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Multilocus Variable-Number Tandem Repeat Typing of *Mycobacterium ulcerans*

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The apparent genetic homogeneity of *Mycobacterium ulcerans* contributes to the poorly understood epidemiology of *M. ulcerans* infection. Here, we report the identification of variable number tandem repeat (VNTR) sequences as novel polymorphic elements in the genome of this species. A total of 19 potential VNTR loci identified in the closely related *M. marinum* genome sequence were screened in a collection of 23 *M. ulcerans* isolates, one *Mycobacterium* species referred to here as an intermediate species, and five *M. marinum* strains. Nine of the 19 loci were polymorphic in the three species (including the intermediate species) and revealed eight *M. ulcerans* and five *M. marinum* genotypes. The results from the VNTR analysis corroborated the genetic relationships of *M. ulcerans* isolates from various geographical origins, as defined by independent molecular markers. Although these results further highlight the extremely high clonal homogeneity within certain geographic regions, we report for the first time the discrimination of the two South American strains from Surinam and French Guyana. These findings support the potential of a VNTR-based genotyping method for strain discrimination within *M. ulcerans* and *M. marinum*.

Buruli ulcer (BU) is an emerging necrotic skin disease caused by *Mycobacterium ulcerans*. Several rural communities in West Africa have recorded dramatic increases in disease incidence over the past decade (7, 12). In some areas in West Africa, BU has replaced tuberculosis and leprosy as the most prevalent mycobacterial disease (1, 7). The foci of BU disease are usually associated with communities living near slow-flowing waters and swamps. On the basis of epidemiological observations it is therefore suggested that aquatic environs could be sources of *M. ulcerans*. Although several attempts to recover *M. ulcerans* in pure culture from the environment have remained unsuccessful (14), the identification of *M. ulcerans*-specific DNA sequences in water and detritus in Australia (18, 23) and in aquatic insects in Ghana and Benin (15), along with recent evidence for association of this mycobacterium with aquatic plants in Ivory Coast (11), suggests that *M. ulcerans* is an environmental pathogen.

Other molecular techniques have been applied with relatively modest gains in further unraveling aspects of the epidemiology of the disease. The genetic relationships among *M. ulcerans* strains from different geographical regions have been inferred from comparisons of insertion sequences and 16S rRNA, housekeeping, and structural gene sequences. These markers have indicated restricted genetic variation in *M. ulcerans*, especially among strains from the same geographic origin (5, 16, 21, 22), strongly hampering the determination of the sources of infection at a local level.

Tandem repeat (TR) DNA sequences are important sources of polymorphism in the genome of many eukaryotes and prokaryotes. Genomic regions showing polymorphism due to different numbers of TR motifs in different strains or individuals are described as variable number tandem repeat (VNTR) loci. Allele-length polymorphism associated with VNTR loci can be indexed by PCR amplification. Especially in highly monomorphic and clonal species such as *Mycobacterium tuberculosis* (8, 13, 17, 19, 20, 25), *Bacillus anthracis* (9), and *Yersinia pestis* (10), VNTR arrays represent polymorphism islets in the otherwise highly conserved genomic backbones and have therefore been very useful in differentiating and studying the genetic relationships of strains of these species.

The main objective of this study was to investigate the existence of VNTR loci in *M. ulcerans* and to assess their utility for the discrimination of strains of this organism.

MATERIALS AND METHODS

Bacterial strains and DNA preparation. A total of 23 *M. ulcerans* isolates, 5 *M. marinum* isolates, and one mycobacterial strain (ITM 00-1026) referred to as an intermediate species (4) were used in this study (Table 1). All isolates were from the culture collection of the Institute of Tropical Medicine, Antwerp (Antwerp, Belgium). The *M. ulcerans* isolates were recovered from tissue fragments of patients suffering from BU. Among the *M. marinum* isolates, two were obtained from granulomatous lesions of patients from Belgium, one was obtained from a fish from Nicaragua, and one (ITM 7732) was obtained from a fish and one (ITM 1717) was obtained from an armadillo in the United States. The isolate from the intermediate species was recovered from a granulomatous lesion of a French patient (4).

