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Comparative Nucleotide Sequence Analysis of Polymorphic Variable-Number Tandem-Repeat Loci in *Mycobacterium ulcerans*

Anthony Ablordey,[1] Markus Hilty,[2] Pieter Stragier,[1] Jean Swings,[3,4] and Françoise Portaels[1]*

Mycobacteriology Unit, Institute of Tropical Medicine, B-2000 Antwerp, Belgium[1]; Swiss Tropical Institute, 4002, Basel, Switzerland[2]; and Laboratory of Microbiology[3] and BCCM/LMG Culture Collection,[4] University Ghent, B-9000 Ghent, Belgium

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We analyzed a set of variable-number tandem-repeat (VNTR) loci to assess their nucleotide sequence diversity in isolates of three *Mycobacterium ulcerans* genotypes. Sequence variants in two loci resulted in intraspecies resolution of Southeast Asian and Asian genotypes in contrast to a homogenous sequence composition among African isolates. Nucleotide sequence polymorphism in repeat units can enhance discrimination of VNTR loci.

Mycobacterium ulcerans causes Buruli ulcer, a necrotizing skin disease in tropical and subtropical regions (4, 10). The epidemiology of Buruli ulcer is poorly understood, due in part to the highly restricted genetic diversity in *M. ulcerans*, especially among isolates with common geographic origins (1, 2, 5, 6, 11, 13, 14), and also to the difficulty in obtaining cultures from environmental specimens (1, 10).

Tandem-repeat (TR) loci have enormous potential as highly evolving genomic regions suitable for typing species with low genetic diversity. Their use in molecular epidemiology studies have contributed significantly to the identification of sources of infection, a better understanding of disease transmission, and strain-trait correlations (8, 9, 12).

To investigate the potential of TRs in providing highly discriminatory markers for studying molecular diversity in *M. ulcerans*, we demonstrated allele-length polymorphism associated with nine variable-number tandem-repeat (VNTR) loci. This allowed inter- and intraspecies differentiation in a representative collection of *Mycobacterium marinum* and *M. ulcerans* (2). Intraspecies discrimination in *M. ulcerans* was, however, limited among isolates within the same geographic region (2). Different isolates from Africa, Southeast Asia, or Asia could not be distinguished by allele-length analysis, after PCR amplification of nine VNTR loci. Such isolates were also not distinguished by multilocus sequence typing (15), mycobacterial interspersed repetitive unit-VNTR typing (16), and IS*2404*-restriction fragment length polymorphism typing (5).

In this study, we carried out a comparative sequence analysis of the VNTR loci to further assess the contribution of nucleotide sequence polymorphism to allelic diversity in isolates belonging to the African, Southeast Asian and Asian *M. ulcerans* genotypes. The investigation involved sequence analysis of nine VNTR loci in three isolates (including sequence strain) belonging to the African genotype, and four loci (8, 9, 18, and

TABLE 1. VNTR profiles of *M. ulcerans* and *M. marinum*

Species	Isolates[a]	Origin	VNTR allelic profile (by locus no.)									VNTR/MLST/IS*2404*-RFLP[b] type	MIRU[c]-VNTR type
			1	4	6	8	9	14	15	18	19		
M. ulcerans	ITM 94-1324	Australia	1	2	1	3	3	1	1	1	2	Southeast Asian	Asian
	ITM 94-1328	Malaysia	1	2	1	3	3	1	1	1	2	Southeast Asian	Asian
	ITM 94-1331	Papua New Guinea	1	2	1	3	3	1	1	1	2	Southeast Asian	Asian
	ITM 98-912	China	1	2	2	3	4	3	1	2	4	Asian	Asian
	ITM 8756	Japan	1	2	2	3	4	3	1	2	4	Asian	Asian
	ITM 97-658	Angola	1	1	1	3	2	1	1	1	2	African	African
	ITM 97-104	Benin	1	1	1	3	2	1	1	1	2	African	African
	Sequence strain	Ghana	1	1	1	3	2	1	1	1	2	African	African
	ITM 842	Surinam	2	1	1	1	2	2	2	1	3		
M. marinum	Sequence strain		5	4	5	2	3	4	3	2	9		

[a] The profile of the Surinam type was included to indicate polymorphism, at loci 1, 8, and 15. ITM, Institute of Tropical Medicine.
[b] MLST, multilocus sequence typing (15); RFLP, restriction fragment length polymorphism (5).
[c] MIRU, mycobacterial interspersed repetitive unit (16).

