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# Constitutive mosaic aneuploidy: a unique genetic feature widespread in the

## *Leishmania* genus

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## **Abstract**

Using fluorescence in situ hybridization, we determined the ploidy of four species of *Leishmania*: *L. infantum*, *L. donovani*, *L. tropica* and *L. amazonensis*. We found that each cell in a strain possesses a combination of mono-, di- and trisomies for all chromosomes; ploidy patterns were different among all strains/species. These results extend those we previously described in *L. major*, demonstrating that mosaic aneuploidy is a genetic feature widespread to the *Leishmania* genus. In addition to the genetic consequences induced by this mosaicism, the apparent absence of alternation between haploid/diploid stages questions the modality of genetic exchange in *Leishmania* sp.

Keywords: ploidy; *Leishmania*; genome; chromosome; aneuploidy; mosaicism; genetic exchange

## 1 Introduction

*Leishmania* are parasitic protozoa responsible for a broad spectrum of diseases affecting different mammals including human. During its lifecycle, the parasite alternates between two stages: one extra-cellular in the digestive tract of the sand fly vector and the other one obligate intracellular in macrophages of the mammalian host. Thus, a rapid adjustment to radically different environments is required for the survival of the parasite. The genomes of these parasites are organized in 36 chromosomes for the Old World species (*L. donovani*, *L. infantum*, *L. major*, *L. tropica*, *L. aethiopica*) [1] and 34 and 35 for the New World *L. mexicana* and *L. braziliensis* respectively [2]. Regarding the ploidy of *Leishmania*, different sets of data have suggested that *Leishmania* are aneuploid rather than strictly diploid [3-9]. Moreover, we have previously shown that *Leishmania major* genome exhibits an unusual feature termed ‘mosaic aneuploidy’ [3]: the population of cells within a strain, or even within a clone, is a mosaic, in that sense that the population is composed of mono-, di- and trisomic cells, in proportions that vary from one chromosome to another and from one strain to another. Taken together, these results strongly suggest that aneuploidy and mosaicism are constitutive features of *L. major*.

The aim of this work was to determine if these features were also present in other species of *Leishmania*. For this purpose, using fluorescence in situ hybridization (FISH), we analyzed the ploidy of individual cells in three Old World species (*L. infantum*, *L. donovani* and *L. tropica*) and one of the New World species (*L. amazonensis*) for six different chromosomes. Our data establish mosaic aneuploidy as a general constitutive feature of *Leishmania*. The consequences of mosaic aneuploidy upon the parasite’s biology will be discussed, with respect to gene dosage, the absence of apparent detrimental effect of aneuploidy, and genetic exchange, in particular as an alternative to overcome the probable lack of gametes/meiosis.

## 2 Materials and methods

### 2.1 Parasites and in vitro cultivation

Four strains of different species of *Leishmania* were analyzed: *L. infantum* (MHOM/FR/95/LEM3049), *L. donovani* (MHOM/IN/2003/LEM4537), *L. tropica* (MHOM/SY/92/LEM2514) and *L. amazonensis* (MHOM/GF/95/LEM3013). Strains were provided by the Biological Resource Centre of *Leishmania* (CRB-*Leishmania*) located in Montpellier ([http://www.parasitologie.univ-montp1.fr/english\\_vers/en\\_cryobanque.htm](http://www.parasitologie.univ-montp1.fr/english_vers/en_cryobanque.htm)).

*Leishmania* promastigotes were grown as described elsewhere, with the difference that supplemented RPMI1640 was replaced with supplemented SDM medium [10]. Parasites were maintained in log phase by splitting the culture every second day. Then, for FISH analysis, in order to minimize the number of dividing cells in the population, they were grown to late log phase and harvested. *L. amazonensis* MHOM/BR/1987/BA276 (BA276) promastigotes (kind gift of Aldina Barral and Manoel Barral-Netto, Fiocruz, Salvador de Bahia, Brazil) were grown in supplemented RPMI1640 [11].