DNA was extracted as described previously (26). Briefly, bacterial suspensions were prepared by scraping three to four loopfuls of colonies in TE (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) and digestion with lysozyme (1 mg/ml). After proteinase K (0.1 mg/ml) and sodium dodecyl sulfate (1%) treatment, the suspensions were incubated with 0.6 M NaCl and 0.27 M *N*-acetyl-*N*,*N*-trimethyl ammonium bromide. The DNA was extracted with chloroform-isoamyl alcohol and precipitated with isopropanol. Alternatively, the DNA was obtained by resuspending bacteria into 100 to 200 μl of TE followed by heat inactivation at

TABLE 1. Strain information

Species	Strain[a]	Origin[b]	Received from[c] (other strain designation)	RFLP type[d]	2426 type[e]	MLST type[f]	VNTR type
M. ulcerans	ITM 5142	Australia	ATCC19423	Australian	Victorian	Victorian	Australian
	ITM 5147	Australia	J. L. Stanford	Australian	ND	ND	Australian
	ITM 94-1324	Australia	176862	ND	ND	ND	SE Asian
	ITM 9537	PNG	D. Dawson (11878/70)	ND	PNG I	SE Asian	PNG
	ITM 94-1331	PNG	D. Dawson (186395)	S. Asia	PNG II	SE Asian	SE Asian
	ITM 94-1328	Malaysia	D. Dawson (186510)	S. Asia	Malaysian	SE Asian	SE Asian
	ITM 98-912	China	W. R. Faber	Asian	Asian	Asian	Asian
	ITM 8756	Japan	ATCC 33728	Asian	Asian	Asian	Asian
	ITM 5114	Mexico	P. Lavalle	ND	Mexican	Mexican	Mexican
	ITM 5143	Mexico	F. Portaels	Mexican	Mexican	Mexican	Mexican
	ITM 7922	F. Guyana	F. Portaels	S. American	ND	ND	F. Guyana
	ITM 842	Surinam	F. Portaels	S. American	Surinamese	Surinamese	Surinamese
	ITM 96-658	Angola	F. Portaels	ND	African	African	African
	ITM 97-111	Benin	F. Portaels	ND	African	African	African
	ITM 97-483	Benin	F. Portaels	African	ND	African	African
	ITM 97-104	Benin	F. Portaels	African	ND	ND	African
	ITM 5152	DRC	F. Portaels	ND	African	African	African
	ITM 5155	DRC	F. Portaels	ND	ND	ND	African
	ITM 97-610	Ghana	F. Portaels	ND	African	African	African
	ITM 97-684	Benin	F. Portaels	ND	ND	ND	African
	ITM 97-680	Togo	F. Portaels	African	African	African	African
	ITM 94-662	Iv. Coast	F. Portaels	ND	ND	ND	African
	ITM 94-511	Iv. Coast	F. Portaels	African	ND	ND	African
M. marinum	ITM 8022	Belgium	F. Portaels	NA	NA	ND	1
	ITM 94-56	Belgium	F. Portaels	NA	NA	ND	2
	ITM 1320	Nicaragua	F. Portaels	NA	NA	ND	3
	ITM 1717	USA	F. Portaels	NA	NA	ND	4
	ITM 7732	USA	ATCC 927	NA	NA	ND	5
Intermediate	ITM 00-1026	France	F. Portaels	ND	ND	ND	MI

[a] ITM, Institute for Tropical Medicine.
[b] PNG, Papua New Guinea; DRC, Democratic Republic of Congo; Iv. Coast, Ivory Coast; USA, United States; F. Guyana, French Guyana.
[c] F. Portaels, Institute of Tropical Medicine, Antwerp, Belgium; J. L. Stanford, School of Pathology, London, United Kingdom; D: Dawson, Laboratory of Microbiology and Pathology, Queensland Health, Brisbane, Australia; P. Lavalle, Centro Dermatologico Pascua, Mexico City, Mexico.
[d] RFLP, genotype designation determined by IS*2404* restriction fragment length polymorphism typing (6). S. Asian, South Asian; S. American, South American. NA, not applicable. ND, not done.
[e] 2426 type, genotype designation determined by *2426*-PCR (22). PNG I and PNG II, Papua New Guinea 2426 types I and II, respectively.
[f] MLST, genotype designation determined by multilocus sequence typing (21). SE Asian, Southeast Asian.

