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Size-polymorphism of mini-exon gene-bearing chromosomes among natural populations of *Leishmania*, subgenus *Viannia*

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Abstract

In order to explore genomic plasticity at the level of the mini-exon gene-bearing chromosome in natural populations of *Leishmania*, the molecular karyotype of 84 *Leishmania* stocks belonging to subgenus *Viannia*, originating mostly from Peru and Bolivia, and differing according to eco-geographical and clinical parameters, was resolved and hybridised with a mini-exon probe. The results suggest that size variation of the mini-exon gene-bearing chromosome is frequent and important (up to 245-kb size-difference), and partially involves variation (up to 50%) in copy number of mini-exon genes. There is no significant size-difference between mini-exon-bearing chromosomes of Peruvian and Bolivian populations of cutaneous and mucosal isolates of *Leishmania (Viannia) braziliensis*, but there is between eco-geographical populations of *Leishmania (Viannia) peruviana*. *Leishmania (V.) peruviana* presented a significantly smaller mini-exon-bearing chromosome than the other species of subgenus *Viannia*. The contrast between the general chromosome size heterogeneity and the homogeneity observed in some Peruvian Andean areas is discussed in terms of selective pressure.

Keywords: Eco-geography; Karyotype plasticity; *Leishmania*; Mini-exon

1. Introduction

Chromosomal size-polymorphism is very frequent among *Leishmania* and other Protozoa, and is being studied for basic purposes [1–4], and less for its contribution to molecular epidemiology [5, 6]. During an extensive survey of Neotropical *Leishmania* belonging to subgenus *Viannia*, we found two interesting chromosomes. The first one, bearing gp63 genes, showed a significantly smaller size in *Leishmania (Viannia) guyanensis* and *Leishmania (Viannia) peruviana* than in *Leishmania (Viannia) braziliensis* [7, 8]. The second one, bearing rDNA genes, permitted identification of eco-geographical populations among *L. (V.) peruviana*; size was significantly smaller in populations isolated from southern Peru than northern ones [7, 9]. In both cases, chromosome size-variation was shown to be due

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partially to variation in copy number of tandemly repeated genes, respectively gp63 and rDNA [8–10].

Amplification/deletion of repeated sequences appears to be an important and general mechanism leading to protozoan genome polymorphism [11]. In addition, if the sequences involved in these phenomena correspond to important genes, their rearrangement might have phenotypic consequences (due to gene dosage [12], deletion of unique interspersed genes [4], or modification at the level of intergenic sequences known to play a role in transcription regulation [13]), and thus be selected by environmental factors. Accordingly, analysing the genomic organisation of important repeated genes might reveal new markers for eco-epidemiology.

Mini-exon genes are tandemly repeated sequences encoding a factor essential for the functionality of mRNA in trypanosomatids [14]. Previous studies on a few laboratory-maintained lines of *Leishmania major* showed that amplification/deletion of the mini-exon gene array was partly responsible for size variation of the bearing chromosome [15]. The aim of the work reported here was to explore this phenomenon in natural populations of *Leishmania*, differing according to eco-geographical and clinical parameters. Therefore, the molecular karyotype of 84 *Leishmania* stocks representing several species of subgenus *Viannia* was resolved and hybridised with a mini-exon probe. Size of hybridising chromosomes was evaluated and compared among the different groups of organisms considered. In addition, the relationship between chromosomal size and hybridisation intensity of the mini-exon probe was studied in order to verify the involvement of mini-exon gene in chromosome plasticity.

2. Materials and methods

2.1. Stocks

Eighty-four stocks previously characterised by isoenzyme analysis (Bañuls, PhD thesis, 1998) were considered in the present study (Table 1). Most stocks were obtained from an allopatric sampling in Peru and Bolivia. With respect to *L. (V.) peruviana*, four bio-geographical units (BGUs, classification of Lamas [16]), constituting a north–south transect along the Peruvian Andes, were considered: Huancabamba (HB), Surco-North, Surco-Centre and Surco-South (SUN, SUC and SUS, respectively). Procedures for cultivation, harvesting and preparation for orthogonal field alternating gel electrophoresis (OFAGE) have been described elsewhere [7].

