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Abstract

The leishmaniases are complex neglected diseases caused by the protozoan parasite *Leishmania*. Cutaneous leishmaniasis is the most common clinical manifestation around the world, and in the Americas the main aetiological agent is *Leishmania braziliensis.* In recent studies, chromosome and gene copy number variations (CNVs) have been highlighted as some mechanisms used by *Leishmania* species to adapt to environmental changes such as host change or drug pressure. However, no studies have described the impact of temperature shifts across the genome of *Leishmania* promastigotes and particularly in *L. braziliensis*. Therefore, we sequenced the genome (DNA-Seq) of *L. braziliensis* promastigotes from cultures subjected to three different temperatures, 24, 28, and 30°C; then, we analysed the aneuploidy, gene CNVs, SNPs and Indels compared with those at the control temperature (26°C). We found that the increase in temperature at 30°C had a negative effect on promastigotes proliferation; although, there were no changes in the somy, SNPs and Indels on the DNA among the three temperatures compared to the control. Only around 3% of the genes having significant copy number variation (CNVs) at each temperature showed some important genes for adaptation to temperature shifts. In conclusion, there is not a relevant genome response to the temperature shift in short-term, therefore the adaptation of this

species to abiotic change could be occurring at transcriptome level. The ecological consequences are herein discussed.

Keywords

Leishmania braziliensis, promastigote, temperature increase, gene copy number variation, aneuploidy.

Introduction

In recent decades, there has been a drastic increase of the global temperature as part of climate change (1), affecting human populations due to the associated scarcity of resources and the emergence and re-emergence of infectious diseases. Several tropical pathologies are relevant in this context, such as zika, chikungunya, malaria and leishmaniases (2,3). The leishmaniases are caused by protozoans belonging to the genus *Leishmania* which feature a variety of clinical manifestations: cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (ML) and visceral leishmaniasis (LV). These diseases are a major public health problem in 98 countries around the world, where 12 million people are infected, more than 350 million people are at risk of infection and 1.3 million new cases occur each year (4). CL is the most common clinical manifestation; between 0.7 and 1.3 million new cases of CL are reported annually and about 90% of them occur in countries such as Afghanistan, Algeria, Brazil, Iran, Syria and Colombia. CL is also the most important and common clinical manifestation in the New World; it is characterised by ulcerative and deformative lesions, for which the most common causative species is *Leishmania braziliensis* (4,5).

Temperature is a factor that determines whether this parasite can develop appropriately, as was reported in a study by Hlavacova *et al*. They evaluated the effect of temperature on the life cycle

of three *Leishmania* species inside two different species of vectors (sandflies); they concluded that the response to temperature is species-specific (6). Other researchers also evaluated the effects of this environmental factor on *Leishmania* in view of the fact that these parasites already encounter temperature shifts during their life cycle (in the sandfly the temperature ranges from 22 to 28°C, while in the mammalian host it ranges from 31 to 37°C) (7,8). These studies reported different results depending on the temperature treatment and the *Leishmania* species, some of the results obtained were an increase in the parasites division, morphological changes (the parasites acquired a round shape), loss of motility (8), changes in parasites differentiation rate and survival rate (7). However, there have been few studies aimed at understanding the biology of *Leishmania* when subjected to an increase of temperature and as far as we know, none of them were subjected to a decrease of this variable. The majority of studies have focused on the impact of temperature shifts on the distribution of the vectors and the association of this with the number of leishmaniasis cases (9–12). Other studies have evaluated this variable on promastigotes of *L. infantum*, *L. major*, *L. tropica*, *L. enrietti* and *L. donovani* in terms of the thermal shock associated with a change of host, in which the specific production of heat shock proteins (HSPs) occurs with an increase of temperature (13–15). Nonetheless, to the best of our knowledge, no study has particularly focused on the effect of temperature on the *Leishmania* genome.

Even though, other studies on *Leishmania* shed light on how the parasite biology changes in response to other stress situations which could be similar under temperature stress. Rastrojo *et al.* found that somy changes, amplifications and deletions are the mechanisms for which the parasite acquire the resistance to antileishmanial drugs (16). Dumetz *et al.*, determined the impact of host change in *L. donovani* where the aneuploidy was higher in promastigotes *in vitro* than in amastigotes *in vivo*. Other important finding of this study is that some chromosomal copy number

variation had a correlation with its transcriptomic profile but also there were a transcriptome response independent to the aneuploidy (17). Bussotti *et al.*, revealed in *L. major*, *L. tropica*, and *L. infantum* two ways of environment adaptation at short term (culture passaging), changes in aneuploidy and telomeric amplification (18); which could play important roles in the adaptation since the chromosomal amplification might be under selection (18,19). At long term adaptation there was a strain specific copy number variation of single genes that may alleviate the necessity of chromosome duplication (18). There are also other studies in *Leishmania* that show the importance of genome and transcriptome changes not only in the response to drugs like trivalent antimony, amphotericin, miltesosine and paramomycin (16,20–23) but also in *Leishmania* variation between strains and species where there are more differences in gene copy number and aneuploidy instead of Single-Nucleotide Polymorphisms (SNP) (24–26); which is why the genotypic plasticity of *Leishmania* plays an important role in the adaptation to some stressful conditions. Although, these kinds of comparisons have not been taken deeply in *L. braziliensis*.

Therefore, we used next generation sequencing (DNA-Seq) to determine the possible genomic impacts that could have *L. braziliensis* promastigotes to shifts of the temperature, taking into account that the prediction of the temperature change during the XXI century is approximately from 1 to 5.8°C (3,27) as a consequence of the climate change. Our study shows for the first time the biological response at genomic level of *L. braziliensis* promastigotes cultivated *in vitro* and subjected to temperature shifts, being the first approach not only for this species but also for the genus, in addition, we report the effect when submitted to a lower temperature, a perspective which have not been studied until the moment.

Materials and methods

Culture conditions and growth curves

Promastigotes of *Leishmania braziliensis* from the strain MHOM/BR75/M2904 were cultivated in the growth medium Roswell Park Memorial Institute (RPMI) (Sigma-Aldrich) supplemented with 10% heat-inactivated foetal bovine serum (Invitrogen). The parasites were incubated at four different temperatures, which were chosen in consideration of the predicted temperature increase during the current century (1–5.8°C); thus, the temperatures assessed were 24, 26, 28 and 30°C. Here, 26°C was used as a control temperature because it is a standard temperature for the *in vitro* culture. We prepared cultures with three replicates per temperature with an initial concentration of 1×10^6 parasites/mL in each. The parasite density for all culture replicates at each temperature was quantified using a Neubauer chamber for 7 consecutive days. This quantification was used to create growth curves for each temperature. From these curves, we determined the beginning of the logarithmic phase (BLP) by defining the day on which a significant difference emerged compared with the initial concentration. Statistical analyses were performed using the program GraphPad Prism, where we analysed normality using the Kolmogorov–Smirnov test and subsequently used a Dunnett test of multiple comparisons to determine the day on which the logarithmic phase started. To determine if there was a difference between the evaluated temperatures, we conducted a two-way ANOVA test. The p values < 0.05 were considered statistically significant.

Isolation of DNA

DNA extraction was performed on the day when the logarithmic phase began, as defined by the parasite growth curves at each temperature. The DNA was extracted from one sample per treatment and was isolated using the Ultraclean Tissue and Cell DNA Isolation kit, following the manufacturer's standard protocol. The concentration and quality of the DNA samples was

quantified using NanoDrop 2000 (Thermo Scientific™), and the integrity was assessed by electrophoresis in a 1% agarose gel. All samples had A_{260}/A_{280} ratios higher than 2.0.

