Mycobacterium komaniense sp. nov., a novel rapidly growing non-tuberculous Mycobacterium species detected in South Africa

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ABSTRACT

Some species of non- tuberculous mycobacteria (NTM) have been reported to be opportunistic pathogens of animals and humans. Recently there has been an upsurge in the number of cases of NTM infections, such that some NTM species are now recognized as pathogens of humans and animals. From a veterinary point of view, the major significance of NTM is the cross − reactive immune response they elicit against *Mycobacterium bovis* antigens, leading to misdiagnosis of bovine tuberculosis. Four NTM isolates were detected from a bovine nasal swab, soil and water, during a NTM survey in South Africa. These were all found using the 16S rRNA gene sequence analysis to be closely-related to *Mycobacterium moriokaense*. The isolates were further characterised by sequence analysis of the partial fragments of *hsp65*, *rpoB* and *sodA*. The genome of the type strain was also elucidated. Gene (16S rRNA, *hsp65, rpoB* and *sodA*) and protein sequence data analysis of 6kDa early secretory antigenic target (ESAT 6) and 10kDa culture filtrate protein (CFP-10) revealed that these isolates belong to a unique *Mycobacterium* species. Differences in phenotypic and biochemical traits between the isolates and closely related species further supported that these isolates belong to novel *Mycobacterium* species. We proposed the name, *Mycobacterium komaniense* sp. nov. for this new species. The type strain is GPK 1020^T (=CIP 110823T=ATCC BAA-2758).

Key words: *Mycobacterium komaniense* sp.nov., non tuberculous *Mycobacterium*, ESAT 6, CFP-10.

Some species of non- tuberculous mycobacteria (NTM) have been reported to be opportunistic pathogens of animals and humans [1]. NTM species like *Mycobacterium szulgai, Mycobacterium marinum, Mycobacterium kansasii, Mycobacterium abscessus* and *Mycobacterium avium* complex (MAC) are now recognised as emerging or opportunistic pathogens of humans and animals causing mycobacteriosis, such that their isolation from clinical samples is regarded as significant [2, 3, 4]. NTM can cause skin lesions, localized lymphadenitis, and pulmonary diseases resembling tuberculosis and disseminated diseases [3]. Recently, there has been an increase in the number of reported cases of NTM disease globally [4, 5, 6]. The upsurge can be associated with increased awareness among physicians and advancement in technology for diagnosis of NTM. However, this may not be the only explanation. Rapidly growing NTM have also received an increased attention because of characteristic multiple antibiotic resistance (MAR) [1, 6]. Among the rapidly growing NTM species, human infections by members of *Mycobacterium chelonae –abscessus* complex including *Mycobacterium abscessus, Mycobacterium massiliense*, and *Mycobacterium bolletii* have been on the rise worldwide [6]. Cases of NTM infection in animals are still under-investigated, as compared to human cases. From a veterinary point of view, the major significance of NTM is the cross-reactive immune responses induced by certain NTM that interfere with the diagnosis of bovine tuberculosis by immuno-assays [7]. These specificity constraints are associated with the presence of shared antigens between certain NTM species and *Mycobacterium bovis.* The ESAT 6 and CFP-10 proteins encoded in RD1 region of mycobacteria are the most studied virulence factors and immunogenic proteins of mycobacteria for their potential as vaccine and diagnostic candidates [8, 9]. Thus it was proposed that characterisation of mycobacteria should include investigation of their RD1 region [10].

Here we describe a novel NTM species represented by four isolates, GPK 1020^T, Pan2S1, Komani and Mbekweni, originating from a bovine nasal swab (GPK 1020^T), water (Komani

and Mbekweni) and soil (Pan2S1) samples. The samples were collected from Queenstown in the Eastern Cape Province (GPK 1020^T, Komani and Mbekweni), and Hluhluwe Imfolozi Park, in KwaZulu Natal Province of South Africa (Pan2S1). These were collected during a NTM survey conducted between the years 2010-2012, investigating the prevalence of NTM in cattle, African buffalo and the environment [11]. Mycobacteria isolation was done as described by Gcebe *et al* [11]. The novel species was identified using PCR-sequencing of the four mycobacterial housekeeping genes namely 16S rRNA , *hsp65, rpoB* and *sodA* genes. Heated culture suspensions from individual isolates were used as DNA templates in the various PCR protocols. 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-antifungal cocktail of polymyxin B, amphotericin B, carbenicillin and trimethoprim (PACT) (Becton Dickinson, USA). *In-vitro* amplification of the 1400 bp fragment of the 16S rRNA gene [12], the 439 bp region of the gene encoding the 65 kDA heat shock protein (*hsp65*) [13], part (764 bp) of the RNA polymerase beta subunit (*rpoB*) gene [14, 15] and part (464 bp) of the superoxide dismutase (*sodA*) gene [16] followed by sequencing, was performed for genetic characterisation of the isolates. Sequencing was done 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 (version 7.0). The resulting consensus sequences were analysed on the NCBI BLAST platform for species identification (www.blast.ncbi.nlm.nih.gov/Blast.cgi) by megablast.

