hsp65*, *sodA*, and *rpoB* was employed to characterize these isolates. Sequence data analysis of the four gene loci revealed that these isolates belong to a unique *Mycobacterium* species. This evidence was further supported by several differences in phenotypic characteristics between the isolates and the closely related species. We proposed the name, *Mycobacterium malmesburyense* sp. nov. for this new species. The type strain is WCM 7299^{T} (ATCC® BAA-2759^{TM} =CIP 110822^{T}). The Genbank accession numbers for the partial gene sequences [16S rDNA , *hsp65*, *rpoB* and *sodA*] for the type strain are as follows: 16S rRNA= KJ 873241; *hsp65*=KJ 873243*; rpoB*= KJ 873245*; sodA*= KJ 873247.

Currently more than 100 non-tuberculous mycobacterial species are listed in public databases (http://www.bacterio.net/). An increasing number of NTM species has recently been isolated and characterized from both humans and animals, highlighting the zoonotic potential of these species (reviewed by Tortoli , 2006, van Helden *et al.*, 2009 and Botha *et al.*, 2013). An increased interest in these organisms has developed in view of the potential impact of host exposure to NTM on cross-reactive immune responsiveness which may affect diagnosis of bovine tuberculosis (BTB) (Michel, 2008; Michel *et al.*, 2011; Schiller *et al.*, 2010) and may also play a role in the variability of the efficacy of *Mycobacterium bovis* BCG vaccination against tuberculosis (Buddle *et al.*, 2002; Brandt *et al.*, 2002; Weir *et al*., 2006). NTM are ubiquitous in the environment and there is no evidence of animal-to-animal or human-to-human transmission (Primm *et al*., 2004; Falkinham, 2002). Characterization of NTM isolates from environmental as well as clinical samples is important as these may also lead to identification of emerging pathogens. For instance, *M. avium* was essentially a rare human pathogen before the acquired immune deficiency syndrome (AIDS) pandemic, and since then this NTM species has become a very important pathogen of AIDS patients (Karne *et al.*, 2012). The reference molecular method for the identification of mycobacteria is the sequencing of the 16S rDNA, which has contributed to the discovery of novel NTM isolates beyond possibilities offered by conventional methods such as phenotypic characterization (Turenne *et al.*, 2001). However, this approach has its limitations as there are instances where the sequence of this gene has been found to be similar, if not identical between species of the genus. It has been shown that multiple locus analysis provides a more detailed and accurate identification of *Mycobacterium* species than the use of a single locus (Devulder *et al.*, 2005). We previously described the prevalence of NTM isolates in cattle, African buffaloes and their environments in South Africa and found a group of isolates (closest related by analysis of 16S rDNA to *Mycobacterium moriokaense*) that were not well described in literature at the

species level (Gcebe *et al.*, 2013). The aim of the present study was to conduct an in depth characterization of this group of isolates closely related to *M. moriokaense*.

Ten isolates used in this study were recovered from soil, water, nasal swabs and lymph nodes of cattle and pharyngeal swabs of buffalo, during a survey conducted between April 2010 and December 2012 to determine the prevalence and distribution of NTM, and analysis of isolates from routine diagnostic samples collected in different geographic and climatic regions of South Africa (Table 1). These isolates were characterized using phenotypic assays, antimicrobial susceptibility profiling, mycolic acid profiling and sequence based analysis of four mycobacterial housekeeping genes *viz* 16S rDNA, *hsp65*, *rpoB*, and *sodA.*

Table 1: Origin of NTM isolates used in the study

NTM Isolate ID	**Sample type**	**Climatic region in South Africa**	**Location and GPS co-ordinates**
Balasi	Water	Escarpment	Bisho: 32°50’58”S, 27°26’17”E
242	Nasal swab	Moderate Eastern Plateau	Frankfort: 27°30’14”S, 27°35’58”E
Middledrift	Nasal swab	Escarpment	Middledrift: 32°49’7”S, 26°59’15”E
WCM 7299^{T}	Bovime Nasal swab	Mediterranean climate	Malmesbury: 33°27’4.7”S, 18°43’19.06”E
Vryburg	Bovine Nasal swab	Semi-arid	Vryburg: 26°58’S, 24°54’E
Uyenvlei	Bovine Nasal swab	Moderate eastern plateau	Lephalale: 23°40′S 27°45′E
C28	Buffalo pharyngeal swab	Subtropical coast	Hluhluwe Imfolozi park: 28°13’11” S, 31°57’07” E
C4	Biffalo pharyngeal swab	Subtropical coast	Hluhluwe Imfolozi park: 28°13’11” S, 31°57’07” E
TB 5612	Bovine lymph node	Sub-tropical coast	Bergville: 28°43’48”S, 29°21’0.2”E
TB 5960A	Bovine lymph node	Escarpment	East London: 32°59’S, 27°52’E