Genome sequencing

The DNA was obtained from cultures of the strain of *Leishmania braziliensis* known as MHOM/BR75/M2904. Once we extracted the DNA, it was sent to be sequenced by Illumina HiSeq X-TEN. The libraries prepared were Microbial Mate-Paired for DNA with insert size of 350 bp. The reads had a size of 2 × 150 bp. The sequencing was performed by Novogene Bioinformatics Technology Co., Ltd, Beijing, China. The software FastQC was conducted to determine the reliability of the sequencing.

DNA read mapping

The reads were mapped to a reference genome, *L. braziliensis* MHOM BR75 M2904, through the software SMALT v0.7.4 (www.sanger.ac.uk/resources/software/smalt/), and using an exhaustive searching option -x and -y 0.8, a reference hash index of 13 bases and a sliding step of 3. We also mapped the reads with an identity threshold y = 0.8 to prevent mapping of non-*Leishmania* reads to the reference. Finally, read file merging, sorting and elimination of PCR duplicates were implemented with the software Samtools v0.1.18 and Picard v1.85.

Genomic data analysis

Evaluation of chromosome and gene copy number variation (CNV)

To obtain the read depth per chromosome, the data from sequencing were normalized by the mean depth for the 35 chromosomes of *L. braziliensis*. The range of somy was determined from the

normalized read depths of the chromosomes. The range of somy (S) is defined as follows: $S < 1.5$ (monosomic), $1.5 \leq S < 2.5$ (disomic), $2.5 \leq S < 3.5$ (trisomic), $3.5 \leq S < 4.5$ (tetrasomic) and $4.5 \leq S < 5.5$ (pentasomic). The heatmaps were created by the R package Heatmap3.

To evaluate gene CNVs, the mean read haploidy per gene was determined, considering or not the impact of somy. The genes were filtered using the thresholds of fold change of Z score > 2 and *p* value < 0.05 compared with the control temperature of 26°C. Then, the filtered genes were analysed according to the shared and unique genes identified at each temperature. The data classified into the genomic location and the gene ontology (GO) terms were obtained through the free database Tritrypdb (http://tritrypdb.org/tritrypdb/). To obtain the GO ontology of the genes evaluated we used the option biological process with a cut off *p* value of 0.05. Statistical analysis was performed using the software GraphPad Prism. We performed tests of normality using the Kolmogorov–Smirnov and Shapiro–Wilks tests and then the Kruskal–Wallis test as a nonparametric test for independent samples.

Single-nucleotide polymorphism (SNP) analysis

To conduct single-nucleotide analysis, the SNPs, small insertions and deletions were called by the software Toolkit v3.4 GATK (https://software.broadinstitute.org/gatk/). Low-quality SNPs were filtered using GATK Variant Filtration and SAMtools was used to avoid false positives. The software SnpEff v4.1 was used to classify the indels and SNPs according to their impact, such as being synonymous or nonsynonymous and having a high or moderate impact.

Results

Growth curves of *L. braziliensis* promastigotes incubated *in vitro* under different temperatures

We determined the growth curves and the BLP for each temperature treatment through a daily count of the parasites, which were maintained under the same conditions. At the control temperature, promastigotes reached the BLP on the sixth day, and we did not see a decrease of the cell density in the control cultures (26°C) during the days assessed (Figure 1). This latter behaviour was also seen in promastigotes incubated at 24°C, which did not exhibit a decrease of cell density during the experiment, but under this temperature the parasites changed the day on which the BLP was reached (third day of the experiment). We also observed that the number of parasites after the third day increased only slightly and slowly during the rest of the experiment (Figure 1). In contrast, the growth curves of the promastigotes at temperatures of 28 and 30°C were similar; in both cases, the BLP was on the second day, followed by a decrease of the cell density on the last days of the experiment (Figure 1). Despite this similarity in pattern between these two highest temperatures, the magnitude of the cell density differed between them, with fewer parasites at 30°C (Figure 1). Notably, the growth curves were significantly different among each temperature (p value < 0.05).

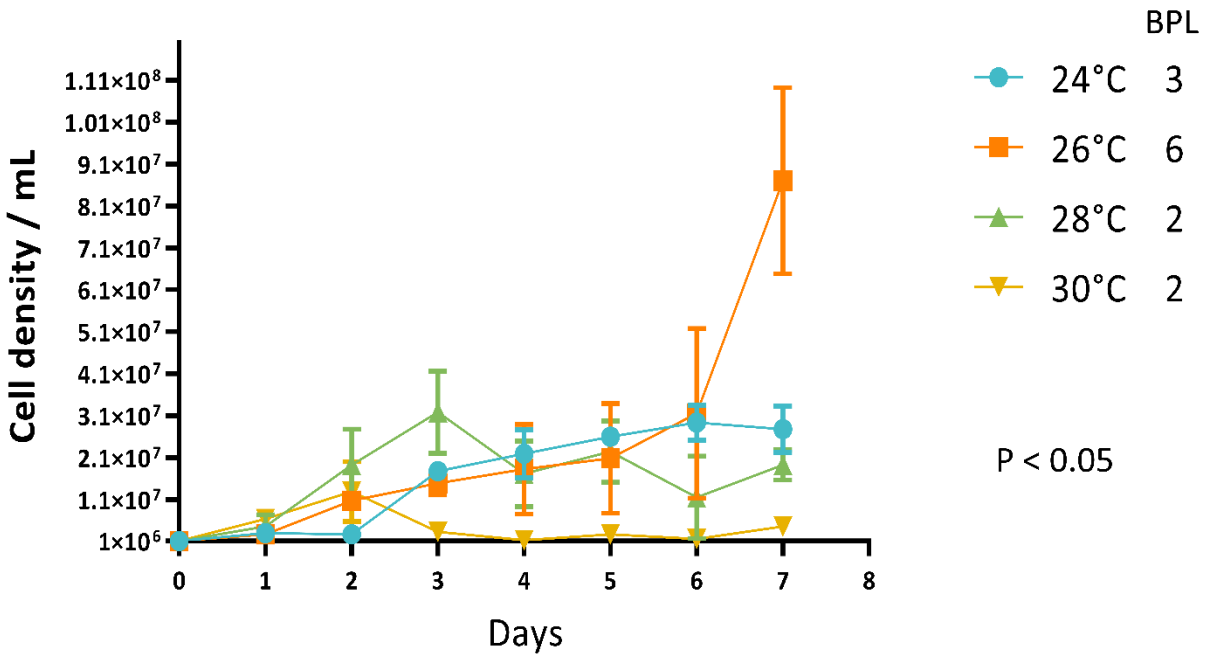

Figure 1. Effect of temperature on the growth curve of *L. braziliensis* promastigotes. Parasites were cultured at four different temperatures: 26°C (orange), as the control temperature; 24°C (light blue); 28°C (light green); and 30°C (yellow). The data of beginning of the logarithmic phase (BLP) and the *p* value for the comparison between the growth curves is mention. The parasite growth was monitored daily for 7 days through a Neubauer chamber. Bars represent standard errors obtained from three independent experiments. The cultures had an initial concentration of 1×10^6 parasites/mL. The days of quantification are represented on the x-axis and the concentration of parasites per millimeter is express on the y-axis.

Evaluation of copy number variation at the chromosomal level

The comparison of chromosomal somy of the samples at different temperatures (24, 26, 28 and 30°C) is illustrated in a heatmap in Figure 2. DNA somy showed no significant difference among the four temperatures evaluated (*p* value = 0.975). This was confirmed by the comparison of somy between the control temperature (26°C) and the three other temperatures studied, for which the *p*

value was not significant (p value > 0.05) for any temperature. Thus, there was no change in the aneuploidy of promastigotes of *L. braziliensis* due to exposure to the temperatures for a short term. In summary, three chromosomes 3, 16 and 24 were found to be disomic; chromosomes 4, 6 and 27 were tetrasomic; and chromosome 31 was pentasomic. The remaining 28 chromosomes were trisomic, as expected (28).

