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Are Variable-Number Tandem Repeats Appropriate for Genotyping *Mycobacterium leprae*?[▽]

Marc Monot,[1]‡ Nadine Honoré,[1]‡ Charlotte Balière,[1] Baohong Ji,[2] Samba Sow,[3] Patrick J. Brennan,[4] and Stewart T. Cole[1,5]*

Unité de Génétique Moléculaire Bactérienne, Institut Pasteur, Paris, France[1]; Bactériologie-Hygiène, Faculté de Médecine Pitié-Salpêtrière, Paris, France[2]; Centre National d'Appui à la Lutte Contre la Maladie, Bamako, Mali[3]; Department of Microbiology, Immunology, and Pathology, Colorado State University, Fort Collins, Colorado 80523-1682[4]; and Global Health Institute, EPFL, Lausanne, Switzerland[5]

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Comparative genomics analysis of the Tamil Nadu strain of *Mycobacterium leprae* has uncovered several polymorphic sites with potential as epidemiological tools. In this study we compared the stability of two different markers of genomic biodiversity of *M. leprae* in several biopsy samples isolated from the same leprosy patient. The first type comprises five different variable-number tandem repeats (VNTR), while the second is composed of three single nucleotide polymorphisms (SNP). Contrasting results were obtained, since no variation was seen in the SNP profiles of *M. leprae* from 42 patients from 7 different locations in Mali whereas the VNTR profiles varied considerably. Furthermore, since variation in the VNTR pattern was seen not only between different isolates of *M. leprae* but also between biopsy samples from the same patient, these VNTR may be too dynamic for use as epidemiological markers for leprosy.

Leprosy remains a public health problem, and on average, 400,000 new cases have been reported annually during the last 5 years (2), although there is no known reservoir for the etiologic agent, *Mycobacterium leprae*, other than human beings. In order to understand better the transmission and epidemiology of leprosy, several investigators have searched for polymorphic markers within the genome of *M. leprae* with the aim of developing robust molecular typing systems for epidemiology. Variable-number tandem repeats (VNTR), such as runs of di- or trinucleotides, have been examined as potential typing markers and found to vary in copy number between strains of *M. leprae* (7, 12, 16, 19, 24), thus arousing interest in their application as epidemiological tools. Other workers have investigated single nucleotide polymorphisms (SNP) and found that despite the reductive evolution and genome decay undergone by the leprosy bacillus (3), the genome sequence is highly conserved and SNP are rare. Three SNP were found to be informative and have been used to reconstitute the evolution and global spread of *M. leprae* (12). While the SNP are useful in studies of long-range transmission of leprosy, the VNTR, owing to their more dynamic nature, appear more appropriate for monitoring the spread of *M. leprae* over shorter epidemiological distances, such as within a region or a large city. A number of reports have appeared that present the findings of VNTR studies of leprosy bacilli from different Asian countries (20, 25) and in one case from patients belonging to the same family (24). Here we present the findings of a study aimed at comparing the performance of three SNP and five VNTR markers in typing strains present in different biopsy samples from the same leprosy patients living in Mali.

* Corresponding author. Mailing address: Global Health Institute, EPFL, Station 15, CH-1015 Lausanne, Switzerland. Phone: 41 21 693 18 51. Fax: 41 21 693 17 90. E-mail: stewart.cole@epfl.ch.

‡ These authors contributed equally.

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MATERIALS AND METHODS

Biopsy samples. The skin biopsy samples were isolated from 45 patients presenting at the Centre National d'Appui à la Lutte Contre la Maladie (CNAM) in Bamako, Mali. Details of the sex, age, type of leprosy, prior treatment history, and biopsy site may be found in Table 1. Generally, three biopsies were taken from each patient, two on the day of arrival at the leprosy clinic (D0) and the third after 1 month of treatment with moxifloxacin (D31) prior to commencement of standard multidrug therapy. At least two biopsies were used in this study (Table 1). All human biopsies were obtained in accordance with a defined protocol, which had been approved by the appropriate institutional review boards, and after informed patient consent.

