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Identification of Variable-Number Tandem-Repeat Loci in *Leptospira interrogans* Sensu Stricto

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***Leptospira interrogans* sensu stricto is responsible for the most frequent and severe cases of human leptospirosis. The epidemiology and clinical features of leptospirosis are usually associated with the serovars and serogroups of *Leptospira*. Because of the difficulties associated with serological identification of *Leptospira* strains, we evaluated a novel PCR-based method for typing *L. interrogans* serovars. Based upon the genome sequence of *L. interrogans* serovar Lai type strain 5660, 44 loci were analyzed by PCR for their variability in size due to the presence of variable-number tandem repeats (VNTR). Seven VNTR loci were found to be powerful markers for serovar identification, epidemiology, and phylogenetic studies of *L. interrogans*. This rapid and easy method should greatly contribute to a better knowledge of the epidemiology of *Leptospira*.**

The genus *Leptospira* consists of a heterogeneous group of pathogenic and saprophytic species belonging to the order *Spirochaetales*. Pathogenic *Leptospira* species, currently classified in seven species based on DNA relatedness (2, 25), are the agents of leptospirosis. Transmission to humans occurs through direct or indirect contacts with urine of infected animals. *Leptospira interrogans* sensu stricto (25) is the main species associated with human leptospirosis. In France, *L. interrogans* sensu stricto is responsible for about 60% of human cases and for the most severe ones. The intraspecies taxonomy of leptospires is well established and based on antigenic determinants. Since the description of serovars in 1915, about 80 serovars have been identified in *L. interrogans* sensu stricto (2); among them, 60 serovars are validly described (12). Since each serovar is usually associated with a particular host, identification of serovars is essential to epidemiological studies and strategies for prevention (5). The reference method for serological identification is the microagglutination test, which is a complex and fastidious test since it requires cross adsorption of many rabbit hyperimmune sera (24).

Antigenically related serovars are grouped into serogroups. However, a given serogroup is often found in several *Leptospira* species. For instance, the nine validly described serovars from serogroup Bataviae are distributed among *L. interrogans* sensu stricto species (two serovars), *L. santarosai* (four serovars), *L. kirschneri* (one serovar), *L. noguchii* (one serovar), and *L. borgpetersenii* (one serovar). Several studies have thus shown that the system of serogroups is not related to molecular classifications. In contrast, serovars can be characterized by different molecular methods, such as restriction fragment length polymorphism-based methods (15, 22), arbitrarily primed PCR (19), and pulsed-field gel electrophoresis (PFGE) (8, 9). However, these techniques are not widely applied, because PFGE and restriction fragment length polymorphism are laborious and require significant volumes of culture and arbitrarily primed PCR results in poor reproducibility and interpretation of results. In addition, lateral genetic transfer among leptospires (18) and large chromosomal rearrangements between serovars (26) prevent the construction of species phylogenetic trees by gene sequencing (7) or discrete whole-genomic data (19).

Analysis of variable-number tandem repeats (VNTR), also called multiple-locus VNTR analysis, has proven to be a highly powerful and discriminant method to study the population structure of bacteria (17) and to characterize isolates even from monomorphic bacterial populations (6, 11, 13). The genome of *L. interrogans* serovar Lai has recently been sequenced (20), and this allowed us to define pairs of primers flanking some VNTR-like loci. Our goal is to determine whether VNTR analysis will be able to differentiate most of the serovar reference strains from *L. interrogans* sensu stricto, providing a practical and simple PCR-based method for the identification of *L. interrogans* serovars.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *L. interrogans* serovars (Table 1), *L. borgpetersenii* serovar Castellonis strain Castellon 3, *L. kirschneri* serovar Grippotyphosa strain Moskva V, *L. kirschneri* serovar Cynopteri strain 3522C, *L. biflexa* serovar Patoc strain Patoc1, and *L. meyeri* serovar Semaranga strain Veldrat were obtained from the strain collection at the National Reference Laboratory for *Leptospira* at the Institut Pasteur, Paris, France. Leptospiral strains used in this study were also isolated from patients (one strain of *L. interrogans* serovar Canicola, six strains of *L. interrogans* serovar Icterohaemorrhagiae, three strains of *L. interrogans* serovar Pomona, and one strain of *L. interrogans* serovar Hardjo), dogs (seven strains of *L. interrogans* serovar Canicola), bovines (two strains of *L. interrogans* serovar Hardjo), horses (one strain of *L. interrogans* serovar Hardjo), and sheep (two strains of *L. interrogans* serovar Hardjo) in the last 20 years. *Leptospira* strains were grown at 30°C in EMJH (4, 10) liquid medium.

