Genomic DNA repeat from *Leishmania* (*Viannia*) *braziliensis* (Venezuelan strain) containing simple repeats and microsatellites

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SUMMARY

In this paper the *Leishmania* (*Viannia*) *braziliensis* complex is defined as containing all species of the actual subgenus *Viannia*. Organisms of the *L.* (*V*) *braziliensis* complex are the causative agents of localized human cutaneous and mucocutaneous leishmaniasis in South America, much of Central America and some ares of North America. In our search for better species and subspecies diagnostic probes we focused our research on repetitive DNA, since it provides a greater number of target sites for hybridization. In this work we report the isolation and sequencing of a 1·8 kb DNA region, LbJ38, which is probably tandemly repeated or dispersed at least 4 times along one chromosome and is naturally present in *L.* (*V*) *braziliensis* genomic DNA. This region contains microsatellites and simple repeat DNA sequences and was isolated by screening a genomic DNA cosmid library with complex- and species- specific probes. No homology was found with other *Leishmania* microsatellite or repetitive DNA. The utility of this repetitive sequence and primers derived from it in the identification of *L.* (*V*) *braziliensis* is demonstrated. As far as we are aware, this is the first report of sequence characterized repetitive microsatellite and GC rich simple repeat DNA from the nuclear genome of New World *Leishmania*.

Key words: *Leishmania* (*V*) *braziliensis*, microsatellite, DNA sequence, PCR.

INTRODUCTION

The *Leishmania* (*Viannia*) *braziliensis* complex is defined in this paper as containing all species of the actual subgenus *Viannia* (Lainson & Shaw, 1987). Organisms of the *L.* (*V*) *braziliensis* complex are the causative agents of localized human cutaneous and mucocutaneous leishmaniasis in South America, much of Central America and some areas of North America. In our search for better species and subspecies diagnostic probes we focused our research on repetitive DNA.

Multicopy genes and repeat sequence DNAs provide extremely useful molecular tools which may allow species differentiation or characterization, diagnosis, the study of genetic diversity or the establishment of phylogenetic relationships. Kinetics studies have shown that around 25% of the *Leishmania* genome (estimated at a total of 6·5 × 107 bp/cell by Leon, Fouts & Manning (1978)) consists of repetitive sequence (Wesley & Simpson, 1973; Tripp, Myler & Stuart, 1991). However, despite the abundance of repetitive nuclear DNAs, few have been identified. They include circular (CD1) (Stuart, 1991; Hamers *et al*. 1989) and linear (LD1) DNA elements (Tripp *et al*. 1991); the mini-exon gene (Fernandez *et al*. 1994); telomeric repeats (Howard *et al*. 1991) and a repetitive DNA sequence isolated from the 250 kb minichromosome of *L.* (*V*) *braziliensis* (Eresh, Mendoza-Leon & Barker, 1993). Multicopy genes such as alpha and beta tubulin (Landfear, McMahon-Pratt & Wirth, 1983), gp63 (Dujardin *et al*. 1994; Espinosa *et al*. 1995) and ribosomal genes (Van Eys *et al*. 1992) have also been characterized.

Repeat sequence DNAs have been extensively used in the study of variation within the *Leishmania* genus: the mini-exon gene has been used to differentiate the major groups of Old and New World *Leishmania* (Fernandez *et al*. 1994; Van Eys *et al*. 1992) telomeric sequences have been used in the characterization of *L.* (*L*) *donovani* (Howard *et al*. 1991); analysis of microsatellites in the genome of Old World *Leishmania* strains showed (CA)n, (GGT)n and (GCA)n to be present on all chromosomes, and revealed similarities in strains from different Old World *Leishmania* complexes (Rossi *et al*. 1994). Primers derived from repetitive sequence have been used to establish phylogenetic

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Table 1. *Leishmania* used in this study

Species		International code
L. (*Viannia*) *braziliensis*		MHOM/BR/75/M2903
L. (*V*) *braziliensis*		MHOM/BR/84/LTB300
L. (*V*) *guyanensis*		MHOM/BR/75/M4147
L. (*V*) *panamensis*		MHOM/PA/71/LS94
L. (*V*) *peruviana*		MHOM/PE/84/LC26
L. (*V*) *lainsoni*		MHOM/BR/81/M6426
L. (*V*) *colombiensis*		IHAR/CO/85/CL500
L. (*Leishmania*) *mexicana*		MHOM/BZ/82/Bel 21
L. (*L*) *mexicana*		MNYC/BZ/62/M379
L. (*L*) *venezuelensis*		MHOM/VE/00/H17300
L. (*L*) *amazonensis*		IFLA/BR/67/PH8
Isolates from infected patients:		
Characterization	Type of lesion	International code
L. (*V*) *braziliensis*	LCL*	MHOM/Ve/93/JJV
braziliensis complex†	LCL	MHOM/Ve/85/EGL
braziliensis complex†	LCL	MHOM/Ve/93/JNR
braziliensis complex†	LCL	MHOM/Ve/85/ADB
braziliensis complex†	LCL	MHOM/Ve/94/JM
braziliensis complex†	LCL	MHOM/Ve/93/JR
braziliensis complex†	LCL	MHOM/Ve/93/ES
braziliensis complex†	LCL	MHOM/Ve/93/JG
braziliensis complex†	LCL	MHOM/Ve/93/AV
braziliensis complex†	LCL	MHOM/Ve/93/AB

* LCL, localized cutaneous leishmaniasis.
† The isolates characterized as the *braziliensis* complex are suspected to be variants from the *L.* (*V*) *braziliensis* species.

relationships between Old World *Leishmania* species (Piarroux *et al*. 1995) and to diagnose visceral leishmaniasis (Piarroux *et al*. 1993). In addition, repetitive sequences may play a role in homologous recombination in *Leishmania* (Fernandez *et al*. 1994), which may explain both inter- and intra-specific chromosome size variation.