SNP and VNTR analysis. Details of the three SNP and five VNTR sites examined and the primers used for their amplification by PCR may be found in Table 2. PCR fragments were generated from *M. leprae* DNA present in human biopsies, processed by standard procedures (22), and then sequenced using an AB3100 DNA sequencer (Applied Biosystems, Foster City, CA).

Briefly, PCR was carried out using 1.25 U of *Taq* DNA polymerase (Q-Biogene, MP Biomedicals) in a 25-μl volume containing 2.5 μl of prepared biopsy samples and 200 nM of custom primers (Table 2) with other reagents supplied by the manufacturer. The mixture was denatured at 94°C for 3 min, followed by 45 PCR cycles (1 min at 94°C, 1 min at 55°C, extension at 72°C for 2 min), with a final extension at 72°C for 10 min in a thermocycler (PTC-100; MJ Research, Inc.). We purified amplified DNA by enzymatic procedures; 8 μl of PCR products were incubated with 2.5 U of exonuclease I (USB Corp., Cleveland, OH) and 0.25 U of shrimp alkaline phosphatase (USB Corp., Cleveland, OH), with a final volume of 10 μl, at 37°C for 15 min before enzyme inactivation at 80°C for 15 min. Then, we added 2 μl of BigDye v3.1, 4 μl of BigDye v3.1 buffer (Applied Biosystems, Foster City, CA), and 200 nM of primer for a final volume of 20 μl. The sequencing mixture was denatured at 96°C for 1 min, followed by 40 cycles of denaturation at 96°C for 30 s, annealing at 56°C for 15 s, and extension at 60°C for 4 min.

In some instances, involving AT-rich regions, PCR products were purified using a QIAquick PCR purification kit (Qiagen, Inc., Valencia, CA) and cloned into the pGEM-T Easy Vector systems (Promega, Madison, WI) prior to sequencing. The complete results of SNP and VNTR analysis are summarized in the text and Table 3.

Bioinformatics. Sequences were compiled and analyzed using Gap4 (http://staden.sourceforge.net/manual/gap4_unix_2.html) and Artemis (13), as previously described (3). Phylogenetic trees were constructed using the QuickTree software (8), which employs the neighbor-joining method (14) to calculate distances that correspond to the square of the number of VNTR differences.

TABLE 1. Patients' details[a]