### 2.2 DNA probes

For *L. donovani*, *L. infantum* and *L. tropica*, chromosome-specific probes (chromosomes 1, 2, 5, 22, 27, 29) were in large part similar to those used in Sterkers *et al.* 2010 [3]. The spliced leader RNA gene probe, specific for chromosome 2, was early gift from S.M. Beverley (Washington University School of Medicine, St. Louis). Specific probes for chromosomes 1, 5, 22, 27 were cosmid (L549) and BAC (LB00822, LB00273, LB00646) clones, respectively, that were kindly provided by Peter Myler (Seattle Biomedical Research Institute, USA) and Christiane Hertz-Fowler and David Harris (Sanger Centre, Hinxton, UK). We also designed a chromosome 29-specific PCR probe. Two successive ORFs (LmjF.29.0110 and LmjF.29.0120) were PCR-

amplified separately from LmjF genomic DNA. PCR products were gel-purified and mixed at equimolar ratios. For *L. amazonensis*, chromosome-specific probes were constructed as follow : a genomic *L. amazonensis* MHOM/BR/1987/BA276 DNA cosmid library was constructed with the pcosTL vector (kind gift of Dr J. Kelly) as described [12] and propagated in the *E. coli* strain STBL-2™ (Invitrogen) ; it contained about 20 000 independent 50 kb insert clones, representing more than 15 haploid genome copies. Two thousand and five hundred individual clones were kept at -80°C in 96 wells microtiter plates in 50% glycerol. Six independent clones (T3, T5, T7.2, T9; T15 and T18) were used in this study. The DNA from BAC and cosmid clones was prepared using Qiagen Large-Construct Kit.

### 2.3 Pulse field gel electrophoresis and Southern blot

The chromosomal specificity of the probes was assessed (i) by end-sequencing of the cosmid insert and sequence alignment (see Sterkers *et al.* 2011 [3] and Table S2); and (ii) by Southern blot analysis of the molecular karyotype of each strain. The molecular karyotypes of all the strains studied here were resolved by pulsed field gel electrophoresis (PFGE). Briefly, chromosomal DNA agarose blocks were prepared and processed for PFGE as described elsewhere [13]). PFGE was performed at 15°C in 1.5% agarose gels with 0.5×Tris-Borate-EDTA (pH 7.4) running buffer on home-made devices [13]). The electrophoretic conditions were the following: pulse/migration times of 300 s/24 h, 130s/24h and 90 s/24 h with 7.5 V/cm. After migration, gels were stained with ethidium bromide and blotted by alkaline transfer onto nylon filters (Hybond N+, Amersham). For Southern blot analysis, probes were labeled and hybridized with the DIG-High Prime DNA Labeling and Detection Starter Kit II according to the manufacturer's procedures (Roche Applied Sciences).

#### 2.4 *Fluorescence in situ hybridization, microscopy and fluorescence imaging*

For each *Leishmania* strain, the preparation of cells for immunofluorescence slides and the hybridization protocol used were as previously described [3]. All probes were labeled with tetramethyl-rhodamine-5-dUTP (Roche Applied Sciences) by using the Nick Translation Mix (Roche Applied Sciences) according to the manufacturer's instructions. Slides were then mounted in Vectashield (Vector Laboratories) with DAPI and microscopically examined [3, 14, 15]. *Leishmania* cells were viewed by bright field, and fluorescence was visualized using appropriate filters on a Zeiss Axioplan 2 microscope with a 100x objective. Digital images were captured using a Photometrics CoolSnap CDD camera (Roper Scientific) and processed with MetaView (Universal Imaging). Z-Stack image acquisitions (15-25 planes of 0.25  $\mu$ m) were systematically performed for each cell analyzed using a Piezo controller, allowing to view the nucleus in all planes and to count the total number of labeled chromosomes. *L. major* reference strain 'Friedlin' (MHOM/IL/81/Friedlin) was used as positive control in all experiments (not shown).

#### 2.5 *Data analysis*

Examination of the bright field and DAPI acquisitions allowed identifying the cells to be counted; mitotic cells were excluded from the analysis. Examination, plane by plane, of a numerically magnified view of each cell allowed the determination of the number of labeled chromosomes per individual cell. Each chromosome was then defined as mono-, di-, tri- or tetrasomic in the cell analyzed, and the ploidy/aneuploidy of the chromosome in the strain expressed as proportions.