Here we describe the isolation and sequence of a useful repetitive DNA region, LbJ38, isolated from a *L.* (*V*) *braziliensis* total DNA cosmid library. LbJ38 is a 1·8 kb genomic fragment containing many simple repeats of between 6 bp and 25 bp in length. These repeat sequences comprise 3 microsatellites (GT) 5 (AG) 8 and (GATC) 4, 20 runs of poly G, C, A, or T and 18 other mixed oligonucleotide repeats. The LbJ38 sequence was found by hybridization experiments to be present in at least 4 genomic bands in digested *L.* (*V*) *braziliensis* DNA, suggesting that this region is itself repeated. We believe this to be the first report of sequence characterized repetitive microsatellite and simple repeat DNAs from the nuclear genome of New World *Leishmania*.

MATERIALS AND METHODS

Parasites

The human infecting *Leishmania* strains used in this study were isolated from Venezuelan patients with cutaneous leishmaniasis and previously characterized with the aid of kinetoplast DNA restriction patterns and monoclonal antibodies. International reference strains were obtained from the Instituto de Biomedicina (Caracas, Venezuela). Promastigotes were grown at 24 °C in blood agar base medium (Difco) supplemented with 10% defibrinated rabbit blood. The parasites used in this study are listed in Table 1.

Isolation of total DNA

Leishmania cells were harvested by centrifugation at 3000 ***g*** at 4 °C, washed 3 times in PBS (phosphate-buffered saline), and resuspended in NET 100 buffer (100 mM NaCl, 100 mM EDTA, 10 mM Tris, pH 8). Lysis of the parasites was carried out in 1% SDS (sodium dodecyl sulfate) and 100 μg/ml Pronase E (Boehringer Mannheim) at 56 °C for 3 h.

DNA was extracted twice with phenol/chloroform (v/v) and precipitated with 4 M LiCl and 100% ethanol at −20 °C overnight. After centrifugation at 4 °C, the DNA was resuspended in TE (10 mM Tris–HCl, pH 7·5, 1 mM EDTA) and stored at 4 °C.

Construction of a DNA cosmid library

A total of 24 μg of *L.* (*V*) *braziliensis* (JJV strain) total DNA (> 100 kb) was partially digested with

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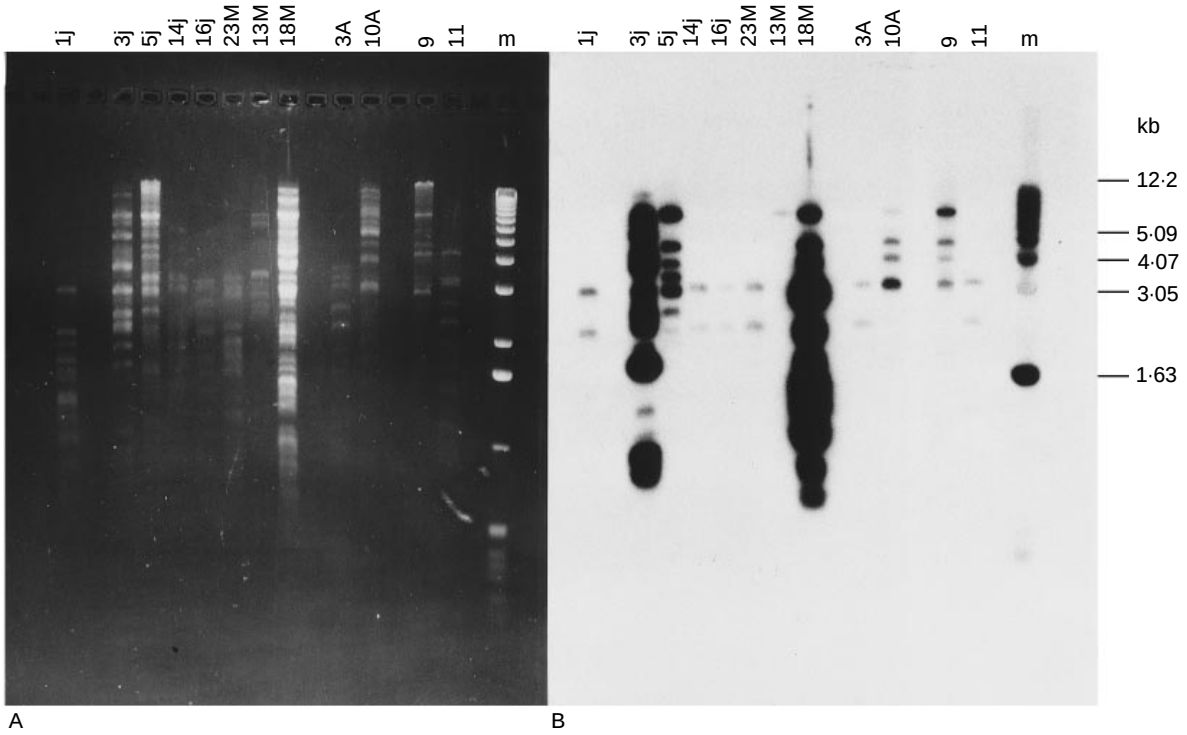