Patient no.	Sex	Yr of Birth	Town of origin	Leprosy type	Diagnosis	Treatment	Biopsy site 1	Biopsy site 2	PCR result
1	F	1940	Koulikoro	LL	RE	D	L elbow	L elbow	+
2	M	1950	Sikasso	LL	RE	D	L lumbar region	L lumbar region	+
3	M	1951	Ségou	LL	RE	D	Abdomen	Back	+
4	M	1975	Kayes	LL	N	U	L scapula	L scapula	+
5	F	1955	Segou	LL	RE	D	L flank	L flank	−
6	F	1982	Ségou	LL	N	U	L scapula	R Lumbar region	+
7	M	1977	Mopti	LL	N	U	L scapula	R lumbar region	+
8	M	1955	Ségou	LL	RE	D	R scapula	R scapula	+
9	M	1980	Ségou	LL	N	U	R back	L arm	+
10	M	1938	Mopti	LL	N	U	L back	R lumbar region	−
11	F	1971	Bamako	LL	N	U	L lumbar region	L lumbar region	+
12	M	1960	Bamako	LL	RE	D	R back	R lumbar region	+
13	M	1940	Gao	LL	RE	D	R lumbar region	R arm	+
14	F	1945	Mopti	LL	N	U	R lumbar region	L back	+
15	M	1938	Mauritania	LL	N	U	R lumbar region	R lumbar region	+
16	F	1970	Koulikoro	LL	N	U	R back	L lumbar region	+
17	F	1960	Ségou	LL	N	U	R back	R lumbar region	+
18	M	1972	Koulikoro	LL	N	U	L back	L back	+
19	F	1960	Bamako	LL	N	U	L lumbar region	L lumbar region	+
20	F	1983	Bamako	LL	N	U	L back	L back	+
21	F	1975	Sikasso	LL	N	U	L back	L back	+
22	F	1944	Koulikoro	LL	RE	D	R back	R lumbar region	+
23	M	1943	Koulikoro	LL	RE	D	L lumbar region	L lumbar region	+
24	M	1980	Kayes	LL	N	U	L lumbar region	L Lumbar region	+
25	F	1940	Sikasso	LL	RE	D	R lumbar region	L lumbar region	+
26	F	1980	Bamako	LL	N	U	L lumbar region	R lumbar region	+
27	M	1975	Kayes	LL	N	U	L back	L lumbar region	+
28	M	1941	Kayes	LL	RE	D	R breast	R breast	+
29	M	1951	Kayes	LL	N	U	R lumbar region	L lumbar region	+
30	F	1951	Guinea Rep	LL	RE	D	L back	L back	−
31	M	1976	Ségou	LL	N	U	R lumbar region	R lumbar region	+
32	M	1972	Kolokani	LL	N	U	R lumbar region	L lumbar region	+
33	F	1976	Kayes	LL	N	U	L lumbar region	L lumbar region	+
34	M	1953	Kayes	LL	N	U	L lumbar region	L lumbar region	+
35	F	1983	Mopti	LL	N	U	R lumbar region	R forearm	+
36	M	1971	Mopti	BL	N	U	L elbow	R elbow	+
37	M	1938	Koulikoro	LL	N	U	R arm	R arm	+
38	M	1978	Ségou	BL	N	U	L arm	L arm	+
39	M	1965	Koulikoro	LL	RE	D	L back	L back	+
40	M	1983	Kayes	BL	N	U	R back	L back	+
41	M	1971	Sikasso	LL	N	U	L arm	R lumbar region	+
42	M	1986	Mopti	LL	N	U	R elbow	L elbow	+
43	M	1956	Mauritania	LL	N	U	R lumbar region	R lumbar region	+
44	M	1965	Kayes	LL	N	U	R lumbar region	R lumbar region	+
45	M	1946	Sikasso	LL	N	U	L lumbar region	L lumbar region	+

[a] M, male; F, female; LL, lepromatous leprosy; BL, borderline leprosy; N, new case; RE, relapse; U, unknown; R, right; L, left.

RESULTS

Setting and characteristics of study population. Mali is a sparsely inhabited West African country (<9 inhabitants per km^2) with a well-functioning leprosy control program that reached the goal of leprosy elimination in April 2001. As part of a chemotherapy trial to test the efficacy of a new regimen including moxifloxacin (5), a group of 45 leprosy patients (Table 1) was constituted at the CNAM. This comprised 29 males and 16 females, and they came from 7 different towns, 5 of which are located on the Niger river; Bamako and Gao are separated by 1,213 km and are the towns that are furthest apart (Fig. 1).

In the current study, leprosy was diagnosed clinically on the basis of skin lesions with impairment of sensation and skin smear positivity. With three exceptions, classed as borderline lepromatous (BL), all patients were diagnosed with lepromatous leprosy (LL). Thirty-two of the patients were new cases with no known history of antileprosy treatment, and 13 patients had relapsed, having previously been treated with dapsone monotherapy. At the time of the study, the patients ranged in age from 21 to 69 years old, with an average age of 44 years old. The average age of relapsed cases was 58 years old. Skin biopsies were taken from lesions at D0 before treatment began and again at D31. The biopsy site was noted, and in the case of large lesions, both D0 and D31 biopsies were taken from the same site. In six cases, two different sites were biopsied at D0 but not at D31.