### **3 Results**

Eleven heterologous chromosomes (Chr.) were analyzed by FISH: Chr. 1, 2, 5, 22, 27 and 29 in three *Leishmania* strains of the Old World, *L. infantum*, *L. donovani* and *L. tropica*; and Chr. 14,

16, 17, 23, 26 and 29 in one New World strain (*L. amazonensis*). The number of homologous chromosomes per individual interphasic cell was determined in all these strains.

### 3.1 Probe efficiency and specificity

Most probes designed for studying *L. major* by FISH [3] were used here with good efficiency in the three Old World species. As regards *L. amazonensis*, new species-specific probes were needed as the probes derived from *L. major* exhibited a too low specificity and sensitivity (not shown). Southern blotting of PFGE gels confirmed chromosome specificity in all cases (Fig. S1 and S2).

### 3.2 FISH analysis

Individual cell analysis showed variable ‘somes’ for all the chromosomes examined, i.e. the population is composed of mono-, di- and trisomic, and less frequently tetrasomic, cells, in proportions that vary depending on the chromosome and on the strain/species examined. For all species, each chromosome was observed in at least two ploidy states in the strain cell population (Fig. 1A–D). A rather diploid pattern was displayed in *L. infantum*, *L. tropica* and *L. amazonensis* strains, as five and four out of the six studied chromosomes for *L. infantum* and *L. tropica* and for *L. amazonensis*, respectively, were predominantly disomic. The chromosome ploidy of the *L. donovani* strain was more heterogeneous: two chromosomes were predominantly trisomic, two monosomic and one disomic. Interestingly, only one chromosome, chr. 2, exhibited a similar ploidy pattern in *L. major* ([3]), *L. infantum*, *L. donovani* and *L. tropica* (these data) strains, with a proportion of about 50% monosomic and 40% disomic. We could not study this chromosome in *L. amazonensis*, since we could not produce any chromosome 2-specific probe for this species; the spliced leader RNA gene probe located on chromosome 2 used for other species showed poor efficiency here.

Similarly to that obtained from high throughput sequencing [4], we calculated a ‘cumulative ploidy’ per chromosome from FISH data in individual cells (Table S1). This varied from 1.6 to 2.8 (mean 2.2) depending on the chromosome and the *Leishmania* strain.

All in all, as all chromosomal contents varied among cells, we inferred that the five *Leishmania* species analyzed display a mosaic structure.

#### **4 Discussion**

Euploidy, mostly haploidy or diploidy, is the common rule in Eukaryotes and the alternation between these two states characterizes sexuality and genetic exchange between individuals. Aneuploidy has most often been associated with disease, lethality or malformation; one exception is a recent natural allopolyploid species, *Tragopogon miscellus* (Asteraceae), which shows extensive chromosomal variation, with 69% of the individuals aneuploid for one or more chromosomes [16]. Here, using FISH analysis, we show that the divergent eukaryote *Leishmania* constitutively displays mosaic aneuploidy across different species, this feature having been observed in four species of the Old and New World, in addition to *L. major* [3]. This has been shown for 11 different chromosomes in total. Data obtained from high throughput sequencing studies [4] further support mosaic aneuploidy as a general feature that affects all heterologous chromosomes of *Leishmania*.

*Leishmania* includes a large number of species which are responsible for a wide spectrum of human or animal diseases (cutaneous, mucosal and visceral forms). Paradoxically, the genome sequencing of these species showed a strong homogeneity in the genome organization, with an extreme degree of synteny observed among species and a remarkably low number of species-specific genes [4, 17]. In addition, *Leishmania*, like other Trypanosomatids, displays several unusual biological features for eukaryotes, among which a unique structural organization of the

genome: indeed, genes are organized in large polycistronic transcription units made of functionally unrelated genes, and for which there is no promoter for RNA polymerase II. The resulting weak level of transcription regulation of protein-coding genes [18] may explain the tolerance to aneuploidy observed in *Leishmania*. Indeed, the changes in gene dosage induced by mosaic aneuploidy seem to have no detrimental effect on cell growth of *Leishmania* promastigotes *in vitro*. However, such a tolerance cannot explain the emergence of aneuploidy in *Leishmania*, since *T. brucei* has the same gene expression regulation system and yet remained diploid [3, 14]. Moreover, one cannot affirm that these parasites are insensitive to gene dosage variations; indeed, (i) several studies have shown that specific gene amplifications are correlated to drug resistance [19-22]; (ii) transcriptome studies do not allow correlating transcript levels to ploidy variations at the cell level, hence we cannot rule out that gene dosage variations do modulate transcript levels in individual cells.