Fig. 1. *Eco*R 1/*Pst* 1 digest of a selection of clones from the *Leishmania* (*V*) *braziliensis* cosmid library, separated by electrophoresis in 0·8 % agarose (A). Clones were selected from the library using the following probes: JJV: 1J, LbJ38, 5J, 14J and 16J; M2903-derived DNA: 23M, 13M, 18M, 3A and 10A; *L.* (*L*) *mexicana*-derived DNA: 9 and 11. DNA was transferred onto a nylon membrane using Southern blotting, and hybridized with LbJ38 (B). The hybridization signal, in general, corresponds to the amount of DNA present which was not exactly the same in each track. Note that the cosmid LbJ38 clone has residual lambda sequences and therefore hybridizes with some bands of the lambda molecular weight marker DNA. JJV probe, is total DNA isolated from *L.* (*V*) *braziliensis* (JJV). M2903-derived DNA: 3 probes were used. The 2903 probe was total DNA isolated from *L.* (*V*) *braziliensis* (M2903). The second probe was a pool of 42 clones isolated from an *L.* (*V*) *braziliensis* (M2903) genomic cosmid library after screening with the highly amplified 250 kb chromosome. The third probe was a *Not* 1 fragment from a specific *L.* (*V*) *braziliensis* cosmid clone, amplified by random primer PCR. *L.* (*L*) *mexicana* derived DNA. This probe was a *Not* 1 fragment from a *L.* (*L*) *mexicana*-specific cosmid clone, amplified by random primer PCR.

Mbo 1, and fragments greater than 40 kb were selected and dephosphorylated with calf intestinal phosphatase (Gibco-BRL). The DNA was ligated to the cosmid PWE15 arms using T4 DNA ligase (Gibco–BRL), at 12 °C overnight. The ligated DNA was packed *in vitro* as described by Bates & Swift (1983). The packaged DNA was transduced into *E. coli* NM554 and plated onto LB/agar medium with ampicillin (50 μg/ml).

Screening of the DNA cosmid library and hybridization

Colonies were screened using hybridization (Sambrook, Fritsch & Maniatis, 1989). Probes were labelled with alpha P32 dATP (3·0 Ci/mmol) using the Prime-It II random priming kit (Stratagene). Filters were pre-hybridized for 4–6 h at 42 °C, in 50 % formamide, 5 × SSC, 1 % SDS; 5 × Denhardts and 50 μg/μl salmon sperm DNA. Hybridization took place for 12–18 h at 42 °C. The filters were washed to a stringency of 2 × SSC, 0·1 % SDS thrice at 65 °C and exposed to photographic film (GRI Ltd) overnight at −70 °C. Colonies exhibiting a strong hybridization signal and retaining a positive signal at a higher stringency wash with 0·1 SSC and 0·1 % SDS were selected for further analysis.

Restriction analysis and probe preparation

Cosmids from recombinant clones were harvested using the Wizard Miniprep kit (Promega) and restricted with *Eco*R I/*Pst* 1 (Gibco–BRL) to release the insert DNA. Fragments were separated by electrophoresis on 0·8 % agarose, and transferred to nylon membranes using the Southern blotting technique (Southern, 1975). A 1·8 kb fragment from one of the recombinant clones was selected for further analysis. The 1·8 kb fragment was isolated from agarose by the freeze and squeeze techniques (Tautz & Renz, 1983) and labelled with alpha P32 dATP as described. The labelled fragment was used for screening clones containing repetitive DNA sequences.

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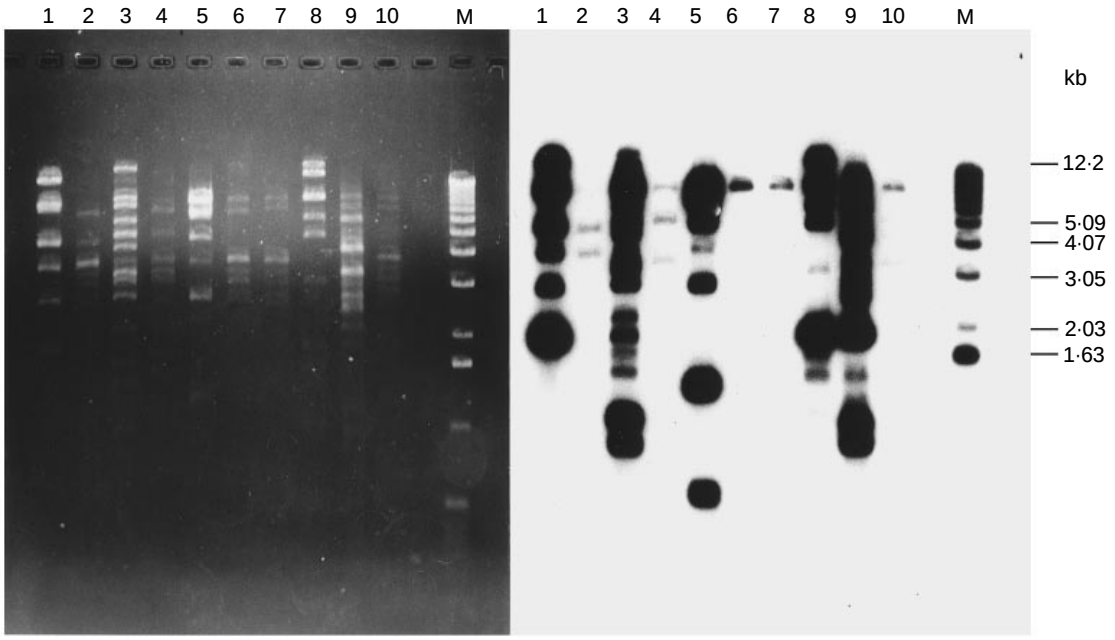

Fig. 2. Recombinant clones 3J and 18M digested with a selection of restriction enzymes (A) and hybridized with LbJ38 (B). Samples were loaded alternately (3J followed by 18M) except in lanes 7 and 8, where the order was reversed. Lanes 1 and 2: *Hin*d III; Lanes 3 and 4: *Sma* 1; Lanes 5 and 6: *Xba* 1; Lanes 8 and 7: *Bam* H1; Lanes 9 and 10: *Pst* 1. M: molecular weight markers (1 kb lambda ladder).