The phylogenetic position of the isolates with respect to other mycobacteria was determined by neighbour joining trees constructed based on the 16S rRNA gene, and concatenated sequences of the four housekeeping genes (16S rRNA gene, *hsp65, rpoB* and *sodA*). Multiple sequence alignments of the individual genes as well as the concatenated sequences were performed using Clustalw [17, 18] from MEGA (version 7), to determine percentage similarity between each of the gene sequences.

Whole genome sequence of strain GPK 1020[T] was determined by Next Generation sequencing (NGS). Genomic DNA paired-end libraries were generated using the Nextera DNA sample preparation kit (Illumina) and indexed using the Nextera index kit (Illumina). Sequencing was performed as paired-end reads (2x250bp) employing the Illumina Miseq system using the Miseq reagent kit v2, at the Agricultural Research Council, as per manufacturer's instructions. *De novo* assembly of the sequenced reads was performed using SPAdes [19]. Each assembly was evaluated using QUAST [20]. The assembled genome was annotated using Prokka annotation pipeline [21].

The most commonly investigated biochemical and phenotypic characteristics namely, ability to grow at different temperatures, colony morphology, pigmentation, acid fastness, growth rate, 5% sodium chloride tolerance, ability to reduce nitrate, urease activity, niacin accumulation, aryl-sulphatase activity, pyrazinamidase Tween 80 hydrolysis, semi-quantitative catalase activity (room temperature), hydrolysis of aesculin and utilisation of citrate as well as the ability to use the following sugars as sole carbon sources: D-mannitol, inositol, L-rhamnose and L-arabinose were tested and compared with those of their closest relatives namely *Mycobacterium moriokaense, Mycobacterium malmesburyense, Mycobacterium novocastrense* and *Mycobacterium flavescens*. 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.

Nitrate reduction, urease activity and niacin accumulation assays were performed using the commercial test strips (Becton Dickinson, USA) according to the manufacturer's instructions. Three days and 14 days arylsulphatase activity test were performed as described in Wayne [22]. The pyrazinamidase assay was carried out as described by Singh *et al* [23], except that Middlebrook 7H11 agar was used and the culture incubated for 4 days. The ability of the isolates to hydrolyse Tween 80 was evaluated using a method described by Kilburn *et al* [24]. Semi quantitative catalase activity of the isolates was tested using 3% hydrogen peroxide and observing formation of gas bubbles [25].

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 up to 3 days at 37°C, after which the zones of inhibition were measured [26]. If a minimum of 3mm zone of inhibition was obtained, it was considered as inhibition.

Mycolic acid profiling of isolate GPK 1020^T as well as that of the most closely related species, *Mycobacterium moriokaense* as determined by the 16S rRNA gene sequencing, was each done in triplicate and analysed separately by LC-MS. Flow injection analysis (FIA) was performed for 3 minutes to produce a total ion count (TIC) chromatogram. Each TIC was scanned to display the spectrum in the 50-1,700 m/z range.

Genetic characterization of the isolates by 16S rRNA gene, *hsp65, rpoB* and *sodA* sequence analysis, revealed that isolates GPK 1020^T, Pan2S1, Komani and Mbekweni shared 97%-99% sequence identity to *M. moriokaense* (16S rRNA); 98% to *Mycobacterium novocastrense* (*hsp65*), 95% -96% to *Mycobacterium flavescens* (*rpoB*) and 92%-93% to *M. flavescens* and *M. malmesburyense* WCM 7299^T (*sodA*) respectively (Table S1). Phylogenetic relatedness of

the isolates and other *Mycobacterium* species, based on the 16S rRNA gene as well as the concatenated sequences of the four gene fragments (16S rRNA gene, *hsp65*, *rpoB* and *sodA*) is illustrated by the neighbour joining trees in Figure 1 and Figure 2, respectively. The highest level of bootstrap support value (up to 100%) supporting the clustering, identified the four isolates as belonging to the same *Mycobacterium* species. The neighbour joining tree based on the 16S rRNA showed very low robustness in which the isolates clustered with *M. malmesburyense* WCM 7299[T]. A robust tree was obtained from concatenated sequences inferred from 16S rRNA, *hsp65, rpoB* and *sodA,* where the isolates did not cluster with any of the close relatives. Trees constructed from concatenation of different housekeeping genes are anyway known to be robust [27]. These results suggest that the isolates belong to a novel species.

