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# New polymorphisms within the variable number tandem repeat (VNTR) 7 locus of *Mycobacterium avium* subsp. *paratuberculosis*



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#### ABSTRACT

Variable number tandem repeat (VNTR) is a frequently employed typing method of *Mycobacterium avium paratuberculosis* (MAP) isolates. Based on whole genome sequencing in a previous study, allelic diversity at some VNTR loci seems to over- or under-estimate the actual phylogenetic variance among isolates. Interestingly, two closely related isolates on one farm showed polymorphism at the VNTR 7 locus, raising concerns about the misleading role that it might play in genotyping. We aimed to investigate the underlying basis of VNTR 7-polymorphism by analyzing sequence data for published genomes and field isolates of MAP and other *M. avium* complex (MAC) members. In contrast to MAP strains from cattle, strains from sheep displayed an "imperfect" repeat within VNTR 7, which was identical to respective allele types in other MAC genomes. Subspecies- and strain-specific single nucleotide polymorphisms (SNPs) and two novel (16 and 56 bp) repeats were detected. Given the combination of the three existing repeats, there are at least five different patterns for VNTR 7. The present findings highlight a higher polymorphism and probable instability of VNTR 7 locus that needs to be considered and challenged in future studies. Until then, sequencing of this locus in future studies is important to correctly assign the underlying allele types.<sup>1</sup>

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#### 1. Introduction

*Mycobacterium avium paratuberculosis* (MAP) is a member of the *M. avium* complex (MAC) and the causative agent of Johne's disease, one of the most economically important diseases in ruminants [1]. The disease is characterized by emaciation and chronic granulomatous enteritis and is suspected to play a role in Crohn's disease in humans [2]. Genotyping of these pathogens helps to better understand the epidemiology of disease and allows sources of infection to be identified, with an ultimate goal of designing more efficient control programs [3]. According to host preference, MAP was originally classified as cattle (C) and sheep (S) types. Later, pulsed-field gel electrophoresis (PFGE) was able to further classify S strains into type I and type III, while assigning solely type II to all C

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Nowadays, mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) genotyping is one of the most widely used typing methods of MAP isolates. This is a rapid PCR-based method that provides higher typing resolution within the main strains, and its results can be readily compared among laboratories using an online database [5].

Ahlstrom et al. [6] described some limitations of MIRU-VNTR typing compared with SNP analysis based on whole genome sequencing data, where MIRU-VNTR over- or under-estimated the phylogenetic variance among MAP isolates. This necessitates a re-evaluation of the loci included in the MIRU-VNTR typing scheme. Among the MIRU-VNTR loci used, polymorphism at the VNTR 7 locus in two closely related isolates from the same farm raises a key question as to the suitability of VNTR 7 for molecular epidemiology. In order to gain a deeper insight into the underlying basis of polymorphism at this locus, we aimed to investigate respective sequence data.





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<sup>&</sup>lt;sup>1</sup> *Note:* Partial VNTR 7 sequences reported here have been deposited in the GenBank database under accession numbers KT824780, KT833325, KT833326, KT833327 and KT833328.

#### 2. Materials and methods

#### 2.1. Analysis of the available MAP genomes

We used an online tool (http://insilico.ehu.es/PCR/index.php? mo=Mycobacterium) for *in silico* amplification of the VNTR 7 locus in MAP strain k10 (GenBank accession number: NC\_002944) using primers designed by Thibault et al. [7]. All homologous sequences of MAP (n = 31) and other MAC (n = 31) origins were obtained by BLAST N analysis and compared using an online multiple sequence alignment software (http://tcoffee.crg.cat/apps/ tcoffee/do:regular) hosted by the Centre for Genomic Regulation, Barcelona [8].