Subcloning and sequencing

LbJ38 was subcloned into pBluescript II SK+ plasmid vector (Stratagene). Sequencing was carried out by the chain termination technique (Sanger, Nicholson & Coulson, 1975) using both the Sequenase Version 2 kit (USB) and automatic sequencing (ABI, Applied Biosystems, Model 373 A Version 1.2.1). Both strains were sequenced.

Sample preparation and PCR

Crude lysates were prepared by centrifugation of 100 μl of a logarithmically growing *Leishmania* promastigote culture at 10000 ***g*** for 10 min at room temperature. The pellet was resuspended in 50 μl of sterile distilled water and lysed for 10 min at room temperature.

A pair of primers to be used in PCR was selected from the sequence of LbJ38: 3J1 5′ TACCTGATGACTCCCAC 3′; 3J2 3′ CCTCATCATACCGTTGATC 5′. Five μl of the culture lysate, containing approximately 1 ng of parasite DNA, was amplified in a total volume of 25 μl containing 50 mM KCl, 10 mM Tris–HCl (pH 8), 1·5 mM $MgCl_2$, 0·01% gelatin, 0·2 mM dNTP mix, 100 ng of each primer and 2·5 units of *Taq* polymerase (Perkin Elmer). Each reaction was overlayed with 30 μl of mineral oil and denatured for 6 min at 95 °C. Amplification took place during 35 cycles of annealing at 60 °C for 1 min, extension at 72 °C for 1 min and denaturation at 95 °C for 30 sec, with a final extension at 72 °C for 10 min. The programme was run on a Trio Block machine (Biometra). PCR products were analysed on 1% agarose gels in 1 × TBE buffer.

RESULTS

Construction and screening of a cosmid library

The number of clones in the library was calculated at 6×10^4, with an average insert size of between 37 and 45 kb. From the expanded library 30000 clones were screened by *in situ* hybridization using alpha P32 dATP-labelled probes.

The Venezuelan strain cosmid library was screened with probes designed to hybridize to repetitive sequence specific to *L.* (*V*) *braziliensis*.

Probe JJV is total DNA isolated from *L.* (*V*) *braziliensis* (JJV). Probe 2903 is total DNA isolated from *L.* (*V*) *braziliensis* (M2903). The probe of DNA derived from high copy number sequences is a pool of 42 clones isolated from another *L.* (*V*) *braziliensis* (M2903) genomic cosmid library after screening with the highly amplified small 250 kb chromosome found in that strain. The N1 probe, is a *Not* 1 fragment from 1 specific *L.* (*V*) *braziliensis* cosmid clone, amplified by random primer PCR. These probes were used to select for repetitive sequences common to both JJV and a reference strain of *L.* (*V*) *braziliensis*.

Thirty-nine clones with strong hybridization signals after overnight exposure were selected for further investigation, specifically to look for re-

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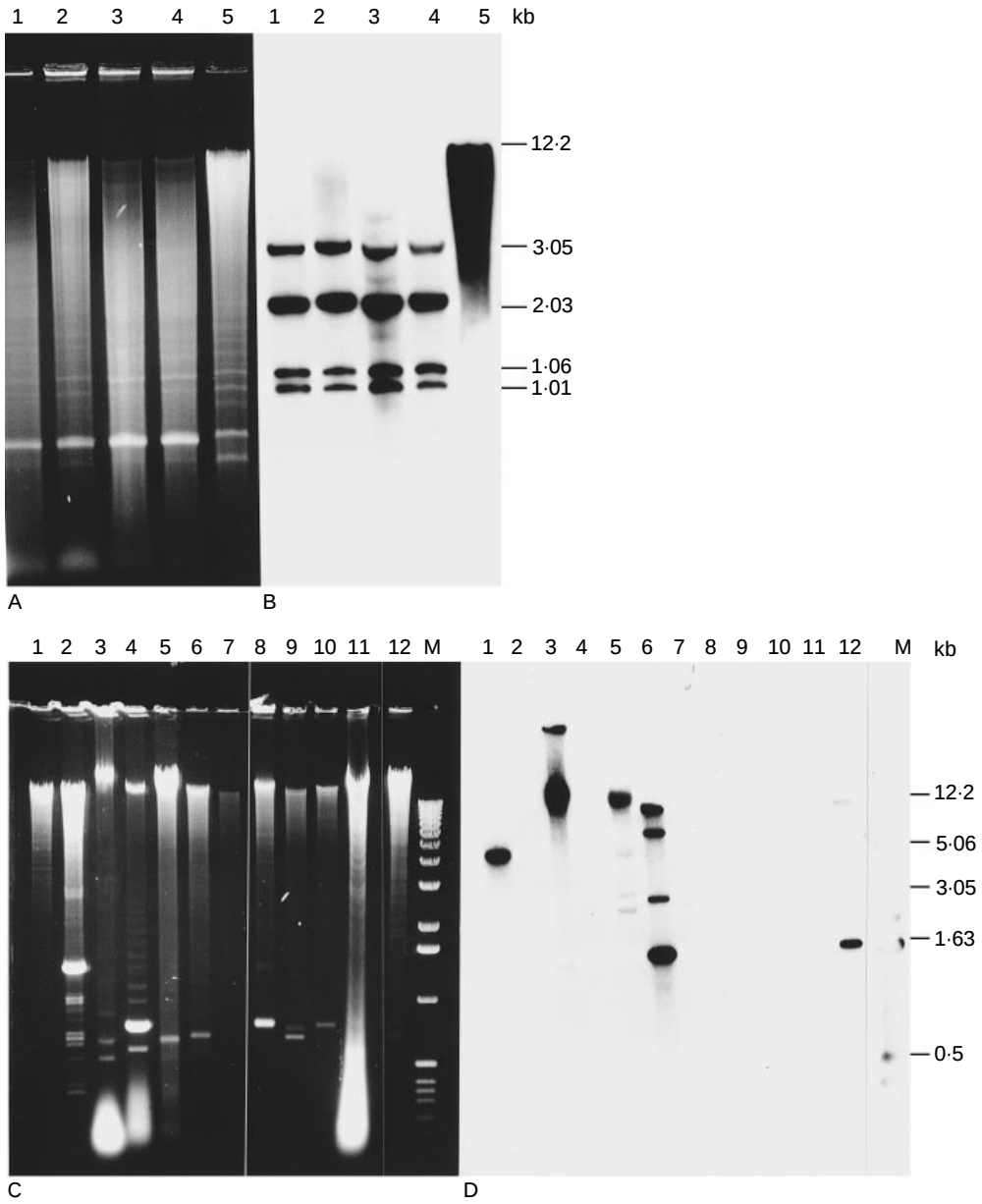