2.2. Molecular karyotyping

Orthogonal field alternating gel electrophoresis-resolved chromosomes were transferred to nylon membranes (Hybond-N, Amersham), processed and hybridised according to the manufacturer's instructions. For each gel, the size of chromosomal bands was estimated using the linear region of a size calibration curve built-up from a reference karyotype of *L. (V.) braziliensis* strain M2903 (loaded in positions 1, 4 and 8 of each gel, [17]). Preparative OFAGE gels for purification of the mini-exon chromosome were made in 1% Nu-Sieve, and digestion within the agarose was performed to completion with an excess of restriction enzymes as described elsewhere [9].

2.3. Calculation of Chromosome Size Difference Index

Quantification of chromosome size-polymorphism was performed by calculation of Chromosomal Size Difference Index (CSDI, [17, 18]). This method considers chromosomes as a continuous variable and introduces a weighing of size variation: two chromosomes showing a 25-kb size-difference are considered to be genomically more similar than two chromosomes presenting a 200-kb size-differences; previous work showed that this way of encoding was better than disjunctive encoding in which no weighing is considered [17, 18]. The index was calculated with a program developed previously [17] and available upon request from the corresponding author. Indices were sub-

Table 1
Origin and designation of the *Leishmania* stocks under study

Species	International code	Lesion	Species	International code	Lesion
L. (V.) braziliensis	MHOM/BR/75/M2903	C	*L. (V.) peruviana* (HB)	MHOM/PE/90/HB22	C
	MHOM/BR/75/M2904	C		MHOM/PE/90/HB31	C
	MHOM/PE/89/LH754	C		MHOM/PE/90/HB39	C
	MHOM/PE/90/LH1013	C		MHOM/PE/90/HB44	C
	MHOM/PE/90/LH1016	C		MHOM/PE/90/HB55	C
	MHOM/PE/91/LC1409	C		MHOM/PE/90/HB56	C
	MHOM/PE/91/LC1412	C		MHOM/PE/90/HB67	C
	MHOM/PE/91/LC1417	C		MHOM/PE/90/HB83	C
	MHOM/PE/92/LC1569	C		MHOM/PE/90/HB86	C
	MHOM/PE/92/LC1577	C		MHOM/PE/89/LC900	C
	MHOM/PE/92/LC1578	C	*L. (V.) peruviana* (SUN)	MHOM/PE/90/LC436	C
	MHOM/PE/92/LC1584	C	*L. (V.) peruviana* (SUC)	MHOM/PE/00/LC106	C
	MHOM/PE/93/LC2123	C		MHOM/PE/89/LH696	C
	MHOM/PE/00/LC2193	C		MHOM/PE/89/LH760	C
	MHOM/PE/94/LC2284	C		MHOM/PE/90/LH807	C
	MHOM/PE/94/LC2292	C		MHOM/PE/90/LH827	C
	MHOM/PE/94/LC2316	C		MHOM/PE/90/LH911	C
	MHOM/PE/94/LC2321	C		MHOM/PE/90/LH925	C
	MHOM/PE/94/LC2353	C		MHOM/PE/90/LH937	C
	MHOM/PE/94/LC2355	C		MHOM/PE/91/LC1015	C
	MHOM/PE/94/LC2367	C	*L. (V.) peruviana* (SUS)	MHOM/PE/90/LCA01	C
	MHOM/PE/94/LC2369	C		MHOM/PE/90/LCA04	C
	MHOM/PE/93/LC2373	C		MHOM/PE/90/LCA05	C
	MHOM/PE/95/LC2711	C		MHOM/PE/90/LCA06	C
	MHOM/BO/92/CUM006	C		MHOM/PE/90/LCA08	C
	MHOM/BO/94/CUM25	C		MHOM/PE/90/LN77	C
	MHOM/BO/94/CUM34	C	*Lb*/*Lp* hybrids	MHOM/PE/91/LC1407	C
	MHOM/BO/94/CUM84	C		MHOM/PE/91/LC1408	C/M
	MHOM/BO/94/CUM129	C		MHOM/PE/91/LC1418	C
	MHOM/PE/84/LC03	M		MHOM/PE/91/LC1419	C
	MHOM/PE/94/LC2320	M	*L. (V.) guyanensis*	MHOM/PE/89/LH691	C
	MHOM/PE/94/LC2368	M		MHOM/PE/91/LC1446	C
	MHOM/BO/94/CUM48	M		MHOM/PE/91/LC1447	C
	MHOM/BO/94/CUM49	M		MHOM/PE/91/LC1448	C
	MHOM/BO/94/CUM50	M		MHOM/PE/91/LC2309	C
	MHOM/BO/94/CUM51	M	*L. (V.) lainsoni*	MHOM/PE/94/LC2398	C
	MHOM/BO/94/CUM52	M		MHOM/BO/94/CUM78	C
	MHOM/BO/94/CUM55	M		MHOM/BO/94/CUM97	C
	MHOM/BO/94/CUM65	M		MHOM/BO/94/CUM106	M
	MHOM/BO/94/CUM67	M		MHOM/BO/94/CUM107	M
	MHOM/BO/94/CUM68	M	*L. (V.) panamensis*	MHOM/PA/00/PRI	
	MHOM/BO/94/CUM123	M			
	MHOM/BO/94/CUM131	M			