All the cultures were maintained on either Middlebrook 7H11 agar plates (Becton Dickinson, USA) supplemented with 0.1% OADC (Merck Chemicals, South Africa), and glycerol as well as on Löwenstein Jensen (LJ) slopes supplemented with glycerol and an antibiotic cocktail of PolymyxinB, AmphotericinB, Carbenicillin and Trimethoprim (PACT) (Becton Dickinson, USA) at 37°C for subsequent biochemical testing and mycolic acid analysis. Phenotypic characteristics of the isolates were compared with those of their closest relatives' *viz. M. moriokaense, M. elephantis, M. novocastrense, M. flavescens,* and *M. arupense.* Slopes and plates were incubated at 37°C, 45°C and 25°C in order to evaluate the ability of each isolate to grow at different temperatures. We also observed colony morphology (rough or smooth), pigmentation and acid fastness and different growth rates of the isolates. Sodium chloride (NaCl) tolerance (5%) of each isolate was evaluated for 2-10 days. The isolates were tested for the ability to reduce nitrate, for urease activity and niacin accumulation, using the commercial test strips (Becton Dickinson, USA) according to the manufacturer's instructions. They were also tested for the 3 day and 14 day arylsulphatase activity (Wayne, 1961). We tested the isolates for pyrazinamidase activity as described by Singh *et al.,* 2007, except that Middlebrook 7H11 agar was used and the culture incubated for 4 days. In addition, the isolates were also tested for the ability to hydrolyse Tween 80 using the method described by Kilburn *et al.,* 1973. Semi quantitative catalase activity of the isolates was tested using 3% hydrogen peroxide and observing formation of gas bubbles (Kent and Kubica, 1985). Finally, the isolates were tested for their ability to hydrolyse aesculin, for citrate utilisation (National Health Laboratory Services, South Africa) as well as their ability to utilise the following sugars as sole carbon sources: D-mannitol, inositol, L-rhamnose and L-arabinose (Selecta media, South Africa). The identified differences in phenotypic characteristics of the isolates with those of the closely related species *i.e. M. moriokaense, M. novocastrense, M. flavescens, M. elephantis* and *M. arupense*, as illustrated in Table 2, include pigmentation,

Table 2: Comparison of phenotypic characteristics of the NTM isolates with those of the closely related species.

NTM ID/ species	Source	Growth rate (days)	Pigment	Growth at:	Biochemical features and Utilisation of sugars														
				25°C .37°C 45°C	Semi quantitative Catalase	5% NaCl tolerance	Tween 80 hydrolysis	Aryl sulphatase (14ays)	Aryl sulphatase (3days)	Urease	Niacin	Nitrate reduction	Pyrazinamidase activity	Citrate	Aesculin	D-mannitol	Inositol	L-rhamnose	L-arabinose
M. moriokaense ATCC 43059^T *(*tested in the lab*)*	Ref	<7	-	+ + + + nd	+	-	+	-	+	+	nd	+	+	-	+	-	-	nd	nd
M. moriokaense (e, f)	Ref	<7	-	+ + nd	-, +	+	nd	nd	-, +	+	nd	+	+	-	-	+, -	+	+	+
M. novocastrense (*a, c*)	Ref	<7	+	+ + +	+	+	+	nd	v	+	-	+	nd	nd	nd	nd	nd	nd	nd
M. flavescens (b, g, h)	Ref	<7	+	+ + -	+	+	+	+	-	+	-	+	+	+	nd	+, v	-	-	nd
M. arupense (d)	Ref	<7	-	+ + -	nd	-	+	+	-	-	-	-	-	nd	nd	nd	nd	nd	nd
M. elephantis a,b	Ref	<7	+	+ +++ + ++ +		+	+	-	-	+		+	+	nd	nd	-	-	nd	nd
NTM isolates (n=10)	**D**	**<7**	**+**	**+ +++ +**	**D (*n=8 /10)**	**-**	**+**	**D (*n= 9/10)**	**D (*n= 1/10)**	**+**	**D (*n= 2/10)**	**D (*n= 9/10)**	**+**	**-**	**D (*n=1 /10)**	**-**	**-**	**-**	**-**

