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TAXONOMIC DESCRIPTION
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Mycobacterium persicum sp. nov., a novel species closely related to *Mycobacterium kansasii* and *Mycobacterium gastri*

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Abstract

Four strains isolated in Iran from pulmonary specimens of unrelated patients are proposed as representative of a novel *Mycobacterium* species. Similarity, at the phenotypic level, with *Mycobacterium kansasii* is remarkable with the photochromogenic yellow pigmentation of the colonies being the salient feature. They differ, however, genotypically from this species and present unique sequences in 16S rRNA, *hsp65* and *rpoB* genes. The average nucleotide identity and the genome-to-genome distance fully support the status of an independent species. The name proposed for this species is *Mycobacterium persicum* sp. nov. with AFPC-000227^T (=DSM 104278^T=CIP 111197^T) as the type strain.

Mycobacterium kansasii is one of the best-known mycobacterial species; it was first described in 1953 and was also the first non-tuberculous mycobacterium (NTM) demonstrated to be responsible for pulmonary disease in humans [1]. The species can be easily identified mainly thanks to its manifest photochromogenicity. Genotypically, the only species really closely related to this species is *Mycobacterium gastri*, but which is clearly distinguishable as it is non-chromogenic and non-pathogenic.

We characterize here four mycobacterial strains phenotypically resembling *M. kansasii* but substantially different from the latter at the genetic level.

The first strain (AFPC-000227^T) was isolated in 2009 from three sputum samples of a 50-year-old female sent to a general hospital in Tehran due to fever, productive cough and shortness of breath. Treatment with sulfonamides, due to an initial diagnosis of nocardiosis, was replaced with an anti-tuberculosis (TB) regimen following the microscopic detection of acid-fast bacilli in her sputum samples. Growth in culture of an NTM led to the addition of imipenem during the last 3 months (from the fourth to the sixth) of the standard anti-TB therapy. The patient was cured and both microscopy and culture of sputum remained negative.

The remaining three strains (NTM209, NTM309 and NTM371) were recovered in 2014 on occasion of a prevalence survey for multi-drug resistant TB in Iran. They were isolated in different provinces of the country (Khorasan, Isfahan and Khuzestan) from unrelated patients. The patients, two males and one female, were classified, on the basis of sputum smear positivity, as new TB cases and were treated with a standard anti-TB regimen.

Rough colonies grew in about 10 days at 37 °C and, a few days later, at 25 °C. No growth was achieved either at 42 °C or on MacConkey agar without crystal violet. Yellow pigmentation developed only after light exposure. Among biochemical tests [2] nitrate reduction, catalase at 68 °C, Tween 80 hydrolysis and semi-quantitative catalase (>45 mm) were positive. Niacin accumulation, urease, tellurite reduction and β-glucosidase were negative. The four strains were able to grow on media supplemented with isoniazid (1 µg ml^{-1}) but were inhibited on those supplemented with *p*-nitrobenzoic acid (400 µg ml^{-1}) or with hydroxylamine (600 µg ml^{-1}). Two of the strains were inhibited also by thiacetazone (10 µg ml^{-1}). Thus, only the lack of urease differentiated the test strains from *M. kansasii*.

With commercial line probe assays (GenoType; Hain Lifescience) [3] the four strains were identified as members of *M. kansasii*: they were assigned to the *M. kansasii*/*M. gastri*

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Keywords: *Mycobacterium persicum*; *Mycobacterium kansasii*; NTM; Iran.
Abbreviations: ANI, average nucleotide identity; NTM, non-tuberculous mycobacteria; TB, tuberculosis.
The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *hsp65* and *rpoB* gene sequences of strain AFPC-000227^T are KX987140, KX987141 and KX987143, respectively; the accession number of the *hsp65* gene sequence of strain NTM209 is KX987142.
Two supplementary figures are available with the online Supplementary Material.

Table 1. MICs of the test strains (AFPC-000227^T, NTM209, NTM309, NTM371) to anti-mycobacterial drugs

	MIC	Interpretation*
Amikacin	2–16	S
Ciprofloxacin	1–4	S-R
Clarithromycin	0.5–2	S
Doxycycline	8->16	I-R
Ethambutol	8->16	I-R
Linezolid	≤1–2	S
Moxifloxacin	≤0.12–1	S-I
Rifabutin	≤0.25	S
Rifampicin	0.5–2	S-I
Streptomycin	8–>64	I-R
Trimethoprim/sulfamethoxazole	>8/152	R

*S, susceptible; I, intermediate; R, resistant.

group by GenoType CM and to *M. kansasii* iii (probes 10 and 12) by GenoType AS.