			*** **
*)gi	347311385	60	AAACAACGTCGCGCCGAGCGTGCACTGAG <mark>C</mark> GCGACAGATCG <mark>CGAAATATTCGCCGTGA</mark>
*)gi	653746667	60	AAACAACGTCGCGCCGAGCGTGCACTGAG <mark>C</mark> GCGACAGATC <mark>GCGAAATATTCGCCGTGA</mark>
*)gi	653746666	60	AAACAACGTCGCGCCGAGCGTGCACTGAG <mark>C</mark> GCGACAGATC <mark>C</mark> G <mark>CGAAATATTCGCCGTGA</mark>
*)gi	756081930	60	AAACAACGTCGCGCCGAGCGTGCACTGAG <mark>C</mark> GCGACAGATCGG <mark>CGAAATATTCGCCGTGA</mark>
*)gi	756072988	60	AAACAACGTCGCGCCGAGCGTGCACTGAGCGCGACAGATCGCCGAAATATTCGCCGTGA
*)gi	494536091	60	
*)gi	41400296  56/100158	60	AAACAACGICGCGCCGAGCGIGCACIGAGCGCGACAGAICGGCGAAAIAIICGCCGIGA
*) ai	1564164441	60	AACAACGTCGCGCCGAGCGTGCACTGAGCGCGACAGATCGGCGAAATATTCGCCGTGA
*) gi	564162510	60	AAACAACGTCGCGCCGAGCGTGCACTGAGCGCGACAGATCGGCGAAATATTCGCCGTGA
*)gi	365918223	60	AAACAACGTCGCGCCGAGCGTGCACTGAG <mark>C</mark> GCGACAGATC <mark>G</mark> CGAAATATTCGCCGTGA
*)gi	365915908	60	AAACAACGTCGCGCCGAGCGTGCACTGAG <mark>C</mark> GCGACAGATC <mark>GCGAAATATTCGCCGTGA</mark>
*)gi	365914711	60	AAACAACGTCGCGCCGAGCGTGCACTGAG <mark>C</mark> GCGACAGATC <mark>GCGAAATATTCGCCGTGA</mark>
*)gi	365913422	60	AAACAACGTCGCGCCGAGCGTGCACTGAG <mark>C</mark> GCGACAGATCGG <mark>CGAAATATTCGCCGTGA</mark>
*)gi	365914760	60	AAACAACGTCGCGCCGAGCGTGCACTGAGCGCGCGACAGATCGGCGAAATATTCGCCGTGA
*)gi *)gi	365914030	60	AAACAACGTCGCCGCCGAGCGTGCACTGAGCGCGACAGATCGGCGAAATATTCGCCGTGA
*)gi	1335993621	60	AAACAACGICGCGCCGAGCGIGCACIGAGCGCGACAGAICGGCGAAAIAIICGCCGIGA
*) ai	335991971	60	AAACAACGTCGCGCCGAGCGTGCACTGAGCGCGACAGATCGGCGAAATATTCGCCGTGA
*) qi	335989921	60	AAACAACGTCGCGCCGAGCGTGCACTGAGCGCGACAGATCGGCGAAATATTCGCCGTGA
*)gi	335989557	60	AAACAACGTCGCGCCGAGCGTGCACTGAG <mark>C</mark> GCGACAGATC <mark>G</mark> CGAAATATTCGCCGTGA
*)gi	334867551	60	AAACAACGTCGCGCCGAGCGTGCACTGAG <mark>C</mark> GCGACAGATC <mark>GCGAAATATTCGCCGTGA</mark>
*)gi	334905600	60	AAACAACGTCGCGCCGAGCGTGCACTGAG <mark>C</mark> GCGACAGATC <mark>GCGAAATATTCGCCGTGA</mark>
*)gi	564224505	60	AAACAACGTCGCGCCGAGCGTGCACTGAG <mark>C</mark> GCGACAGATCGG <mark>CGAAATATTCGCCGTGA</mark>
*)gi	347311386	60	AAACAACGTCGCGCCGAGCGTGCACTGAG <mark>C</mark> GCGACAGATC <mark>G</mark> CGAAATATTCGCCGTGA
#)gi	564210004	60	AAACAACGTCGCGCCGAGCGTGCACTGAG <mark>C</mark> GCGACAGATCAG <mark>CGAAATATTCGCCGTGA</mark>
#)gi	564172308	60	AAACAACGTCGCGCCGAGCGTGCACTGAG <mark>C</mark> GCGACAGATCAG <mark>CGAAATATTCGCCGTGA</mark>
#)gi	365912005	60	AAACAACGTCGCGCCGAGCGTGCACTGAG <mark>C</mark> GCGACAGATCAG <mark>CGAAATATTCGCCGTGA</mark>
#)gi	365911984	60	AAACAACGTCGCGCCGAGCGTGCACTGAG <mark>C</mark> GCGACAGATCAG <mark>CGAAATATTCGCCGTGA</mark>
#)gi	334868597	60	AAACAACGTCGCGCCGAGCGTGCACTGAG <mark>C</mark> GCGACAGATCAG <mark>CGAAATATTCGCCGTGA</mark>
#)gi	336459548	60	AAACAACGTCGCGCCGAGCGTGCACTGAG <mark>C</mark> GCGACAGATCAG <mark>CGAAATATTCGCCGTGA</mark>
^)gi	557950927	60	AAACAACGTCGCGCCGAGCGTGCACTGAGTGCGACAGATCAG <mark>CGAAATATTCGCCGTGA</mark>