* Corresponding author. Mailing address: Mycobacteriology Unit, Prince Leopold Institute of Tropical Medicine, B-2000 Antwerp, Belgium. Phone: 32-3-247-63-17. Fax: 32-3-247-63-33. E-mail: portaels@itg.be.

TABLE 2. Multiple sequence alignment of repeat unit[a]

Locus	Sequence	Variant	Species occurring
1	ATCGCCCGACTCCTCCTCCGGCCTCACCGGCCGGTATCGTCGCCGCGCACCACCCCA	A_1	MM
	.....C.....---------	B_1	MU
	.....C.....	C_1	MM
	.....T.....	D_1	MM
	.....T.....---------	E_1	MM
4	GGTCGCCTCGCTCCCATCACTCGCCAAGCTCGCTCTGCTCGCTCGGCTCCCAAACCCAACA	A_4	MM
	.....C.....	B_4	MM
	.....GG.....--	C_4	MU
	.....GG.....	D_4	MM
	.....---------	E_4	MM
6	GTGGTGGTCGCGAAACCGGCGAAGCCGGGCGAAGCGGGCCACCACCGACAAGCCCC	A_6	MM
	.....----------	B_6	MM
	.....T.....----------	C_6	MU
	----------.....	D_6	MM
8	AGTGGTGACCGCCAGCGCGGCGGGGAGCCGGGCGCAGCGGGTCGCCACCATCAAATCC	A_8	MM/MU
	.....A.....	B_8	MU
	.....A.....	C_8	MU
9	GTGGCGATCGCAAGCGCGGCCCAGCCGGGGGCAGCGGGTCGCCACCAAGGTGGCGGC	A_9	MM/MU
	.....----------	B_9	MM
	.....T....T.....---------.T....----------	C_9	MU
	.....T....T.....---------.T....----------	D_9	MU
	.....G.....T.....	E_9	MU
	.G.....T....T.....---------.....----------	F_9	MU
14	GCCCTCGGTCGCGACCCGCCGCGCCCGGCTCCGCCGCGCTCGCGATCGCTCCAC	A_{14}	MM
	.....A.....	B_{24}	MM
	.....----------	C_{14}	MM
	.A.....----------	D_{14}	MU
15	AGCCGGCTCCGCTCAGCCGGCTCCGGCTCAATTCGCCGACTTCGCTCGCCGGCC	A_{15}	MM
	.....----------	B_{15}	MM
	..A.....----------	C_{15}	MU
18	CCGGTTCCCCCGGTATCACCAGTACCGCTCCCCGTACCACCCGTATCACCGGTACCGCCGCTC	A_{18}	MM
	...T.G.....C.....	B_{18}	MU
	..TT.G.....CGGCACC.....TGGC..C.....	C_{18}	MU
	.GT.AC.....CGGCAC.....TGGC.ATGGTGGTG..	D_{18}	MU
	.GTT.G.....TGGC.ATGGT-G....	E_{18}	MU
	.G..GA.....GG..C.GG..A.....GTG.....C....CTG...CTG.TG.TGA	F_{18}	MU
	.G..GA.....C.GG..C.G.....GGTG.....C....CTG...C.G.TG....	G_{18}	MU
	...TAG....C.TGG.G.TA..GG...A.....A....GAA.CGG.G.....G..G.---..T	H_{18}	MU
	...T.G....A.TGG.G.T...G....A.....A....GAA.CGG.G.....G..G.---..T	I_{18}	MU
19	GGGGATCGCAAGCCCGGCGACGCCGGGCGCCGCGGGTCACCACCAACAATTCCCGC	A_{19}	MM
	.....G.....	B_{19}	MM
	.....T	C_{19}	MM
	.....T...C.....	D_{19}	MM
	.....G.....GA..-------	E_{19}	MM
	.....A.....G.....T...C.....	F_{19}	MU
	.....G.....T...C.....	G_{19}	MU
	.....G.....----------	H_{19}	MU
	.....GC.....A.....G.....	I_{19}	MU
	.....C.....	J_{19}	MU
	.....G.....----------	K_{19}	MU

[a] −, base deletion; ., identical nucleotide position; MM, *M. marinum*; MU, *M. ulcerans*.