a, data retrieved from Tortoli , 2003; b, Data retrieved from Turenne *et al.*, 2002; c, Shojaei et al., 1997; d, data taken from Cloud *et al.*, 2006; e, data retrieved from Tsukamura *et al.*, 1986; f, data taken from Adékambi *et al.*, 2006b; g, data taken from Tortoli *et al.*, 2004; h, data taken from Bojalil *et al.*, 1962; v, variable results based on a and c; +, positive; -, negative; +++, optimum growth; D; strain dependant; *, ratio of positive results .

growth rate as well as other biochemical traits. The most apparent feature that distinguished the isolates from both *M. moriokaense,* and *M. arupense* was pigmentation, as all the isolates appeared to be scoto- chromogens whereas both these NTM were previously reported to be non-chromogenic (Cloud *et al.,* 2006; Turenne *et al.,* 2002; Tortoli, 2003). Other characteristics that further segregated the isolates from *M. arupense* was their ability to degrade urea as well as to reduce nitrate to nitrite (except for isolate 242). *M. arupense* was previously reported to lack these traits (Cloud *et al.,* 2006). Contrary to what is reported for *M. novocastrense, M. flavescens* as well as *M. elephantis,* all the isolates were unable to grow in the presence of 5% NaCl (Tortoli, 2003; Shojaei et al., 1997; Bojalil *et al.,* 1962; Turenne *et al.,* 2002; Tortoli *et al.,* 2004). In addition, the ability of the isolates to grow at 45°C further separated them from *M. flavescens* as well as *M. arupense,* which were previously reported not to grow at this temperature (Tortoli *et al.,* 2004; Cloud *et al.,* 2006). The phenotypic characteristics of the individual isolates are presented in the supplementary Table 1.

Antibiotic susceptibility to amikacin (30 µg), cefoxitin (10 µg), ciprofloxacin (10 µg), clarithromycin (15 µg) , doxycycline (30 µg) , imipenem (10 µg), amoxylin (30 µg) and tobramycin (10 µg) (Oxoid LTD, UK) was determined for all the isolates, using a modified Kirby Bauer disk diffusion method on Middlebrook 7H11 agar plates supplemented with 0.1% OADC and incubated for 2-5 days at 37°C, after which the zones of inhibition were measured (Brown-Eliot and Wallace, 2002).If a minimum of 3 mm zone of inhibition is obtained, that was interpreted as inhibition. The isolates presented quite a uniform pattern with all susceptible to amikacin, ciprofloxacin and doxycycline. With the exception of isolate Uyenvlei, Vryburg and Middledrift all were also susceptible to clarithromycin. With the exception of WCM 7299^{T} which was susceptible to tobramycin as well as TB 5960, TB 5612

and C4 which were susceptible to amoxylin, all the other isolates were resistant to imipenem, cefoxinin and tobramycin and amoxylin.

Genetic characterisation included PCR and sequence analysis of the 1400 bp fragment of the 16S rDNA gene (Turenne *et al.*, 2001), the 439 bp region of the gene encoding the 65 kDA heat shock protein (*hsp65*) (Telenti *et al.*, 1993), part (764 bp) of the RNA polymerase beta subunit (*rpoB*) gene (Adékambi *et al.*, 2003; 2006a) and part (464 bp) of the superoxide dismutase (*sodA*) gene (Adékambi and Drancourt, 2004). Boiled culture suspensions from individual isolates were used as DNA template in the various PCR protocols. The amplicons were sequenced at the Central Analytical Facility of Stellenbosch University and Inqaba biotechnologies (South Africa) using the same primer sequences that were used for amplification of the respective gene fragments. Sequences from both strands were edited manually and pairwise alignments undertaken using the BioEdit Sequence alignment editor (version 7.1.9) and Molecular Evolutionary Genetics Analysis (MEGA) platform (www.megasoftware.net) (version 6) (Tamura *et al.*, 2013). The resulting consensus sequences were analysed on the NCBI BLAST platform for species identification (www.blast.ncbi.nlm.nih.gov/Blast.cgi) by megablast. For phylogenetic analysis, all the sequences were first trimmed at both the 5' and the 3' ends to encompass the most corresponding gene fragment sequences of mycobacteria deposited in Genbank database. The resulting fragment used for the concatenated nucleotide sequences was in the following order (16S rDNA, *hsp65*, *rpoB*, and *sodA*). Multiple sequence alignments of the individual genes as well as the concatenated sequences were performed using Clustalw (Thompson *et al.*, 1994) from MEGA (version 6), to determine percentage similarity between each of the gene sequences. Evolutionary divergences between the sequences were estimated using the maximum composite likelihood method. Phylogenetic trees resulting from individual gene fragment sequences as well as concatenated sequences of the isolates and those of other