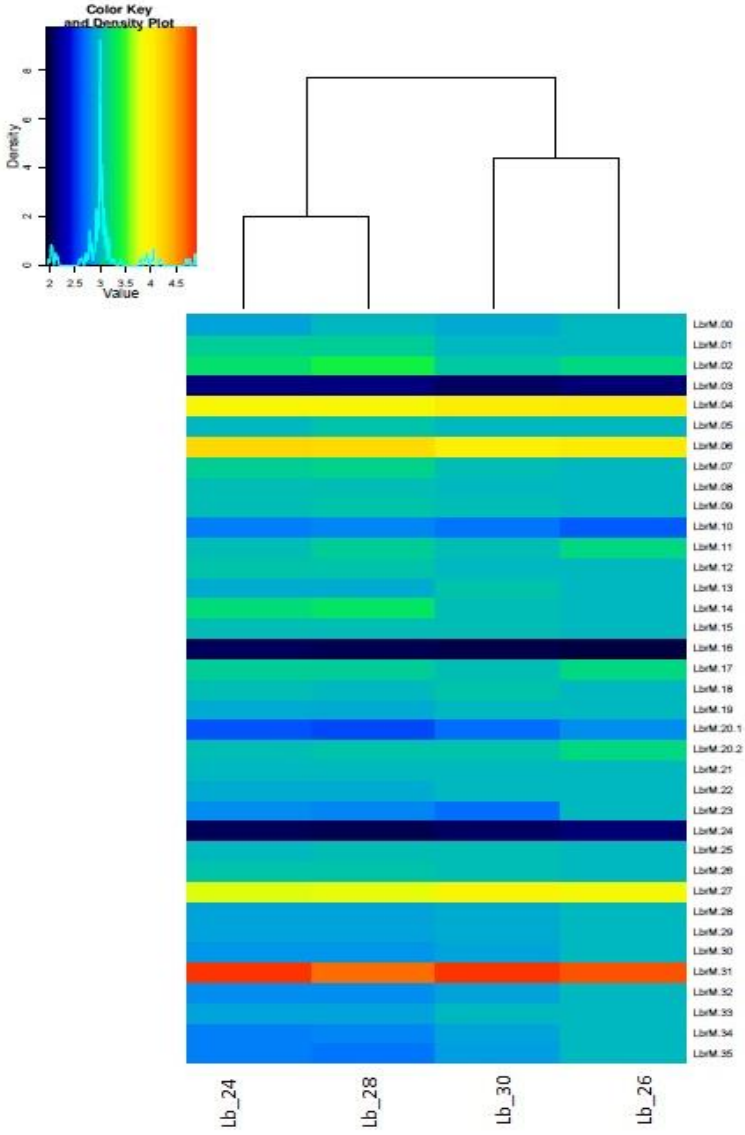

Figure 2. DNA-based somy values. Heatmap show the aneuploidy of each of the 35 chromosomes calculated through the results obtained by high-throughput DNA sequencing. The colour key indicates the somy value (S), which ranges from 1 to 5, where monosomy is when $S < 1.5$, disomy $1.5 \leq S < 2.5$, trisomy $2.5 \leq S < 3.5$, tetrasomy $3.5 \leq S < 4.5$ and pentasomy $4.5 \leq S < 5$, as previously described. The heatmap include the three assessed temperatures along with the control temperature. In addition, the letters "Lb" (abbreviation for *L. braziliensis*) and the last number (the temperature assessed) are the way in which the results are label; for instance, Lb_30 is the sample result at the temperature of 30°C.

Evaluation of the copy number variation (CNV) at the gene level

Gene CNVs was evaluated from the sequence of DNA extracted from *L. braziliensis* parasites under four different temperatures. A total of 8507 genes were assessed. At the temperatures of 24, 28 and 30°C, 253 (2.97%), 247 (2.9%) and 260 genes (3.05%), respectively, had CNVs compared with the status at the control temperature of 26°C ($-2 >$ Z score > 2, equivalent to a p value <0.05). A mean of 61.33% ± 1.5% of these genes at each temperature had an increase in copy number compared with that at 26°C; from the genes with such an increase, a mean of 41.84% ± 1.7% for each temperature encoded hypothetical proteins. The remaining 38.67% ± 1.5% of genes showed a decrease in gene copy number compared with the control. For these genes, a mean of 32.6% ± 3.6% for each temperature encoded hypothetical proteins.

Figure 3 shows the distribution of genes with a fold change of 2 in CNVs by temperature compared with the level in the control (26°C). For all temperatures, the chromosomes with more than ten genes with copy variation were chromosomes 14, 20.1, 27, 31 and 35 (Figure 4A), where chromosome 31 had the highest number of genes with copy number variation in the three temperatures. In terms of the percentage of the total genes showing copy number variation per

chromosome, the one with the highest rate was chromosome 2. In this chromosome, all sequenced genes with a change in read depth increased at 24°C compared with the level in the control.


01
(4 genes) 231,164 bp
(6 genes)
(5 genes)
02
(7 genes) 290,514 bp
(8 genes)
(5 genes)
03
(4 genes) 362,421 bp
(1 gene)
(0 genes)
04
(6 genes) 444,124 bp
(5 genes)
(/ genes)
05
(5 genes) 451,537 bp
(4 genes)
(7 genes)
06
(3 genes) 512,965bp
(10 genes)
(8 genes)
07
(4 genes) 563,954 bp
(3 genes)
(3 genes)
08
(2 genes) 386,780 bp
(4 genes)
(0 genes)
09
(9 genes) 556,679 bp
(5 genes)
(6 genes)
10
(2 genes) 564,221 bp
(6 genes)
(6 genes)
11
(2 genes) 558,541 bp
(1 gen)
(2 genes)
12
(0 genes) 456,321 bp
(1 gen)
(1 gen)
13
(3 genes) 616,006 bp
(2 genes)
(4 genes)
14
(5 genes) 648,278 bp
(11 genes)
(10 genes)
15
(4 genes) 606,906 bp
(4 genes)
(3 genes)
16
(5 genes) 688,705 bp
(2 genes)
(4 genes)
17
(3 genes) 694, 698 bp
(4 genes)
(5 genes)
20.1
(17 genes) 1,662,735 bp
(17 genes)
(15 genes)
20.2
(3 genes) 724,111 bp
(2 genes)
(3 genes)
21
(3 genes) 687,743 bp
(5 genes)
(3 genes)
22
(5 genes) 641,930 bp
(5 genes)
(6 genes)
23
(11 genes) 799,329 bp
(8 genes)
(0 genes)
24
(3 genes) 829,282 bp
(2 genes)
(1 gen)
25
(5 genes) 712,314 bp
(7 genes)
(9 genes)
26
(9 genes) 992,961 bp
(6 genes)
(7 genes)
27
(12 genes) 1,157,752 bp
(12 genes)
(13 genes)
28
(7 genes) 1,159,459 bp
(6 genes)
(9 genes)
29
(6 genes) 1,175,128 bp
(4 genes)
(3 genes)
30
(10 genes) 1,327,198 bp
(9 genes)
(8 genes)
31
(27 genes) 1,550,184 bp
(14 genes)
(19 genes)
32
(3 genes) 1,573,228 bp
(4 genes)
(5 genes)
33
(9 genes) 1,504,364 bp
(8 genes)
(14 genes)
34
(11 genes) 2,012,582 bp
(9 genes)
(8 genes)
35
(19 genes) 2,686,643 bp
(11 genes)
(15 genes)
Gene Copy Variation
Increase
Decrease
Temperatures
30 °C
28 °C
24 °C

Figure 3. Genomic distribution of multicopy genes (genes with copy number variation) across the chromosomes of *L. braziliensis* under different temperatures. Multicopy genes are colored by increase (blue) or decrease (green) compared to the control temperature of 26°C. The genomic results for 30°C, 28°C and 24°C are represented in chromosomes with colors black, brown and gray, respectively.