DNA manipulations. Genomic DNA of *Leptospira* was isolated as previously described (16). Amplification was achieved with *Taq* polymerase (Amersham), using one cycle of denaturation (94°C for 5 min) followed by 35 cycles of amplification consisting of denaturation (94°C for 30 s), annealing (55°C for 30 s), and primer extension (72°C for 1 min 30 s) and a final extension of 10 min at 72°C. The amplified products were analyzed by 1.5% agarose gel electrophoresis. The sizes of the amplified products were estimated by comparison with a 100-bp ladder (Invitrogen). Some of the amplified products were sequenced at the Genomic Platform (Institut Pasteur).

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TABLE 1. Strains used in this study

Serovar	Strain	Serogroup[c]	Abbreviation	Country	Source
Australis[a]	Ballico[b]	Australis	A1	Australia	Human
Bangkok	Bangkok-D92	Australis	A2	Thailand	Dog
Bratislava[a]	Jez-Bratislava[b]	Australis	A3	Czechoslovakia	Hedgehog
Fugis[a]	Fudge[b]	Australis	A4	Malaysia	Human
Jalna[a]	Jalna[b]	Australis	A6	Czechoslovakia	Mouse
Muenchen[a]	Munchen C 90[b]	Australis	A8	Germany	Human
Autumnalis[a]	Akiyami A[b]	Autumnalis	Au1	Japan	Human
Carlos[a]	C-3[b]	Autumnalis	Au4	Philippines	Toad
Mooris[a]	Moores[b]	Autumnalis	Au5	Malaysia	Human
Bataviae[a]	Van Tienen	Bataviae	B1	Indonesia	Human
Benjamini[a]	Benjamin[b]	Canicola	C1	Indonesia	Human
Bindjei[a]	Bindjei[b]	Canicola	C2	Indonesia	Human
Broomi[a]	Patane[b]	Canicola	C3	Australia	Human
Canicola[a]	Hond Utrecht IV[b]	Canicola	C4	Netherlands	Dog
Jonsis[a]	Jones[b]	Canicola	C6	Malaysia	Human
Kuwait[a]	136/2/2[b]	Canicola	C7	Kuwait	Rat
Portlandvere[a]	MY 1039[b]	Canicola	C8	Jamaica	Human
Schueffneri[a]	Vleermuis 90C[b]	Canicola	C10	Indonesia	Bat
Sumneri[a]	Sumner[b]	Canicola	C11	Malaysia	Human
Djasiman[a]	Djasiman[b]	Djasiman	D1	Indonesia	Human
Gurungi[a]	Gurung[b]	Djasiman	D2	Malaysia	Human
Grippotyphosa[a]	Andaman	Grippo	G1	ND[d]	ND
Liangguang	1880	Grippo	G2	China	Rat
Muelleri[a]	RM 2[b]	Grippo	G3	Malaysia	Rat
Valbuzzi[a]	Valbuzzi[b]	Grippo	G4	Australia	Human
Hebdomadis[a]	Hebdomadis[b]	Hebdomadis	H1	Japan	Human
Kremastos[a]	Kremastos[b]	Hebdomadis	H2	Australia	Human
Birkini	Birkin[b]	Ictero	I1	Malaysia	Human
Copenhageni[a]	M20[b]	Ictero	I3	Denmark	Human
Copenhageni	Fiocruz L1-130	Ictero	I3b	Brazil	Human
Copenhageni	Wijnberg	Ictero	I3c	Holland	Human
Gem[a]	Simon[b]	Ictero	I4	Sri Lanka	Human
Icterohaemorrhagiae[a]	RGA	Ictero	I6	Belgium	Human
Lai[a]	Lai[b]	Ictero	I7	China	Human
Naam[a]	Naam[b]	Ictero	I11	Indonesia	Human
Smithi[a]	Smith[b]	Ictero	I13	Malaysia	Human
Lanka[a]	R 740[b]	Louisiana	L0	Sri Lanka	Human
Kennewicki	LT 1026	Pomona	P2	United States	Bovine
Pomona[a]	Pomona[b]	Pomona	P4	Australia	Human
Abramis[a]	Abraham[b]	Pyrogenes	PY1	Malaysia	Human
Biggis[a]	Biggs[b]	Pyrogenes	PY2	Malaysia	Human
Camlo[a]	LT 64-67[b]	Pyrogenes	PY3	Vietnam	Human
Manilae[a]	LT 398[b]	Pyrogenes	PY5	Philippines	Rat
Pyrogenes[a]	Salinem[b]	Pyrogenes	PY6	Indonesia	Human
Robinsoni[a]	Robinson[b]	Pyrogenes	PY7	Australia	Human
Evansi[a]	267–1348[b]	Ranarum	R0	Malaysia	Water
Geyaweera[a]	Geyaweera[b]	Sejroe	SE1	Sri Lanka	Human
Haemolytica[a]	Marsh[b]	Sejroe	SE2	Malaysia	Human
Hardjo[a]	Hardjoprajitno[b]	Sejroe	SE3	Indonesia	Human
Jin	A81	Sejroe	SE4	China	Human
Ricardi[a]	Richardson[b]	Sejroe	SE7	Malaysia	Human
Romanica[a]	LM 294[b]	Sejroe	SE8	Romania	*Mus musculus*
Wolffi[a]	3705[b]	Sejroe	SE10	Indonesia	Human

[a] Serovar present in the serovar list of 1992 (12). Other serovars are mentioned in reference 2 but are not yet validated.
[b] Serovar reference strain.
[c] Ictero, Icterohaemorrhagiae; Grippo, Grippotyphosa.
[d] ND, not determined.