Fig. 3. *Eco*R I digested total DNA (A and C) hybridized with LbJ38 (B and D). (A and B) Lane 1: *Leishmania* (*V*) *braziliensis* M23903; Lane 2: *L.* (*V*) *braziliensis* JJV; Lane 3: *braziliensis* complex JNR; Lane 4: *braziliensis* complex AV; Lane 5: *L.* (*V*) *peruviana* LC26. (C and D) Lane 1: *L.* (*V*) *lainsoni* M6424; Lane 2: *Trypanosoma cruzi*; Lane 3: *L.* (*V*) *peruviana*; Lane 4: *L.* (*V*) *guyanensis* M4147; Lane 5: *L.* (*V*) *panamensis* LS94; Lane 6: *L.* (*V*) *braziliensis* LTB300; Lane 7: *L.* (*L*) *venezuelensis* H17 300; Lane 8: *L.* (*L*) *pifanoi* LL1; Lane 9: *L.* (*L*) *amazonensis* PH8; Lane 10: *L.* (*L*) *mexicana* M379; Lane 11: *L.* (*L*) *infantum* IPT1; Lane 12: *L.* (*L*) *colombiensis* LC500. M: molecular weight markers (1 kb DNA ladder).

petitive DNA. The *L.* (*L*) *mexicana*-derived sequence probe was a *Not* 1 fragment from a *L.* (*L*) *mexicana*-specific cosmid clone, amplified by random primer PCR and was used as a negative control, to enable us to eliminate repetitive sequences which may have been common to both the *mexicana* and *braziliensis* complexes. No signal was observed with this *L.* (*L*) *mexicana*-specific probe.

Isolation of repetitive DNA probes

DNA from each individual clone selected above was single and double digested with 5 units of *Eco*R I, *Not* 1 and *Pst* 1. A 1·8 kb fragment (LbJ38) derived from clone 3J was chosen for further analysis, based on an intense signal after hybridization with JJV total DNA. The fragment was isolated from agarose, and used as a probe.

Characterization of L. (V) braziliensis *genomic DNA repetitive sequence*

Hybridization of *Eco*R I-digested clones with LbJ38 revealed numerous bands extending from 0·8 to 12 kb in clones 3J and 18M (Fig. 1A and B). The other clones shown in Fig. 1 (A and B) were not used