Amplification of *M. leprae* DNA by PCR. To prepare DNA suitable for sequencing purposes, as part of the SNP and VNTR analysis, a variety of PCR primers were used to amplify the eight corresponding loci (Table 2). Samples from three relapsed patients (patients no. 5, 10, and 30) consistently

TABLE 2. Primers used in this study

Polymorphism	Locus[a]	Primer sequence
SNP-14676-F	ML0009–ML0010 IG	AATGGAATGCTGGTGAGAGC
SNP-14676-R	ML0009–ML0010 IG	CAATGCATGCTAGCCTTAATGA
SNP-1642875-F	ML1378	TGCTAGTTTAACCGAGTACTGCTA
SNP-1642875-R	ML1378	GTAGTAGTCTTCCAAGTTGTGGTG
SNP-2935685-F	ML2462	ATCTGGTCCGGGTAGGAATC
SNP-2935685-R	ML2462	ACCGGTGAGCGCACTAAG
VNTR 21_TTC-F	ML2345	ACTCGATCGAAGAACCAACC
VNTR 21_TTC-R	ML2345	GGACCTAAACCATCCCGTTT
VNTR 9_GTA-F	ML2172–ML2173 IG	CTCGATTAGTGCGATCAACG
VNTR 9_GTA-R	ML2172–ML2173 IG	GAGCCAGCGGTAGTACTGGA
VNTR 14_AT-F	ML0235–ML0236 IG	AGCGTTATGAGCCGTAAGGA
VNTR 14_AT-R	ML0235–ML0236 IG	CGAACCACTAACCTGGCAAC
VNTR 15_AT-F	ML079–ML0799 IG	TGATCAATATGCGGGTTGG
VNTR 15_AT-R	ML0798–ML0799 IG	GGTTATGTTCGGCATCCATC
VNTR 17_AT-F	ML2183	TTGAGCGAAAGAAAGCAGGT
VNTR 17_AT-R	ML2183	TGCATTTAGCAGGACGATTG

[a] IG, intergenic region.

yielded no products (Table 1) despite multiple efforts, including the highly sensitive RLEP amplification procedure (4, 21, 22), suggesting that either they contained no *M. leprae* DNA or inhibitors of *Taq* polymerase were present. The great majority of samples from both the D0 and D31 biopsies generated PCR samples suitable for DNA sequencing, and in most cases the sequencing reaction was successful, yielding unambiguous results. To eliminate possible technical errors, all experiments were performed in duplicate by three different experimentalists with the same result.

SNP analysis. On sequence interrogation of the three polymorphic genomic positions, which are the basis of the SNP typing system, it was found that all 42 PCR-positive *M. leprae* strains belonged to the same group, SNP type 4 (12). This SNP type is characterized by the nucleotides T, T, and C at genome positions 14676, 1642875, and 2935685, respectively, and has been found mainly in West Africa but also in the Americas, where it was believed to have been introduced during the era of the slave trade. Since these isolates could not be distinguished by SNP typing, five VNTR loci were amplified, sequenced, and compared.

VNTR analysis. PCR was used to amplify fragments suitable for sequencing from five genomic loci known to harbor VNTR, namely, two trinucleotide repeats (21_TTC and 9_GTA) and three dinucleotide repeats (14_AT, 15_AT, and 17_AT), as described in Table 2. These repeats were chosen since, like the three SNP, they are located in pseudogenes or intergenic regions and thus should not be functionally constrained. All loci were readily amplified and sequenced well, with the exception of 15_AT, which occasionally yielded fragments that could not be sequenced directly. Some of the larger 15_AT-repeat-bearing fragments were cloned prior to sequencing. In these cases the same repeat copy number was obtained from all clones examined, but in some instances, when the copy number was >25, the exact number of repeats could not be established.