Mosaic aneuploidy appears to be a major feature of the *Leishmania* genus which has numerous consequences on its genetics, hence on its biology. One of these consequences is that population genetics studies on this parasite need to be revisited. In particular, microsatellite data have to be analyzed in the light of aneuploidy of *Leishmania*. In our view, the conclusion that microsatellite data support diploidy rather than aneuploidy [23] is not justified. Indeed, PCR amplification of a given locus reveals either one or two bands that correspond to a phenotype and not a genotype. This, for example when applied to microsatellites, does not allow determining the number of allele (hence chromosome copies) present in the genome. As previously described in *Leishmania*, constitutive mosaic aneuploidy results from the over-replication of one homologue and/or under-replication events [24]. Over-replication of one homologue generates two identical alleles and those will be undistinguishable by PCR amplification.

We already described another consequence of mosaic aneuploidy, which is that the cell population is essentially made of homozygous cells, yet retaining the global genetic heterogeneity of the strain [24]. As regards the heterozygote deficit in *Leishmania* 'populations', two events further contribute to the homozygosity of the strains : automixy [25] and selective pressures. These consequences are increased in low endemicity areas where reduced chances of encounters of different genotypes obviously make automixy predominant, as opposed to the formation of hybrids.

A more speculative issue may be addressed from our data. In most eukaryotes, the alternation of haploid/diploid stages, associated with gene shuffle and fusion of the gametes, is a hallmark of sexual reproduction. In *Leishmania*, genetic exchange during cyclical development in the sand fly vector has been experimentally demonstrated and is now believed to be a normal (although non obligatory) aspect of the parasite's biology [26, 27]; yet, the existence of meiosis remains unclear in *Leishmania*. As stated by Akopyants *et al.* [26], two hypotheses may explain this genetic exchange: either a fusion of parental cells which would be followed with a progressive chromosome number reduction [16] as seen in *Candida albicans* [28]; or a passage through gametic cells, *i.e.* classical meiosis. Indeed, the fusion of parental cells cannot be distinguished from the fusion of gametic cells during a meiotic process with the methods used hitherto. Fusion of *in vitro* grown promastigotes have previously been recorded [29]. Inbar *et al.* [27]. found chromosomal inheritance of both parental alleles at multiple unlinked loci, which they assume supports the existence of a meiotic process; they also stated the presence of so-called 'meiotic genes' which are known to be expressed in *T. brucei* [30] and exist in the *Leishmania* genome, such as DMC1 (Tb09.211.1210), HOP1 (Tb10.70.1530), MND1 (Tb11.02.3380) and SPO11 (Tb927.5.3760). However, as shown previously in *C. albicans* [28], the expression of meiosis-specific genes is not necessarily associated with meiosis. Moreover deletion of SPO11 prevented genetic recombination between homologous chromosomes during the *C. albicans* parasexual cycle

[28]. Actually, as underlined by others [31], aneuploidy excludes classical meiosis and is compatible only with parasexual processes. In sexual reproduction, the genotypes of each given individual are stable/fixed for at least one generation. On the contrary, the mosaic aneuploidy system allows maintaining a dynamic genotypic diversity, with multiple possible genetic combinations [24] within the same cell population, which theoretically avoids the need for gametes, while still maintaining the adaptive potential of the parasite.

In total, mosaic aneuploidy, first described in only one species of *Leishmania* (*L. major*), now appears as a constitutive feature across the genus of this protozoon. This phenomenon has multiple and major consequences upon the biology of this parasite, which remain essentially unexplored. It constitutes one more example of the highly unusual biological features of this organism that challenge models derived from classical model organisms and often adopted as dogma in life sciences.

