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PERMANENT GENETIC RESOURCES

A set of 12 microsatellite loci for genetic studies of *Leishmania braziliensis*

V. ROUGERON,*‡§ E. WALECKX,*‡ M. HIDE,* T. DE MEEÛS,* J. AREVALO, † A. LLANOS-CUENTAS† and A. L. BAÑULS*

**Genetique et Evolution des Maladies Infectieuses, IRD/CNRS (UMR2724), Centre, IRD, 911, Avenue Agropolis BP 64501, F-34394, Montpellier Cedex 5, France, †Instituto de Medicina Tropical 'Alexander von Humboldt', Lima, Peru*

Abstract

Twelve microsatellite loci of *Leishmania braziliensis* were examined, nine of which were developed in this work. Fifty-six *Leishmania braziliensis* were genotyped with these microsatellite loci. The 12 loci studied were polymorphic with the number of alleles ranging from five to 19, with a mean of 9.7 ± 4.1 and the observed heterozygosity averaging 0.425 ± 0.202. The important heterozygote deficits we observed (F_{IS} = 0.41, P value = 0.004) appear incompatible with the heterozygote excess expected in clonal diploids. This last result could revive the clonality/sexuality debate regarding *Leishmania*. This work validates the potential use of these microsatellites for population genetics analysis.

Keywords: *Leishmania braziliensis*, microsatellites, population genetics, population structure

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Leishmaniases are severe human and veterinary diseases caused by *Leishmania* protozoan parasites and transmitted by sandfly bites. About 14 million people are infected worldwide, with an incidence of 2.357 million new cases per year (WHO 2002, http://who.int/zoonoses/diseases/leishmaniasis/en). More information for diagnostic and epidemiological inquiries and for drug or vaccine elaboration are needed (Wolday *et al*. 2001). In this respect, the use of molecular markers can prove very useful for such organisms (De Meeus *et al*. 2007). In this note, we detail the technique used for studying a *Leishmania braziliensis* sample with 12, microsatellite loci, three of which have already been published by Russel *et al*. (1999).

Fifty-six strains of *Leishmania* (*Viannia*) from Peru were previously characterized as *L. braziliensis* using isoenzymes (Bañuls, unpublished data). Genomic DNA was extracted from parasite mass cultures (promastigotes) using the phenol/chloroform extraction technique.

Microsatellites were either taken from Russel *et al*. (1999) or designed using genomic libraries of *L. braziliensis* detailed in NCBI (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide), using the parasite BLAST server (http://www.ebi.ac.uk/blast2/parasites.html) or in the *Leishmania* genome project (http://www.genedb.org/genedb/leish/index.jsp). Primers were designed on flanking sequences using the PRIMER 3 software package (Rozen & Skaletsky 2000). A minimum length of 20–22 bp and a GC content greater than 60% were selected to guarantee primer stability. Sixteen different primer pairs were tested using a temperature gradient thermal cycler on a *Leishmania* reference strain (MHOM/BR/75/M2904) and one strain of our sample (LC 2148 from Peru). Reaction products were visualized on a 1.5% agarose gel stained with ethidium bromide. The selection of primer pairs and of optimal annealing was done on the basis of polymerase chain reaction (PCR) profiles. The best amplifications were obtained for the locus and primer pairs listed in Table 1. Once the PCR protocol was designed, the forward primer from each set was the 5′ fluorescent labelled with different dyes (Applied Biosystems) for detection with an automated DNA sequencer (ABI PRISM 310 Genetic Analyser, Applied Biosystems; Table 1).

The 56 strains under study were amplified according to the following conditions. Every 30 μL reaction mix was composed of 1.2 μL of each primer (10 μM), the forward being labelled, 100 ng template DNA, 0.9 μL dNTP mix (5 mM), 3 μL buffer 10× and 0.3 μL *Taq* polymerase (Roche Diagnostics, 5UI/μL). Amplifications were carried out in a

‡These two authors contributed equally to the work
§Correspondence: Virgine Rougeron, Fax: (33)4.67.41.62.99; E-mail: rougeron@mpl.ird.fr

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Table 1 Description of the 12 microsatellite loci from 56 strains of *Leishmania braziliensis* (name; GenBank Accession number to reference clone; primer sequences; fluorescent dye; structure of repeated array), thermocycling conditions (annealing temperature, T_a), number of cycles, N_c and genetic variation (alleles number), N_a; allele size range; observed heterozygosity H_O; average estimate of within-sample gene diversity computed by FSTAT as $H_S = [\tilde{n}/(1 - \tilde{n})] \cdot [1 - \Sigma p^2_{ik} - H_O/2\tilde{n}]$ where ñ is the harmonic mean of sample sizes, and deviation from panmixia measured as (F_{IS}). The three loci noted by '*' were developed by Russel *et al*. (1999)