19) in isolates of the Asian and Southeast Asian type (Table 1). The African isolates were of Angolan, Beninese, and Ghanaian (sequence strain) origins. The Southeast Asian genotype comprises isolates of Australian, Papua New Guinean, and Malaysian origins, while isolates from Japan and China formed the Asian genotype. All *M. ulcerans* isolates were subcultured (from frozen stocks of the collection of the Institute of Tropical Medicine, Antwerp [ITM]) onto Löwenstein-Jensen medium and incubated at 32°C for 4 weeks. Isolates were further characterized phenotypically and tested for the presence of

TABLE 3. Sequence profiles of *M. ulcerans* isolates and the *M. marinum* sequence strain

Locus	Sequence profile
1	
M. marinum (sequence strain)	$A_1C_1C_1D_1E_1$
M. ulcerans (sequence strain)	B_1
ITM 96-658	B_1
ITM 97-104	B_1
4	
M. marinum (sequence strain)	$A_4B_4D_4E_4$
M. ulcerans (sequence strain)	C_4
ITM 96-658	C_4
ITM 97-104	C_4
6	
M. marinum (sequence strain)	$A_6A_6A_6B_6D_6$
M. ulcerans (sequence strain)	C_6
ITM 96-658	C_6
ITM 97-104	C_6
8	
M. marinum (sequence strain)	A_8A_8
M. ulcerans (sequence strain)	$A_8A_8B_8$
ITM 96-658	$A_8A_8B_8$
ITM 97-104	$A_8A_8B_8$
ITM 94-1324	$A_8A_8B_8$
ITM 94-1328	$A_8A_8B_8$
ITM 94-1331	$A_8A_8B_8$
ITM 8756	$A_8C_8C_8$
ITM 98-912	$A_8C_8C_8$
9	
M. marinum (sequence strain)	$A_9A_9B_9$
M. ulcerans (sequence strain)	A_9C_9
ITM 96-658	A_9C_9
ITM 97-104	A_9C_9
ITM 94-1324	$A_9A_9D_9$
ITM 94-1328	$A_9A_9C_9$
ITM 94-1331	$A_9A_9D_9$
ITM 8756	$A_9A_9E_9F_9$
ITM 98-912	$A_9A_9E_9F_9$
14	
M. marinum (sequence strain)	$A_{14}A_{14}B_{14}C_{14}$
M. ulcerans (sequence strain)	D_{14}
ITM 96-658	D_{14}
ITM 97-104	D_{14}
15	
M. marinum (sequence strain)	$A_{15}A_{15}B_{15}$
M. ulcerans (sequence strain)	C_{15}
ITM 96-658	C_{15}
ITM 97-104	C_{15}
18	
M. marinum (sequence strain)	$A_{18}A_{18}$
M. ulcerans (sequence strain)	B_{18}
ITM 96-658	B_{18}
ITM 97-104	B_{18}
ITM 94-1324	C_{18}
ITM 94-1328	D_{18}
ITM 94-1331	E_{18}
ITM 8756	$F_{18}H_{18}$
ITM 98-912	$G_{18}I_{18}$
19	
M. marinum (sequence strain)	$A_{19}B_{19}B_{19}B_{19}C_{19}C_{19}D_{19}D_{19}E_{19}$
M. ulcerans (sequence strain)	$F_{19}G_{19}H_{19}$
ITM 96-658	$F_{19}G_{19}H_{19}$
ITM 97-104	$F_{19}G_{19}H_{19}$
ITM 94-1324	$F_{19}H_{19}$
ITM 94-1328	$F_{19}H_{19}$
ITM 94-1331	$F_{19}H_{19}$
ITM 8756	$F_{19}I_{19}J_{19}K_{19}$
ITM 98-912	$F_{19}I_{19}J_{19}K_{19}$

IS*2404* and IS*2606* insertion sequences as previously described (13, 18).

TR loci were bioinformatically identified by applying the TR Finder algorithm on *M. marinum* genome sequences (available *M. ulcerans* genome sequences not accessible for TR Finder analysis), which also generated a consensus pattern for each locus. Details of TR discovery, DNA extraction, PCR primers, and amplification conditions have been previously described (2). Purified PCR products were sequenced by using the ABI 310 genetic analysis system.

For each locus, TR sequences of the different isolates were aligned and compared with the consensus pattern. All nine loci were found to consist of heterogeneous arrays of repeat units (variants) with deletions and/or nucleotide substitutions (Table 2). Locus 8 was the most conserved in both species, with no nucleotide deletion and two substitutions in sequence variants among all *M. ulcerans* isolates.