Whole genome sequence analysis of the type strain (GPK 1020[T]) was published in another study [28]. The European Nucleotide (ENA)/ Genbank accession numbers for the sequences are CVTA01000001-CVTA0100065 [28]. The genome was found to be 5 370 343bp long with a high G+C content of 67.33%. BLAST searches using the largest contig (298 570 bp) revealed the closest relative of the type strain to be *Mycobacterium* sp. JLS, displaying 81% similarity at amino acid level. This further supports that the sequenced strain belong to a unique species whose genome sequence has not been published before. The amino acid sequence analysis of ESAT 6 and CFP-10 of the type strain (GPK 1020[T]) showed sequence similarities of 94% for ESAT 6 as well as 99% for CFP-10 with *M. malmesburyense* WCM 7299[T] (Table S1) further suggesting that GPK 1020[T] belongs to a novel species .

The identified differences in phenotypic characteristics of the isolates with those of the closely related species *i.e. M. moriokaense, M. novocastrense* and *M. flavescens ,* illustrated in Table 1, include pigmentation, growth rate as well as other biochemical traits. The most apparent feature that distinguished the isolates from *M. moriokaense,* was pigmentation, as all the
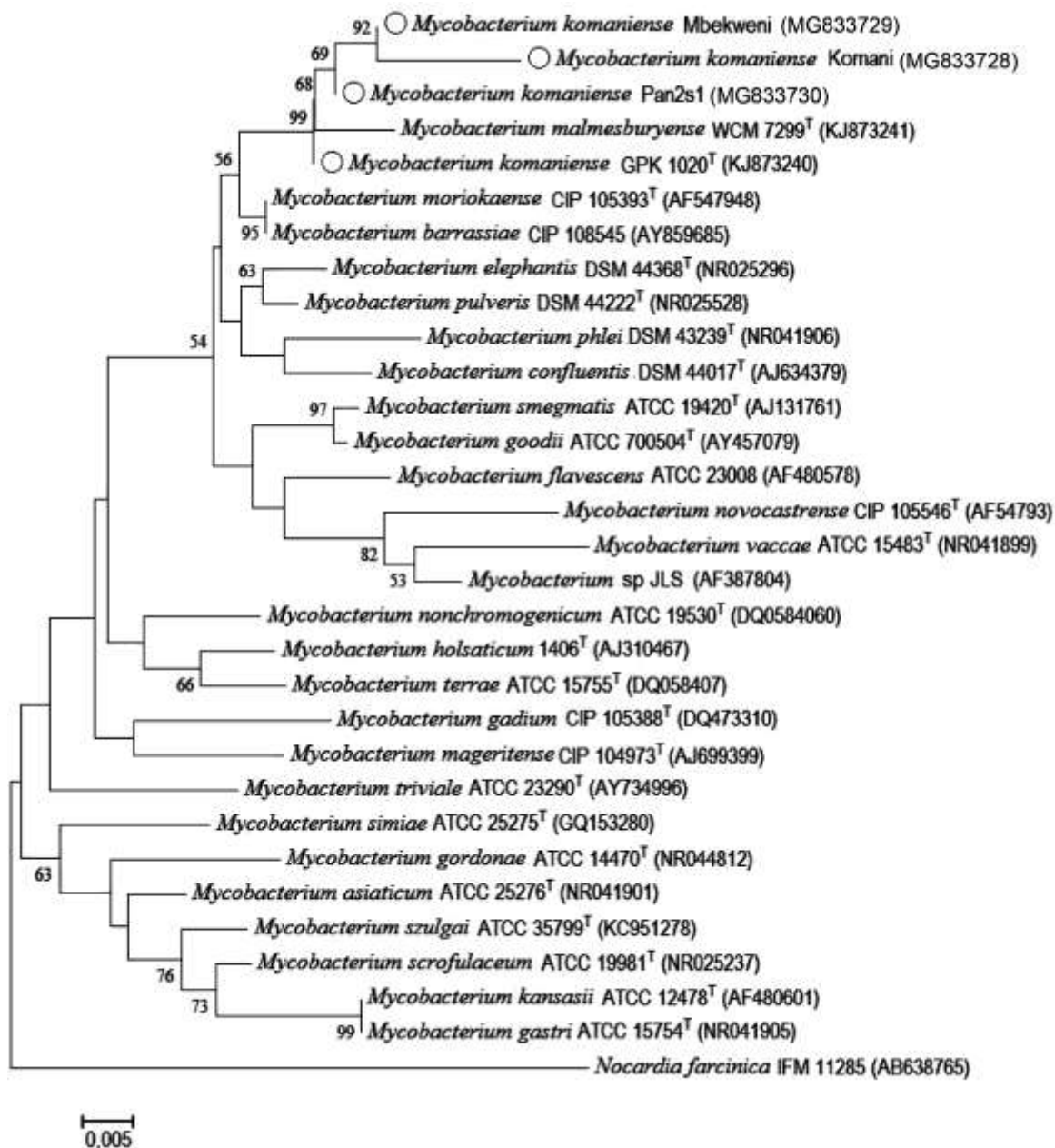
Fig. 1: Neighbour joining tree illustrating the phylogenetic relationship of the isolates and other Mycobacterium species. The *M. komaniense* sp. nov. isolates are indicated with circles in front of each strain. The tree is based on the partial 16S rRNA gene sequences. Genbank accession numbers for the sequences are shown in parenthesis. The percentage of replicate trees (more than 50%) in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. *Nocardia farcinica* was used as an outgroup sequence. Bar, 0.005 substitutions per nucleotide.
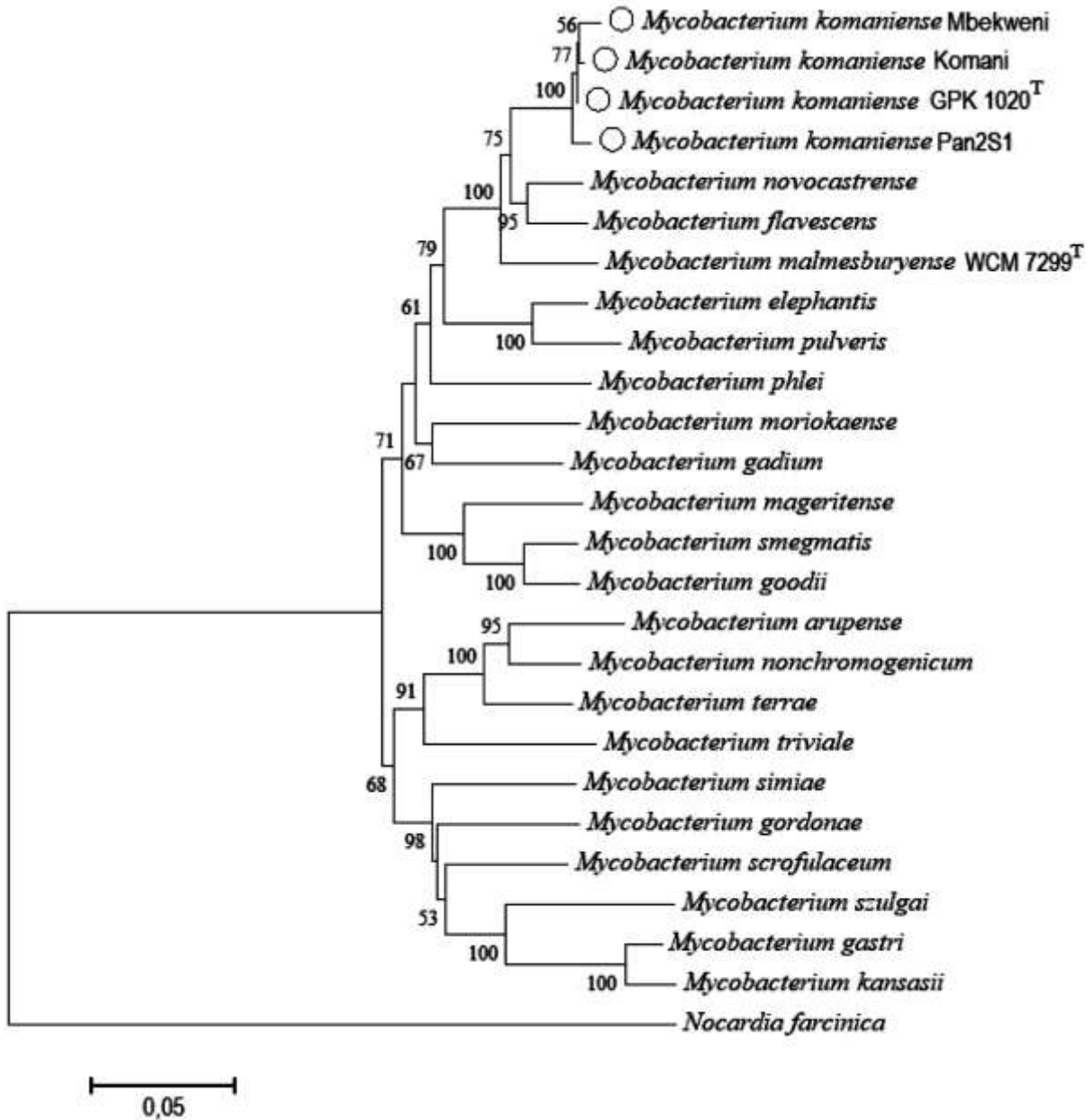
Fig. 2: Neighbour joining tree illustrating the phylogenetic relationship of the isolates and other Mycobacterium species. The *M. komaniense* sp. nov. isolates are indicated with circles in front of each strain. The tree is based on concatenated sequences of: 16S rRNA gene, *hsp65* , *rpoB* and *sodA* gene fragments. The percentage of replicate trees (more than 50%) in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches . *Nocardia farcinica* was used as an outgroup sequence. Bar, 0.05 substitutions per nucleotide