In our study population, eight alleles of the 21_TTC repeat were found, and the number of TTC repeats ranged from 10 to 17, with 14 being the most common (Table 3). Likewise, seven alleles of the 9_GTA repeat were detected, and the range was from 8 to 14, with 9 the most common type. Most allelic diversity was seen at the 14_AT locus, where 13 different versions were observed, ranging from 8 to 25 copies, with 14 being by far the most abundant form. The numbers of alleles at the 15_AT and 17_AT loci were 12 and 9, respectively, and in both cases 13 was the most commonly occurring repeat unit. Full details of the frequency of the alleles at these five VNTR loci are reported in Table 3. The *sigA* (*rpoT*) locus (11) of the Malian strains was also examined for variability in its hexanucleotide repeat region, but none was found (data not shown).

Phylogenetic analysis. To determine whether any links could be established between the *M. leprae* strains and the town of origin of the 33 corresponding patients for which complete data sets were available, we used the neighbor-joining method (14) to produce phylogenetic trees. In no case was the same tree obtained, nor was convincing evidence for clustering associated with the geographical origin of the strain detected (Fig. 2), irrespective of whether VNTR loci were used individually (Fig. 2B to F) or collectively (Fig. 2A).

Intrapatient variability in VNTR type. To establish whether there was any copy number variation at selected VNTR loci taken from different sites on the same patient or from the same site at two different times, we screened our biopsy collection using PCR and sequencing of the four most-robust sites. Owing to its lower reliability in PCR, the 15_AT locus was excluded. This comparative analysis revealed no variation in 36 of the 42 *M. leprae* isolates where 2 samples were available. However, in six cases, reproducible copy number differences were seen (Table 4); four strains varied at a single locus, one at three loci, and the remaining isolate at all four. Greatest variability was seen at the 21_TTC locus (5 out of 11), followed by the 17_AT repeat (3 out of 11). Most of the differences were due to a loss or gain of a single repeat unit (9 out of 11), with strain 15 showing two and three unit differences at the 14_AT and 9_GTA loci, respectively.

When SNP analysis was performed on both samples from all of the 42 *M. leprae* specimens, no variation was seen at any of the three sites examined.

TABLE 3. Alleles present at VNTR loci in *M. leprae* from patients in Mali

Locus	Allele	No. of isolates	Frequency (%)	Total no. of isolates for locus
21_TTC	10	4	9.5	42
	11	5	11.9	
	12	6	14.3	
	13	6	14.3	
	14	11	26.2	
	15	5	11.9	
	16	2	4.8	
	17	3	7.1	
9_GTA	8	6	14.3	42
	9	18	42.9	
	10	9	21.4	
	11	5	11.9	
	12	2	4.8	
	13	1	2.4	
	14	1	2.4	
14_AT	8	1	2.4	42
	12	2	4.8	
	13	4	9.5	
	14	11	26.2	
	15	5	11.9	
	16	4	9.5	
	17	2	4.8	
	18	3	7.1	
	19	2	4.8	
	21	2	4.8	
	22	3	7.1	
	24	1	2.4	
	25	2	4.8	
15_AT	11	2	5	40
	12	6	15	
	13	10	25	
	14	5	12.5	
	15	4	10	
	16	3	7.5	
	17	1	2.5	
	18	2	5	
	19	1	2.5	
	20	1	2.5	
	23	1	2.5	
	>25	4	10	
17_AT	9	1	2.4	42
	10	1	2.4	
	11	7	16.7	
	12	11	26.2	
	13	12	28.6	
	14	5	11.9	
	15	2	4.8	
	16	2	4.8	
	20	1	2.4	

DISCUSSION

The aim of this study was to compare the performances of selected SNP and VNTR as potential epidemiological markers for transmission of *M. leprae* within a defined setting by taking advantage of a set of serial biopsy samples from a patient cohort participating in a trial of a new regimen for the treatment of leprosy. Although the SNP analysis was technically easier than VNTR analysis, since only interrogation of a single position is required, this failed to detect any differences between the *M. leprae* isolates, all of which belong to SNP type 4. This finding was not unexpected in light of the known stability of this type of marker and the confinement of the study to a single country. By contrast, extensive variation was seen at all five VNTR loci that were examined in this work, both between patient isolates and within samples from the same patient. Consistent with the results of earlier studies (7, 19, 25), we observed greatest allelic variation in the dinucleotide repeats (in descending order of allelic variation, 14_AT, 15_AT, and 17_AT) and then the trinucleotide repeats (21_TTC followed by 9_GTA).