C, cutaneous; M, mucosal; HB, Huancabamba; SUN, SUC, SUS, respectively, Surco-North, Centre and South.

sequently agglomerated by the UPGMA method and dendrograms were built up using the program TAXO (E. Serres, Laboratoire d'Ecologie médicale et de Pathologie parasitaire, Faculté de Médecine, Montpellier, France).

2.4. Probes and hybridisation

The mini-exon probe consisted of a 39-nt oligonucleotide (AACTAACGCTATATAAGTATCAGTTTCTGTACTTTATTG [19]) syn-

thesised commercially (Eurogentec). Probe pLb-149G is derived from pLb-149 and contains a 2-kb random genomic fragment isolated from *L. (V.) braziliensis* M2904 [20], corresponding to a low copy number sequence (unpublished data). The last washing after hybridisation was performed at 2×SSC (mini-exon probe) or 1×SSC (pLb-149G). The mini-exon probe was end-labelled by a kinase reaction with ^{32}P-dATP, and pLb-149G was labelled with ^{32}P-dCTP, by random prime labelling [21]. Hybridisations were performed according to the manufacturer's instructions at 65°C.

2.5. Densitometric scanning and measuring of hybridisation intensity

Densitometric scanning of autoradiograms was performed in duplicate with a Pharmacia set-up, using a SHARP JX-330 scanner and Diversity one™ software package for the analysis of polymorphic system (PHARMACIA). For each isolate, the average density of the mini-exon hybridising band was then normalised to the quantity of DNA present for that chromosome (as estimated by the density after hybridisation with pLb-149G), using *L. (V.) braziliensis* strain M2903 as reference standard. The following formula allowed inter-gel comparisons: $NDmex_a = (MDmex_a \times MD149G_r / MD149G_a)/MDmex_r$, where NDmex, MDmex and MD149G stand for normalised density and measured density of mini-exon hybridising band, and measured density of the pLb-149G hybridising band in isolate (*a*) or in the reference strain M2903 (*r*). For example, $NDmex_a > 1$ if isolate *a* shows a higher density of mini-exon hybridising band(s) than strain M2903, for the same quantity of chromosomal material; this method is thus independent of eventual differences in the ploidy of that chromosome, and its robustness was demonstrated elsewhere [9].