isolates appeared to be scoto-chromogens whereas *M. moriokaense* was previously reported to be non-chromogenic [29, 30]. Contrary to what is reported for *M. novocastrense* and *M.*

Table 1: Phenotypic and biochemical identification results

Test	*M. moriokaense* ATCC 43059 (tested in the lab)	*M. moriokaense* [29; 30]*	*M. novocastrense* [31; 32]*	*M. flavescens* [33, 34, 35]*	*M. malmesburyense* WCM 7299 [36]*	NTM isolates from this study (n=4)
Growth rate (days)	<7	<7	<7	<7	<7	<7
Pigmentation	-	-	+	+	+	+
Growth at :						
25°C	+	+	+	+	+	+
37°C	+++	+++	+	+	+++	+++
45°C	ND	ND	+	-	+	+
5% NaCl tolerance	+	+	+	-	-	-
Semi-quantitative catalase	+	-,+	+	+	+	D (n=2/4)‡
Tween 80 hydrolysis	+	ND	+	+	+	+
Arylsuphatase (14 days)	-	ND	ND	ND	+	+
Aryl sulphatase (3 days)	+	-,+	V	-	-	-
Urease	+	+	+	+	+	+
Niacin	ND	ND	-	-	-	-
Nitrate reduction	+	+	+	+	+	+
Pyrazinamidase activity	+	+	ND	+	+	+
Citrate	-	-	ND	+	-	-
Aesculin	+	-	ND	ND	-	-
D-Mannitol	-	+,-	ND	+, V	-	-
Inositol	-	+	ND	-	-	-
L-Rhamnose	ND	+	ND	-	-	-
L-Arabinose	ND	+	ND	ND	-	-

V, variable results ; +, positive; -, negative; +++, optimum growth, D; strain dependant. * Data retrieved from Tsukamura *et al.,* [29];   Adékambi *et al.,* [30]; Tortoli. [31] ;

Shojaei *et al*., [32];   Turenne [33]; Tortoli *et al.,* [34]; Bojalil *et al.,* [35]; Gcebe *et al.,* [36]; ND, not determined; ‡, ratio of positive results

flavescens , all the isolates were unable to grow in the presence of 5% NaCl [31; 32, 33,34,35]. In addition, the ability of the isolates to grow at 45°C further separated them from *M. flavescens,* which was previously reported not to grow at this temperature [35]. The isolates could not be separated from *M. malmesburyense* by the tested phenotypic traits buy by differences in nucleotide sequences and the phylogenetic relationship inferred from the concatenated sequences as well as differences of their ESAT 6 and CFP-10 sequences.