The antimicrobial pattern of the four strains based on MIC determination [4] revealed susceptibility to amikacin, clarithromycin, linezolid and rifabutin and resistance to ethambutol and trimethoprim/sulfamethoxazole; MICs of other drugs were borderline (Table 1).

The HPLC profile of cell-wall mycolic acids [5] of the four strains was investigated in cells grown on Middlebrook 7H10 agar. Following saponification, extraction and derivatization, the mycolic acids were separated using a gradient of methanol and 2-propanol as recommended for the Sherlock Mycobacteria Identification System (SMIS; MIDI). The pattern of the strains was characterized by a cluster of six major peaks eluting between 7 and 9 min, a profile most similar to that of *M. kansasii* (Fig. 1) as certified by the Sherlock software (similarity index 0.633).

With regard to the 16S rRNA gene (1527 bp) [6], the test strains presented identical sequences; the sequence was most closely related to *M. kansasii* and *M. gastri*, but from which they differed by 7 bp (99.5 % similarity). In the hypervariable fragment (401 bp) of the *hsp65* gene [7] two sequevars were present, both presenting the best resemblance to *M. gastri*; from this species strain AFPC-000227^T differed by 7 bp (98.2 %) and the other three strains by 5 bp (98.7 %). Again *M. kansasii* was the closest related species based on the *rpoB* gene (669 bp) [8], differing by 12 bp (97.0 %); in this region, too, the four strains presented identical sequences.

Phylogenetic analysis was conducted on the sequences above aligned with the type strains of the most closely related *Mycobacterium* species retrieved from GenBank. The neighbor-joining method [9] bootstrapped 1000 times, supported by the MEGA 6 software [10], was used with *Mycobacterium tuberculosis* H37Rv chosen as the outgroup (Fig. 2). In different phylogenetic thees based on the *hsp65*

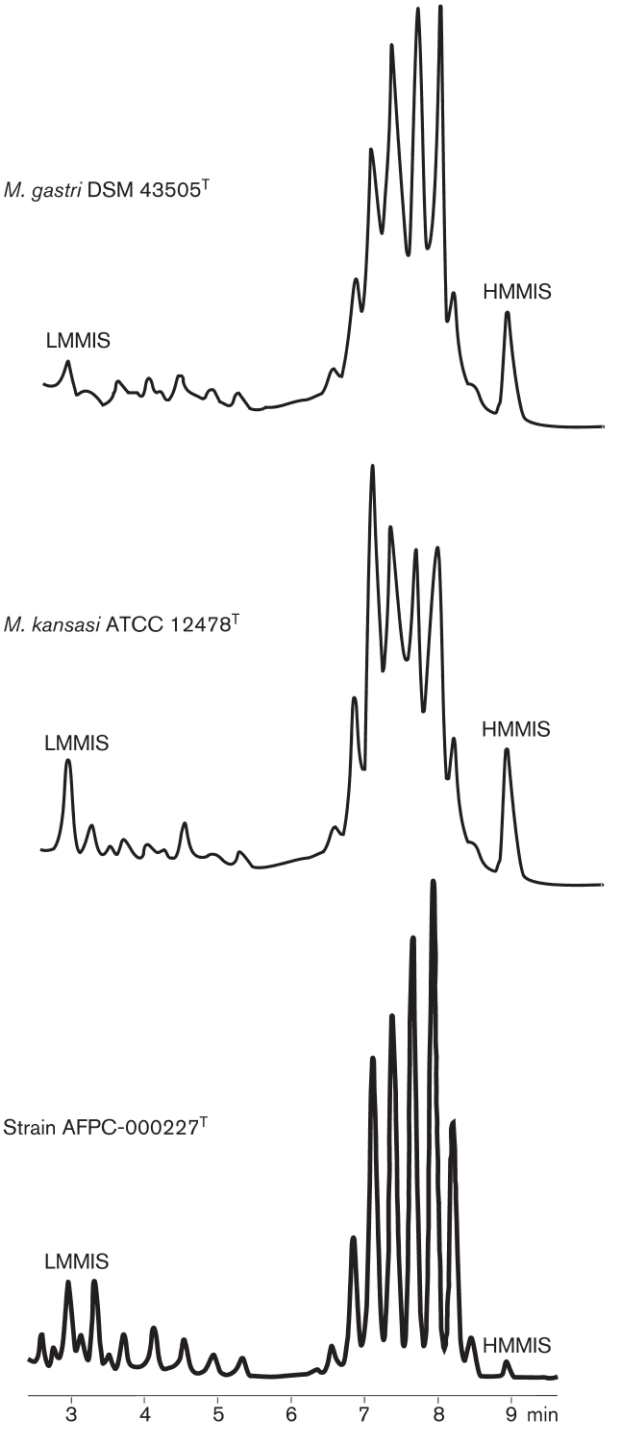

Fig. 1. Representative mycolic acid patterns of *M. gastri* DSM 43505^T, *M. kansasii* ATCC 12478^T and strain AFPC-000227^T. LMMIS, low molecular mass internal standard; HMMIS, high molecular mass internal standard.