3. Results

3.1. Chromosome hybridisation patterns of mini-exon genes

The karyotype of 84 stocks belonging to subgenus *Viannia* was hybridised with a mini-exon gene probe. As expected, patterns were highly polymorphic, with chromosomal size ranging between 360 and 605 kb. In addition, in several stocks, double hybridising bands were encountered (Fig. 1A). Identical patterns were observed when hybridising with pLb-149G (Fig. 1B), a random genomic probe not cross-hybridising with mini-exon genes (not shown). This suggests homology between the different hybridising chromosomes; accordingly, double bands would

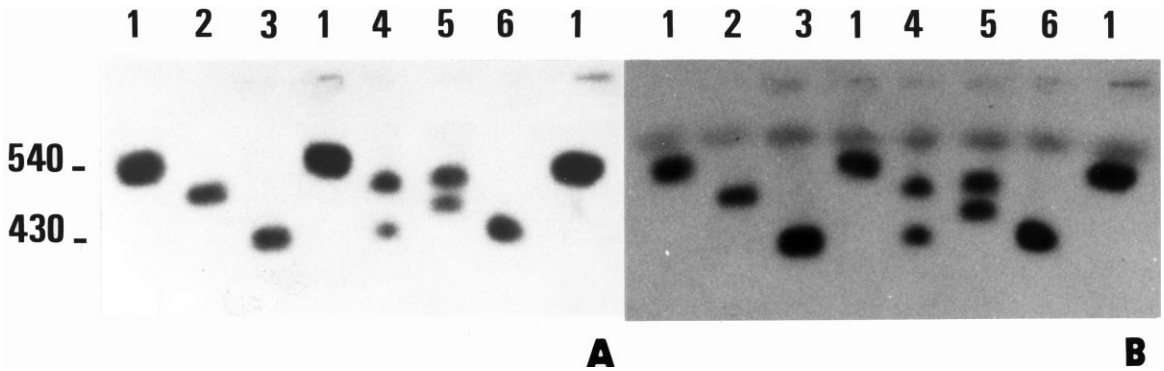

Fig. 1. Molecular karyotype after hybridisation with mini-exon (A) and pLb-149G (B) probes; stocks: *L. (V.) braziliensis* M2903 (1), *L. (V.) guyanensis* LH691 (2), *L. (V.) peruviana* LH696 (3), LH760 (4), LH827 (5), LH925 (6); size in kb.

be explained as two different size-variants of a pair of mini-exon gene-bearing homologues.

3.2. Size comparison of mini-exon gene-bearing chromosomes

The size of the mini-exon gene-bearing chromosomes was estimated in each stock and used to calculate the Chromosome Size Difference Index (CSDI [17]) between each pair of stocks. Then the CSDI values were agglomerated by the UPGMA method; this generated a dendrogram illustrating chromosomal size dissimilarity between the different stocks (Fig. 2). For five stocks, data were obtained from independent gels and integrated into the analysis; this allowed evaluation of the reproducibility of size estimation and deduction of a threshold of significance of measured differences. The Chromosome Size Difference Index between duplicates of the five stocks was always lower than 50 kb, and this value was thus adopted as threshold of significance on the dendrograms. A simplified version of the dendrogram, where only branching above 50 kb is indicated, is shown in Fig. 2. This revealed five significant clusters, each of them containing representatives of several species, except cluster V, which was composed largely of *L. (V.) peruviana* stocks.