mycobacteria, (both slow and rapidly growing) retrieved from Genbank (www.ncbi.nlm.gov/genbank), were constructed using the neighbour-joining method (Saitou and Nei, 1987) and validated using the maximum composite likelihood method. One thousand bootstrap replicates were run and *Nocardia farcinica* and *Nocardia brasiliensis* were used as outgroups.

Sequence analysis of the isolates' *hsp65* gene fragments, showed 95% sequence similarity to *Mycobacterium novocastrense*. Partial sequence analysis of the *rpoB* gene identified these isolates to share 95% sequence similarity with *M. novocastrense*, *M. flavescens* or *M. arupense.* Characterization of the isolates by sequencing of the partial fragment of the *sodA* gene showed 8/10 isolates to share between 92% and 93% sequence similarity with *M. flavescens* and *M. novocastrense.* Two isolates, *viz* Balasi and TB 5612 could not be amplified with the *sod*A primers used. Sequence analysis of the partial 16S rDNA gene showed the isolates to share between 97%-98% similarity with *M. moriokaense.*

Phylogenetic relatedness of the isolates and other *Mycobacterium* species, based on the 16S rDNA as well as the concatenated sequences of the four gene fragments (16S rDNA, *hsp65*, *rpoB* and *sodA*) is illustrated by the phylogenetic tree in Figure 1 and Figure 2 respectively. Phylogenetic analysis of the isolates based on the 16S rDNA gene sequences as well as the concatenated sequences revealed the isolates to form a single cluster. The highest level of bootstrap support value (up to 100%) supporting the clustering identified these isolates as belonging to the same *Mycobacterium* species. Despite that these isolates showed 97% - 98% 16S rDNA similarity to *M. moriokaense*, they did not cluster phylogenetically with this species and neither did they cluster with the other closest relatives. These isolates clearly represented a novel species not previously described in literature.

Mycolic acid profile determination of isolate WCM 7299^T was done and compared to those of *Mycobacterium fortuitum* ATCC 6148^T and that of *Mycobacterium moriokaense* ATCC

Fig. 1: Phylogenetic tree constructed using neighbour joining method, illustrating the genetic position of the isolates. Genbank accession numbers for the sequences are shown in parenthesis. The tree is based on the partial 16S rDNA gene sequences. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). Nocardia farcinica was used as an out group sequence