Sequence analysis. The large chromosome *CI* sequence (4,332 kb) of *L. interrogans* serovar Lai (20) was analyzed by using the Repeat Finder software (1) and the Tandem Repeats Database (http://iech5.igmors.u-psud.fr/GPMS/) (3). The copy number of repeats of each VNTR locus was deduced from sequencing data and sizes of the amplified products. The data were then imported into the Bionumerics software package (Applied Maths, Kortrijk, Belgium), and a phylogenetic tree was constructed by using the neighbor-joining method. The multiple phylogenetic methods showed similar tree topology. The ClustalX program (23) was used to generate nucleotide sequence alignments.

Nucleotide sequence accession numbers. The sequences of the VNTR loci described in this report can be found in GenBank under accession numbers AY766398, AY766399, AY766400, AY766401, AY766402, and AY766403.

RESULTS

Computer-assisted analysis of VNTR-like regions in the *L. interrogans* genome. At the beginning of our work, only one

TABLE 2. VNTR loci from the *L. interrogans* serovar Lai genome used in this study

VNTR locus	Primers (5′→3′)	Position in *CI* (bp)	Unit length (bp)	Copy no.	Total length of PCR product (bp)	No. of alleles/51 serovars	Copy no. range in *L. interrogans* serovars
VNTR4	4a (CAAAATCAGTCACTACCCTG) 4b (CTTTGTTGGAGCGCAATCTC)	1122221–1122580	34	5	362	10	0–23
VNTR7	7a (TCATCTGCTCCGGAGATTCG) 7b (TCCCTCCACAGGTTGTCTTG)	3312338–3312035	46	3	304	15	0–14
VNTR9	9a (TCGCTCTACAGGTCGGTGTT) 9b (GGTGAAGAGCAAACCTTTGG)	2652531–2652151	46	4	381	13	1–13
VNTR10	10a (TCCAAAATTCAGCCCTCAAG) 10b (GACGCTTGGCATTTGTATCC)	1666395–1666157	45	2	239	15	1–18
VNTR11	11a (ACAGAAGCCGTCTCATTTTG) 11b (CACAGGTCGGAATTTGTCA)	167476–167184	45	4	293	7	1–11
VNTR19	19a (CAGAAACAAGAGGGAAGATTC) 19b (ACTCTCATTTAAGAGTGGCTG)	2877449–2877029	47	6	421	15	1–18
VNTR23	23a (TTTCCAAATATACTTACTCGG) 23b (GCAAGAGAATTATTGGGATGG)	2179070–2178732	46	5	339	13	0–14
VNTR31	31a (TTCATGAAGGTCCCGAAAAC) 31b (ACGTGAGTTCGACCATGATTC)	2729699–2730370	77	4	671	ND[a]	ND

[a] ND, not determined (VNTR31 was used to differentiate between *L. interrogans* serovars Canicola and Portlandvere from serogroup Canicola).

Leptospira genome sequence was available (at the time of this writing, another genome, that of *L. interrogans* serogroup serovar Copenhageni, has been sequenced [14]), preventing strain comparison and prediction of polymorphic loci. Analysis of the genome sequence of *L. interrogans* serovar Lai (20) enabled the rapid identification of loci containing repetitions of short sequences. Among more than 1,000 VNTR-like regions, an initial selection of 44 loci was chosen after comparing the lengths of repeats (repeats of 30 to 75 bp), sequence identities (nucleotide sequence identity between repeats of >85%), and copy numbers (between two and eight copies of the unit repeat).

PCR analysis of VNTR-like regions in *L. interrogans* senso stricto. Forty-four primer pairs (sequences of the primers are available on request) were tested for their usefulness with a set of six well-characterized *L. interrogans* strains (strains A1, I3, I7, C4, PY6, SE3) (Table 1). The primers used for the PCR correspond to the VNTR flanking regions identified in the *L. interrogans* serovar Lai genome. Analysis of the amplified products by agarose gel electrophoresis revealed size variations in most of the loci. However, either no amplification or amplification of several faint bands was obtained for several loci, which were therefore excluded from this study (data not shown). This could be due to low conservation of the VNTR flanking regions among serovars. The seven most discriminative VNTR loci (i.e., VNTR4, VNTR7, VNTR9, VNTR10, VNTR11, VNTR19, and VNTR23) that exhibited a single PCR product whose size could be easily determined in 1.5% agarose gel electrophoresis for the six reference strains were further evaluated with a large collection of strains.