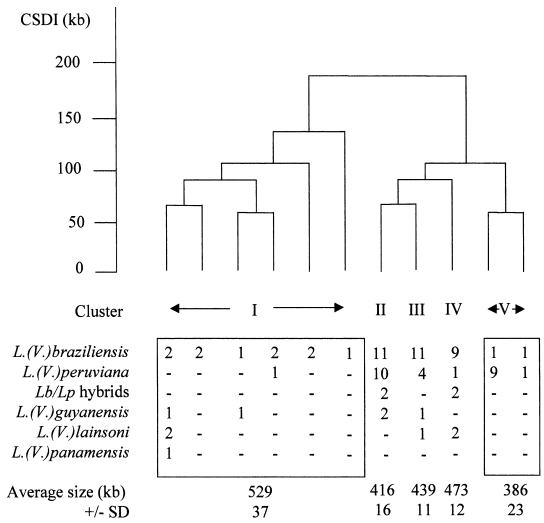

Fig. 2. Simplified dendrogram (considering only branchings superior to 50 kb) illustrating size dissimilarities (CSDI method) of mini-exon gene-bearing chromosomes among the population under study. Under the dendrogram are given: the cluster number, the number of stocks of the respective species present in the different branchings, the average chromosomal size and S.D. in each cluster.

Table 2
Size (expressed in kb) comparison of mini-exon gene-bearing chromosomes in several *Leishmania* spp. of subgenus *Viannia*

Species	Size			
	N stocks	Range	Average	S.D.
L. (V.) braziliensis	43	380–605	448	51
Cutaneous	29	380–605	450	50
Mucosal	14	382–605	441	52
Peruvian	25	382–605	442	49
Bolivian	16	380–604	458	52
L. (V.) peruviana	26	354–518	405	35
HB	10	354–408	375	16
SUC/SUN	10	380–518	433	36
SUS	6	394–406	402	5
Lb/*Lp* hybrids	4	434–482	457	22
		385–409	400	11
L. (V.) guyanensis	5	431–508	485	60
L. (V.) lainsoni	6	410–526	476	41
L. (V.) panamensis	1		506	

HB, SUC/SUN and SUS correspond to different eco-geographical populations; in hybrids, size data for large and small variants are mentioned.

Comparison of the size-distribution between species (Table 2) revealed that the average size was (i) similar in *L. (V.) braziliensis*, *L. (V.) guyanensis*, *Leishmania (Viannia) lainsoni* and *Leishmania (Viannia) panamensis*, but (ii) significantly smaller ($P < 0.01$) in *L. (V.) peruviana*. *Leishmania (V.) braziliensis*/*L. (V.) peruviana* hybrids [22] all presented two bands, with an average size similar to the one found in *L. (V.) braziliensis* and *L. (V.) peruviana*, respectively. Intra-specific heterogeneity was further explored in *L. (V.) braziliensis* and *L. (V.) peruviana*. Within *L. (V.) braziliensis*, no significant difference was encountered between stocks isolated from cutaneous and mucosal lesions (average size of 450 and 441 kb, respectively; Table 2) nor between Peruvian and Bolivian populations (average size of 442 and 458 kb, respectively; Table 2) which were present in each of the five clusters identified above. However, in *L. (V.) peruviana*, differences were observed according to the eco-geographical origin of the stocks. Stocks originating from the northern and southern extremes of Peru (BGU of Huancabamba and Surco-South) constituted two distinct but homogeneous populations; they were mostly restricted to clusters V and II, respectively, and standard deviation of chromosomal size was very low in both populations. In contrast, stocks isolated in the central part of the country showed a wider range of size variation and were consequently dispersed in several clusters.

3.3. Involvement of mini-exon genes in chromosome size-variation

In order to check if size variation of the mini-exon-bearing chromosome was due to variation in copy number of the mini-exon gene themselves, relative quantification of chromosome hybridisation with the mini-exon probe was performed by densitometric scanning using the hybridisation intensity with linked probe pLb-149G as internal control (see Materials and methods and Ref. [9]). A significant correlation ($P < 0.001$) was observed between normalised hybridisation intensity of the mini-exon probe and size of the hybridising chromosome (Fig. 3).