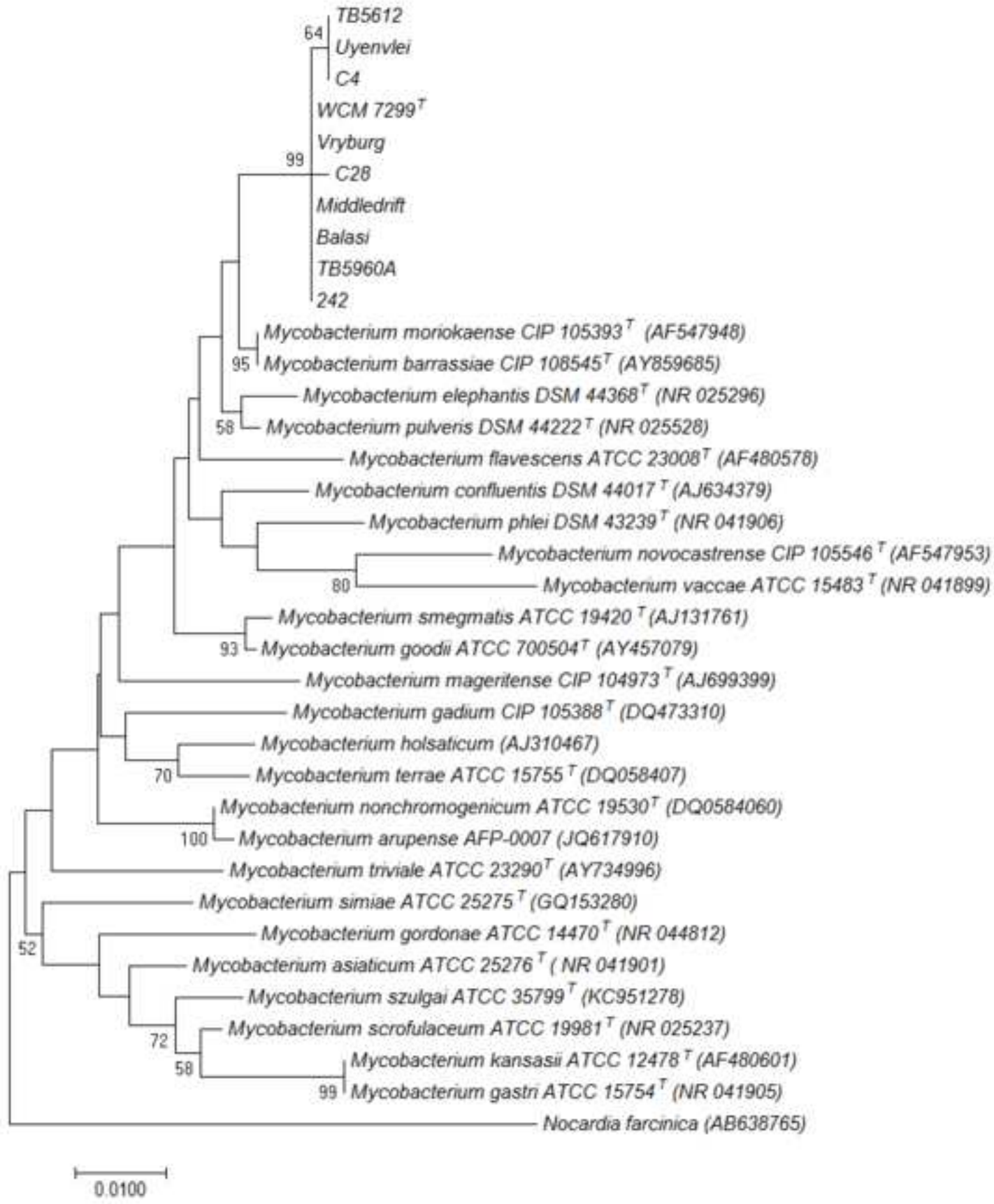


Fig. 2: Phylogenetic tree constructed using neighbour joining method, illustrating the genetic position of the isolates. The tree is based on concatenated sequences of: 16S rDNA (1366bp), hsp65 (424bp), rpoB (711bp) and sodA (411bp) gene fragments. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). Nocardia farcinica was used as an out group sequence