For each locus, the individual repeat variants were assigned designations (Table 2). While some repeat variants were found exclusively either in *M. ulcerans* (e.g., G_{19}, H_{19}, or D_{18}) or in *M. marinum* (e.g., A_{18} or B_{19}), others variant occurred in both species (e.g., A_8 or A_9).

Sequence profiles were generated at each locus for the isolates by combining these designations. Comparison of the sequence profiles (which defines an allele at a given locus) facilitates the identification of sequence types (Table 3).

Among the African isolates, corresponding loci featured 100% TR sequence identity; consequently, intraspecies differentiation within this genotype was not possible. Among Southeast Asian isolates, nucleotide sequence homology was complete in all except for loci 9 and 18, for which point mutations resulted in different allelic states. In locus 9, a single-nucleotide deletion in a repeat variant in the Malaysian isolate (ITM 94-1328, with profile $A_9A_9C_9$) differentiated it from the Australian isolate (ITM 94-1324) and Papua New Guinean isolate (ITM 94-1331), both with the $A_9A_9D_9$ sequence profile. Each of the isolates, however, harbored a unique sequence variant at locus 18 (D_{18}, C_{18}, and E_{18}, respectively, for isolates ITM 94-1328, ITM 94-1324, and ITM 94-1331), permitting the complete resolution of the Southeast Asia genotype. Locus 18 also resolved the Asian type into China and Japan genotypes (Table 3).

Although polymorphism at TR loci can occur either as a result of variation in the number of repeat units (length polymorphism) or as a result of nucleotide sequence changes between individual repeat units (sequence polymorphism) (12), the practical ease and lower cost of analyzing length polymorphism (by agarose gel electrophoresis) over sequencing have promoted the use of the former approach for routine typing purposes. Few studies on sequence polymorphism in TR loci have yielded mixed results. While some studies have indicated incremental gain in strain discrimination when length polymorphism data were complemented with sequence analysis (3, 7), this has not been realized in others (9, 17).

In this study, we showed the occurrence of sequence polymorphism in two TR loci, which exhibit no length polymorphism among isolates of two *M. ulcerans* genotypes. A general trend of TR sequence conservation in isolates from the same geographic region was noticed. This was most pronounced among the African isolates, which displayed complete se-

quence homology across the nine VNTR loci. Consistent with previous data (1, 2, 5, 6, 11, 14–16), the lack of sequence variants in this investigation further emphasizes the clonal homogeneity and recent evolutionary origin and distribution of the African genotype (15).

In contrast, sequence analysis revealed three Southeast Asian alleles and two alleles within the Asian genotype. Notably, the discrimination of these genotypes corroborates the data from IS*2404*-Mtb2 PCR (which differentiates between the isolates from China and Japan and also among the three Southeast Asian isolates) (1) and 2426 PCR (14), which discriminates among the Southeast Asian but not between the Asian isolates. Isolates of these two genotypes show limited differences in their repetitive-sequence-based PCR profiles. Differences in their VNTR sequence profiles therefore are significant in further highlighting differences among these isolates. A combination of the sequence and length polymorphism data results in a total of 11 *M. ulcerans* alleles compared to 8 indexed by length polymorphism analysis alone and 10 alleles by IS*2404*-Mtb2 PCR on the same set of isolates. The conservation of TR loci in the two *Mycobacterium* species and with much sequence degeneration in *M. ulcerans* is consistent with the proposed origin of *M. ulcerans* from *M. marinum* through a reductive genome evolution (15).

Sequence polymorphisms among *M. ulcerans* isolates involved single-nucleotide substitutions and microdeletions. For clonal organisms, and also across VNTR loci, such point mutations are often not considered major sources of genetic variation among isolates. However, data accruing from whole-genome sequence analyses of a number of organisms and also from sequence analysis of several genetic markers indicate that even in highly clonal species like *Mycobacterium tuberculosis*, *Bacillus anthracis*, and *Yersinia pestis*, many thousands of point mutations can be discovered when large portions of genomes are investigated (8). This theme is thus further reinforced by sequence data from this investigation. Complementation of sequence and length polymorphism data should potentially increase the discriminatory power of the VNTR-typing method. It is envisaged that this approach would be more useful for genotyping *M. ulcerans* and other highly monomorphic species.

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