Other workers have compared the stability of these VNTR in strains of *M. leprae* that have been passaged through animals (19, 25) and in clinical specimens taken directly from patients (24, 25). On the basis of the animal passage work, it was concluded that with the exception of strain Thai-53, the VNTR genotypes were stable during serial passaging, which augured well for their use in epidemiological studies of humans (19). However, the situation changes when the results of the relatively few studies pertaining to leprosy patients belonging to the same household are examined. In a study of 3 different households involving 11 patients, no variation was seen at 9 VNTR loci from 9 patients, whereas the 2 samples from the third household differed in the 17_AT and 9_GTA loci by 1 repeat unit (25). Similar findings of VNTR differences were reported by others when skin and nerve biopsies from the same patient were compared (24).

The present work involves the largest number of serial biopsy samples (42) yet examined by VNTR analysis and thus has considerably greater statistical significance than previous studies involving only three to four patients. We find that as many as 15% (6/42) of our paired samples differ in 1 or more VNTR loci when they come from two distinct biopsy sites or two separate sites within the same large lesion (Table 4). VNTRs are known to be highly prone to slipped-strand mispairing during replication (10), and the finding of copy number differences in two separate lesions probably reflects the fact that following infection with an isolate of *M. leprae* with a

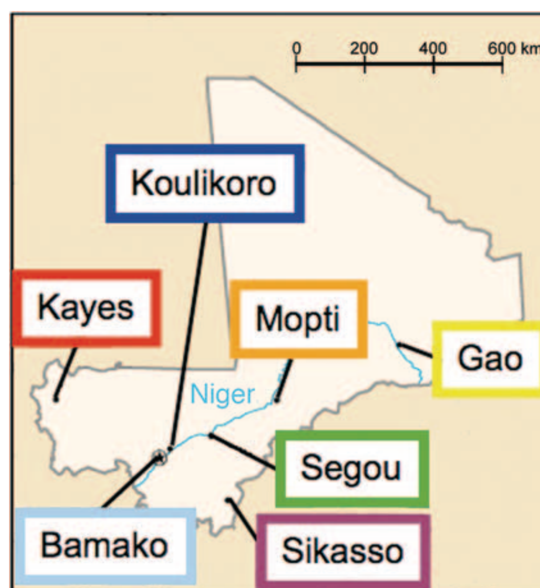

FIG. 1. Map of Mali showing major towns and cities. The patient's town of origin is colored and boxed; the same color scheme applies to Fig. 2.