Among the DNA reads for parasites at a temperature of 24°C, there were no genes with significative change in the copy number on the chromosomes 3, 8 and 23, whereas in the other temperatures there was at least one gene with CNV; for example, the chromosome 23 had more than 7 genes with copy number variation in 28 and 30°C and most of them increased their number compared with the control. Also in 24°C there were chromosomes with only genes with increase in copy number compared with the control. These chromosomes were 2, 7 and 14. The last two chromosomes had the same pattern in the temperature of 28°C as well as the chromosomes 13, 15, 16 and 17. In this temperature and in 30 °C there was a unique chromosome with only genes with decrease copy number compared with the control, it is the chromosome 18 with 5 and 6 genes respectively for each temperature (Figure 3). The chromosomes with only increase genes in copy number for 30°C, were 3, 8, 15, 22 and 32. The read depth of genes for parasites at 30°C had not significant change in the chromosome 12. The other chromosomes with only one gene with copy variation included 24, with LbrM.24.1600 (which encode an amastin-like surface protein-like) showing an increase in copy number at 24°C; and chromosomes 3 and 11 at a temperature of 28°C, with the gene LbrM.03.ncRNA3 (which encode an unspecified product) showing an increase and LbrM.11.0910 (which encode a 60S ribosomal protein L28, putative) showing a decrease compared with the DNA sequencing result from parasites at 26°C (Tables S1–3).

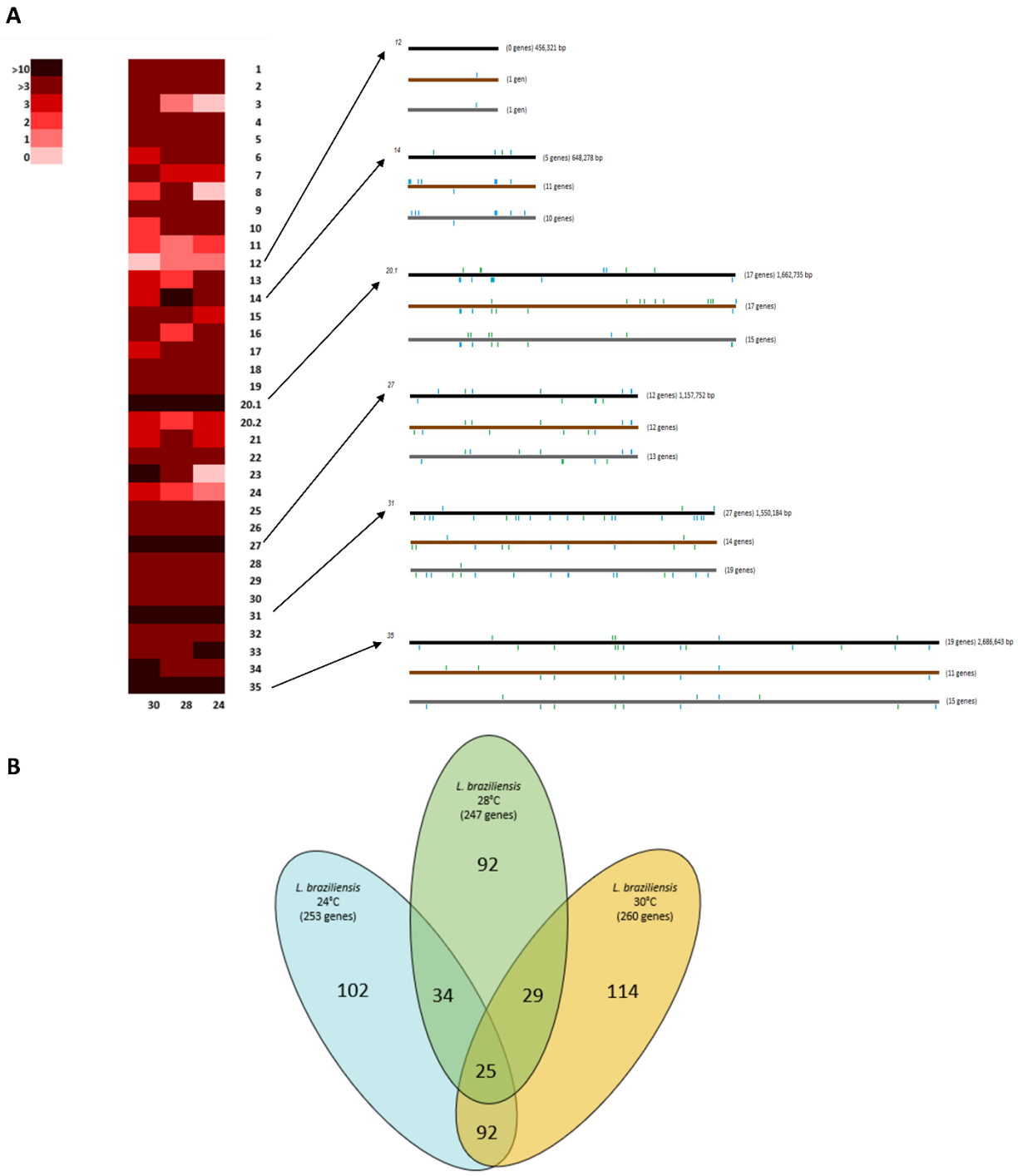

Figure 4. (A) Heatmap representing the number of genes per chromosome with copy number variation (CNVs) compared with the control. It shows the genomic distribution of CNVs for the chromosomes with more than ten genes with copy variation, and the chromosome 12 as the one

with the less genes. Multicopy genes are colored by increase (blue) or decrease (green) compared to the control temperature of 26°C. The genomic results for 30, 28 and 24°C are represented in chromosomes with colors black, brown and gray, respectively. (B) Number of genes with copy number variation compared with the control. Venn diagram that shows overlapping genes of *L. braziliensis* under different temperatures 24°C (light blue), 28°C (light green) and 30°C(yellow) with a fold change of Z score >2 compared with the copy number of control temperature (26°C). The numbers in brackets are the total amount of genes with copy number variation in each temperature.

Among the three non-control temperatures, there were 92 genes sharing gene copy number variation compared with the level at 26°C (Figure 4B). All of these 92 genes increased or decreased in copy number in the same way at all temperatures; there was also no significant difference in the read depth of the shared genes (*p* value = 0.971 for genes with increased read depth and *p* value = 0.982 for genes with decreased read depth). Among the shared genes (Figure 5A) that increased in copy number compared with the control, these included genes that encode an elongation factor 1-alpha and a surface antigen protein. The genes with decreased copy number (Figure 5B) included one that encode an amastin-like surface protein and another that encode NADH-dependent fumarate reductase. Moreover, genes that encode to amastin like proteins, and to beta and alpha tubulins increase or decrease in copy number. The genes for which findings were shared only between two temperatures are presented in Figure 6. At 28 and 30°C, there were more genes with a decrease in copy number compared with the level in the control (Fig. 6B). Those identified included genes that encode an ubiquitin-conjugating enzyme E2, tRNAs and an amastin surface protein. At 24 and 28°C, there were more genes with an increase in copy number (Fig. 6C), such

as the ones that encode for a peptidase M20/M25/M40 and a receptor-type adenylate cyclase. Finally, between 24 and 30°C, fewer genes were shared, genes that encode a HSP 83-1 and a HSP 70 for a decrease in copy number (Fig. 6F). The unique genes numbered 114 genes for 30°C, 102 genes for 24°C and 92 genes for 28°C (Fig. 4B). For these genes, we obtained the associated ontology terms through the database tritrypdb.org, choosing the option biological process to determine the genes' functions.

