RESEARCH NOTE

Identification of a new variable number tandem repeat locus in *Mycobacterium ulcerans* for potential strain discrimination among African isolates

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ABSTRACT

Intra-species discrimination in the highly clonal pathogen *Mycobacterium ulcerans* was studied in a diverse collection of isolates by PCR amplification of a short sequence repeat locus containing heterogeneous arrays of tri-nucleotide repeats with an ACC consensus pattern. Post-amplification analysis indicated excellent typeability and identified five *M. ulcerans* alleles, including a unique Angolan type differentiated for the first time among a genetically conserved cluster of African isolates. These results are consistent with previously investigated independent markers, and provide an additional locus for variable number tandem repeat-based typing of *M. ulcerans*.

Keywords Discrimination, *Mycobacterium ulcerans*, PCR, typing, variable number tandem repeat

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Mycobacterium ulcerans causes Buruli ulcer (BU), a necrotic skin disease that often results in loss of limbs or limb function, contracture deformities and oesteomyelitis. BU is the third most prevalent mycobacterial disease of public health importance, after tuberculosis and leprosy, in several tropical and sub-tropical countries [1,2]. *M. ulcerans* is a highly clonal species, with limited or no detectable genetic diversity among isolates within the same geographical region [3–13]. Together with the difficulty in isolating the bacterium from the environment, this hinders the determination of the local route(s) of transmission and identification of reservoir(s) of infection. About 16–28% of treated BU patients relapse [14], and in the absence of an effective epidemiological typing tool, it is difficult to know the extent to which relapse is caused by re-infection with a different strain or by reactivation of the previous infection. Minisatellite loci (tandem repeats (TRs) with a unit size >10 bp) containing variable number tandem repeats (VNTRs) have been identified as possible polymorphic targets for strain differentiation in *M. ulcerans*. Most notable has been the resolution of VNTR alleles among African isolates that are refractory to other genotyping techniques [12,13]. The contribution of another class of TRs, referred to as short sequence repeats (SSRs) or microsatellites (TRs with unit size < 10 bp), to genetic diversity in *M. ulcerans* has yet to be explored.

The present study investigated a SSR locus as a potential target for intra-species discrimination in *M. ulcerans* by determining variation in tandem copies of repeat units in a geographically diverse collection of isolates of this organism. The locus was initially identified *in silico* in the *M. ulcerans* genome sequence (http://genolist.pasteur.fr/BuruList/) using the TR Finder program of the Department of Biomathematical Science of Mount Sinai School of Medicine, New York, NY, USA (http://c3.biomaths.mssm.edu/trf.html), and contains 34 copies of heterogeneous arrays of tri-nucleotide repeat motifs with an ACC consensus pattern and 80% sequence identity.

Using the Artemis genome viewer and annotation tool, this repeat region was found to be located in MUL_0583, encoding a conserved threonine-rich hypothetical protein. PCR primers (forward, 5′-GCCGTTCCCCACCATAAC; reverse, 5′-ATCATCCAGCTTGCCCAT) were designed, based on the flanking sequences of the TR locus, using Oligo v.5.0 software (National Biosciences Inc., Plymouth, MN, USA).

The study investigated 57 clinical isolates of *M. ulcerans* cultured from tissue fragments of BU

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Table 1. Isolates of *Mycobacterium ulcerans* included in the study

Isolate	Year of isolation	Origin	No. of repeats
ITM 04-2767, ITM 04-2768, ITM 04-2769	2004	French Guiana	14
ITM 04-2770, ITM 04-2271	2004	French Guiana	14
ITM 7922	1990	French Guiana	14
ITM 842	1984	Surinam	14
ITM 96-0658	1996	Angola	28
ITM 04-1292	2004	PNG	31
ITM 03-524	2003	PNG	31
ITM 5150, ITM 5145, ITM 5122	1962	DRC	34
ITM 5149	1962	DRC	34
ITM 5146, ITM 9119	1963	DRC	34
ITM 5152	1975	DRC	34
ITM 5153	1995	DRC	34
ITM 5155	1976	DRC	34
ITM 9096	1979	DRC	34
ITM 03-0952	2003	DRC	34
Sequence strain (Agy99)	1999	Ghana	34
ITM 97-0483	1997	Ghana	34
ITM 97-0104, ITM 97-0680	1997	Benin	34
ITM 98-0239	1998	Benin	34
ITM 99-0826, ITM 99-1567	1999	Benin	34
ITM 00-0040, ITM 00-0358, ITM 00-0945	2000	Benin	34
ITM 00-1213, ITM 00-1240, ITM 00-1441	2000	Benin	34
ITM 01-0499	2001	Benin	34
ITM 02-0280, ITM 02-1081	2002	Benin	34
ITM 94-0622, ITM 94-0511	1994	Ivory Coast	34
ITM 02-0279	2002	Cameroon	34
ITM 9537	1971	PNG	34
ITM 94-1331	1994	PNG	34
ITM 5142	1967	Australia	34
ITM 9540	1978	Australia	34
ITM 9546	1988	Australia	34
ITM 9550	1983	Australia	34
ITM 94-339, ITM 94-1324, ITM 94-1327	1994	Australia	34
ITM 94-1328	1994	Australia	34
ITM 99-1642	1999	Australia	34
ITM 98-912	1998	China	34
ITM 8756	1980	Japan	34
ITM 94-1328	1994	Malaysia	34
ITM 9099	1964	DRC	45
ITM 5144	1961	DRC	45
ITM 5143	1961	Mexico	45