100°C for 10 min and by subsequent centrifugation (10,000 × *g* for 20 min at 4°C) to remove cellular debris.

Location of tandem repeats in *M. marinum* and *M ulcerans*. The unassembled *M. marinum* genome sequence available as of August 2003 at the Sanger Centre website (http://www.sanger.ac.uk/Projects/M_marinum/) was screened for the presence of TR loci by using the Tandem Repeat Finder program of the Department of Biomathematical Science of Mount Sinai School of Medicine (http://c3 .biomath.mssm.edu/trf.html).

BLAST searches were performed on the *M. ulcerans* genome sequences available as of August 2003 on the BURULIST Web server of the Pasteur Institute (http://genopole.pasteur.fr/Mulc/BuruList.html) to establish the presence of homologous TR loci in the *M. ulcerans* genome sequence. The sequences were also aligned with the *M. tuberculosis* genome sequence available on the TUBERCU-LIST Web server (http://genolist.pasteur.fr/TubercuList/).

VNTR PCR. The primers for PCR amplification (Table 2) were designed on the basis of the flanking sequences of each TR locus of *M. marinum* by the use of Oligo 5.0 software (National Biosciences). The PCR was carried out using a Hotstar Taq DNA polymerase kit (QIAGEN). Sample DNA (purified or crude extract) (3 μl) was added to 27 μl of a PCR mix containing 15.5 μl of water, 3 μl of 10× PCR buffer (containing 1.5 mM MgCl$_2$ at the final concentration), 0.4 μM of each primer, 0.2 mM of each deoxynucleotide triphosphate (Roche), 1× Q-solution, and 0.1 μl of HotstarTaq DNA polymerase (0.5 U).

The PCRs were run on a PTC 100 thermocycler (MJ Research, Waltham, Mass.) at 95°C for 15 min, followed by 40 cycles of 94°C for 30 s, 59°C for 1 min, and 72°C for 1 min 30 s and a final extension at 72°C for 10 min. A total of 3 μl of the PCR products was electrophoretically separated using a 3% small-fragment agarose gel (Eurogentec, Seraing, Belgium) in 0.5× TAE (20 mM Tris-acetate, 0.5 mM EDTA at the final concentration) buffer at 100 V, and the gel

was then stained with ethidium bromide. The sizes of the amplicons were estimated by comparison with a 50- and 100-bp stepladder (Promega, Leiden, The Netherlands).

Genetic distance analysis. The analysis of the genetic relationships on the basis of the VNTR amplicon size profiles was performed using the neighbor-joining algorithm included in the PAUP software package (D. Swofford, phylogenetic analysis using parsimony, 4.0 beta version; Sinauer Associates, Inc., Sunderland, Mass.).

RESULTS

Location of tandem repeat loci in *M. marinum* and *M ulcerans*. The presence of TR loci was initially investigated in sequences of the closely related *M. marinum* genome (21), because the *M. ulcerans* genome sequences were not directly accessible for Tandem Repeat Finder analysis. Numerous tandem repeat loci of different sizes were found in the *M. marinum* genome. We focused on TRs of the minisatellite category, classically defined by a repeat unit size between 10 and 100 bp. This choice was motivated by the relative ease with which allelic differences can be resolved by agarose gel electrophoresis and by the relatively large range of variability of sequences of this type in *M. tuberculosis* (13, 25). Among the 59 TR loci with period size ranges between 40 and 100 bp identified, 19

