A battery of 12 microsatellite markers for genetic analysis of the *Leishmania* (*Viannia*) *guyanensis* complex

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(*Received 6 October 2009; revised 27 December 2009 and 7 April 2010; accepted 12 April 2010; first published online 7 July 2010*)

SUMMARY

We used 12 microsatellite markers developed for *Leishmania braziliensis* to genotype 28 strains of the main species of the *Leishmania guyanensis* complex (*i.e. L. guyanensis* and *L. panamensis*) collected in Ecuador and Peru. The important heterozygote deficits observed in these populations are similar with the previous data obtained in *L. braziliensis* and raise again the debate on the reproductive mode of these protozoan parasites. The data showed genetic polymorphism and geographical differentiation giving information on population structure of the *L. guyanensis* complex. Regarding the two species, this study enhances again the debate on the taxonomic status of the different isolates belonging to *L. guyanensis* s.l. since the results showed substantial heterogeneity within this species complex. In conclusion, this study increases the number of available microsatellite loci for *L. guyanensis* species complex and raises fundamental biological questions. It confirms that microsatellite markers constitute good tools for population genetic studies on parasites of this complex.

Key words: *Leishmania guyanensis* complex, microsatellite markers, population genetics, population structure.

INTRODUCTION

Leishmaniases are a serious public health problem caused by *Leishmania* protozoan parasites and transmitted by sandfly bites. About 14 million people are infected world-wide, with an incidence of 2·357 million new cases per year (WHO, 2002). Parasites of the *Leishmania* subgenus (*Viannia*) cause the majority of cutaneous and mucocutaneous leishmaniases cases in South America. More information is needed on the population biology of these pathogens for diagnostic and epidemiological inquiries and for drug and vaccine elaboration (Wolday *et al*. 2001). In this respect, the use of molecular markers can prove very useful (De Meeûs *et al*. 2007*a*). For the present study, we tested the usefulness of 12 microsatellite loci published for *Leishmania braziliensis* (Rougeron *et al*. 2008) for studying the *Leishmania guyanensis* complex. In the light of the results obtained, we discuss future possible applications of these markers for the study of this complex.

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MATERIALS AND METHODS

Ten strains from Peru and 18 strains from Ecuador were previously characterized as belonging to the *L. guyanensis* complex and especially either to *L. panamensis* or *L. guyanensis* species using isoenzymes (Bañuls *et al*. unpublished data; Bañuls *et al*. 1999). The Peruvian sample was composed of 10 *L. guyanensis* and the Ecuadorian sample of 4 *L. guyanensis* and 14 *L. panamensis* strains (Table 1, Fig. 1). We also added 5 reference stocks: MHOM/BR/75/M2904 (*L. braziliensis* from Brazil), MHOM/BR/78/M5378 (*L. guyanensis* from Brazil), MHOM/FG/84/H166 (*L. guyanensis* from French Guiana), MHOM/FG/83/CAYA116 (*L. guyanensis* from French Guiana) and MCHO/PA/00/M4039 (*L. panamensis* from Panama) in order to validate the technique for the two species. For all the isolates, genomic DNA was extracted from parasite mass cultures (promastigotes) with the DNeasy Blood and Tissue Kit (Qiagen, Venlo, The Netherlands) and eluted in 50 μl.

Microsatellites were either taken from the publications by Russell *et al*. (1999) or Rougeron *et al*. (2008). The 33 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

doi:10.1017/S0031182010000776

Table 1. Data set of the 33 *Leishmania* strains studied indicating the World Health Organization code, the country and the department of collection and the species characterized using isoenzymes for each strain

(The 5 reference stocks are noted by '*' and the unknown collection departments are noted by '?'.)