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0001  ATCGAATTCA  AGAAAGACAA  AGGAAAGGAG  AGCGAAACGG  AAAGCGAAGA
0051  GAGTGAGACG  GTCGACCCGA  CCTCAGCCTC  AGTCCCCAGT  CCCTCTAGCC
0101  TCTCCCTTCA  TGTAAAGTCG  CAGTGGCTTA  CGCTTTTCAG  TCCTAGAGAG
0151  AGGCGTATTG  TATGATCAAC  GGTATGATGA  GGAGCTTGAA  GACCGAACGC
0201  TGAGAACCGA  AGCGTAACAT  TAGCTAGAGT  CAGAGAGAGA  GAGAGAGCAC
0251  TGCAGAGCTG  TCCTTCGCGC  AGTGCTAGGT  ACTAGATCCG  ACCCGGTTCA
0301  CCCTTGGGAA  TAAAAATTTT  TTTCCCAAAT  GGCCAAAAAG  GGGCAGGGGC
0351  CCCCAAGGCC  GGGGGGGGT   AGGGGCTTTT  CCAAAAGGTT  CCCCCCCCCA
0401  AAGAGAGCAA  AAATTTACCC  AAAAAAGGG   GGGGGCCCCC  CCCCCCCCCC
0451  CCTTTGCGGT  TGGGCGTTGG  ACGCTTTCCC  TGGCCCAAGG  CAGCGGTATG
0501  TTATGTTTTA  ACAGTGGATG  GATGGAGGGG  GGGTGGACCA  TTCAGGGCAC
0551  CTTTCCTCTC  CCCTTTGCCG  GCTCCCCATG  GGAAACTTTT  TATTCTTACA
0601  ATATATTTAC  CCTGGATCTC  TCGCCTTCAC  GTCTTTTTTA  CACCCCATCC
0651  CCCCCGAGGT  GGACAAGGCC  TTCGGGGGTC  TGTTCTAGGT  TATTTTGTGG
0701  GTGCCCCCCC  ATCCCTTTTT  TACCCCCTCG  CCCGCCCCCG  TCAAACCCTC
0751  TCCCTCCTCA  CCCTTACCTG  ATGACTCCCA  CGGCCCACTT  TCCCATACTC
0801  ACTTCCTTTT  CCTCTTGTTT  CATCCCTTCC  CCGCCCCTCC  CCCCCCGAAA
0851  TCTTCCTCCC  TTCACTAATT  TCCCCCTTGC  CATTACTAAA  TCGTACCTTC
0901  CTCCGGCCGG  CCCTCCGGCT  CCCTCATGTT  CCTCCCGTTA  CCCTCTAATG
0951  GGGGTGGGAG  CCCCCCCCTT  CGGCGCCTTT  CCGTACTCCC  CCTTACGATT
1001  GGCACCGCTC  CACCAGGGCG  GGGATTCCTT  CCTTTCTTCC  ACTCTCTATT
1051  TTTCGTTCTT  CAAACCTCTT  CCCTTCTCTC  GGCTTGCCTC  TCCCTTCATG
1101  TACGAGCTGC  ACCTGCCCGG  GTCTGTGGTG  ATGACGGCGG  TCGAGAGCGC
1151  GCCTGGCAAC  GGCAAGAACA  GTGAGGAGTA  TGTCTAACGT  CTTCTTGGCG
1201  TAGGAGTAGC  TTTTTGGACG  AGAGCGATGT  CGTTCTCGAG  CGAGGCCTAC
1251  GCCTCACGCC  ATCGCACGTA  CCAGCTCGCT  GCAGGTTGAC  ATACCCCTTG
1301  CTTGAAGGGC  AGTGGCGTCT  CTTCCCACCC  CCCCTTCTTT  AAGTTTCTTT
1351  CCCCTTTTTT  TAGTAAGGGC  CTGCGCTTGT  GCGTCCGTTG  TGTGTGTGTT
1401  GTTGTCAATT  GAATTGACTT  GACTGACTTC  GGTCAAGATA  AGCGAACGCC
1451  CGCGCTTATT  GTTCCCCCCT  CCCGCTTCCA  CGATGGTGCA  ATTTCGGGTT
1501  TACGAATTAC  GCCCCTTCCC  CATTACCCTT  GGGAAAATTA  ATATCTTCCC
1551  CCTTGGGAGG  CTTCGATTAA  ATGGGGAAAA  AGGTTTCCTC  TTCTTTTTGC
1601  CCCAAAAAAA  GGAAAGTCCC  AAAAGTCTTA  CGCTTTTTTC  AGTCACTTAG
1651  AGAGGAAGGC  GGTATGTTAT  GTACAACAGT  GGATGGATCT  AGGAGATCGA
1701  TCGATCGATC  GATAGGTGGA  AAGCACGAAA  CGCCTGAGGA  CACGAGACGT
1751  ATACAATTTT  ATGAAAAAAA  GTTCCTCTTC  TTTTCCGCCC  CAAAAAAAGC
1801  CTTCCTGTCG  GGCCGGTTCC  TGGTCCGGTC  C
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Fig. 4. Nucleotide sequence of LbJ38. Microsatellites are in *bold italics*; Runs of poly(G) and poly(C) are in *bold and underlined*; runs of poly(A) and poly(T) are *underlined*. The primers 3J1 and 3J2 are indicated by bold type at positions 564 and 164 respectively. 3J2 is the complimentary sequence to the reverse sequence 164–182.

for further analysis. In order to confirm the presence of repetitive sequence, 3J and 18M were digested with a panel of restriction enzymes. The results in Fig. 2 (A and B) indicate that only 3J contains a high dispersion of LbJ38. LbJ38 is repeated 4 times with respect to *Eco*R I (Fig. 3) but is also internally restricted with respect to other enzymes such as *Hae* III, *Pst* 1, *Rsa* 1 and *Bcl* 1.

Repetitive DNA among Leishmania *species*

Total DNA from different *Leishmania* strains was digested with *Eco*R I, and hybridized with labelled LbJ38 (Fig. 1 A, B,C and D). The LbJ38 sequence is present in all *L.* (*V*) *braziliensis* variants investigated, showing at least 4 intense hybridization bands of 3 kb, 2 kb and 2 other bands of approximately 1 kb, although *L.* (*V*) *braziliensis* (LTB 300) shows 4 bands at 10, 8, 3 and 1·6 kb. The restriction enzyme digestion releases fragments of minicircles from kinetoplast DNA below 700 bps which do not hybridize with this probe. Only 1 strong hybridization band was detected in *L.* (*V*) *peruviana*, *L.* (*V*) *lainsoni*, *L.* (*V*) *colombiensis* and *L.* (*V*) *panamensis* at 12, 5, 1·6 and 12 kb respectively. No hybridization signal was observed in *T. cruzi*, *L.* (*L*) *infantum* (IPT1) nor in any *L.* (*L*) *mexicana* isolates. In addition no hybridization was observed in *L.* (*V*) *guyanensis* (M4147). In order to be certain of this result strain M4147 total DNA was digested with a number of restriction enzymes and hybridized with LbJ38 with negative results. Professor J.-P. Dedet kindly sent us 9 other strains of *L.* (*V*) *guyanensis* characterized by isoenzymes. Restriction enzyme digests of total DNA of these strains also failed to be hybridized with LbJ38 (results not shown).

Sequence data analysis

The complete sequence for LBJ38 was obtained (Fig. 4). It was analysed at EMBL gene bank, using the Fasta GCG program.

Our results show that LbJ38 contains 3 dispersed microsatellites: (AG)8, (GATC)4 and (GT)5 and 18 other oligonucleotide repeats from 6 to 16 bp in length, the majority of which are GC-rich (Table 2). Contained within the sequence are also 20 homo-nucleotide runs which vary in size from 6 to 25 bp.