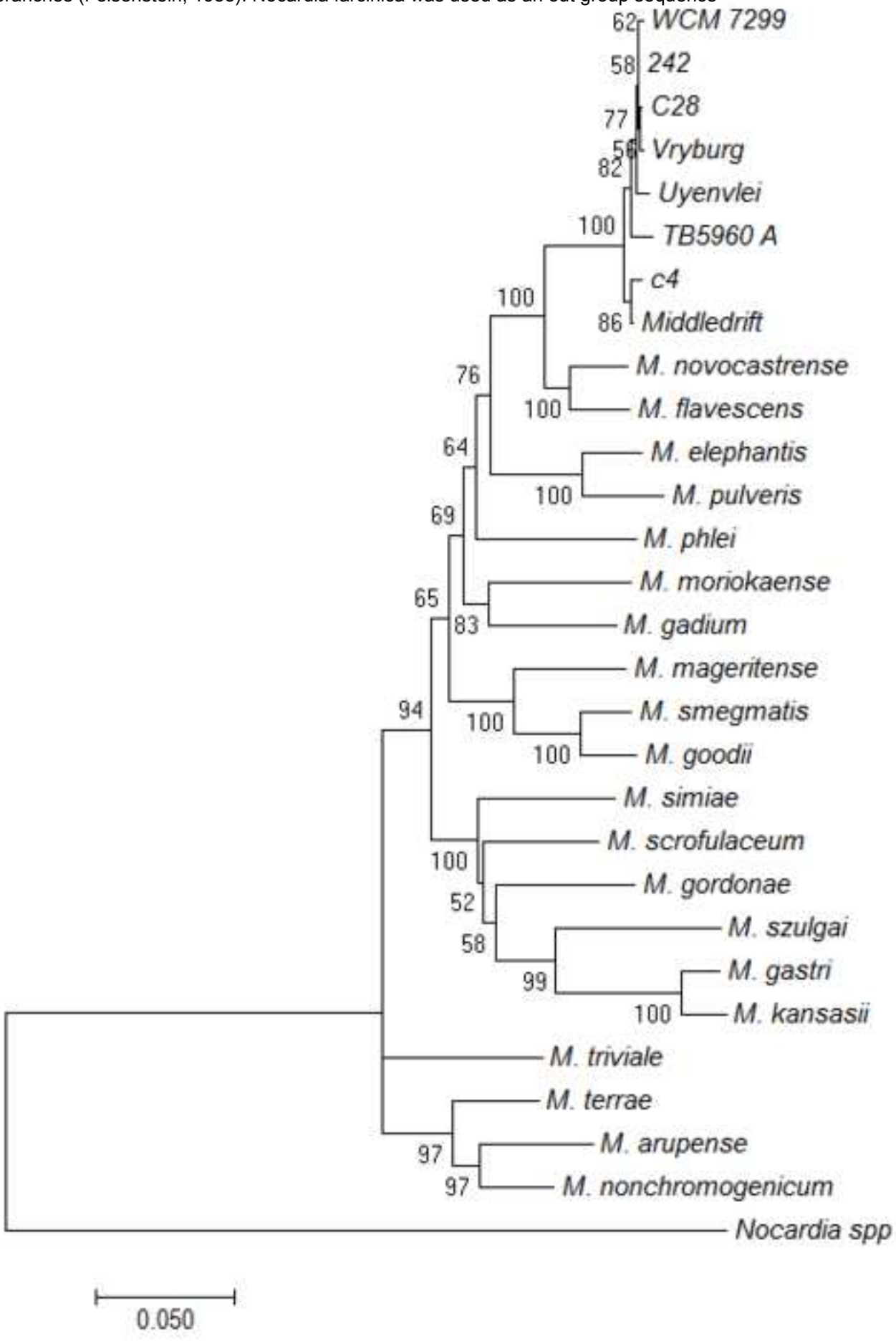
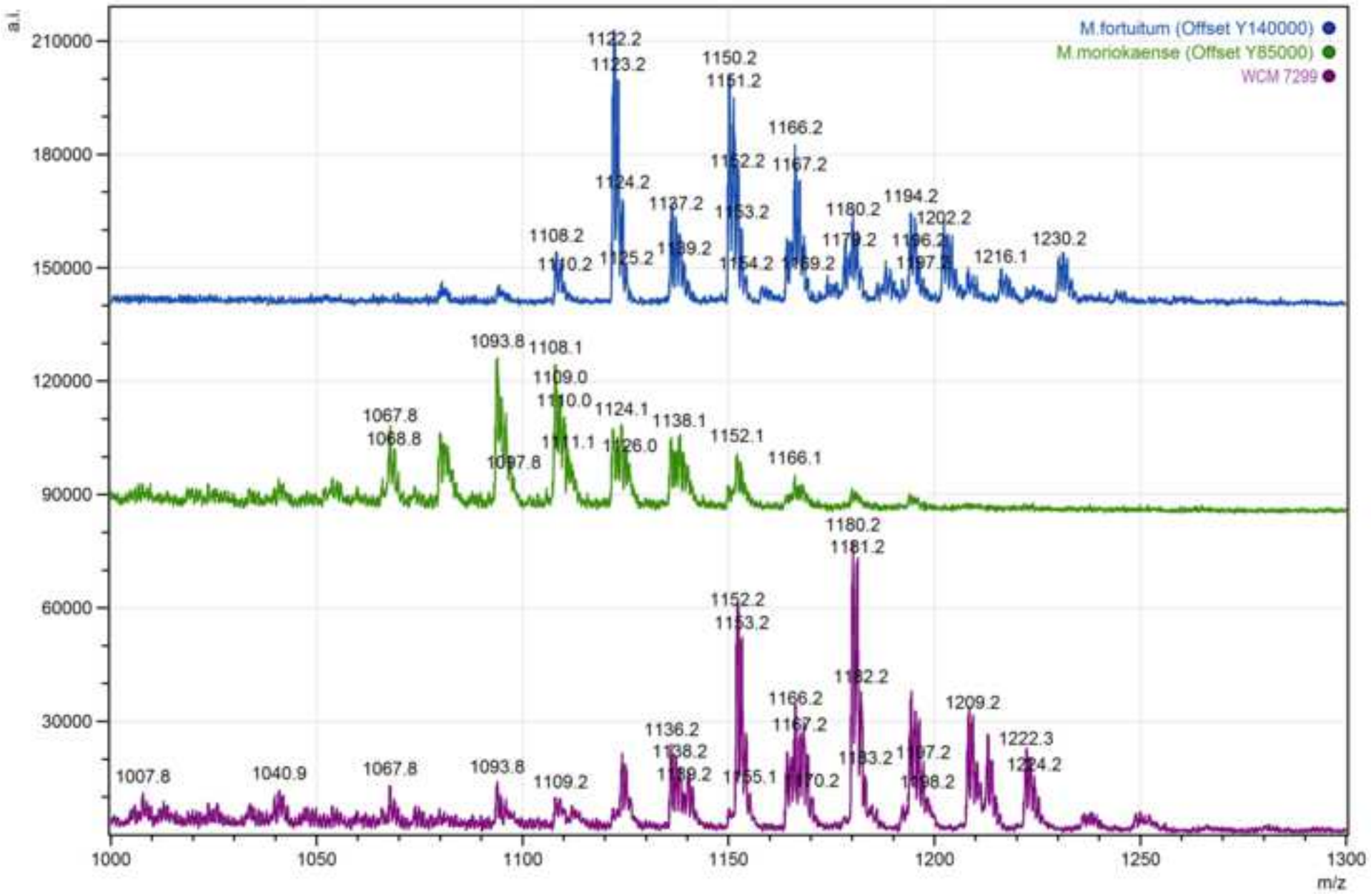