The isolates presented quite a uniform pattern of antibiotic profiles with all susceptible to amikacin, ciprofloxacin, doxycycline and clarithromycin. The isolates were resistant to imipenem, cefoxinin, tobramycin and amoxylin.

The comparative mass spectrometric analysis of isolate GPK 1020^T representing the proposed novel type strain and *M. moriokaense* is presented in the 1,000-1,250 m/z range as this is the typical mass range in which mycolic acids are found when analysed using liquid chromatography-mass spectrometry [36] (Fig 3).
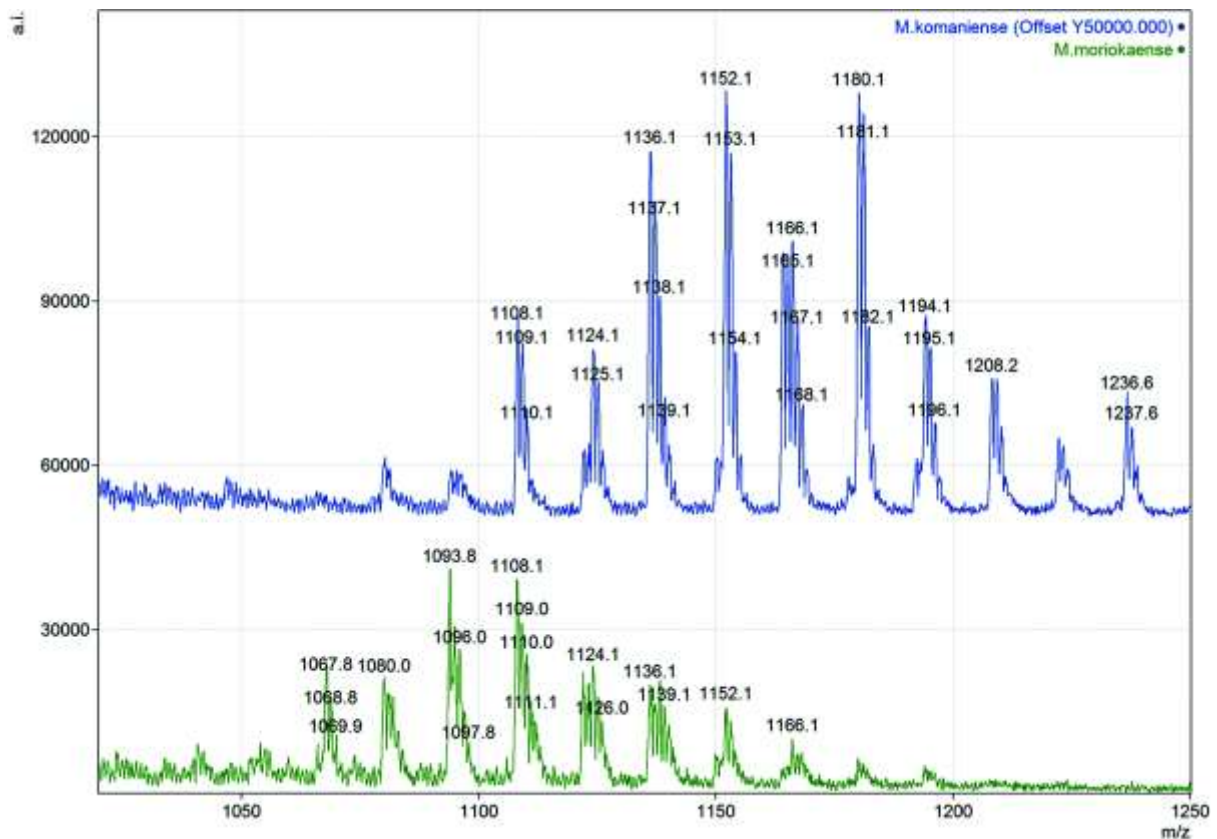
Fig. 3: **FIA of *M. komaniense* sp. nov. and *M. moriokaense* in the 1,000-1,250 m/z range using liquid chromatography-mass spectrometry .**

As the isolates clearly represented a novel species not previously described in literature, we propose the name *Mycobacterium komaniense* sp. nov.

Description of *Mycobacterium komaniense* sp. nov.

Mycobacterium komaniense [ko.ma.ni.en'se. N.L. neut. adj. *komaniense* pertaining to Komani, a Xhosa name for Queenstown, South Africa, where the type strain was isolated].