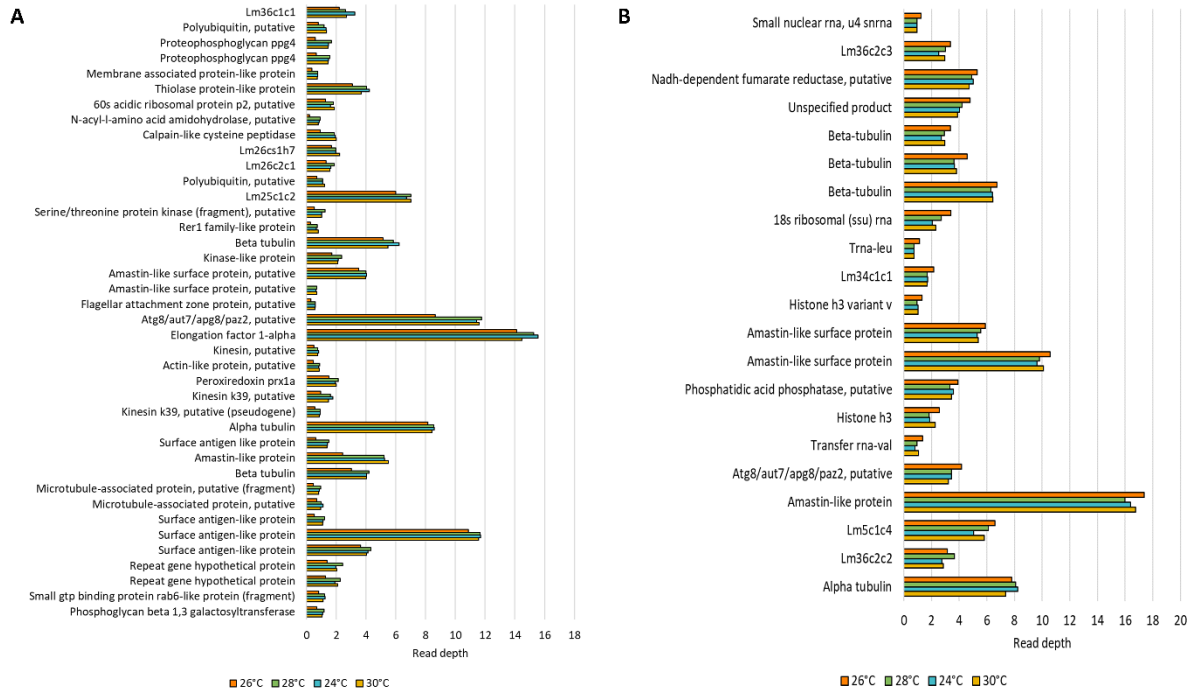

Figure 5. Read depth of genes compared with control temperature. These genes are shared by the three temperatures evaluated. The genes of increase read depth compared with the control by temperature (A) and the genes of decrease (B).

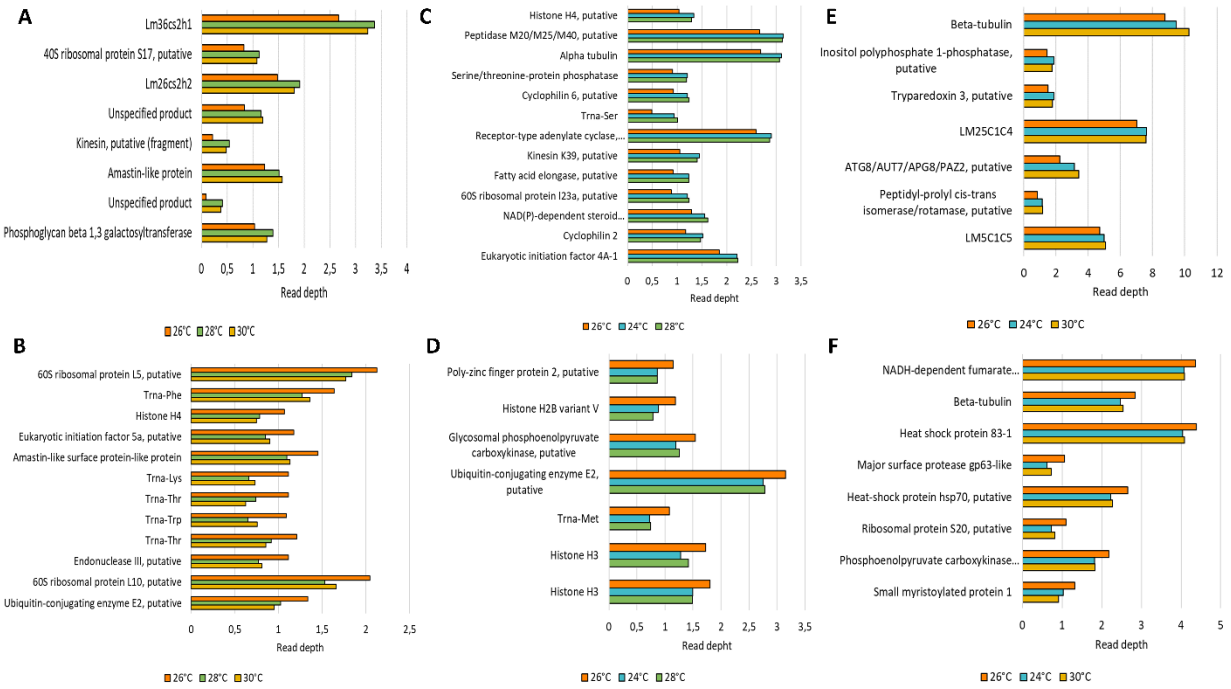

Figure 6. Read depth of genes compared with control temperature (26°C). These genes are the ones shared by only two temperatures. The unique genes shared by 28°C and 30°C with increase copy number (A) and decrease copy number (B). The genes shared by 24°C and 28°C with increase (C) and decrease (D) copy number and the genes shared by only 24°C and 30°C with increase (E) and decrease (F) copy number.

Figure 7 shows the ontology terms associated with large numbers of genes. Some of the ontology terms of genes with an increased copy number for unique genes at each temperature were shared. For example, findings for 24 and 28°C showed the sharing of terms associated with biological process and host modification of morphology and physiology by symbiotic interaction. In contrast, findings for 30 and 28°C shared ontology terms such as biological regulation, regulation of biological process, cellular protein localization and intracellular protein transport. Only the term biological adhesion was shared among the three temperatures and no other term was shared only between 30 and 24°C. Numerous unique terms were associated with temperatures. The ontology

terms unique to 28°C were phosphorylation, regulation of cellular process, organonitrogen compound biosynthetic process and cellular nitrogen compound metabolic process. For 30°C, there were unique terms associated with cellular, macromolecule and protein localization and peptide, amide and intracellular protein transport (Figure 7A). Otherwise, the ontology of genes associated with a decrease gene in copy number compared with the control differed from the ontology terms associated with increase copy number. Some ontology terms for the genes with decrease copy number shared among the three temperatures were associated with amide and peptide metabolic and biosynthetic process and organonitrogen compound biosynthetic process. The ontology terms shared only by 30 and 24°C were about cellular and organic substance metabolic processes. The term shared only by 30 and 28°C was cellular amino acid metabolic process and there were no terms shared only by 28 and 24°C. The unique terms from 24°C were related to metabolic, cellular and biosynthetic processes. For 28°C, the unique ontology terms were protein folding, post-transcriptional regulation of gene expression and cellular protein metabolic process. Finally, for 30°C, the only unique term was L-serine metabolic process (Figure 7B).