Fig. 3: FIA of M. fortuitum ATCC 6841, M. moriokaense ATCC 43059 and WCM 7299. in the 1,000-1,300 m/z range.



43059^T. Triplicate culture plates of each of the three Mycobacterium strains were analysed separately by Liquid Chromatography –Mass Spectrometry (LC-MS). The comparative mass spectrometric analysis between *M. fortuitum, M. moriokaense* and isolate WCM 7299^T is presented in the 1,000-1,300 m/z range as this is the typical mass range in which mycolic acids are found when analysed using LC-MS (Crick et al. 2016). The number of molecular ions of significantly high intensity is ten for *M. fortuitum*, seven for *M. moriokaense*, and eight for WCM 7299^T (Figure 3). Although the ion clusters between all three isolates show a different m/z pattern from their respective FIAs, further studies using liquid or gas chromatography may be required to determine their mycolic acid class ratio (Figure 3). Gcebe *et. al.,* 2016 determined the G+C content of the type strain WCM 7299^T in a separate study as 67.4% (Gcebe *et al.,* 2016).

The data presented here revealed that the 10 isolates belong to a novel NTM species and we proposed *Mycobacterium malmesburyense* sp. nov. as the name for the new species.

Description of *Mycobacterium malmesburyense* sp. nov.

Mycobacterium malmesburyense (malmes.bu.ry.en'se. N.L. neut. adj. *malmesburyense* pertaining to Malmesbury*)* is named after a town (Malmesbury) in South Africa, where one of the isolates (the type strain) of this species originated from.

The organisms are acid fast bacilli. Colonies form a yellow-orange pigment in the dark, and grow on LJ slants supplemented with PACT, as well as on Middlebrook 7H11 at 25°C, 37°C and 45°C, within 2-5 days but optimally at 37°C. The bacteria are positive for semi quantitative catalase test (>45 mm foam), Tween 80 hydrolysis, nitrate reductase, urease and pyrazinamidase activity tests. Different isolates show variable activity for the 3 day and 14 day arylsulphatase tests, as well as niacin production, but most were negative for the 3 day

test, positive for the 14 day test and negative for niacin accumulation. The bacteria do not grow in the presence of 5% NaCl and neither do they utilise D-mannitol, inositol, L-rhamnose and L-arabinose as sole carbon sources. The organisms are negative for the aesculin hydrolysis and citrate activity tests. Positive or negative reaction are regarded as those exhibited by 80% or more strains. Growth of most strains is not inhibited by imipenem, tobramycin, cefoxitin and amoxylin, but are inhibited by ciprofloxacin, clarithromycin and doxycycline. The bacteria have unique 16S rDNA, *hsp65, sodA* and *rpoB* gene sequences that are clearly different from any other mycobacterial species with *M. moriokaense, M. flavescens, M. novocastrense* and *M. arupense* being the most closely related species. Phenotypic differences that separated this species from its closest relatives include pigmentation, biochemical, growth characteristics as well as the mycolic acid profile. These bacteria were mainly found in the environment at the escarpment, semi-arid plateau, moderate eastern plateau, subtropical coastal regions, as well as areas with mediterranean climate in South Africa. Isolates from bovine tissue samples have been encountered in multiple parts of the country. No clinical relevance has been defined for this species.