Sequence features of selected VNTR loci. The positions of these VNTR loci were scattered in different locations in chromosome *CI* of *L. interrogans* (Table 2). Further sequence analysis indicated that none of the selected VNTR loci were located in open reading frames. However, some of the VNTR loci could contain small open reading frames. In addition, despite obvious sequence similarities between unit repeats, a closer look at the selected loci revealed sequence similarities among VNTR7, VNTR9, VNTR10, and VNTR19 (Fig. 1). These four loci share a 47-bp consensus sequence which is repeated in tandem (Fig. 1). Sequences of the repeats from the VNTR4 and VNTR23 loci also display significant similarities

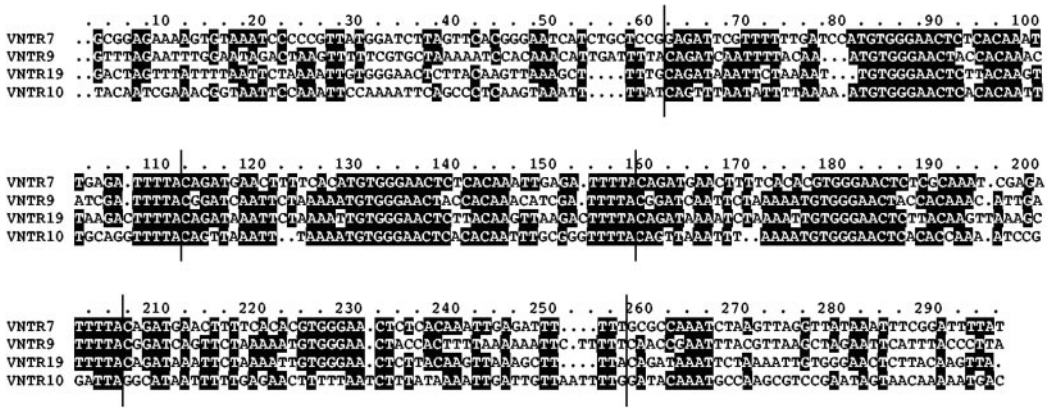


FIG. 1. Nucleotide sequence alignment of the VNTR7, VNTR9, VNTR10, and VNTR19 loci from *L. interrogans* serovar Lai. The 47-bp repeated units are delineated by vertical bars.

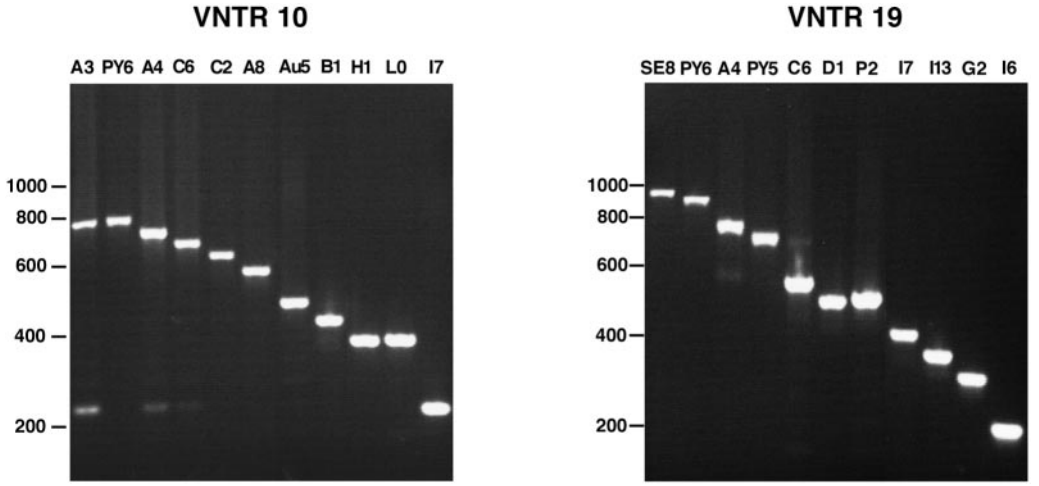


FIG. 2. PCR analysis of the polymorphism of two representative VNTR loci. Amplification was performed on the VNTR10 and VNTR19 loci of *L. interrogans* strains. Lanes indicate *Leptospira* serovars (Table 1).

with repeats in other locations of the *L. interrogans* genome (data not shown). These results suggest that VNTR loci could be grouped in distinct families of tandem-repeat-containing loci.