TABLE 2. Characteristics of VNTR loci in *M. marinum* and *M. ulcerans*

Locus	Primer sequence (5′ to 3′)[a]	Repeat length (bp)[b]	ORF[c]	Function[d]
1	F GGCAGTGGGTGACGTCTCAGT	57	Rv2188c	Glycosyltransferase
	R TCGAGGCGATCTACACCAAGGATTA		FadD15	Long-chain fatty acid coenzyme A ligase
4	F GCCTTGCTTACCGTCGTGCCAA	61	Rv1864c	Conserved hypothetical protein
	R CGAGCCAAGTTGGACCGTCAACACAT		NF	NA
6	F GACCGTCATGTCGTTCGATCCTAGT	56	PfkA	Phosphofructokinase
	R GACATCGAAGAGGTGTGCCGTCT		gatB	Glutamyl-tRNA amidotransferase
8	F CGGATGACGTCGGAACTCTGA	58	cobB	Cobalamine biosynthesis
	R GGACGCGGTAGCACGTTTTGT		cysG	Cobalamine biosynthesis
9	F GGTGGATCTCCGCGTCATTTG	57	Rv3200c	Transmembrane cation transporter
	R CGACCGCCCTCGAGACAG		nudC	NADP pyrophosphate
10	F ACAAGCCACGGCGAGATATAG	71	NF	NA
	R GCGGGGCTTTTATCTGCTTA		NF	NA
13	F CAGGTATTCCAGGAGATCAAA	53	RegX3	TCS regulator
	R GGCGACAAGGCTCGTT		SenX3	TCS sensory protein
14	F CCTTGTATCCGAGTTTCAGTT	54	fgd1	Glucose-6 phosphate dehydrogenase
	R GTCGACCAGATATGAGCAAT		Rv0406c	β-lactamase-like protein
15	F GCCACCGGTCAGGTCAGGTT	54	MgtE	Mg2+ transmembrane transporter
	R TCACCAACTACGACGGCGTTC		fbA	Fructose biphosphate aldolase
16	F CCAACGCTCCCCCAACCAT	59	NF	NA
	R GCTCACAGGCCTTCGCTCAGA		Rv0238	Transcriptional regulator
18	F CCCGGAATTGCTGATCGTGTA	63	NF	NA
	R GGTGCGCAGACTGGGTCTTA		NF	NA
19	F CCGACGGATGAATCTGTAGGT	56	DeaD	Cold shock protein
	R TGGCGACGATCGAGTCTC		IprE	Lipoprotein

[a] F, forward primer, R, reverse primer.
[b] For a full length consensus repeat in *M. marinum*. Lengths could not be determined in several loci of the *M. ulcerans* sequence strain that only include a single copy of repeat element.
[c] ORF, homologous open reading frame in *M. tuberculosis* H37Rv genome. NF, not found.
[d] As predicted from Tuberculist. TCS, two-component system; NA, not applicable.

loci with more than 95% nucleotide identity between individual repeat units and with two or more copies of repeats were selected for experimental analysis. These two criteria were used on the basis of the observation that the presence of at least two identical or nearly identical repeats is necessary and sufficient to generate TR variability in the case of *M. tuberculosis* minisatellites (25).

The loci were given numerical labels 1 to 19. A total of 13 of them were found to be polymorphic in a small collection of *M. marinum* strains. Table 2 shows their locus information. Most of them, with the exception of locus 18, contained repeats with complete consensus sizes plus one partial repeat, with lengths ranging from half to nearly complete units. An alignment of the sequences of each locus with the homologous sequences in the *M. ulcerans* genome revealed complete identity of TR unit sequences among both mycobacterial species at loci 8, 9, and 14. In the other loci, there were nucleotide deletions of from 2 to 17 bp and/or from 2 to 14 nucleotide substitutions in the *M. ulcerans* units compared to those of *M. marinum*. The sequences flanking the repeat sequences were found to be highly conserved in both species and homologous to *M. tuberculosis* open reading frames encoding products of diverse functional categories (Table 2). Most of the corresponding TR sequences were found in intergenic positions in these regions. At least one of these homologous regions (locus 13) corresponds to a known VNTR locus in *M. tuberculosis*, namely, the *senX3-regX3* locus (25).