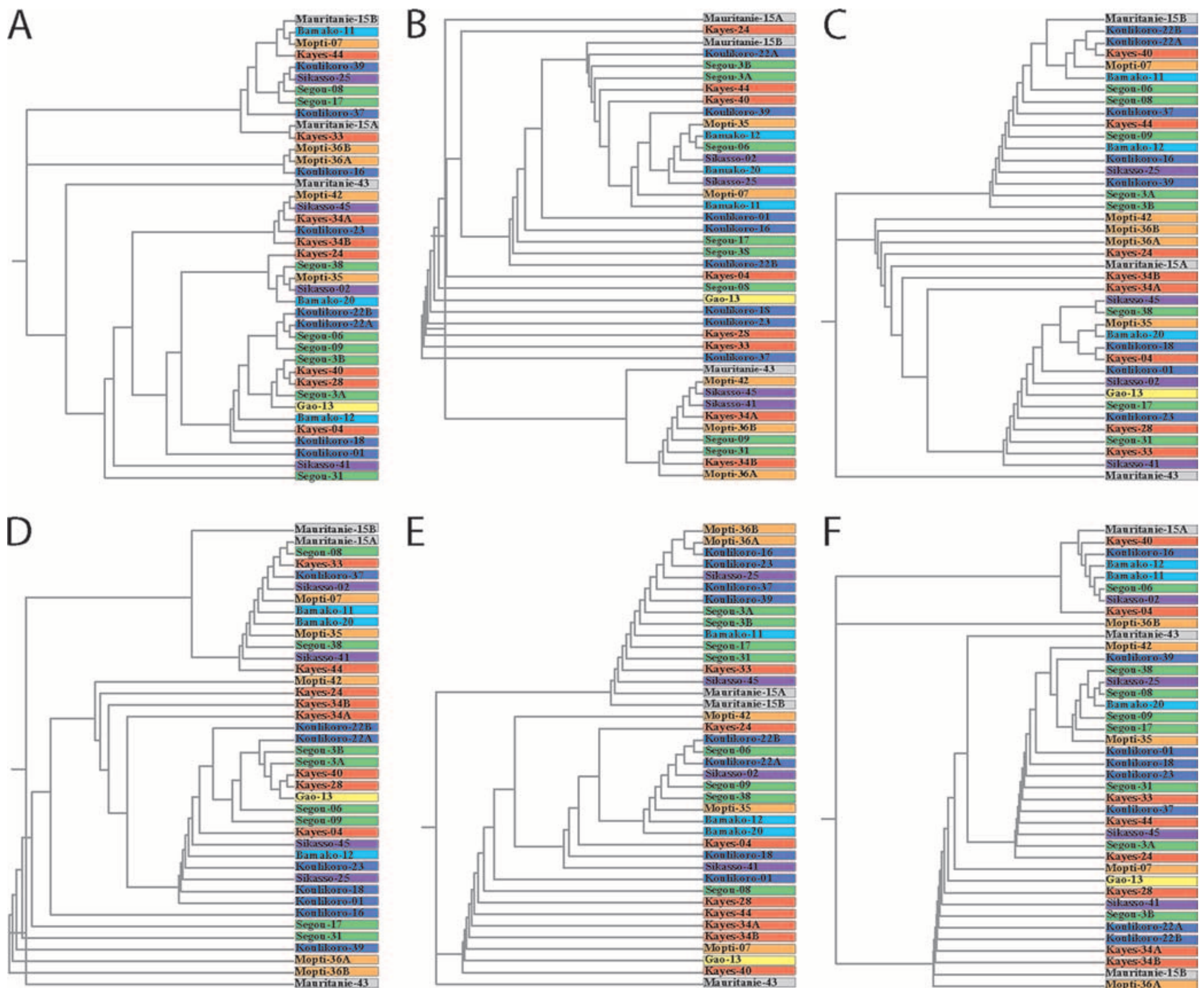

FIG. 2. Phylogenetic trees generated by the neighbor-joining method. Trees were obtained using all VNTR (A), 21_TTC (B), 9_GTA (C), 14_AT (D), 15_AT (E), or 17_AT (F). Note the widely different arrangements between all trees. Samples labeled A and B correspond to different specimens from the same patient.