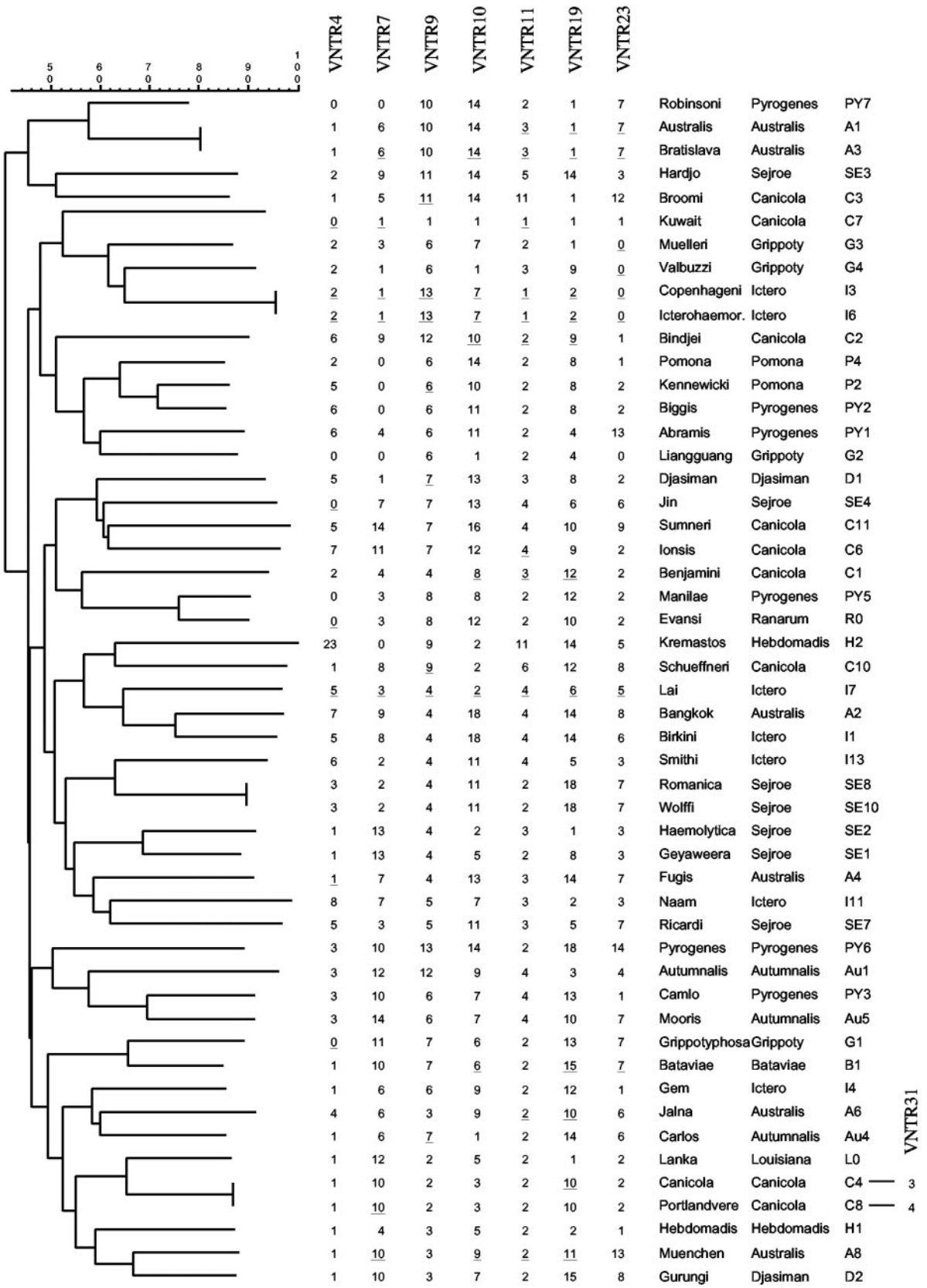
Characterization of *L. interrogans* serovars by VNTR polymorphism analysis. PCR was performed with the seven selected VNTR loci and a total of 51 serovars, clustered in 13 distinct serogroups (Table 1). The sizes of the amplified products displayed a wide range of polymorphism, suggesting variation in tandem-repeat copy numbers in the seven VNTR loci (Fig. 2). This was confirmed by sequencing of 60 amplified products from the seven loci (Fig. 3). Figure 4 shows sequences of VNTR19, which contains variable number of perfectly identical repeats. For each VNTR locus, sequence analysis of amplified products indicated a high conservation of repeat units and flanking regions among *L. interrogans* serovars (data not shown). Although the presence of multiples of a full-length repeat was the general rule, an absence of the unit repeat, insertions, and/or deletions (4 out of the 60 sequenced amplicons) could result in a size of the amplified product which is not compatible with the variation in copy number of a full-length repeat.

For each locus, the number of tandem repeats was calculated by measuring the sizes of the amplified products. The strains were typed by the numbers of variable tandem repeats in each of the seven VNTR loci. It should be noted that the value of 0 was used for amplified fragments shorter than a one-copy VNTR locus (this is the case in VNTR4, VNTR7, and VNTR23). These data could then be easily stored in databases and imported in Bionumerics for analysis.