gene (Fig. S1, available in the online Supplementary Material), *rpoB* gene (Fig. S2) and the concatenated sequences of the 16S rRNA, *hsp65* and *rpoB* genes the test strains clustered close to *M. kansasii* and *M. gastri* (Fig. 3), justifying

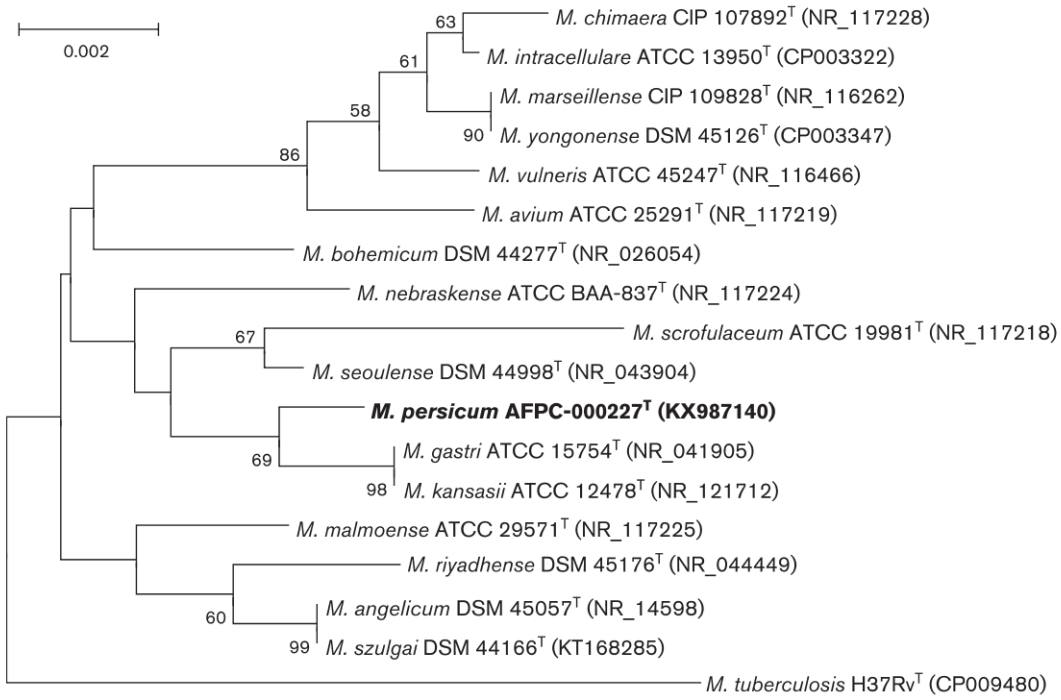

Fig. 2. Phylogenetic tree based on 16S rRNA gene sequences, reconstructed using the neighbor-joining method bootstrapped 1000 times. Bootstrap values >50 % are given at nodes. Bar, 0.002 substitutions per nucleotide position.

their inclusion in a groping for which we propose the name *M. kansasii* complex.

PCR restriction analysis [7] with the *Bst*EII enzyme produced a pattern compatible with *M. kansasii* (fragments of 231, 131 and 79 bp), while the restriction pattern with *Hae*III was characterized by three major fragments (127, 103 and 101 bp) not previously reported for *M. kansasii* or for any other mycobacterium (http://app.chuv.ch/prasite/index.html).

No strain with the genotypic features above has been reported so far. To clarify whether they represented a novel species, we determined the whole genome sequence of two of them (AFPC-000227^T and NTM371) and calculated their average nucleotide identity (ANI) [11] in