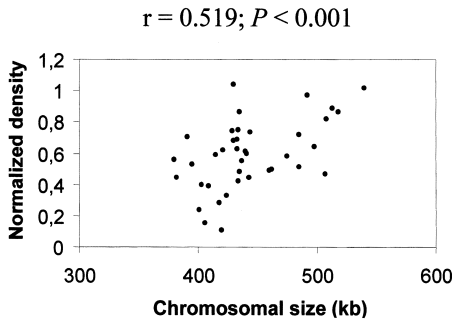

Fig. 3. Relationship between the normalised density of mini-exon hybridising chromosomes and size of the respective chromosomes.

Confirmation of the role of mini-exon genes in chromosome size-polymorphism (and validation of the densitometric method) was provided by chromosomal digestion with restriction enzymes not cutting within the mini-exon gene array (*Pst*I and *Eco*RI). Double digestion of purified chromosomes of *L. (V.) braziliensis* M2903 (540 kb) and *L. (V.) peruviana* LCA06 (400 kb) with these two enzymes allowed evaluation of the maximal size occupied by the mini-exon gene

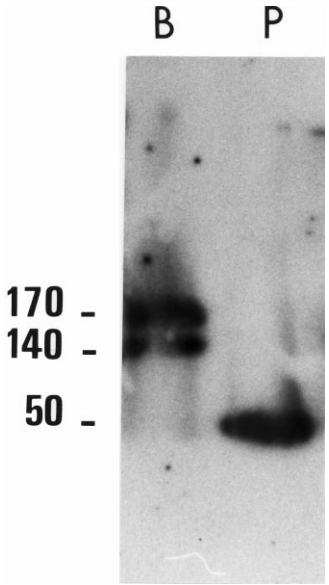

Fig. 4. Hybridisation of a mini-exon probe on *Pst*I/*Eco*RI double digests of mini-exon gene-bearing chromosomes of *L. (V.) braziliensis* M2903 (B) and *L. (V.) peruviana* LCA06 (P); orthogonal field alternating gel electrophoresis; size in kb.

array; two mini-exon hybridising fragments of 140 and 170 kb, respectively, were observed in strain M2903 (probably two size-variants of the mini-exon gene array in the two unresolved sister chromosomes of that stock), and a single 50 kb in strain LCA06 (Fig. 4). Taken together with the difference in relative hybridisation intensity encountered between mini-exon chromosomes of these two strains (1 and 0.56, respectively), restriction patterns thus show that a 50% expansion/contraction of the mini-exon gene array is at least partially responsible for the size variation of the mini-exon gene-bearing chromosome of the corresponding stocks; however, comparing the size differences of the whole chromosomes (140 kb) and restriction fragments (90–110 kb), we cannot exclude the involvement of other sequences.

4. Discussion

In previous work, Iovannisci and Beverley [15] showed that size variation of the mini-exon gene-bearing chromosome was partly due to expansion/contraction of the mini-exon gene array for a few laboratory-maintained strains. In the present study, we explored this phenomenon in natural populations of Neotropical *Leishmania* (subgenus *Viannia*). Our results show that size variation of the mini-exon gene-bearing chromosome is frequent and important (up to 245 kb size difference), chromosomal plasticity involves variation in copy number of mini-exon genes (up to 50%), but other sequences are probably also involved. It should be noted that genomic rearrangements of the mini-exon gene array appears to be a classical phenomenon among trypanosomatids, but that it involves different mechanisms specific to the organism, like translocation in *Trypanosoma cruzi* [23] or transposition in African trypanosomes [24].