The type strain is WCM 7299^{T} (ATCC® BAA-2759^{TM} =CIP 110822^{T}) which was isolated from bovine nasal swab. Isolates Balasi, Vryburg, Uyenvlei, TB 5612, TB 5960A, 242, Middledrift, C4 and C8 are additional strains of this species.

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Supplementary Table 1: Comparison of phenotypic characteristics of the NTM isolates with those of the closely related species.

NTM ID/ species	Source	Growth rate (days)	Pigment	Growth at:	Biochemical features and Utilisation of sugars														
				25°C .37°C 45°C	Semi quantitative Catalase	NaCl 5% tolerance	Tween 80 hydrolysis	Aryl sulphatase (14ays)	Aryl sulphatase (3days)	Urease	Niacin	Nitrate reduction	Pyrazinamidase activity	Citrate	Aesculin	D-mannitol	Inositol	L-rhamnose	L-arabinose
M. moriokaense ATCC 43059^T *(*tested in the lab*)*	Ref	<7	-	+ + + + nd	+	-	+	-	+	+	nd	+	+	-	+	-	-	nd	nd
M. moriokaense (e, f)	Ref	<7	-	+ + nd	-, +	+	nd	nd	-, +	+	nd	+	+	-	-	+, -	+	+	+
M. novocastrense (*a, c*)	Ref	<7	+	+ + +	+	+	+	nd	v	+	-	+	nd	nd	nd	nd	nd	nd	nd
M. flavescens (b, g, h)	Ref	<7	+	+ + -	+	+	+	+	-	+	-	+	+	+	nd	+, v	-	-	nd
M. arupense (d)	Ref	<7	-	+ + -	nd	-	+	+	-	-	-	-	-	nd	nd	nd	nd	nd	nd
M. elephantis a,b	Ref	<7	+	+ + ++ +		+	+	-	-	+		+	+	nd	nd	-	-	nd	nd
TB 5960A	Bo tissue	<7	+	+ + ++ +	+	-	+	+	-	+	-	+	+	-	-	-	-	-	-
C28	Bu swab	<7	+	+ + ++ +	+	-	+	+	-	+	-	+	+	-	-	-	-	-	-
C4	Bu swab	<7	+	+ +++ +	+	-	+	+	-	+	--	+	+	-	-	-	-	-	-
TB 242	Bu swab	<7	+	+ +++ +	+	-	+	+	-	+	+	-	+	-	-	-	-	-	-
WCM 7299^T	Bo swab	<7	+	+ +++ +	+	-	+	+	-	+	-	+	+	-	-	-	-	-	-
TB 5612	Bo tissue	<7	+	+ +++ +	+	-	+	+	-	+	-	+	+	-	-	-	-	-	-
Vryburg	Bo swab	<7	+	+ +++ +	-	-	+	-	-	+	-	+	+	-	--	-	-	-	-
Balasi	water	<7	+	+ +++ +	-	-	+	+	-	+	-	+	+	-	-	-	-	-	-
Middledrift	Bo swab	<7	+	+ +++ +	+	-	+	+	-	+	+	+	+	-	-	-	-	-	-
Uyenvlei	Bo swab	<7	+	+ +++ +	+	-	+	+	+	+	-	+	+	-	+	-	-	-	-

a, data retrieved from Tortoli , 2003; b, Data retrieved from Turenne *et al.,* 2002; c, Shojaei et al., 1997; d, data taken from Cloud *et al.,* 2006; e, data retrieved from Tsukamura *et al.,* 1986; f, data taken from Adékambi *et al.,* 2006b; g, data taken from Tortoli *et al.,* 2004; h, data taken from Bojalil *et al.,* 1962; v, variable results based on a and c; +, positive; -, negative; +++, optimum growth; Bo, bovine; Bu, buffalo; Ref, reference strain; swab, nasal swab.