WHO code	Country	Department	Species
MCHO/FG/83/A116*	French Guiana	—	*L. guyanensis*
MHOM/FG/84/H166*	French Guiana	—	*L. guyanensis*
MHOM/BR/78/M5378*	Brazil	—	*L. guyanensis*
MCHO/PA/00/ LBP4039*	Panama	—	*L. panamensis*
MHOM/BR/75/M2904*	Brazil	—	*L. braziliensis*
MHOM/EC/92/EK91	Ecuador	Santo Domingo	*L. panamensis*
MHOM/EC/92/PR1	Ecuador	Zumba	*L. guyanensis*
MHOM/EC/92/A8044	Ecuador	La Tablada	*L. guyanensis*
MHOM/EC/91/E67	Ecuador	Zumba	*L. panamensis*
MHOM/EC/–/E50	Ecuador	?	*L. panamensis*
MHOM/EC/91/E3	Ecuador	La Tablada	*L. guyanensis*
MHOM/EC/91/E19	Ecuador	La Tablada	*L. panamensis*
MHOM/EC/92/E94	Ecuador	Santo Domingo	*L. panamensis*
ITRA/EC/92/EK665	Ecuador	Paraiso Escundido	*L. panamensis*
MHOM/EC/91/E4	Ecuador	La Tablada	*L. panamensis*
MHOM/EC/91/E1	Ecuador	La Tablada	*L. panamensis*
MHOM/EC/–/E73	Ecuador	?	*L. panamensis*
ITRA/EC/92/EK649	Ecuador	Paraiso Escundido	*L. guyanensis*
MHOM/EC/91/E30	Ecuador	Paraiso Escundido	*L. panamensis*
MHOM/EC/91/E12	Ecuador	La Tablada	*L. panamensis*
MHOM/EC/91/E747	Ecuador	La Tablada	*L. panamensis*
MHOM/EC/91/E18	Ecuador	La Tablada	*L. panamensis*
ITRA/EC/92/EK112	Ecuador	?	*L. panamensis*
MHOM/PE/91/LH1041	Peru	Huanuco	*L. guyanensis*
MHOM/PE/00/LH904	Peru	Junin	*L. guyanensis*
MHOM/PE/00/LH1102	Peru	Pasco	*L. guyanensis*
MHOM/PE/00/LH705	Peru	San Martin	*L. guyanensis*
MHOM/PE/00/LH941	Peru	Ucayali	*L. guyanensis*
MHOM/PE/91/LC1447	Peru	Lambeyeque	*L. guyanensis*
IPRN/PE/87/LP52	Peru	Ancash	*L. guyanensis*
MHOM/PE/91/LH837	Peru	Huanuco	*L. guyanensis*
MHOM/PE/00/LH835	Peru	Ucayali	*L. guyanensis*
MHOM/PE/91/LC1448	Peru	Lambeyeque	*L. guyanensis*

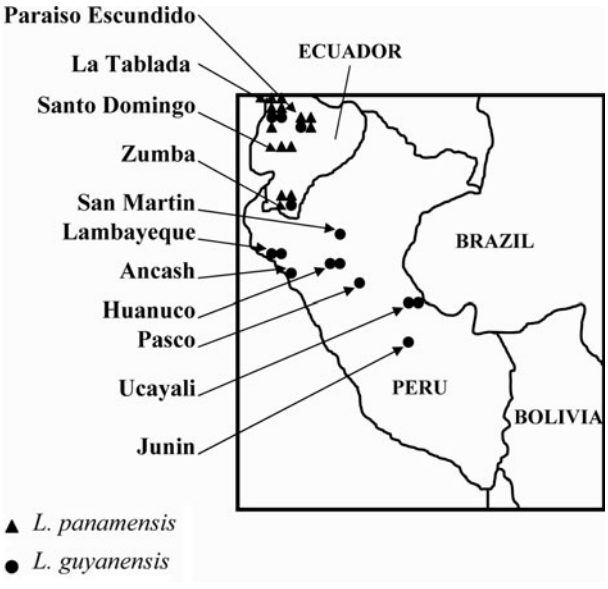

Fig. 1. Localization of each strain in Peru and Ecuador.