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Table 2. Repetitive elements found within LbJ38 sequence

(Searched at EMBL/GENBANK using the GCG-repeat program.)

Element type	Position(s)	Sequence
Microsatellite	232–247	(AG) 8
	1695–1710	(GATC) 4
	1390–1400	(GT) 5
Direct repeat	80–93	(CAGTCCC) 2
Dispersed repeat	160–166, 172–178	GTATGAT
(AT-rich)	690–696, 594–598	TTATTTT
	1531–1537, 1574–1580	GGGAAAA
	1577–1583, 1606–1612	AAAAAGG
	1601–1607, 1618–1624	CCCAAAA
Dispersed repeat	338–344, 370–376	AAGGGGC
(GC-rich)	350–356, 395–401	CCCCCAA
	396–402, 418–424	CCCCAAA
	823–829, 857–863	TCCCTTC
	852–859, 897–904	CTTCCTCC
	898–904, 929–935	TTCCTCC
	900–907, 912–919	CCTCCGGC
	964–970, 988–994	CCCCCTT
	1070–1076, 1091–1097	TCCCTTC
	1174–1180, 1202–1208	AGGAGTA
	1515–1521, 1545–1551	CTTCCCC
	1526–1534, 1550–1558	CCCTTGGGA
Poly(A/T)	1604, 1764, 1792, 1355	(A) 7 or (T) 7
	634, 716, 1634	(T) 6
	309–323	(A) 5 (T) 7
Poly (G/C)	527, 1328, 649, 704	(G) 7 or (C) 7
	1464–1469	(C) 6
	960–968	(C) 8
	391–400	(C) 9
	428–450	(G) 8 (C) 15
	1016–1023	GGGCGGGG
	1115–1121	GCCCGGG
	730–740	CGCCCG (C) 5G
	346–368	(G) 4 (C) 5

Some of the repeats have micro-homology with human microsatellites and others have micro-homology with repetitive DNA sequences previously described in prokaryotic and eukaryotic organisms. The Fasta search did not reveal any homology with other repetitive sequence previously described in *Leishmania*, nor did it reveal any significant homologies in other organisms to our sequence once the monomeric runs were removed. Several small open reading frames were found, the longest of which was about 233 amino acids in length and proline-rich. A BLAST search of the SwissProt database revealed no significant homologies.

Specificity of PCR

Amplification of genomic DNA from lysed *L.* (*V*) *braziliensis* (M2903) parasites using primers 3J1 and 3J2 gave a product of 617 bp (Fig. 5A). This product was the same when DNA from other braziliensis complex strains, suspected to be variants of *L.* (*V*) *braziliensis*, was used as the template. No amplification occurred with *L.* (*V*) *panamensis* (LS 94). *L.* (*L*) *amazonensis* (PH8), *L.* (*L*) *mexicana* (Bel 21), *Trypanosoma cruzi* or *L.* (*V*) *guyanensis* (M4147). *L.* (*V*) *peruviana* (LC 26) gave a product of 300 bp. Labelled LbJ38 DNA hybridized specifically to the 617 bp fragment (Fig. 5B).

The sensitivity of the primer pair 3J1 and 3J2 has been tested and we are able to detect 0·1 ng in dilution experiments using purified parasite DNA and approximately 5 parasites using serial dilutions of cultured promastigotes of *L.* (*V*) *braziliensis* variants (results not shown).

DISCUSSION

In this work, we report the construction of a *L.* (*V*) *braziliensis* genomic DNA cosmid library and its use in isolating a repetitive DNA sequence. Screening of 30000 clones (an estimated 30× haploid coverage) facilitated isolation of a repeat DNA sequence from *L.* (*V*) *braziliensis*, a 1·8 kb fragment called LbJ38. Restriction enzyme digestion of total *L.* (*V*) *braziliensis* DNA with *Eco*R I, *Eco*R V, *Bam*H 1, *Kpn* 1 and *Hin*d III released multiple fragments to which LbJ38 sequence hybridized, which indicated

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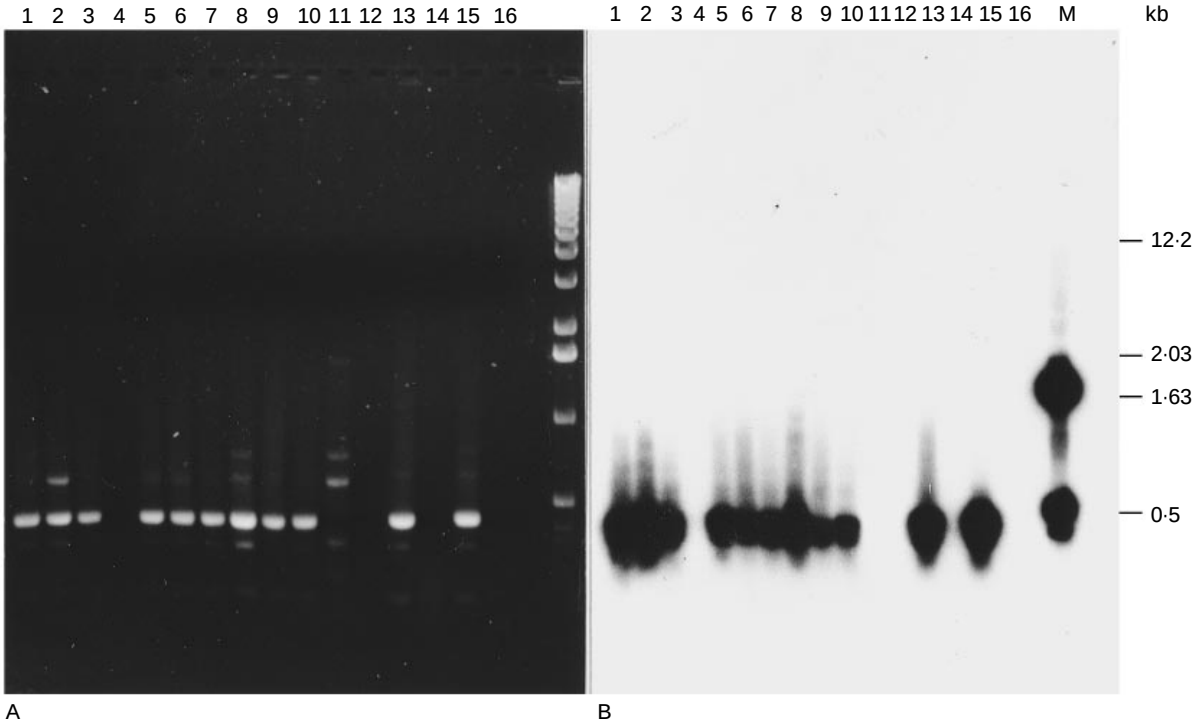