There was no significant size-difference between mini-exon-bearing chromosomes of cutaneous and mucosal isolates of *L. (V.) braziliensis*, nor between Peruvian and Bolivian populations (despite being separated by 750 km) of that species. However, significant size differences were encountered between eco-geographical populations of *L. (V.) peruviana*. Moreover, *L. (V.) peruviana* presented a significantly smaller mini-exon-bearing chromosome than the other species of subgenus *Viannia*. A similar eco-geographical structuring in *L. (V.) peruviana* has already been described for two other chromosomes [7, 9], for the restriction fragment length polymorphism patterns of gp63 genes [10, 25], for one isoenzymatic and three random amplified polymorphic DNA loci (AL Bañuls, Analyse génétique d'isolats naturels sud-américains de *Leishmania* montrant de possibles phénomènes de recombinaison. DEA thesis, Université de Montpellier II, 1993). Such a strong structuring constitutes a unique feature in Neotropical *Leishmania* and is thought to be influenced by the insulated nature of Andean valleys where that species is endemic [7, 16]. The contrast between genomic homogeneity in northern and southern populations on one hand, and the heterogeneity in the central populations on the other, was also characteristic of other markers [7], but is more difficult to understand in the case of a chromosome generally submitted to frequent rearrangements in the mini-exon gene array. Such a phenomenon might be the product of strong and homogeneous selective factors in the northern and southern BGUs. The presence of a single sandfly vector species in the South (*Lutzomyia ayacuchensis*; Caceres and Dujardin, unpublished results) compared with two different species in the centre (*Lutzomyia peruensis* and *Lutzomyia verrucarum*, [26, 27]) might constitute a source of selective homogeneity. In addition, the correlation between mini-exon gene copy number and growth behaviour observed in other *Leishmania* species [15] certainly represents an important selectable characteristic.

The observation of a smaller mini-exon gene-bearing chromosome, coupled with a lower copy number of mini-exon genes in *L. (V.) peruviana* compared with its closest taxon [28], *L. (V.) braziliensis*, deserves particular attention. Indeed, it constitutes the third chromosome for which a small variant was found to characterise the former species. We previously showed that the gp63 and rDNA gene bearing chromosomes were

smaller (100 and up to 200 kb, respectively) in *L. (V.) peruviana*; in both cases, the mechanism leading to chromosomal size-variation was also the same (amplification/deletion of tandemly repeated genes) and copy number of essential genes (gp63 and rDNA) was smaller in *L. (V.) peruviana* [8–10]. Both species are considered to have diverged recently [7, 28], an event concomitant with an important difference in pathogenicity: *L. (V.) braziliensis* causes severe cutaneous lesions that may lead to disfiguring mucosal metastases in up to 10% of cases (EA Llanos-Cuentas, Tratamiento de leishmaniasis mucosa: analisis de los factores asociados con la repuesta terapeutica a los antimoniales pentavalentes. Tesis de Doctorado, Universidad Peruana Cayetano Heredia, Lima, 1991), while *L. (V.) peruviana* produces benign and selfhealing ulcers [29]. Rearrangement of tandemly repeated genes may have phenotypic consequences due to gene dosage itself [12] or to modifications in the intergenic sequences shown to play a role in transcription regulation in the case of gp63 [13] and mini-exon [30] genes. Accordingly, it is tempting to speculate that the accumulation of deletions reaching genes that code for general (mRNA splicing for the mini-exon gene [14] or protein translation for rDNA) and specific (invasion and survival within macrophages for the gp63 [31]) functions might have a synergistic effect and play a role in the lower pathogenicity of *L. (V.) peruviana*. Analyses at the RNA level and genetic manipulations in an appropriate experimental model should allow this hypothesis to be tested.

The relationship between chromosome size-polymorphism and rearrangement of tandemly repeated genes demonstrated here illustrates two important implications of genome dynamics in natural populations of *Leishmania*. Firstly, it stresses the need to further look at genomic organisation of other tandemly repeated genes; their genomic plasticity might allow testing for population structure and would make them potential polymorphic targets for PCR assays such as that developed for the three genes mentioned here [19, 32, 33]. Second, it paves the way for the identification of potential direct markers for polymorphic phenotypes by exploring functional consequences of chromosome rearrangement.

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