Locus	Primer sequence (5′–3′)	GenBank Accession no.	Repeat type	T_a (°C)	Labelling dye	N_a	Allele size (bp)	H_O	H_S	F_{IS}
AC01*	5′-GGGGAGGGAAACACACTAGC-3′° 5′-TCTCCCACCCTTAGTGCTTG-3′	AF139110	$(CA)_{13}$	63.4	6FAM	8	198–212	0.432	0.77	0.442
AC16*	5′-TCCTTGGAGGACTTCTCCTG-3′° 5′-TAGTGCCATTAGGGGCTCAT-3′	AF139112	$(GT)_{21}$	58.5	NED	11	147–161	0.646	0.8	0.192
AC52*	5′-CATCTACGGCTGATGCAGAA-3′° 5′-CGTCTGGCTAAAGTGGGAAT-3′	AF139111	$(CA)_{18}TA(CA)_{14}$	61	PET	19	098–126	0.589	0.921	0.361
ITSbraz	5′-GGGGAGGCTTGTGTTTTCTA-3′° 5′-CGTCGATCGGCCTTTTTCTA-3′	AJ300483	$TA(TG)_2(TA)_8$	59.1	VIC	6	100–108	0.417	0.726	0.417
LRC	5′-CTGCCTCTGCCTCACCTACt-3′° 5′-CTAACCCTCACACTCCCCATC-3′	BX544585	$(GT)_{17}$	61	PET	12	118–134	118–134	0.787	0.059
EMI	5′-CGCTGAAGCACGGCGAATG-3′° 5′-CGTAGCTCCTCTGTCCGTTC-3′	BX541508	$(GT)_{20}$	61	VIC	12	183–189	0.161	0.821	0.803
GO9	5′-CAAGCAGGCAAGAGTCTGAAA-3′° 5′-GTCTCCCGTATTGCTCTCTCTA-3′	BX539509	$(CA)_3(GA)_{12}$	57.5	NED	7	148–168	0.589	0.785	0.25
E11	5′-TGCGTAGGGCAAAGGAGTT-3′° 5′-GGGTGTCTGCCTGCATTC-3′	BX542509	$(GA)_{10}$	57.5	VIC	6	096–108	0.208	0.723	0.712
ARP	5′-GGCTTCGGTCTGTTCGACTA-3′° 5′-CACCCACTCGCATCCGTA-3′	AF045249	$(GT)_{10}$	56	6FAM	15	121–157	0.531	0.887	0.401
Ibh3	5′-GGAGAGGCTGCGATGTATCT-3′° 5′-CAGGGCTGTCTTGACGAAG-3′	AF044682	$(GT)_2GG(GT)_2$	56	6FAM	5	116–136	0.167	0.44	0.621
CAK	5′-TGGAAGAAGGCAGTGGTACA-3′° 5′-TGGAGAGGACACCTTTTTCC-3′	BX544561	$GG(GT)_4(GA)_{15}$	56	6FAM	8	152–170	0.307	0.774	0.603
LBA	5′-CCTCTGTGAGAAGGCAAGGA-3′° 5′-GCTGCACATGCATTCTCTCGT-3′	BX539885	$(GA)_{11}$	56	PET	8	168–180	0.766	0.806	0.05

thermal cycler using the following reaction conditions: 30 cycles of 94 °C for 30 s, annealing temperature of each locus (see Table 1) for 1 min, 72 °C for 1 min and a final extension step of 72 °C for 7 min.

Genotyping in the automated sequencer was undertaken with 1 µL each of the PCR-amplified DNA sample added into a standard loading mix: 0.5 µL of an internal standard-size GENESCAN 500LIZ, (Applied Biosystems) and 13.5 µL of formamide (Hi-Di) (Applied Biosystems). Fragment size was determined using ABI PRISM GENESCAN Analysis 3.7 and GENOTYPER 3.7 (both from Applied Biosystems). Only clear electrophoregrams with peaks of the expected size were considered in the study. All electrophoregrams displayed only one or two peaks. Each *L. braziliensis* strain was genotyped at the 12 loci studied. Several trials showed that multiplex was possible, for PCR products characterized by different sizes (no overlapping zones) or labelled with different fluorochromes.

The 12 loci studied were polymorphic, with an average of (±SD) 9.7 (±4.1) alleles, ranging from five to 19 alleles. Observed heterozygosity (H_O), mean genetic diversities (H_S) and deviation due to nonrandom union of gametes (measured by Wright's F_{IS}) were computed using the FSTAT software, version 2.9.3.2 (updated from Goudet 1995). Mean H_O and H_S were 0.425 ± 0.202 and 0.77 ± 0.119, respectively (Table 1). A strong F_{IS} appeared in our sample (F_{IS} = 0.41, P value = 0.004). The weak variance of F_{IS} across loci and the total absence of blanks in the data exclude null alleles as a possible explanation. This result seems incompatible with the heterozygote excess expected in clonal diploids (De Meeûs *et al*. 2007, Balloux *et al.* 2003) and, if confirmed, will probably revive the clonality/sexuality debate regarding *Leishmania* (see Bañuls *et al*. 2007).

Aneuploidy, the existence of which was proven in the *Leishmania* genome (Cruz *et al*. 1993), did not interfere with the microsatellite loci analysed in the present study, as no electrophoregram contained either three or four peaks, suggesting that the 12 loci analysed did not belong to tetraploid or triploid chromosomes, and F_{IS} were homogeneous across loci, which is incompatible with the occurrence of aneuploidy at some loci.

These 12 microsatellite markers studied therefore represent good tools for *L. braziliensis* population genetic studies and may constitute a source for essential information on the biology and the epidemiology of these protozoan parasites.

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