Fig. 5. PCR-amplified genomic DNA fragments using primers 3J1 and 3J2 derived from the LbJ38 sequence (A), hybridized with LbJ38 (B). PCR products were separated by electrophoresis in 1% agarose. Lane 1: *Leishmania* (*V*) *braziliensis* (M2903); Lane 2: *L.* (*V*) *braziliensis* JJV; Lane 3: *braziliensis* complex AV; Lane 4: *L.* (*L*) *amazonensis* (PH8); Lane 5: *braziliensis* complex ADB; Lane 6: *braziliensis* complex JNR; Lane 7: *braziliensis* complex EGL; Lane 8: *braziliensis* complex JM; Lane 9: *braziliensis* complex JG; Lane 10: *braziliensis* complex JR; Lane 11: *L.* (*L*) *mexicana* (Bel 21); Lane 12: *Trypanosoma cruzi*; Lane 13: *braziliensis* complex ES; Lane 14: *L.* (*V*) *guyanensis* (M4147); Lane 15: *braziliensis* complex AB; Lane 16: Negative control (human DNA); M: Molecular weight marker (1 kb DNA ladder).

that the LbJ38 sequence is itself repeated in the genome, making it an ideal target for PCR amplification. A telomeric probe does not hybridize to LbJ38, unlike the other repetitive sequence previously described (Ellis & Crampton, 1988, 1989). The LbJ38 sequence is located in the large chromosomal region (both homologues of the 925 kb chromosome band) of the *L.* (*V*) *braziliensis* JJV genome (result not shown). The function of LbJ38 is unknown.

The LbJ38 sequence when used as a probe revealed differences between and within species. LbJ38 hybridized to a varying number of bands in digested genomic DNA from different *L.* (*V*) *braziliensis* variants suggesting polymorphism of the LbJ38 sequence in *L.* (*V*) *braziliensis* isolates from human patients (manuscript in preparation). Polymorphism of repeat DNA with the large size of LbJ38 is of interest. Other repetitive subtelomeric and telomeric sequences have been implicated in chromosome size polymorphism in *L.* (*V*) *peruviana* (Dujardin *et al.* 1993).

LbJ38 is present in a single *Eco*R I fragment in all other *braziliensis* complex species investigated but absent from the DNA of the 10 isolates of *L.* (*V*) *guyanensis* we have tested. This could be exploited as a useful test to distinguish *L.* (*V*) *guyanensis* from other members of the *braziliensis* complex. LbJ38 is not present in *T. cruzi* nor any *L.* (*L*) *mexicana* strain.

Within the LbJ38 sequence are simple repeats and microsatellites between 6 and 16 bp in length. The microsatellites (GATC)n and (GA)n have not previously been described in *Leishmania*, while polymorphisms of (CA)n have been characterized extensively in *L.* (*L*) *infantum* (Rossi *et al.* 1994).

The short microsatellites within LbJ38 could be useful for typing variants of *L.* (*V*) *braziliensis* although this has not yet been investigated. The simple repeats in LbJ38 are characteristic of 'cryptic simplicity' a phrase coined by Tautz, Trick & Dover (1986) to describe scrambled arrangements of repetitive motifs. Also within LbJ38 are a large number of homo-nucleotide sequences, the majority of which are GC-rich. No function can at present be ascribed to these sequences, although they could be involved in secondary structure (for example A or T rich homo-nucleotide in Z-DNA have been described by Wang *et al.* 1979). Secondary structures could be predicted especially between nt 345 and 452, which contains a high proportion of (C)n and (G)n adjacent to each other.

In the last few years genomic repeat sequences have been very useful both in the study of

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relationships between and within *Leishmania* species (Fernandez *et al*. 1994; Van Eys *et al*. 1992), and in diagnosis and identification of *Leishmania* isolates by PCR. Their high representation provides a greater sensitivity than single copy sequences (Howard, 1991; Wahl *et al*. 1987). The primers derived from the repetitive DNA sequence amplified a 617 bp fragment in both *L*. (*V*) *braziliensis* isolates and isolates suspected to be variants of the *L*. (*V*) *braziliensis* species. A manuscript on PCR with our genomic and kDNA primers is in preparation. We consider LbJ38 and the primers derived from it to be specific for the *L*. (*V*) *braziliensis* species and its variants. These repeats may be very useful in genetic studies of *Leishmania* populations and for epidemiological studies of New World cutaneous and mucocutaneous leishmaniasis.

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