Typing of strains and cluster analysis. The size polymorphism of the 19 TR loci was analyzed by PCR using a collection of 23 *M. ulcerans* isolates, 5 *M. marinum* isolates, and an intermediate isolate. This latter strain contains a low copy num-

ber of the *M. ulcerans*-specific insertion sequence IS*2404*, while many other of its phenotypic and genetic characteristics are similar to those of *M. marinum* (4). The amplification was robust and yielded reproducible results for all loci and all isolates. A total of 13 of these loci were polymorphic among the *M. marinum* control strains; nine were also polymorphic in *M. ulcerans* (Fig. 1). (Only the results obtained for the nine polymorphic loci in both species are shown).

For the *M. ulcerans* reference strain (ATCC 19423), the experimentally determined sizes were consistent with the predicted sizes determined from the sequence, except for loci 4, 8, and 14. These discrepancies in the amplicon sizes could be due to inaccuracies in the incomplete *M. ulcerans* genome sequence available. In view of these discrepancies, the differences in repeat unit sizes between *M. ulcerans* and *M. marinum*, and the apparent involvement of incomplete repeat units in the variation in some loci (see below), we used the experimentally determined amplicon sizes (Fig. 1) instead of repeat copy numbers to generate profiles for indexing strain differences. For each locus, amplicon sizes of the different strains were found in most cases to differ by nearly exact multiples of complete repeat unit lengths predicted for the respective loci in *M. ulcerans* and *M. marinum*. However, smaller differences in amplicon sizes were also observed in some loci, suggesting the involvement of shorter repeat variants in the variability of these loci. These features are consistent with polymorphisms associated with VNTRs.

Eight different *M. ulcerans* genotypes, five *M. marinum* genotypes, and a unique intermediate strain genotype were found by VNTR typing (Fig. 1). Although the test panel is of limited size, the results suggest differential variability among the nine
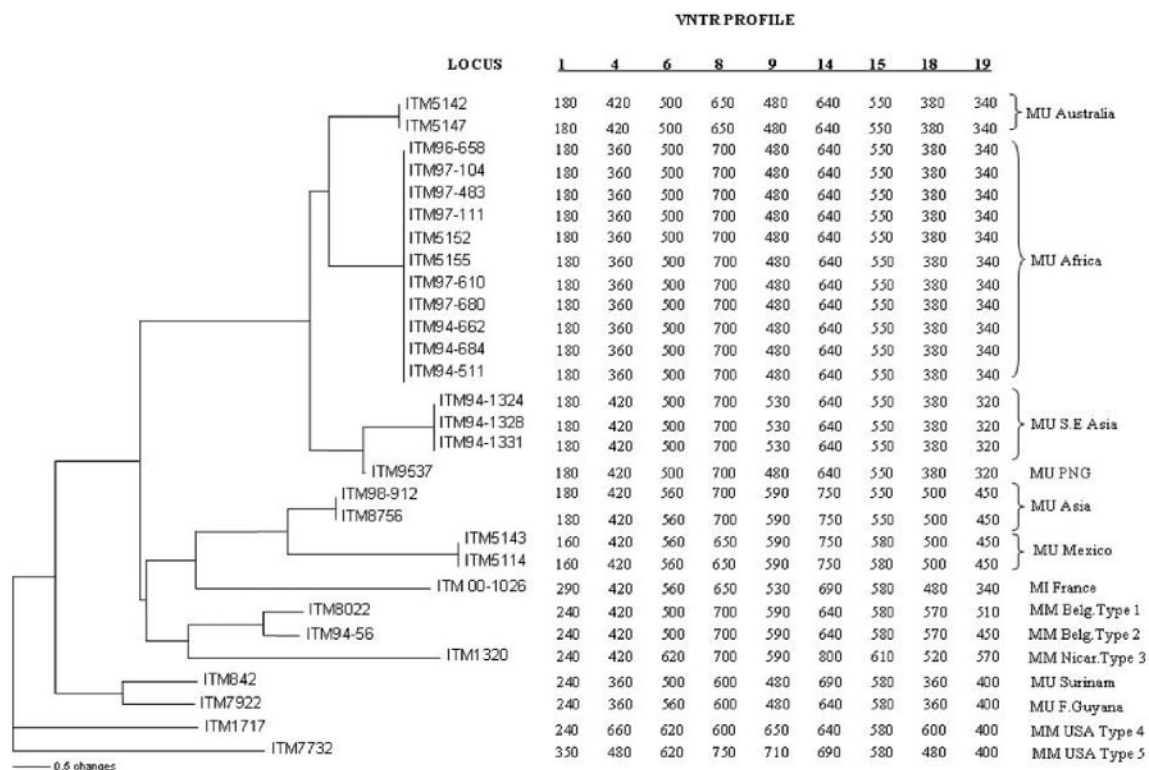
FIG. 1. Dendrogram showing the genetic relationships of the *M. marinum* and *M. ulcerans* isolates determined using nine VNTR loci. The dendrogram was built using the neighbor-joining algorithm and ITM 1717 and 7732 as outgroups, as described in Material and Methods. The VNTR amplicon sizes and the corresponding genotype designations are indicated at the right. The linkage distance scale and the VNTR locus numbers are indicated at the bottom. MM, *M. marinum*; MU, *M. ulcerans*; MI, intermediate isolate.