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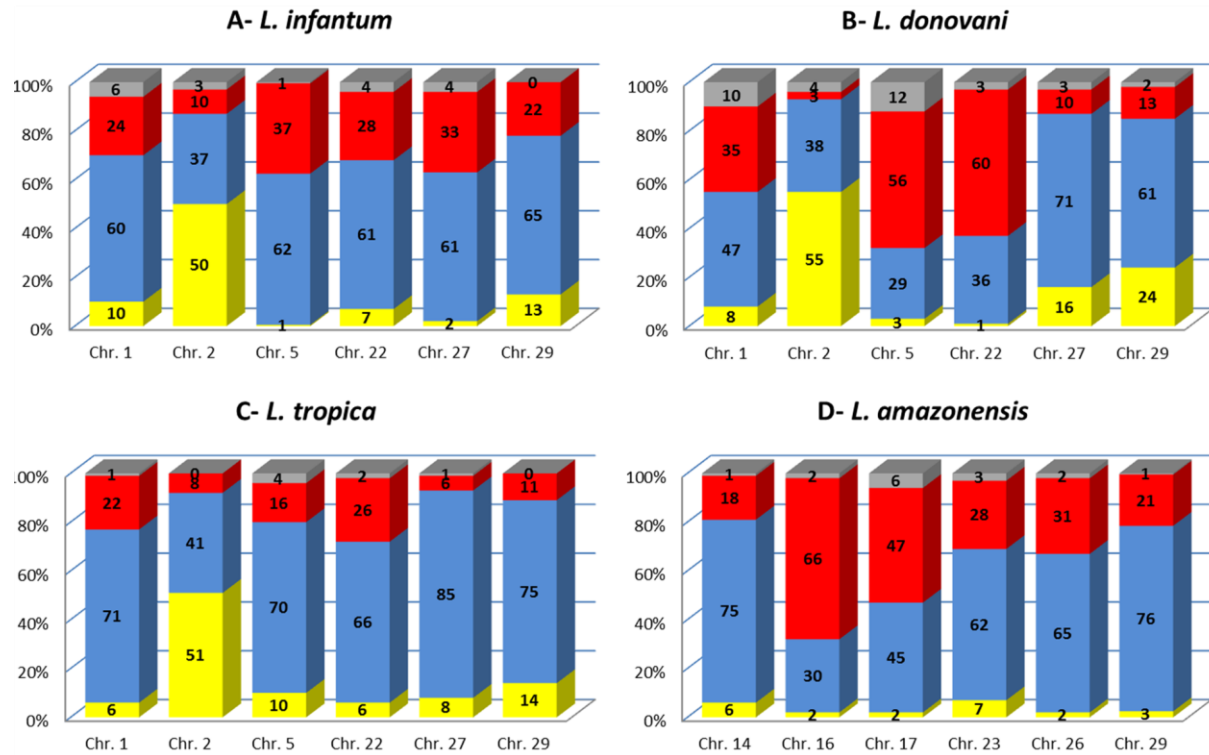
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**Figure 1. Mosaic aneuploidy in four *Leishmania* species of the Old and New World**

Histograms show the proportions (percentages in ordinate) of mono- (yellow), di- (blue), tri- (red) and tetrasomic (grey) chromosomes in the different *Leishmania* species studied: A- *L. infantum* (MHOM/FR/95/LEM3049), B- *L. donovani* (MHOM/IN/2003/LEM4537), C- *L. tropica* (MHOM/SY/92/LEM2514) and D- *L. amazonensis* (MHOM/GF/95/LEM3013). Each bar represents one of the six chromosomes studied (abscissa). The ploidy of each chromosome was calculated from the examination of 3D views of a mean of 244 labeled cells for each probe and strain; a total of 5846 cells were examined. Mosaicism was observed for all the chromosomes analyzed; nevertheless, the proportions of mono-, di-, tri- or tetrasomic chromosomes vary depending on the chromosome and on the strain examined.

## Supporting information

**Table S1. Percentages of nulli-, mono-, di-, tri-, and tetrasomic cells observed for six chromosomes in in four *Leishmania* species of the Old and New World (raw data).**