^)gi	576372154	60	AAACAACGTCGCGCCGAGCGTGCACTGAGTGCGACAGATCAGCGAAATATTCGCCGTGA
^)gi	576381702	60	AAACAACGTCGCGCCGAGCGTGCACTGAGTGCGACAGATCAGCGAAATATTCGCCGTGA
^)gi	5/6354835	60	AAACAACGTCGCGCCGAGCGTGCACTGAGTGCGACAGATCAGCGAAATATTCGCCGTGA
)gi	564210907	60	AAACAACGTCGCGCCGAGCGTGCACTGAGTGCGACAGATCAGCGAAATATTCGCCGTGA
)gi	564190103	60	AAACAACGTCGCGCCGAGCGTGCACTGAGTGCGACAGATCAGCGAAATATTCGCCGTGA
~)gi	564150584	60	AAACAACGTCGCGCCGAGCGTGCACTGAGTGCGACAGATCAGCGAAATATTCGCCGTGA
) gi	642002204	60	
)gi	1042993304	60	AAACAACGICGCGCCGAGCGIGCACIGAGIGCGACAGAICAGCGAAAIAIICGCCGIGA
)gi	15763536600	60	AAACAACGICGCGCGAGCGIGCACIGAGIGCGACAGAICAGCGAAAIAIICGACGIGA
) gi	1576552591	60	AAACAACGICGCGCCGAGCGIGCACIGAGIGCGACAGAICAGCGAAAIAIICGACGIGA
) g I	1504150512	60	
)gi	576442216	60	
)gi	504172999	60	AAACAACGICGCGCCGAGCGIGCACIGAGIGCGACAGAICAGCGAAAIAIICGCCGIGA
)gi	1564210905	60	AAACAACGICGCGCCGAGCGIGCACIGAGIGCGACAGAICAGCGAAAIAIIICGCCGIGA
) gi	1564180504	60	AAACAACGICGCGCCGAGCGIGCACIGAGIGCGACAGAICAGCGAAAIAIIICGCCGIGA
/gr	1560163/93	60	AAACAACGICGCGCCGAGCGIGCACIGAGIGCGACAGAICAGCGAAAIAIIICGCCGIGA
/91 ^) gi	1560164440	60	
/91 ^) gi	1642994477	60	
/91 ^) gi	1643003469	60	
) gi	1365917474	60	AAACAACGTCGCGCGGGGGGGGCGCGGGGGGGGGGGGGG
^) gi	221741470	60	AAACAACGTCGCCGCGAGCGTGCACTGAGTGCGACAGATCAGCGAAAATATTCGCCGTGA
') di	1564226128	60	AAACAACGTCGCGCGAGCGTGCACTGAGTGCGACAGATCAGCGAAAATATTCGCCGTGA
) gi	1643004606	60	A A C A A C GT C C C C C C C C C C C C C C C C C
/91 ^) / (1	1508732639	60	A A C A A C GT C C C C C C C C C C C C C C C C C
/91 /) //i	1701188573	60	AAACAACGTCGCCGCGCGTGCACTGAGTGCGACAGATCAGCGAAAATATTCGCCGTGA
191 10(^	1690324916	60	AAACAACGTCGCCGCGAGCGTGCACTGAGTGCGACAGATCAGCGAAAATATTCGCCGTGA
-191 in(^	690305170	60	AAACAACGTCGCGCGAGCGTGCACTGAGTGCGACAGATCAGCGAAAATATTCGCCGTGA
') ni	1118163506	60	AAACAACGTCGCGCGGAGCGTGCACTGAGTGCGACAGATCAGCGAAAATATTCGCCGTGA
/91 ^)ai	625351599	60	AAACAACGTCGCGCCGAGCGTGCACTGAGTGCGACAGATCAGCGAAATATTCGCCGTGA

**Fig. 1.** Multiple sequence alignment of published partial sequences of VNTR 7 locus of *Mycobacterium avium* complex (MAC) origin (GenBank accession numbers are provided next to each sequence). In contrast to (MAP) cattle (C) genomes (one asterisk), which always displayed either one or two 22 bp perfect repeats (highlighted with red and green colors), all MAP sheep (S) genomes (#) had a 22 bp imperfect repeat with a length of 12 bp (highlighted in blue), which was identical to all other MAC genomes ( $^{-}$ ). A (C > T) SNP at position 89 bp (three asterisks) clearly differentiates between MAP and all other MAC isolates, while a (G > A) SNP at position 100 bp (Two asterisks) differentiates between MAP C and S types (positions were calculated relative to a 203 bp PCR product amplified usin primers described by Thibault et al. (2007). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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