DNA, 0·9 μl of dNTP mix (5 mM), 3 μl of buffer 10X and 0·3 μl of *Taq* Polymerase (Roche Diagnostics, 5UI/μl). Amplification was carried out in a thermal cycler using the following reaction conditions: 35 cycles of 94 °C for 30 s, annealing temperature of each locus (see Table 2) for 1 min, 72 °C for 1 min and a final extension step of 72 °C for 7 min. Reaction products were visualized on 1·5% agarose gel stained with EnVISION™ DNA Dye as loading buffer (Ambresco). Genotyping in the automated sequencer (ABI PRISM 310 Genetic Analyser, Applied Biosystems, Foster City, CA, USA) 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 (HiDi) (Applied Biosystems). Fragment size was determined using ABI PRISM Genescan Analysis 3.7 and Genotyper 3.7 (both from Applied Biosystems). Several trials

Table 2. Description of the 12 microsatellite loci of *Leishmania (V.) guyanensis* complex name; primer sequences; GenBank Accession number to reference clone; structure of repeat array; thermocycling conditions (annealing temperature), *Ta*; labelling dye noted '°'; allele size (bp); genetic variation (alleles number), *Na*; average estimate within-sample gene diversity computed by FSTAT as $H_s=[\tilde{n}/(1-\tilde{n})]\cdot[1-\Sigma p_{ik}^2-Ho/2\tilde{n}]$ where ñ is harmonic mean of sample sizes, and deviation from panmixia measured as F_{IS}.

(The 3 loci noted by '*' were developed by Russell *et al*. (1999) and the 9 others by Rougeron *et al*. (2008).)

Locus	Primer sequence (5′–3′)	GenBank Accession no.	Repeat type	*Ta* (°C)	Labelling dye	Allele size (bp)	No. of alleles	H_s	F_{IS}
AC01*	5′-ggggagggaaacacactagc-3′° 5′-tctcccacccttagtgcttg-3′	AF139110	$(CA)_{13}$	63·4	6FAM	198–212	10	0·735	0·968
AC16*	5′-tccttggaggacttctcctg-3′° 5′-tagtgccattaggggctcat-3′	AF139112	$(GT)_{21}$	58·5	NED	147–161	3	0·513	0·881
AC52*	5′-catctacggctgatgcagaa-3′° 5′-cgtctggctaaagtgggaat-3′	AF139111	$(CA)_{18}TA(CA)_{14}$	61	PET	098–126	4	0·364	0·457
ITSbraz	5′-ggggaggcttgtgttttcta-3′° 5′-cgtcgatcggccttttttcta-3′	AJ300483	$TA(TG)_2(TA)_8$	59·1	VIC	100–108	5	0·689	0·781
LRC	5′-ctgcctctgcctcacctact-3′° 5′-ctaaccctcacactccccatc-3′	BX544585	$(GT)_{17}$	61	PET	118–134	8	0·590	0·951
EMI	5′-cgctgaagcacggcgaatg-3′° 5′-cgtagctcctctgtccgttc-3′	BX541508	$(GT)_{20}$	61	VIC	183–189	9	0·760	0·427
G09	5′-caagcaggcaagagtctgaaa-3′° 5′-gtctcccgtattgctctctcta-3′	BX539509	$(CA)_3(GA)_{12}$	57·5	NED	148–168	4	0·250	1·000
E11	5′-tgcgtagggcaaaggagtt-3′° 5′-gggtgtctgcctgcattc-3′	BX542509	$(GA)_{10}$	57·5	VIC	096–108	7	0·699	0·929
ARP	5′-ggcttcggtctgttcgacta-3′° 5′-cacccactcgcatccgta-3′	AF045249	$(GT)_{10}$	56	6FAM	121–157	8	0·670	0·779
Ibh3	5′-ggagaggctgcgatgtatct-3′° 5′-cagggctgtcttgacgaag-3′	AF044682	$(GT)_2GG(GT)_2GG(GT)_4$	56	6FAM	116–136	8	0·674	0·898
CAK	5′-tggaagaaggcagtggtaca-3′° 5′-tggagaggacacctttttcc-3′	BX544561	$(GA)_{15}$	56	6FAM	152–170	6	0·759	1·000
LBA	5′-cctctgtgagaaggcaagga-3′° 5′-gctgcacatgcattctctcgt-3′	BX539885	$(GA)_{11}$	56	PET	168–180	7	0·741	0·537