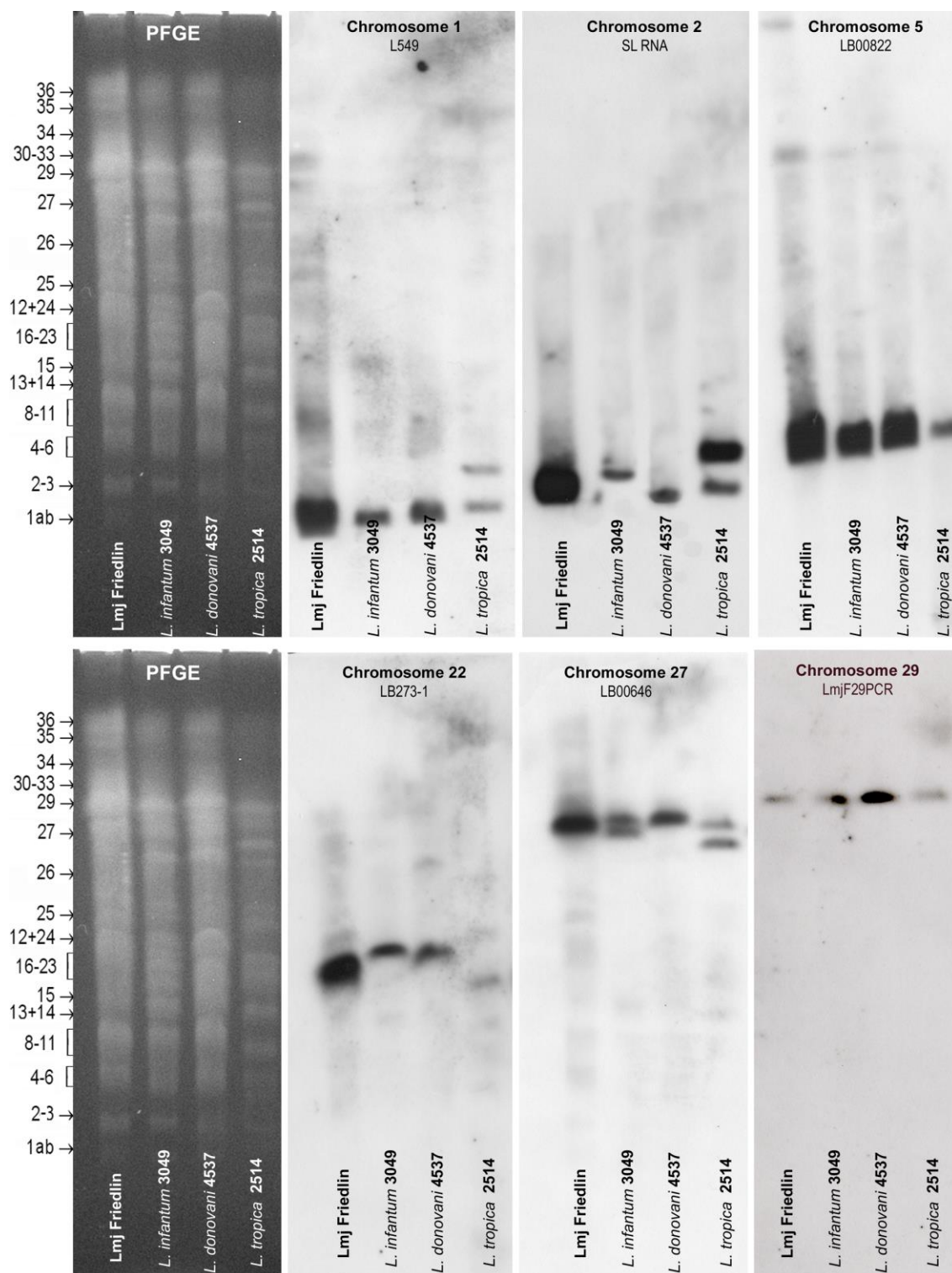
Species	Chromosome	Probe	% monosomic <sup>a</sup>	% disomic <sup>a</sup>	% trisomic <sup>a</sup>	% tetrasomic	Total number of cells	Cumulative ploidy
<b><i>L. infantum</i></b> MHOM/FR/95/LEM3049	Chr. 1	L549	10	60	24	6	147	2.3
	Chr. 2	SL RNA	50	37	10	3	301	1.7
	Chr. 5	LB00822	0.5	62	37	0.5	183	2.4
	Chr. 22	LB00273_1	7	61	28	4	330	2.3
	Chr. 27	LB00646	2	61	33	4	204	2.4
	Chr. 29	LmjF29PCR	13	65	22	0	185	2.1
<b><i>L. donovani</i></b> MHOM/IN/2003/LEM4537	Chr. 1	L549	8	47	35	10	393	2.5
	Chr. 2	SL RNA	55	38	3	4	285	1.6
	Chr. 5	LB00822	3	29	56	12	415	2.8
	Chr. 22	LB00273_1	1	36	60	3	133	2.7
	Chr. 27	LB00646	16	71	10	3	145	2.0
	Chr. 29	LmjF29PCR	24	61	13	2	486	1.9
<b><i>L. tropica</i></b> MHOM/SY/92/LEM2514	Chr. 1	L549	6	71	22	1	264	2.2
	Chr. 2	SL RNA	51	41	8	0	112	1.6
	Chr. 5	LB00822	10	70	16	4	180	2.1
	Chr. 22	LB00273_1	6	66	26	2	338	2.2
	Chr. 27	LB00646	8	85	6	1	158	2.0
	Chr. 29	LmjF29PCR	14	75	11	0	183	2.0
<b><i>L. amazonensis</i></b> MHOM/GF/95/LEM3013	Chr. 14	T7.2	6	75	18	1	233	2.1
	Chr. 16	T3	2	30	66	2	203	2.7
	Chr. 17	T5	2	45	47	6	230	2.6
	Chr. 23	T15	7	62	28	3	263	2.3
	Chr. 26	T18	2	65	31	2	226	2.3
	Chr. 29	T9	2.5	76	21	0.5	249	2.2