TABLE 4. Allelic variation at VNTR loci in *M. leprae* isolated from the same patient

Patient no.	Site(s) of biopsy	Allele at VNTR locus[a]			
		21_TTC	9_GTA	14_AT	17_AT
3	Abdomen, back	12, 12	10, 10	22, 22	**13, 12**
34	L lumbar region, L lumbar region	**16, 15**	9, 9	16, 16	12, 12
36	L elbow, R elbow	**15, 16**	9, 9	15, 15	12, 12
26	L lumbar region, R lumbar region	**15, 14**	10, 10	14, 14	12, 12
21	L back, L back	**14, 13**	10, 10	**14, 13**	**12, 13**
15	R lumbar region, R lumbar region	**14, 12**	**9, 12**	**12, 14**	**11, 12**

[a] The left number corresponds to VNTR copy number in the biopsy sample at day 0, the right at day 31, with allele numbers shown in bold differing between them.

particular VNTR genotype, the bacilli are transported within circulating phagocytes to different niches in the body, where they replicate their DNA and multiply in number independently. We consider that in five of these six cases, the variability corresponds to clonal instability within VNTR rather than coinfection with two different strains, for the following reasons. First, although the allelic range is quite large, the copy number difference is only a single repeat unit. Second, the time frame of 31 days between sampling is too short to generate diversity since it corresponds to only two generations in the life of *M. leprae* (9). In the sixth case, patient 15 (Table 4), infection with two different strains of *M. leprae* appears more probable because four VNTR differ simultaneously and three of these by two, two, and three units, respectively. While coinfection with different strains has been reported in pulmonary tuberculosis (15), it has not been described previously for leprosy, to our knowledge.

An interesting observation was made when comparing the allelic diversity of four of the five VNTR surveyed in this work with that of their counterparts in 68 strains of *M. leprae* isolated from patients in the Yunnan Province of the People's Republic of China (20). The Chinese strains were all members of SNP type 3 and displayed far greater diversity at the trinucleotide VNTR loci than those from Mali. For instance, there were 22 and 24 alleles at the 21_TTC and 9_GTA loci, respectively, compared to 8 and 9 in the Malian population. Furthermore, the median repeat length was longer in type 3 than in type 4, with 14 versus 19 copies at the 21_TTC locus and 13 versus 16 copies at the 15_AT locus, while the other two VNTR (9_GTA and 17_AT) that could be compared showed the same median repeat length in both populations. This apparent shortening of the VNTR and the resultant decrease in allelic diversity are consistent with the finding that SNP type 4 strains are descended from type 3 and might therefore be expected to have undergone further genome reduction. This hypothesis could be tested by performing large-scale analysis of VNTR loci in strains of SNP types 1 and 2, which in turn precede types 3 and 4 in the evolutionary scheme for the leprosy bacillus (12).

From the different phylogenetic analyses conducted here, it was not possible to establish any convincing relationships between the *M. leprae* strains, even those isolated from patients living in the same town, where one might have expected a common source of infection to occur. Other workers were also unable to establish a link between the VNTR genotype and the geographic origin of *M. leprae* or its hosts (19). It is possible, however, that by sampling a larger number of VNTR or additionally by developing new algorithms to interpret the findings, more-robust phylogeographic lineages may be established, as has been done for *Mycobacterium tuberculosis* (1, 17).

While our work was in review, Young and coworkers (23) published the findings of a VNTR study of *M. leprae* in which eight short tandem repeats were surveyed (of which only one was common to the set used in our study) in serial biopsy samples from the same four patients. These investigators found that none of the collective profiles, based on five VNTR, from the same patient were identical over time and observed that genotypic differences were more likely between bacilli from different tissues. This is reminiscent of our own findings, especially the variation in the VNTR profiles of leprosy bacilli isolated from different lesions on the same patient. Taken together, these 2 studies indicate that the 12 VNTR examined may not be suitable for epidemiological purposes owing to their excessive variability. Indeed, in a phylogeographic study of *M. tuberculosis*, Filliol et al. found that microsatellites, such as the mycobacterial interspersed repetitive units (18), another kind of VNTR, are less sturdy and informative as phylogenetic markers than SNP (6). It is ironic to note that, by contrast, the SNP in *M. leprae* are too stable for short-range studies of leprosy transmission, meaning that further genome mining will be required in order to find appropriate sites of biodiversity for epidemiological tool development.

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