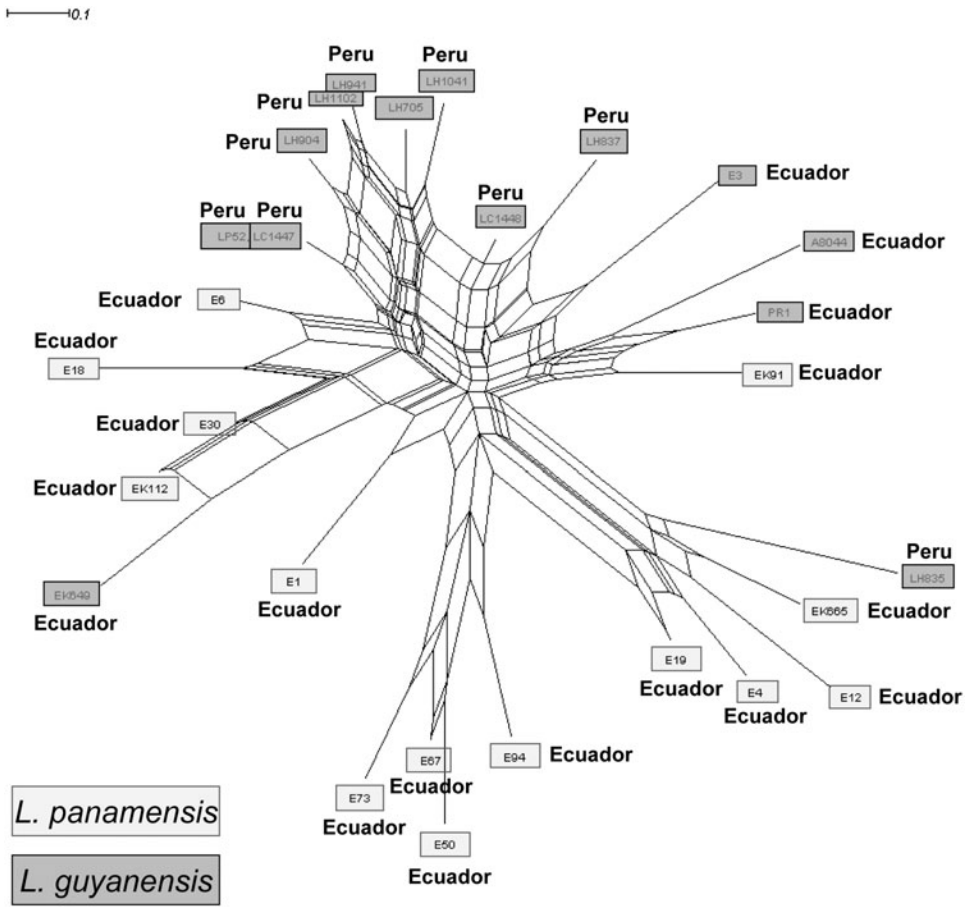

Fig. 2. Neighbor-joining network depicting genetic distance relationships based on Cavalli-Sforza's chord distances among 28 strains of the *Leishmania guyanensis* complex. The network was built using PHYLIP version 3.5c from raw allelic frequencies and performed by SplitsTree software.

showed that multiplex was possible, for PCR products characterized by different sizes (no overlapping zones) or labelled with different fluorochromes.

Some technical problems can be met, such as existence of null alleles (Paetkau and Strobeck, 1995). Thus, null allele frequencies were computed using the software Micro-Checker (Van Oosterhout *et al.* 2004). We used both Van Oosterhout's and Brookfield's methods (Brookfield, 1996). This was used to compute the total number of expected blanks in the whole data set and this value was compared to the observed one (0) with an exact unilateral (H_1: there are less blanks than expected) binomial test under the software R (R-Development-core-team, 2008).