<sup>a</sup>The predominant ploidy is indicated by a colored box (same color code as Fig. 1).

**Table S2. Genomic localization of the DNA cosmids used to determine the ploidy of *L. amazonensis***

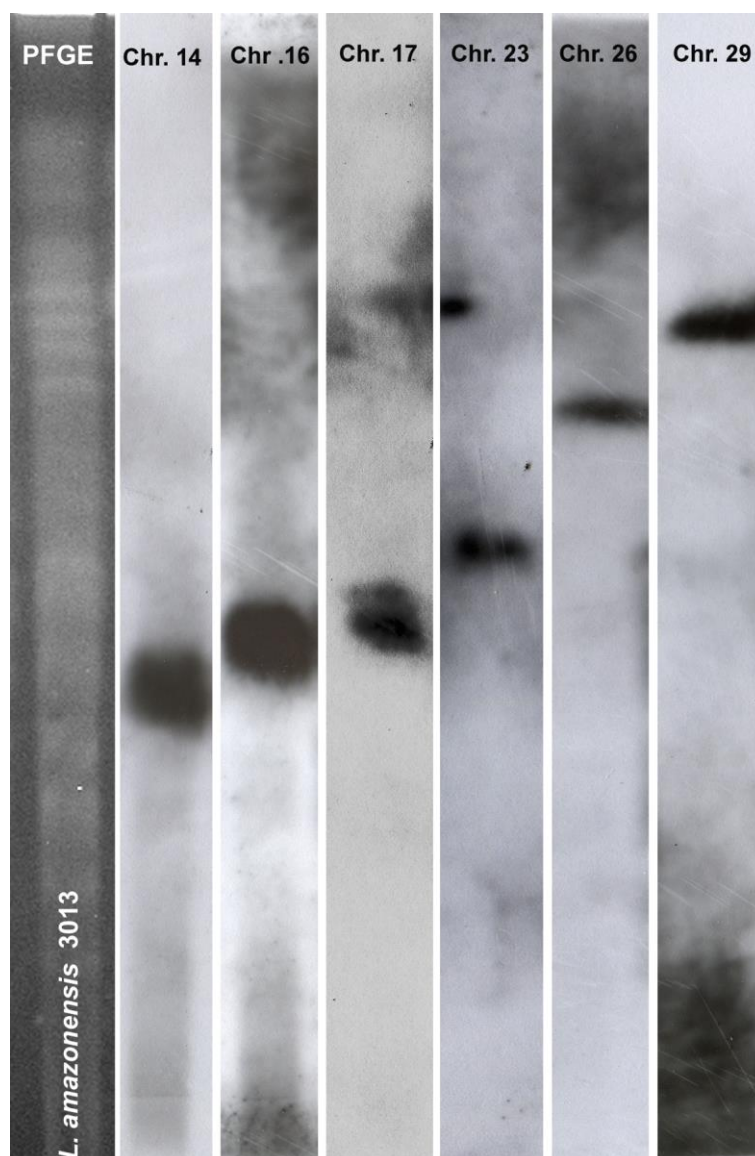
Chromosome	Cosmid code name	Primer <sup>a</sup>	Sequence ID <sup>a</sup>	Identities <sup>c</sup>	Gaps <sup>c</sup>	Query start <sup>c</sup>	Query end <sup>c</sup>	Subject start <sup>c</sup>	Subject end <sup>c</sup>	Score <sup>c</sup>	P <sup>c</sup>
Chr. 14	T7.2	T3	emb FR799567.1	977/1003 (97%)	13/1003 (1%)	14	1009	233213	232217	1701 bits (921)	0.0
Chr. 14	T7.2	G071	emb FR799567.1	851/863 (99%)	3/863 (0%)	1	860	193609	194471	1526 bits (826)	0.0
Chr. 16	T3	T3	emb FR799569.1	383/406 (94%)	5/406 (1%)	2	406	514159	513758	628 bits (340)	8,00E-177
Chr. 16	T3	G071	emb FR799569.1	387/395 (98%)	3/395 (1%)	1	392	474159	474553	686 bits (371)	0.0
Chr. 17	T5	T3	emb FR799570.1	663/668 (99%)	0/668 (0%)	7	674	397498	398165	1206 bits (653)	0.0
Chr. 17	T5	G071	emb FR799570.1	593/605 (98%)	0/605 (0%)	1	605	435914	435310	1059 bits (573)	0.0
Chr. 23	T15	T3	emb FR799576.1	801/844 (95%)	24/844 (3%)	1	824	94697	95536	1299 bits (703)	0.0
Chr. 23	T15	G071	emb FR799576.1	858/887 (97%)	8/887 (1%)	12	898	130562	129684	1480 bits (801)	0.0
Chr. 26	T18	T3	emb FR799579.1	883/889 (99%)	1/889 (0%)	22	910	882720	883607	1613 bits (873)	0.0
Chr. 26	T18	G071	nd	nd	nd	nd	nd	nd	nd	nd	nd
Chr. 29	T9	T3	FR799582.1	831/844 (98%)	0/844 (0%)	29	872	530572	529729	1500 bits (812)	0.0
Chr. 29	T9	G071	FR799582.1	64/67 (96%)	0/67 (0%)	6	72	502416	502482	113 bits (61)	1,00E-22