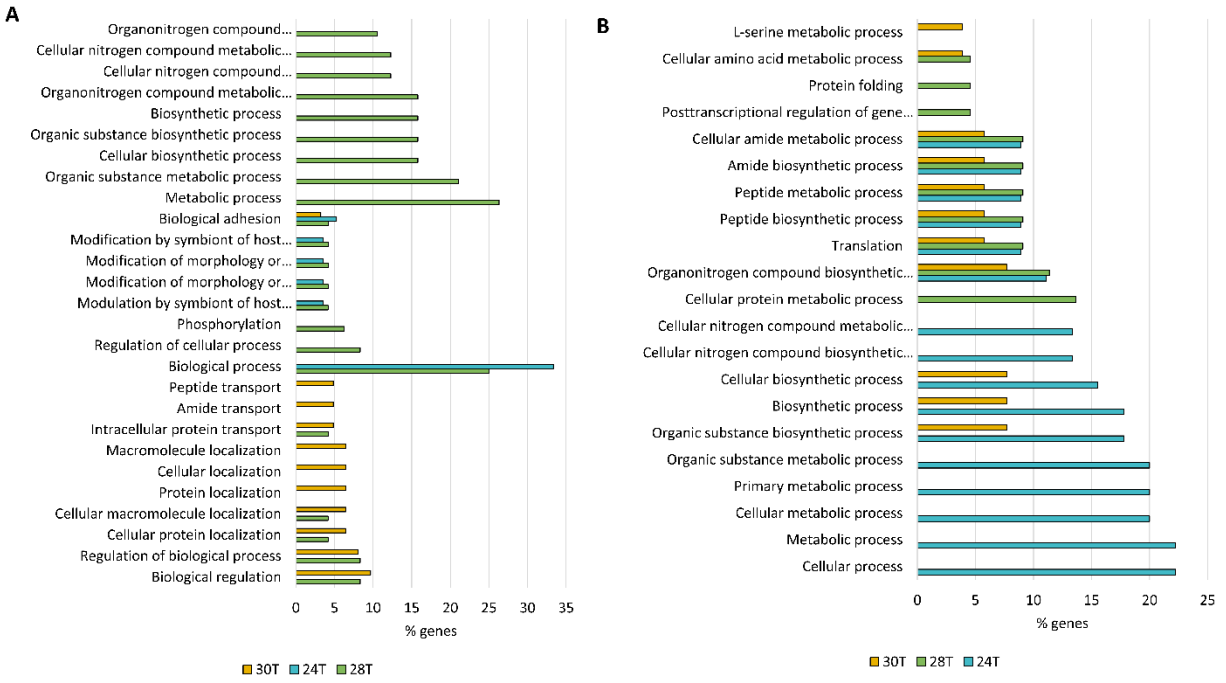

Figure 7. Percentage of genes per ontology term in each temperature. Unique genes with fold change of 2 in copy number compared with 26°C for each temperature 24°C (102 genes), 28°C (92 genes) and 30°C (114 genes) were evaluated to obtain the ontology terms. Only the first ten terms were used for each temperature. (A) The ontology of increase read depth compared with the control (B) the ontology of decrease read depth compared with 26°C. The x-axis corresponds to the percentage of genes classified on the ontology term (percentage was calculated with the total of genes in the category of temperature and increase or decrease read depth).

Next, we illustrated the genes associated with the ontologies described above. Figure 8 shows the genes with an increase in copy number compared with that at the control temperature. At 24°C, the gene with the highest read depth encoded for a HSP 70, putative. This was followed by iron/zinc transporter protein-like (Fig. 8A). For 28°C, one gene with an increase in read depth was one that encode for GP63 leishmanolysin (Fig. 8C) and a gene with a decrease in read depth was heat shock 70-related protein 1 (Fig. 8D). For 30°C, the notable genes with an increase in copy number were

heat shock protein 83-1, beta-tubulin and metallopeptidase (Fig. 8E). In contrast, some of the genes with a decrease in copy number encoded for a serine-threonine dehydratase and a translation elongation factor 1-beta (Fig. 8F).

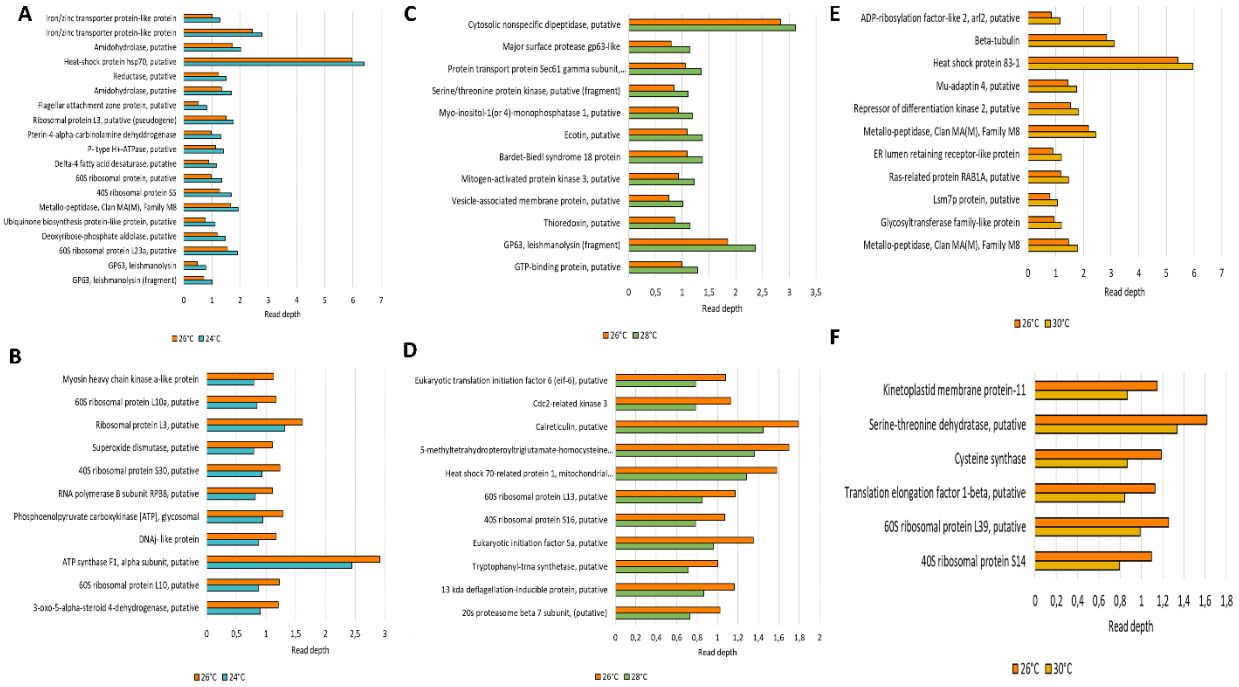

Figure 8. Read depth of genes compared with that at the control temperature. These genes have ontology classifications illustrated in Figure 7. The genes for 24°C with increased (A) and decrease read depth (B). The genes for 28°C with increase (C) and decrease (D) copy number and the unique genes for 30°C with increase (E) and decrease (F) in copy number compared with the control.