loci. Loci 18 and 19 were the most variable loci, with a total of seven and six alleles overall, respectively. Locus 19 was the most discriminatory at the intraspecies level, showing four different alleles in *M. ulcerans* and four in *M. marinum* (Fig. 2A and B, respectively). At the other extremity, locus 6 was among the least discriminatory, with only three alleles in total, of which two and three were found in *M. ulcerans* and *M. marinum*, respectively. Nevertheless, only loci 6 and 14 could be used to differentiate between the Surinam and French Guyana *M. ulcerans* isolates (Fig. 1).

Genetic relationships of the *M. marinum* and *M. ulcerans* isolates. A dendrogram (Fig. 1) of the VNTR profiles of the 29 isolates was built using the neighbor-joining algorithm.

A first group in the dendrogram included one Australian genotype (alias Victoria) (22), two genotypes designated Southeast Asian and Papua New Guinea of *M. ulcerans*, and one
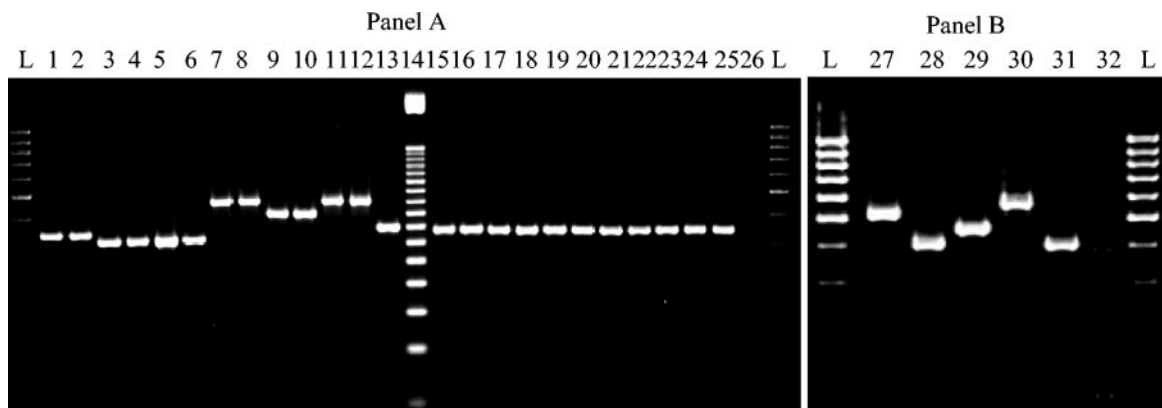