The seven markers were able to differentiate 43 of 51 *L. interrogans* serovars (Fig. 3). An identical level of discrimination was obtained with only three markers, i.e., VNTR7, VNTR10, and VNTR19, that displayed the widest range of polymorphism with 15 distinct alleles among the 51 serovars (Table 2). Only four strain pairs were not differentiated whatever VNTR locus was used. The two strains within each pair belong to the same serogroup (*L. interrogans* serovars Copenhageni and Icterohaemorrhagiae from serogroup Icterohaemorrhagiae, *L. interrogans* serovars Australis and Bratislava from serogroup Australis, *L. interrogans* serovars Romanica and Wolffi from serogroup Sejroe, and *L. interrogans* serovars Canicola and Portlandvere from serogroup Canicola). *L. interrogans* serovars Copenhageni and Icterohaemorrhagiae were not differentiated with the 44 VNTR loci, as they were initially used as reference strains for the screening of the markers. We have undertaken PCR with VNTR loci that were previously excluded from the study to differentiate serovars that gave identical results with the seven selected markers. VNTR31 (four copies of a 77-bp repeat in *L. interrogans* serovar Lai) was able to differentiate between *L. interrogans* serovars Canicola (three copies) and Portlandvere (four copies) from serogroup Canicola. The three other pairs of strains were not differentiated whatever other VNTR was used.

Interestingly, three strains belonging to a same serovar (serovar Copenhageni strain M20 from Denmark, serovar Copenhageni strain Wijnberg from Holland, and serovar Copenhageni strain Fiocruz L1-130 from Brazil) exhibited identical results with the seven VNTR loci (data not shown). Since *L. interrogans* is phylogenetically related to other pathogenic species, we performed the VNTR assay with a few strains from *L. kirschneri* and *L. borgpetersenii*. Analysis of the PCR products of two of the seven markers, i.e., VNTR11 and VNTR19, exhibited a single band, variable in size, with the four strains of *L. kirschneri* and *L. borgpetersenii*. The size variation corresponded to multiples of the unit repeat identified in *L. interrogans* serovar Lai. These results suggest that *L. interrogans*, *L. kirschneri*, and *L. borgpetersenii* shared similar VNTR loci. In contrast, no amplification was obtained with DNAs from the saprophytic species *L. biflexa* and *L. meyeri* with the seven markers.

Application of our VNTR-based method to clinical strains. To assess our PCR-based method for genotyping, we analyzed 23 clinical strains (including 11 strains isolated from humans) with the seven most discriminative VNTR loci (Table 1). The serovars of these isolates (*L. interrogans* serovars Icterohaemorrhagiae, Pomona, Hardjo, and Canicola) were previously identified by NotI restriction and PFGE (data not shown). All



VNTR4	VNTR7	VNTR9	VNTR10	VNTR11	VNTR19	VNTR23				VNTR31
0	0	10	14	2	1	7	Robinsoni	Pyrogenes	PY7	
1	6	10	14	3	1	7	Australis	Australis	A1	
1	6	10	14	3	1	7	Bratislava	Australis	A3	
2	9	11	14	5	14	3	Hardjo	Sejroe	SE3	
1	5	11	14	11	1	12	Broomi	Canicola	C3	
0	1	1	1	1	1	1	Kuwait	Canicola	C7	
2	3	6	7	2	1	0	Muelleri	Grippoty	G3	
2	1	6	1	3	9	0	Valbuzzi	Grippoty	G4	
2	1	13	7	1	2	0	Copenhageni	Ictero	I3	
2	1	13	7	1	2	0	Icterohaemor.	Ictero	I6	
6	9	12	10	2	9	1	Bindjei	Canicola	C2	
2	0	6	14	2	8	1	Pomona	Pomona	P4	
5	0	6	10	2	8	2	Kennewicki	Pomona	P2	
6	0	6	11	2	8	2	Biggis	Pyrogenes	PY2	
6	4	6	11	2	4	13	Abramis	Pyrogenes	PY1	
0	0	6	1	2	4	0	Liangguang	Grippoty	G2	
5	1	7	13	3	8	2	Djasiman	Djasiman	D1	
0	7	7	13	4	6	6	Jin	Sejroe	SE4	
5	14	7	16	4	10	9	Sumneri	Canicola	C11	
7	11	7	12	4	9	2	Ionsis	Canicola	C6	
2	4	4	8	3	12	2	Benjamini	Canicola	C1	
0	3	8	8	2	12	2	Manilae	Pyrogenes	PY5	
0	3	8	12	2	10	2	Evansi	Ranarum	R0	
23	0	9	2	11	14	5	Kremastos	Hebdomadis	H2	
1	8	9	2	6	12	8	Schueffneri	Canicola	C10	
5	3	4	2	4	6	5	Lai	Ictero	I7	
7	9	4	18	4	14	8	Bangkok	Australis	A2	
5	8	4	18	4	14	6	Birkini	Ictero	I1	
6	2	4	11	4	5	3	Smithi	Ictero	I13	
3	2	4	11	2	18	7	Romanica	Sejroe	SE8	
3	2	4	11	2	18	7	Wolffi	Sejroe	SE10	
1	13	4	2	3	1	3	Haemolytica	Sejroe	SE2	
1	13	4	5	2	8	3	Geyaweera	Sejroe	SE1	
1	7	4	13	3	14	7	Fugis	Australis	A4	
8	7	5	7	3	2	3	Naam	Ictero	I11	
5	3	5	11	3	5	7	Ricardi	Sejroe	SE7	
3	10	13	14	2	18	14	Pyrogenes	Pyrogenes	PY6	
3	12	12	9	4	3	4	Autumnalis	Autumnalis	Au1	
3	10	6	7	4	13	1	Camlo	Pyrogenes	PY3	
3	14	6	7	4	10	7	Mooris	Autumnalis	Au5	
0	11	7	6	2	13	7	Grippotyphosa	Grippoty	G1	
1	10	7	6	2	15	7	Bataviae	Bataviae	B1	
1	6	6	9	2	12	1	Gem	Ictero	I4	
4	6	3	9	2	10	6	Jalna	Australis	A6	
1	6	7	1	2	14	6	Carlos	Autumnalis	Au4	
1	12	2	5	2	1	2	Lanka	Louisiana	L0	
1	10	2	3	2	10	2	Canicola	Canicola	C4	3
1	10	2	3	2	10	2	Portlandvere	Canicola	C8	4
1	4	3	5	2	2	1	Hebdomadis	Hebdomadis	H1	
1	10	3	9	2	11	13	Muenchen	Australis	A8	
1	10	3	7	2	15	8	Gurungi	Djasiman	D2	