Finally, we evaluated a total of 38,703 SNPs, among which 44 (0.11%) SNPs have a high impact and 7611 SNPs (17.33%) have a moderate impact on DNA. Among the three temperatures evaluated, there were no significant differences in the SNPs (p value = 0.947). We also evaluated a total of 18,755 indels, where 245 (1.30%) have a moderate impact and 387 (2.06%) have a high

impact on DNA. The indels did not differ significantly among the three temperatures (*p* value = 0.939).

Discussion

The aim of this study was to evaluate the changes at the genomic level in *L. braziliensis* promastigotes under different incubation temperatures to determine the cellular and biological changes in possible future climate change scenarios. In first place we found that temperature affects the growth curves of *L. braziliensis* with 30°C having the highest negative effect in parasite concentration. This could be explained by the general response of promastigotes to higher temperatures as a consequence to host change, when it has been found that the *in vitro* promastigotes lost their motility, become rounded and express heat shock proteins (13,15). We illustrated for the first time the response of the growth curves at a lower temperature than 26°C and we found a continuing growth in parasite concentration but with a lower parasite density. Also, we observed that the BLP at 24°C was comparable to 28 and 30°C (Figure 1), this could be associated with the effect of low temperatures in microorganism and cells where there are physiology changes like decrease of membrane fluidity, decrease efficiency in transport proteins and a growth with slower rate (29,30).

We also evaluated changes at the somy level and we did not find variation in the form of aneuploidy in any of the 35 chromosomes. In other studies, it has been seen that the change in aneuploidy is an average response to environmental change, and drug resistance (16–18,20–23,31), although we did not find changes in aneuploidy, which means that the variation on chromosome copy number is not an alternative for short term adaptation to temperature in *L. braziliensis*. Even though we

report a different somy value for the strain MHOM/BR75/M2904 where three chromosomes (3 ,16 and 24) were disomic; chromosomes 4, 6 and 27 were tetrasomic, chromosome 31 was pentasomic and 28 of 35 chromosomes were trisomic for the four temperatures (Figure 2) compared to the data reported by Rogers *et al.* (28), where there were two different tetrasomic chromosomes (5 and 29); the chromosome 31 was hexasomic, the chromosome 14 had and ambiguous profile been more like a tetrasomic chromosome and the rest were trisomic. It could be a consequence of the variable aneuploidy characteristic of *Leishmania* that is not just at strain level but also a clone and single cell level (32–34).

Similarly, there is not a big effect on gene CNVs, around 3% of the genes evaluated had significant copy number difference compared to the control temperature and most of them were found in genes encode to hypothetical proteins. In the same way this means that gene CNVs might not be relevant for short term adaptation to temperature. However, there were some patterns regarding the chromosomes with change in gene copy number. There were chromosomes with high number of these genes compared to the control in which the chromosome 31 stands out, as having the highest number of CNVs (Figure 3). This chromosome is known to be supernumerary in a lot of *Leishmania* species such as *L. major* and *L. peruviana* (24) and in all sequenced *Leishmania* has been reported more than two copy number in all species and strains (32). Hence this chromosome might be important for *Leishmania* adaptation. When we analyzed the ontology of the genes related to this chromosome, we found that most of them were associated to iron metabolism, which have been previously associated to the iron uptake under oxidative stressed environment (35). This can explain the increase in copy number especially in 30°C considering that this was the temperature more stressful for *L. braziliensis* according to the growth curves constructed in this study. It is important to consider that there were also chromosomes of low importance in gene copy number

like the chromosome 12 (Figure 4)and there were different distributions of genes between the three temperatures as described in results.

On the contrary, genes shared with CNVs compared to the control have the same behavior in the three temperatures, so, the genes increase or decrease in copy number in the same way for the three temperatures. The ones that are increased in gene copy number compared with the control encode for elongation factor 1 alpha (Figure 5), which function is involved in cellular translation, degradation or cytoskeletal movements (36), and a surface antigen protein which are essential for parasite survival and has been seen to be overexpressed in metacyclic promastigotes (35). This means that the shared genes with increase copy number encode proteins that are essential for parasite survival. Genes with decrease copy number for the three temperatures were amastin proteins which function is to mediate the interaction between host and parasite that allows infection and survival, and this decrease behavior has also been seen in *L. peruviana* which lacks of the mucocutaneous clinical manifestation (17,24). Therefore, it could be considered that the change in temperature may affect the capacity of infection of *L. braziliensis.* For the extreme temperatures 24°C and 30°C the genes shared with decrease copy number are heat shock proteins HSP70/83 (Figure 6).

For the genes unique at each temperature with CNVs compared to the control, there were characteristic ontology terms and genes associated. For 24°C the ontology of the unique genes with decrease copy number where associated with metabolic, cellular and biosynthetic process (Figure 7), which is also related to the general response of cells and microorganism to low temperatures where there is a reduction in the metabolism (29,30). For this temperature there is a high read depth in the copy number of genes that encode heat-shock protein hsp70, putative and transporter like protein (Figure 8). The first one is knowing to be a chaperone protein that is expressed for

environment adaptation (13,15), and the second one is important for the uptake of essential nutrients from the environment of growth, some of them such as FT1 transporters are implicated in drug resistance (38) so the increase of copy number in these kind of genes could be intend for survival in this extreme temperature.

For 28°C the ontology terms for increase gene copy number were associated to nitrogen compound metabolic process (Fig.7), Among these genes there was one that encode for GP63, which is a virulence factor in *Leishmania* promastigotes because is associated with the protection of the parasite to host immune responses by the production of this molecule into the cytoplasm of the macrophage promoting infection (24,39). For genes with decrease copy number the ontology terms are about posttranscriptional regulation and protein metabolic process. Also there was a decrease in the copy number of a gene that encode heat shock 70 opposite (Fig.8) to the response at other higher temperatures found in *Leishmania* promastigotes (13,15). At 30°C, the ontology term for decrease gene copy number was L-serine metabolic process (Fig.7) that is important for cell proliferation and basic necessities such as the synthesis of deoxythymidine monophosphate (40). In the same way 30°C had a decrease copy number in genes that encode for serine-threonine dehydratase (Fig.8) this kind of proteins are also important for the course of infection in the macrophages (41). Therefore, at this temperature, the genes mentioned above and the increase of copy number in the genes that encode heat shock protein 83-1 might play an important role in the response to higher temperatures.

In conclusion, we found specific responses conducted by CNVs in each temperature evaluated, even though, it is just around the 3% of the genes evaluated. There were not differences in the aneuploidy by temperature and we did not find significant differences between the SNPs and Indels

of the three temperatures. Therefore, there is not a relevant effect of temperature in the genome of *Leishmania braziliensis*.

This study provides evidence that *L. braziliensis* promastigotes exhibit a short-term response to heat stress, generating copy number of a range of genes associated with processes such as infection, virulence, survival, growth, cell proliferation and host–parasite interaction. Even though, we cannot draw definitive conclusions on whether the temperature shifts will have an impact on *L. braziliensis*. We found that temperature has a negative effect on the growth curves of *L. braziliensis*, at least for a short term. These findings confirm the important role played by this abiotic factor in the biological processes of the parasite and encourage the planning of more related studies exploring whether temperature has a long-term impact on genome aneuploidy, gene CNVs and transcriptome profiles. This is important because it is possible that, in a long-term study maintaining the parasites under this stress for several generations, adaptation would be generated through CNVs, as has been reported in drug resistance in *Leishmania*.

Supporting Information

Table S1. Genes resulted by the DNA sequence of *L. braziliensis* under 24°C with a significative increase or decrease in copy number variation compared with the control temperature of 26°C.

Table S2. Genes resulted by the DNA sequence of *L. braziliensis* under 28°C with a significative increase or decrease in copy number variation compared with the control temperature of 26°C.