FIG. 2. PCR analysis of VNTR locus 19. (A) *M. ulcerans* and the intermediate strain. Lanes: L, 100-bp DNA ladder; 1, ITM 5142; 2, ITM 5147; 3, ITM 94-1324; 4, ITM 9537; 5, ITM 94-1331; 6, ITM 94-1328; 7, ITM 98-912; 8, ITM 8756; 9, ITM 842; 10, ITM 7922; 11, ITM 5114; 12, ITM 5143; 13, ITM 00-1026; 14, 50-bp ladder (locus 19); 15, ITM 96-658; 16, ITM 97-111; 17, ITM 97-483; 18, ITM 97-104; 19, ITM 5152; 20, ITM 5155; 21, ITM 97-610; 22, ITM 97-648; 23, ITM 97-680; 24, ITM 94-662; 25, ITM 94-511; 26, negative control. (B) *M. marinum*. Lanes: L, 100-bp DNA ladder; 27, ITM 8022; 28, ITM 1717; 29, ITM 94-56; 30, ITM 1320; 31, ITM 7732; 32, negative control.

African genotype. The Australian genotype included two isolates from Australia (Victoria), while the Southeast Asian genotype comprised the third isolate from Australia (Queensland), the Malaysian isolate, and one Papua New Guinea isolate (ITM 94-1331). The genotype of the other Papua New Guinea isolate (ITM 9537) was distinct from the Southeast Asian genotype by only one locus. Strikingly, the African genotype included the 11 *M. ulcerans* isolates from the seven different African countries tested; these isolates did not display any differences among the nine VNTR loci investigated. Similarly, the isolates from Japan and China had identical profiles at all the nine loci and constituted the Asian genotype. The two Mexican isolates had also identical profiles at all nine loci and were designated the Mexican genotype. This Mexican type was found to be closely related to the Asian type. The two South American isolates from Surinam and French Guyana both gave unique but closely related genotypes, referred to as the Surinam and Guyana genotypes, respectively. These strains appeared to be more distant from the other *M. ulcerans* strains.

The five *M. marinum* isolates had five unique genotypes but could be classified into two subgroups, showing some correlation with their source of isolation. These two groups included the two isolates from Belgium and the one from Nicaragua as one group and the two isolates from the United States as the other group. The isolate of the intermediate species (ITM 00-1026) was found to be distantly related to the *M. ulcerans* Asian-Mexican genotype.

DISCUSSION

M. ulcerans features low variability in house-keeping gene sequences and other genetic elements, especially among isolates from the same geographic region (5, 16, 21, 22). Given the informative value of VNTRs for many other genetically homogeneous organisms, the identification of VNTRs in *M. ulcerans* may be useful for phylogenetic and epidemiological studies of this pathogen. In this study, we have identified 9 VNTR loci in *M. ulcerans* and 13 in *M. marinum* and assessed their usefulness as new molecular targets to study genetic relationships in these two species.

The set of nine VNTR loci revealed eight genotypes among the 23 *M. ulcerans* reference isolates tested. Using largely overlapping sets of isolates, six genotypes have previously been found by multilocus sequence typing (MLST) analysis of eight structural and house-keeping genes (21) and by restriction fragment length polymorphism (RFLP) of IS*2404* (6), whereas nine genotypes were found with *2426*-PCR, a repetitive-element PCR targeting IS*2404* and IS*2606* (22) (Table 1). In contrast, amplified fragment length polymorphism (AFLP) revealed only three *M. ulcerans* subtypes (6). Although the different typing methods were not applied to exactly the same strain collections, these results suggests that the typing method used on the basis of the set of nine VNTR loci described here is at least as discriminatory as the previous typing methods.

VNTR typing groups *M. ulcerans* isolates according to their geographic origin similarly to MLST results. Both methods cluster the Malaysian and Papua New Guinean isolates ITM 94-1331 as the Southeast Asian type. VNTR analysis provided additional resolution by discriminating one Papua New Guinean (strain 11878/70 or ITM 9537) genotype, but this genotype

was consistently closely related by a single-locus difference to the South East Asian genotype. Numerical analyses of the profiles generated by both methods indicate the closest evolutionary link between strains from Southeast Asia, Australia, and Africa and a comparatively more distant relation among genotypes from other regions. However, at variance with *2426*-PCR results (22) but consistent with MLST results (21), multilocus VNTR analysis shows the Asian genotype (Japan-China) to be much more closely related to the Mexican genotype than to the Surinam genotype. Considering that the most reliable method for determining genetic relatedness is sequence comparison, the congruence between VNTR-based and MLST-based (21) groupings indicates that reliable phylogenetic inferences can be made from this set of markers over the scale of evolutionary divergence considered. Furthermore, such congruence is consistent with the clonal population structure of *M. ulcerans* apparent from MLST analysis (21).