ITM, Institute for Tropical Medicine; DRC, Democratic Republic of Congo; PNG, Papua New Guinea.

patients. All of the isolates formed part of the culture collection of the Institute of Tropical Medicine, Antwerp, Belgium, and were selected to reflect both spatial and temporal diversity (Table 1). Methods and conditions used for the cultivation of *M. ulcerans*, extraction of DNA, PCR and sequencing of amplicons have been described previously [9,15].

PCR yielded amplification products from all isolates tested. Multiple sequence alignment revealed almost identical (except for a few nucleotide substitutions) flanking regions, and insertion and/or deletion mutations that were confined to the repeat region. However, in a few cases, some mutations were detected in the 5′ end of the repeat region (results not shown). The number of repeat units was determined and used as the allele designation for each isolate. Overall, the number of tri-nucleotide repeats varied from 14 to 45 copies, corresponding to five *M. ulcerans* alleles (Table 1). The majority of the isolates (including 30 of 33 isolates from five African countries, all nine isolates from Australia, and one isolate each from China, Japan, Malaysia and Papua New Guinea) had an identical allelic designation in this locus. Two of the three alleles identified among African isolates were not unique to the African continent, as they were also found in isolates from other geographical regions. The third allele, found in ITM 96-0658, an isolate from Angola, was unique (Table 1). Diversity was also evident among isolates from Papua New Guinea, with two alleles being discriminated. A final *M. ulcerans* type with the least number of repeat units (14) was represented by an isolate from Surinam and by six isolates from French Guiana. Point mutations in the repeat and flanking regions resulted in the discrimination of three sequence types, comprising isolates from Japan and China, isolates from Papua New Guinea, and those of African and Australian origin, within the cluster of isolates with 34 repeats.

The study focused on this locus because of the presence of a high copy number of repeats, with relatively high sequence identities, of the constituent repeat units in the genome of the *M. ulcerans* sequence strain. These two intrinsic features correlate with high mutation rates across TR loci [16], and a high level of intra-species discrimination within this locus was therefore expected. However, the level of resolution achieved was modest and was comparable to that obtained with other minisatellite loci in an overlapping set of isolates investigated previously. Nevertheless, the discrim-

Table 2. Profiles of *Mycobacterium ulcerans* of African origin[a]

	ML-VNTR loci									MIRU-VNTR loci								
	1	4	6	8	9	14	15	18	19	1	2	5	7	9	20	33	ST1	ACC
Profile A[b]	180	360	500	700	480	640	150	380	340	3	0	1	1	1	0	3	2	34
Profile B[c]	180	360	500	700	480	640	150	380	340	3	0	1	1	1	0	3	2	28

VNTR, variable number of tandem repeats.
[a]Numerals in the table are amplicon sizes (for multilocus (ML)-VNTR loci) and repeat copy numbers (for mycobacterial interspersed repetitive unit (MIRU)-VNTR, STI and ACC loci).
[b]Profile of 30 *M. ulcerans* isolates from 12 African countries.
[c]Profile of Angolan isolate (ITM 96-0658).

ination of the Angolan isolate was not achieved with previous methods, including 2426 PCR [4], multilocus sequence typing [5], IS*2404* restriction fragment length polymorphism [6], amplified fragment length polymorphism [7], IS*2404*-Mtb2 PCR [8], multilocus (ML)-VNTR [9], mycobacterial interspersed repetitive unit (MIRU)-VNTR [11] and ST1-VNTR [13], in which this isolate formed part of a single cluster designated in each respective assay as the African genotype (Table 2).

SSR regions have been exploited, especially in several highly clonal species, for studying phylogenetic relationships, and as targets for tracking epidemiological events, because of their high level of intra-species resolution. Examples include the TTC locus in *Mycobacterium leprae* [17], $(GTG)_5$ in *Mycobacterium tuberculosis* [18], and several mononucleotide, di-nucleotide and tri-nucleotide repeat sequences in *Yersinia pestis* [19], *Mycobacterium paratuberculosis* [20] and *Bacillus anthracis* [16]. The identification of allelic diversity in this locus in *M. ulcerans* is a promising step towards establishing the utility of an equivalent SSR-based VNTR typing approach for intra-species discrimination in this highly clonal species. Given the excellent typeability, concordance with previous independent markers, and the modest gain in discrimination, this study underscores the importance of investigating SSRs as potentially useful markers for molecular epidemiological studies of BU disease.

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