Acid fast bacillus Colonies form a yellow 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 Tween 80 hydrolysis, nitrate

reductase, urease, pyrazinamidase, niacin, and 14 day aryl sulphatase activity tests. Different isolates show variable activity for the semi quantitative catalase test (>45 mm foam) They are negative for the 3 day arylsulphatase test, citrate and aesculin hydrolysis tests. 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. 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 rRNA, *hsp65, sodA* and *rpoB* gene sequences that are clearly different from any other mycobacterial species, with *M. moriokaense, M. malmesburyense, M. flavescens* and *M. novocastrense* being the most closely related species. The type strain also has different ESAT 6 and CFP-10 protein sequences from their closest relatives, with *M. malmesburyense* WCM 7299^T displaying the closest sequence similarities. Phenotypic differences that separated this species from its close relatives include pigmentation, biochemical characteristics and growth characteristics. They have been isolated from bovine nasal membrane, water and soil. No clinical relevance has been defined for this species.

The type strain is GPK 1020^T (ATCC$^®$ BAA-2758^{TM} =CIP 110823^T) which was isolated from bovine nasal membrane. Isolates Pan2S1, Komani and Mbekweni are additional strains of this species.

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Conflict of interest

The authors declare that there are no conflicts of interest.

Ethical Statement

Animal Ethics approval for the study was obtained from University of Pretoria: Animal Ethics Committee (S4285-15).

Abbreviations

CIP: Collection de l'Institut Pasteur; **EMBL:** European Molecular Biology Laboratory; **ENA:** European Nucleotide Archive; **ESAT-6:** 6 kilodalton early secretory antigenic target**; CFP-10:** 10 kilodalton culture filtrate protein**; NTM:** Non tuberculous Mycobacterium; **sodA:** superoxide dismutase; **hsp 65:** 65 kilodalton heat shock protein; **rpoB:** β subunit of RNA polymerase; **MAC:** Mycobacterium avium complex; **MAR:** multiple antibiotic resistance; **RD1**: Region of difference 1; MEGA: Molecular evolutionary genetics analysis; **FIA:** Flow injection analysis; **NCBI-BLAST**: National Center for Biotechnology Information -Basic Local Alignment Search Tool; **NGS:** Next generation sequencing; **QUAST:** Quality assessment tool for genome assemblies; **SPades**: St. Petersburg genome assembler; **LJ:** Löwenstein

Jensen; **PACT:** polymyxin B, amphotericin B, carbenicillin and trimethoprim; **USA**: United States of America; **UK**; United Kingdom; **OADC;** Oleate-albumin-dextrose-catalase; **LTD;** Limited; **TIC**, total ion count; **NaCl**; Sodium Chloride; **NWO-WOTRO;** Netherlands organisation for Scientific Research, **kDa**: kilodalton; **m/z;** mass to charge; **Prokka:** Prokaryotic genome annotation **bp:** base pairs; **mm:** millimetres**; LC-MS;** liquid chromatography-mass spectrometry; **µg:** micrograms.

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Supplementary Material

Table S1: Sequence analysis of the different Mycobacterium house -keeping genes from novel NTM isolates

NTM strain	Highest BLAST hits for the different gene and protein sequences					
	16S rRNA	*hsp5*	rpoB	sodA	ESAT 6	CFP-10
GPK 1020[T]	99% M. moriokaense (KC494311)*	98% *M. novocastrense* (AF5478620)*	96% M. flavescens (AY85698)*	93% M. flavescens (AY54482)*/ M. malmesburyense (KJ873247)*	94% M. malmesburyense WCM7299 (CRL79347)*	99% malmesburyense WCM7299 (CRL79345)*
Komani	97% M. moriokaense (KC494311)*	98% M. novocastrense (AF5478620)*	95% M. flavescens (AY859698)*	93% M. flavescens (AY54482)*	ND	ND
Mbekweni	98% M. moriokaense (KC494311)*	98% M. novocastrense (AF5478620)*	95%M. flavescens (AY859698)*	93% M. flavescens (AY54482)/ M. malmesburyense (KJ873247)*	ND	ND
Pan2S1	98% M. moriokaense (KC494311)*	98% M. novocastrense (AF5478620)*	95% M. flavescens (AY859698)*	92% M. flavescens (AY54482)*	ND	ND

NTM, non-tuberculous mycobacteria; ND, not determined; *, Genbank/ENA/EMBL sequence ID.