FIG. 3. Dendrogram of the VNTR-typed serovars of *L. interrogans*. The copy number of each VNTR locus is indicated. The serovars and serogroups of reference strains are also indicated. PCR products that were sequenced are underlined. VNTR31was used to differentiate between *L. interrogans* serovars Canicola and Portlandvere from serogroup Canicola.

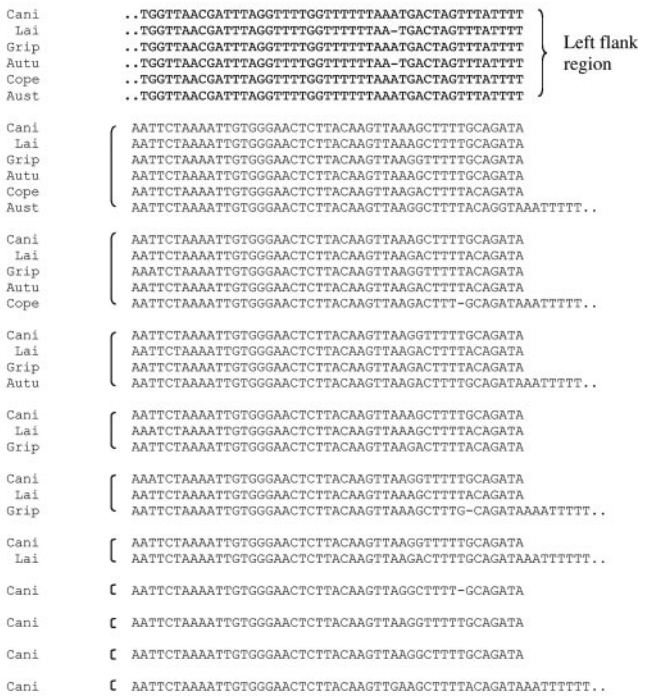


FIG. 4. Sequence alignment of VNTR19 loci. Brackets on the left indicate the repeat units. Cani, *L. interrogans* serovar Canicola; Lai, *L. interrogans* serovar Lai; Grip, *L. interrogans* serovar Grippotyphosa; Autu, *L. interrogans* serovar Autumnalis; Cope, *L. interrogans* serovar Copenhageni; Aust, *L. interrogans* serovar Australis.

isolates displayed a pattern identical to that of the corresponding reference strain, suggesting the stability of PCR patterns in strains belonging to the same serovar. Again, the use of only three markers, i.e., VNTR7, VNTR10, and VNTR19, was enough to identify these strains at the serovar level.