<sup>a</sup> Cosmid end sequences were obtained using the universal primer T3 and primer G071 (5'-CTT CAG ATG CCT GGT TG-3'). <sup>b</sup> BLAST analysis was performed in NCBI: <http://blast.ncbi.nlm.nih.gov/Blast.cgi> to localize the cosmids on a specific chromosome. <sup>c</sup> nd = not determined.



**Figure S1. Specific localization of the DNA probes used for this study onto chromosomes of three *Leishmania* species of the Old World.**

Molecular karyotypes of *Leishmania infantum* (MHOM/FR/95/LEM3049), *L. donovani* (MHOM/IN/2003/LEM4537), *L. tropica* (MHOM/SY/92/LEM2514) were resolved by PFGE and transferred on nylon membranes for Southern blot analysis. The position and number of the 36 *LmjF* chromosomes are shown on the left of each molecular karyotype (Ravel *et al.*, *Parasitol Today* 1998). Each probe proved specific for a given chromosome, confirming end-sequence data (Sterkers *et al.* *Cell Microbiol* 2011). SL RNA = spliced leader RNA (or mini-exon).



**Figure S2. Specific localization of the DNA probes used for this study onto chromosomes of *Leishmania amazonensis*.**

Unsheared chromosomal DNA was prepared from *L. amazonensis* (MHOM/GF/95/LEM3013) and separated by PFGE as described (Ravel *et al.*, *Parasitol Today* 1998). The ethidium bromide-stained PFGE gel (molecular karyotype) is shown on the left and the autoradiograms of the specific probe hybridizations are shown on the right. Each probe proved specific for a given chromosome (Chr. 14, Chr. 16, Chr. 17, Chr. 23, Chr. 26 and Chr. 29), confirming end-sequence data (Table S2).