For the population genetic study, data were analysed with the software Fstat Version 2.9.3.2 (Goudet, 2002) updated from Goudet (1995), which computes, estimates and tests the significance of various population genetic parameters. These parameters are H_s, Nei's unbiased estimator of genetic diversity, Weir and Cockerham's (1984) unbiased estimator of Wright's *F*-statistics (Wright, 1965) F_{IS}, which measures deviation from Hardy-Weinberg genotypic frequencies within subsamples, and F_{ST}, which measures differentiation between subsamples. The last was also estimated with its standardized version corrected for polymorphism ($F_{ST}' = F_{ST}/(1 - H_s)$) (e.g. De Meeûs *et al.* 2007*a*).

A Neighbor-joining network (NeighborNet) (Saitou and Nei, 1987), constructed from Cavalli-Sforza genetic distance, was used to cluster the strains from allelic frequencies. Data were computed using the PHYLIP software to build the distance matrix (version 3.5c; J. Felsenstein, Department of Genetics, University of Washington, Seattle, 1993) and network was performed using SplitsTree (Huson, 1998) (Fig. 2).

RESULTS

We obtained clear electropherograms for all genotypes at all loci investigated, with only 1 or 2 alleles per strain at each locus. This observation excluded events of aneuploidy (for which we could have expected individuals with no allele, 3 or 4 alleles). No blank was ever observed in the genotypes (no missing data, i.e. all individuals were amplified at all loci), suggesting the absence of a null allele. According to Brookfield's and van Oosterhout's methods, null allele frequencies necessary to explain the observed F_{IS} should have generated, under

random mating assumption, 33 and 44 null homozygotes (blanks) across the entire sample (among the 336 loci × sample combinations) for Brookfield's and Van Oosterhooot's methods respectively. The difference with the absence of blanks in observed data was highly significant (exact unilateral binomial tests, *P*-value <0·0001 in both cases). The presence of null alleles is thus highly unlikely.

There was a high genetic diversity, with an average (±S.D.) of 6·58 (±2·19) alleles per locus, ranging from 3 (locus AC16) to 10 (locus AC01). Nei's mean unbiased genetic diversity (Nei and Chesser, 1983) $H_s = 0{\cdot}62$ (±0·1) was reasonably high. The mean genetic diversity was high either for samples collected in Ecuador ($H_s = 0{\cdot}69$, ±0·17) or for samples collected in Peru ($H_s = 0{\cdot}55$, ±0·22). There was strong and significant (*P*-value ⩽0·002) heterozygote deficits compared to Hardy-Weinberg expectations in each subsample at all loci. The mean F_{IS} ranged from 0·73 in Peru to 0·89 in Ecuador. For individual loci, the average values ranged from $F_{IS} = 0{\cdot}43$ for locus EMI to $F_{IS} = 1$ for loci G09 and CAK. According to the species, the F_{IS} values range from $F_{IS} = 0{\cdot}726$ for *L. guyanensis* to $F_{IS} = 0{\cdot}882$ for *L. panamensis*. The overall mean value is $F_{IS} = 0{\cdot}85$ (95% CI = 0·77–0·92). Between *L. guyanensis* and *L. panamensis* in Ecuador, we obtained a moderate and marginally significant genetic differentiation ($F_{ST} = 0{\cdot}097$; *P*-value = 0·051). If corrected for polymorphism (De Meeûs *et al.* 2007*a*; Hedrick, 2005), this differentiation appears more substantial ($F_{ST}' = F_{ST}/(1 - H_s) = 0{\cdot}257$). Finally, results showed a high and significant geographical differentiation between Peru and Ecuador for the sample composed of only *L. guyanensis* strains ($F_{ST} = 0{\cdot}324$, *P*-value = 0·01). We have calculated the genetic differentiations between *L. braziliensis* and the two species of the *Leishmania guyanensis* complex, *L. panamensis* and *L. guyanensis*. To estimate this value, we used the *L. braziliensis* dataset published in our previous publication (Rougeron *et al.* 2008). We observed significant genetic differentiations between *L. guyanensis* and *L. braziliensis* ($F_{ST} = 0{\cdot}181$, *P*-value = 0·01) and between *L. panamensis* and *L. braziliensis* ($F_{ST} = 0{\cdot}179$, *P*-value = 0·01). In the same way, if we corrected these values for polymorphism (De Meeûs *et al.* 2007*a*; Hedrick, 2005), these differentiations appear more substantial (between *L. guyanensis* and *L. braziliensis* $F_{ST}' = F_{ST}/(1 - H_s) = 0{\cdot}722$ and between *L. panamensis* and *L. braziliensis* $F_{ST}' = 0{\cdot}704$). It is worth noting that geographical localization of the strains cannot entirely explain the structuring of the sample (see Fig. 1).