DISCUSSION

Recently, a giant leap forward has been achieved with the completion of the genome sequence of *L. interrogans* serovar Lai (20). *L. interrogans* contains a 4.33-Mb large circular chromosome, a 359-kb small circular chromosome, and no extrachromosomal element (14, 20). Numerous repeated sequences have been found in its genome. For example, about 50 insertion sequences have been identified. The *L. interrogans* genome also contains abundant small repetitive DNA sequences. Among these DNA repeats, the structure of short sequence repeats is typical of tandem repeats. Tandem repeats consist of head-to-tail repetitions of short sequence motifs of about 10 to 100 bp. Polymorphic tandem repeats, also called VNTR, have been extensively used for fingerprinting in higher eukaryotes, including humans. Recently, the use of VNTR has also been described for phylogenetic and epidemiological studies of pathogenic bacteria.

A database of tandem repeats in more than 140 completely sequenced genomes is available (3). By using this database (http://iech5.igmors.u-psud.fr/GPMS/), genome analysis indicates that most of the tandem repeats (901 of 1,100; 82%) in *L. interrogans* strain Lai are between 15 and 50 bp, which is convenient for observation of polymorphism by analyzing PCR products of polymorphic loci on agarose gels. Compared to those of other bacteria, the *L. interrogans* genome exhibits a high number of tandem repeats with a total length greater than 100 bp (27 and 29 per Mb for strains Fiocruz and Lai, respectively). Comparative genomics shows that 131 VNTR loci (length of unit repeat, between 5 and 500 bp) were shared between the two available *L. interrogans* genomes (3, 14, 20). For strains within a species or genus, this number varies from 4 (strains from *Chlamydia pneumoniae*) up to 163 (strains from *Yersinia pestis)*. Tandem repeats predicted to be polymorphic by genome comparison between the two serovars from serogroup Icterohaemorrhagiae indeed exhibit size variations by PCR typing of *Leptospira* strains.

The function of repetitive elements in bacteria is not fully understood. Similar to the *Mycobacterium tuberculosis* mycobacterial interspersed repetitive units (21), tandem-repeat-containing loci in *L. interrogans* are located in intergenic regions, are dispersed throughout the genome, and constitute subfamilies based on sequence similarities. The dissemination of homologous VNTR loci in the genome may suggest frequent intragenomic rearrangement or that these elements are (or have been) mobile elements. Studies on the structural and functional properties of these families of repetitive DNA should improve our knowledge of the role of these abundant repeat sequences in *Leptospira* spp.

All molecular tools described so far for the diagnosis of *Leptospira* suffer from significant drawbacks, such as a low discrimination level, lack of reproducibility, and requirement for large quantities of purified DNA. We therefore took advantage of the presence of VNTR-like regions to design a PCR-based test. Our data showed that VNTR typing was able to differentiate 45 out of 51 serovars of *L. interrogans*. Our VNTR assay shows that serovars from either the same serogroup or the same geographical area are not grouped together (Table 1; Fig. 3). This was true when the seven markers were used in combination but also when only three markers (VNTR7, -10, and -19) were used. This result confirms the heterogeneity among serovars of a given serogroup previously found by DNA relatedness (2) and PFGE (8, 9). Most serovar reference strains from *L. interrogans* have been isolated in Asia (2), mainly in southeast Asia (Table 1). It is noteworthy to find that strains originating from distant continents can be grouped together in the dendrogram. We also show that VNTR analysis was able to differentiate serovars among *L. interrogans* clinical strains, therefore demonstrating the usefulness of this PCR-based method for the identification of *L. interrogans* serovars.

A current method for typing *Leptospira* strains is macrorestriction by PFGE. This method is labor-intensive and not accessible to most laboratories in tropical and subtropical countries, where the incidence of the disease is the highest. Similar to PFGE, VNTR typing was not able to discriminate between *L. interrogans* serovars Icterohaemorrhagiae and Copenhageni. However, *L. interrogans* serovars Muenchen, Jalna, and Bratislava from serogroup Australis, which gave identical NotI macrorestriction patterns by PFGE (9), were differentiated by VNTR analysis. The use of only one VNTR locus, VNTR19 or VNTR23, was enough to discriminate serovars Muenchen, Jalna, and Bratislava (Fig. 3). In contrast, VNTR analysis of *L. interrogans* serovars Bratislava and Aus-

tralis from serogroup Australis gave identical results (Fig. 3), but the two serovars were differentiated by PFGE (9). Distinct macrorestriction profiles of closely related strains could be due to large genomic rearrangements. For example, comparative genomics between two *L. interrogans* serovars from serogroup Icterohaemorrhagiae revealed a one-Mb chromosomal inversion (14).

In conclusion, this method based on VNTR polymorphism provides rapid typing as well as a highly discriminant assay to identify *L. interrogans* serovars. In addition, VNTR typing could be widely accessible for research and public health laboratories, particularly in developing countries. This method should also be suitable for sharing results and for the generation of databases. Further studies should include the development of a VNTR typing test with biological materials. The genome sequences of other *Leptospira* pathogenic strains are at different stages of completion. These sequences would greatly facilitate the development of multiple-locus VNTR assays for pathogenic *Leptospira* spp.

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