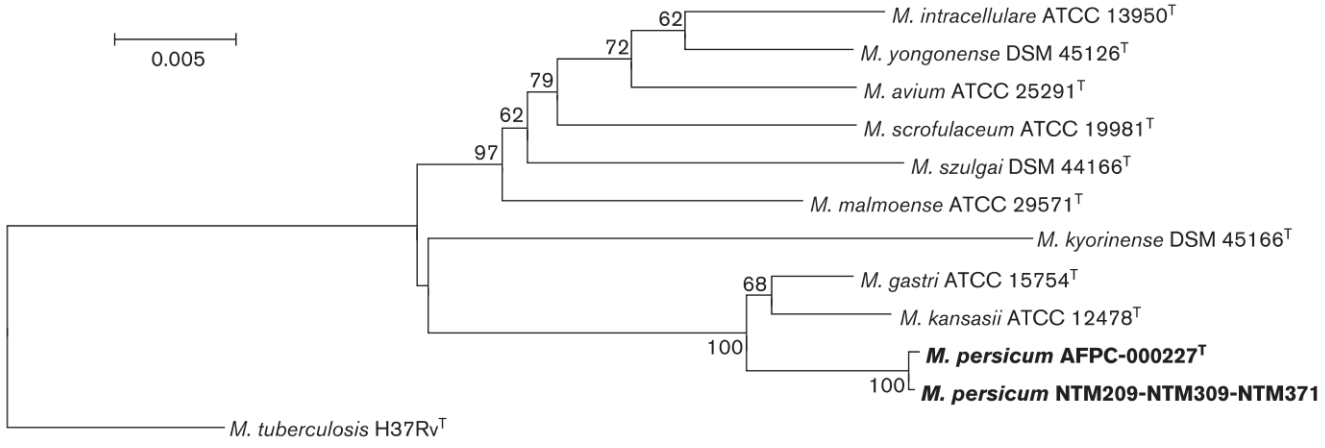

Fig. 3. Phylogenetic tree based on concatenated sequences of 16S rRNA, *hsp65* and *rpoB* genes, reconstructed using the neighbor-joining method bootstrapped 1000 times. Bootstrap values >50 % are given at nodes. Bar, 0.005 substitutions per nucleotide position.

Table 2. ANI values between *M. kansasii* ATCC 12478^T, *M. gastri* DSM 43505^T, AFPC-000207^T and NTM371; values <95–96 % characterize strains belonging to independent species

	***M. kansasii* ATCC 12478^T**	***M. gastri* DSM 43505^T**	**AFPC-000227^T**	**NTM371**
M. kansasii ATCC 12478^T				
M. gastri DSM 43505^T	91.44			
AFPC-000227^T	92.65	92.22		
NTM371	92.7	92.21	99.73	

comparison with *M. kansasii* ATCC 12478^T and *M. gastri* DSM 43505^T. The ANI between orthologous genome fragments [12] of the two test strains was, in comparison with *M. kansasii* ATCC 12478^T and *M. gastri* DSM 43505^T, clearly below the cutoff (95–96 %) while the ANI between them indicated that they belong to the same species (Table 2). The genome-to-genome distance [13], the equivalent *in silico* of DNA–DNA hybridization, confirmed the status of a species independent from both *M. kansasii* and *M. gastri* (Table 3).

On the basis of the data presented, the four strains are considered to represent a novel species of the genus *Mycobacterium*, for which the name *Mycobacterium persicum* sp. nov. is proposed.

DESCRIPTION OF *MYCOBACTERIUM PERSICUM* SP. NOV.

Mycobacterium persicum (per'si.cum. L. neut. adj. *persicum* of, or belonging to, Persia, the ancient name of Iran, from where the test strains originated).

Table 3. Genone-to-genome distance between *M. kansasii* ATCC 12478^T, *M. gastri* DSM 43505^T, AFPC-000207^T and NTM371; values >0.0170 characterize strains belonging to independent species (corresponding DNA–DNA hybridization values are given in parentheses)

	***M. kansasii* ATCC 12478^T**	***M. gastri* DSM 43505^T**	**AFPC-000227^T**	**NTM371**
M. kansasii ATCC 12478^T				
M. gastri DSM 43505^T	0.0860 (44.4 %)			
AFPC-000227^T	0.0756 (48.3 %)	0.0787 (47.1 %)		
NTM371	0.0757 (48.3 %)	0.0791	0.0029 (98.1%)	

The novel species is phenotypically barely differentiable from *M. kansasii* with which it shares growth rate (approximately 2 weeks at 37 °C), photochromogenicity, pattern of mycolic acids and most biochemical features with the exception of urease. Positive for nitrate reduction, catalase at 68 °C, Tween 80 hydrolysis and semi-quantitative catalase >45 mm but negative for niacin accumulation, urease, tellurite reduction and β-glucosidase. Susceptible *in vitro* to amikacin, clarithromycin, linezolid, moxifloxacin and rifamycins. In the 16S rRNA, *hsp65* and *rpoB* genes, although more closely related to *M. kansasii* and/or to *M. gastri*, it is distinguishable from these species. The PCR restriction pattern is unique. *M. persicum* is therefore a novel member of the *M. kansasii* complex along with *M. kansasii* and *M. gastri*.

The type strain, isolated from pulmonary specimens of Iranian patient with pulmonary disease, is AFPC-000227^T (=DSM 104278^T=CIP 111197^T). NTM209, NTM309 and NTM371 are additional strains of the species.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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