It is particularly noteworthy that despite the resolution power of these markers, we did not find any size differences in any of nine VNTR loci tested among the 11 African isolates coming from the seven different countries. Consistent with the absence of any sequence variation among African strains identified so far (21, 22), these findings highlight the extremely high clonal homogeneity of *M. ulcerans* in Africa and lend support to the hypothesis of a recent distribution of the organism across this continent (21). The level of genetic conservation is not so extreme within other geographic regions. In addition to discriminating the isolates from Papua New Guinea by one locus (see above), VNTR analysis could discriminate between the isolates from French Guyana and Surinam by two loci, in contrast to IS*2404* RFLP results (5) and AFLP results (6). This finding identifies for the first time two closely related but different *M. ulcerans* genotypes in South America. Furthermore, the difference between these two isolates and the Mexican isolates, also apparent from MLST analysis (21), was much greater (at seven loci out of nine) than that between the two South American isolates. This is the most extensive difference among *M. ulcerans* strains originating from a same continent observed so far by VNTR analysis and suggests that the corresponding genotypes may have arisen independently in the Americas.

Beyond this comparison with the Mexican isolates, the South American isolates appeared distantly related to the *M. ulcerans* strains from the other geographic regions in general. Consistently, the isolate from Surinam appeared as the most distal geographical genotype among the *M. ulcerans* isolates tested by MLST (21) and was distinct from *M. ulcerans* isolates from the other geographic regions by the sequence of the 3′-terminal end of its 16S rRNA gene, which is identical to that of *M. marinum* strains (16). Similarly, the isolate of the intermediate species (ITM 00-1026) was found to be distantly related to the Asian-Mexican *M. ulcerans* genotypes and one of the *M. marinum* subgroups. However, the analysis of more deep-rooted relationships should be considered with some caution because of the limited degree of confidence inherent in investigations conducted using a set presently restricted to nine VNTR loci.

In conclusion, this study supported the potential of a VNTR-based genotyping method for *M. ulcerans* and *M. marinum*. Since VNTR genotyping has also been successfully applied to the *M. tuberculosis* (8, 17, 19, 20, 25) and *M. avium* (2, 3)

complexes, it is likely that they can be used for the typing of other species of the mycobacterial genus as well. This method offers several practical advantages over AFLP- or RFLP-based typing methods, such as easier DNA extraction (from boiled colonies) suitable for typing and easier interpretation of the banding patterns. The panel of nine markers identified here permitted the discrimination of *M. ulcerans* isolates within some but not all geographic regions and offers a resolution power comparable to or slightly higher than that of these methods and of MLST. The identification of these nine loci can therefore be considered as a first promising step towards the potential use of VNTRs as markers for molecular epidemiological studies of BU within given geographic regions. This set of loci most likely represents only a fraction of the VNTR patrimony of *M. ulcerans*, as numerous and diverse arrays of TRs identified in *M. marinum* have homologs in *M. ulcerans* which have not been explored yet. Conversely, it is possible that in *M. ulcerans*, some VNTR loci, such as in the recently identified giant plasmid encoding its macrolide toxin (24), exist that have no counterparts in the *M. marinum* genome and thus could not be detected by our homology-based strategy. Hence, the publication of the complete *M. ulcerans* genome sequence will provide an opportunity for the discovery of additional tandem repeat loci, which should enhance strain resolution. Higher resolution will be especially useful to further assess the genetic homogeneity of African *M. ulcerans* isolates.

ACKNOWLEDGMENTS

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