The network reflects the important genetic heterogeneity within our sample and conflicting phylogenetic signals, suggesting a strong disagreement between loci (Fig. 2). We can also notice that neither geography nor current systematic (i.e. distinction between *L. guyanensis* and *L. panamensis*) satisfactorily describes the structuring found in our sample. More especially, it suggested that the NeighborNet network is not sufficiently informative to conclude about the taxonomic distinction between *L. panamensis* and *L. guyanensis*.

DISCUSSION

The data point to 3 fundamental purposes relating to the biology, the taxonomy and the population structure of these parasites.

Concerning the biology and particularly the reproduction mode, our findings reveal extreme homozygosity at all loci studied, in agreement with the data obtained by Oddone *et al.* (2009) for *L. guyanensis*. As discussed for *L. braziliensis* (Rougeron *et al.* 2008, 2009), these results seem to be incompatible with the heterozygote excesses that would have arisen in a predominantly clonal diploid organism (Balloux *et al.* 2003; De Meeûs *et al.* 2007*b*). Thus, the reproductive mode of these protozoan parasites is yet again questioned (Bañuls *et al.* 2007; Rougeron *et al.* 2009).

Regarding the population structure, the data showed an important genetic diversity which could be related to the great ecosystem richness, in terms of vector and/or reservoir diversities involved in the transmission cycle of the parasite in the two countries (e.g. Amazonian forest). Bigger and more accurate sampling (taking into account as many parameters as possible) will improve our understanding of the population biology of the different species belonging to this complex in the two countries.

On a taxonomical point of view, several reports questioned the distinct species status of *L. panamensis* and *L. guyanensis* (Bañuls *et al.* 1999, 2007). Regarding microsatellite data, we found a marginally significant differentiation between the isolates belonging to the two taxa and a significant genetic differentiation ($F_{ST} = 0{\cdot}156$, *P*-value = 0·01) between the *L. guyanensis* complex and the *L. braziliensis* species. Given the very small sample sizes regarding the *L. guyanensis* complex, and the substantial F_{ST}' value between the two strain types, this result suggests the separation between these two entities. Nevertheless, due to the extreme heterogeneity illustrated by the NeighborNet, it is difficult to reach a conclusion and the taxonomic organization of the *L. guyanensis* complex appears challenging. Our sample sizes do not allow clear conclusions and more extensive studies will be necessary to improve our knowledge on the taxonomy of this group.

In summary, this study allows the addition of 12 other polymorphic microsatellite markers, initially designed for *L. braziliensis* (Rougeron *et al.* 2008), to the 13 loci developed by Odonne *et al.* (2009) (13 out of 15 because 2 loci are in common with those of Oddone and coworkers and with the present paper) for the genetic characterization of the *L. guyanensis*

complex. The present data demonstrate that microsatellites can significantly help in raising numerous questions about the biology, population structure and taxonomy of strains belonging to the *Leishmania guyanensis* complex.

ACKNOWLEDGEMENTS

We acknowledge F. Prugnolle, C. Barnabé and H. Gómez Díaz for helpful discussions. We thank the unit of Molecular Parasitology of the Institute of Tropical Medicine, Antwerpen, Belgium, for their help in obtaining Peruvian parasite isolates and C. Barrera, I. Espinel, R. Guderian, R. Echeverria and F. Le Pont for obtaining Ecuadorian isolates. We acknowledge the genotyping platform of the IFR 119 Montpellier Environment Biodiversité, Montpellier, France. We are grateful to the Institut de Recherche pour le Développement, Centre National de la Recherche Scientifique and the 2 CEE projects: CI1*-CT91-0901 and TS3*-CT92-0129 for their financial support. This work was also supported by a French National Project, ANR 06-SEST-20 IAEL.

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