Table S3. Genes resulted by the DNA sequence of *L. braziliensis* under 30°C with a significative increase or decrease in copy number variation compared with the control temperature of 26°C.

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Table S1. Genes resulted by the DNA sequence of *Leishmania braziliensis* under 24°C with a significative increase or decrease in copy number variation compared with the control temperature of 26°C.

Chromosome	# Total genes	# Genes with CNVs	Gene IDs with increase CNVs	Gene IDs with decrease CNVs
1	78	5	LbrM.01.0170 LbrM.01.0270 LbrM.01.0640 LbrM.01.0740 LbrM.01.0760	
2	68	5	LbrM.02.0070 LbrM.02.0090 LbrM.02.0140 LbrM.02.0220 LbrM.02.0290	
3	96	0		
4	125	7	LbrM.04.0210 LbrM.04.0220 LbrM.04.0230 LbrM.04.0650 LbrM.04.1080	LbrM.04.0740 LbrM.04.1020
5	129	7	LbrM.05.0380 LbrM.05.0390 LbrM.05.snoRNA3	LbrM.05.0520 LbrM.05.snRNA1 LbrM.05.snoRNA1 LbrM.05.snoRNA2
6	130	8	LbrM.06.0090 LbrM.06.0100 LbrM.06.0330 LbrM.06.0580 LbrM.06.0590 LbrM.06.1050 LbrM.06.1170 LbrM.06.1240	
7	129	3	LbrM.07.0510 LbrM.07.1060 LbrM.07.1080	
8	97	0		
9	169	6	LbrM.09.0180 LbrM.09.1070	LbrM.09.0170 LbrM.09.tRNA3

			LbrM.09.1220 LbrM.09.1490	
10	156	6	LbrM.10.0490 LbrM.10.1260	LbrM.10.1070 LbrM.10.1071 LbrM.10.1080 LbrM.10.1090
11	123	2	LbrM.11.0760	LbrM.11.0220
12	94	1	LbrM.12.0710	
13	158	4	LbrM.13.0190 LbrM.13.0200 LbrM.13.1330	LbrM.13.1070
14	169	10	LbrM.14.0060 LbrM.14.0070 LbrM.14.0150 LbrM.14.0210 LbrM.14.0660 LbrM.14.1110 LbrM.14.1120 LbrM.14.1300 LbrM.14.1310 LbrM.14.1510	
15	164	3	LbrM.15.1080 LbrM.15.1280	LbrM.15.tRNA2
16	174	4	LbrM.16.0030 LbrM.16.1510	LbrM.16.0470 LbrM.16.0790
17	166	5	LbrM.17.0090 LbrM.17.0110 LbrM.17.1440 LbrM.17.1570	LbrM.17.tRNA2
18	171	8	LbrM.18.0850 LbrM.18.1270 LbrM.18.1550 LbrM.18.1620	LbrM.18.0450 LbrM.18.0460 LbrM.18.0470 LbrM.18.1720
19	192	4	LbrM.19.0100 LbrM.19.1180 LbrM.19.1730	LbrM.19.0950
20.1	451	15	LbrM.20.0620 LbrM.20.0780 LbrM.20.2480 LbrM.20.4280 LbrM.20.4290	LbrM.20.0720 LbrM.20.0760 LbrM.20.1.snoRNA2 LbrM.20.1.tRNA3 LbrM.20.1.tRNA4 LbrM.20.1.tRNA5 LbrM.20.1010 LbrM.20.1110

				LbrM.20.1460 LbrM.20.4300
20.2	181	3	LbrM.20.5550 LbrM.20.5910	LbrM.20.5530
21	212	3	LbrM.21.0620 LbrM.21.tRNA1	LbrM.21.1140
22	163	6	LbrM.22.0520 LbrM.22.1330 LbrM.22.1370 LbrM.22.1560	LbrM.22.0330 LbrM.22.0640
23	217	0		
24	248	1	LbrM.24.1600	
25	203	9	LbrM.25.0320 LbrM.25.0340 LbrM.25.0410 LbrM.25.1010 LbrM.25.1290 LbrM.25.1690 LbrM.25.1730 LbrM.25.1960	LbrM.25.0990
26	278	7	LbrM.26.0680 LbrM.26.1890 LbrM.26.snoRNA13 LbrM.26.snoRNA6	LbrM.26.snoRNA10 LbrM.26.snoRNA4 LbrM.26.snoRNA9
27	286	13	LbrM.27.0250 LbrM.27.0810 LbrM.27.2330 LbrM.27.2751 LbrM.27.2810 LbrM.27.2830	LbrM.27.0760 LbrM.27.1480 LbrM.27.1730 LbrM.27.1920 LbrM.27.1940 LbrM.27.1950 LbrM.27.2530
28	328	9	LbrM.28.1020 LbrM.28.2100 LbrM.28.2990 LbrM.28.3240	LbrM.28.0220 LbrM.28.0850 LbrM.28.1080 LbrM.28.1660 LbrM.28.2970
29	291	3	LbrM.29.2700 LbrM.29.2720	LbrM.29.1690
30	383	8	LbrM.30.0440 LbrM.30.1491 LbrM.30.3640 LbrM.30.3760	LbrM.30.0770 LbrM.30.1790 LbrM.30.2740 LbrM.30.2970

31	376	19	LbrM.31.0280 LbrM.31.0380 LbrM.31.0920 LbrM.31.1330 LbrM.31.1660 LbrM.31.1670 LbrM.31.1800 LbrM.31.1840 LbrM.31.2190 LbrM.31.2920 LbrM.31.3290 LbrM.31.3480 LbrM.31.3490	LbrM.31.0080 LbrM.31.2250 LbrM.31.2770 LbrM.31.ncRNA1 LbrM.31.snRNA1 LbrM.31.tRNA1
32	428	5	LbrM.32.2500 LbrM.32.4200	LbrM.32.0630 LbrM.32.0780 LbrM.32.3420
33	367	14	LbrM.33.0960 LbrM.33.1580 LbrM.33.2100 LbrM.33.tRNA1 LbrM.33.tRNA2	LbrM.33.0330 LbrM.33.0920 LbrM.33.0950 LbrM.33.0990 LbrM.33.1010 LbrM.33.1570 LbrM.33.1690 LbrM.33.3080 LbrM.33.ncRNA3
34	545	8	LbrM.34.0020 LbrM.34.0050 LbrM.34.0530 LbrM.34.0540	LbrM.34.1100 LbrM.34.1110 LbrM.34.3460 LbrM.34.4460
35	761	15	LbrM.35.0270 LbrM.35.3760 LbrM.35.7310 LbrM.35.snoRNA6	LbrM.35.1320 LbrM.35.1790 LbrM.35.1960 LbrM.35.4000 LbrM.35.4800 LbrM.35.6690 LbrM.35.snRNA1 LbrM.35.snoRNA1 LbrM.35.snoRNA3 LbrM.35.snoRNA4 LbrM.35.snoRNA5

Table S2. Genes resulted by the DNA sequence of *Leishmania braziliensis* under 28°C with a significative increase or decrease in copy number variation compared with the control temperature of 26°C.

Chromosome	# Total genes	# Genes with CNVs	Gene IDs with increase CNVs	Gene IDs with decrease CNVs
1	78	6	LbrM.01.0170 LbrM.01.0300 LbrM.01.0640 LbrM.01.0680 LbrM.01.0740 LbrM.01.0760	
2	68	8	LbrM.02.0140 LbrM.02.0160 LbrM.02.0220 LbrM.02.0290 LbrM.02.0650 LbrM.02.0660 LbrM.02.0680	LbrM.02.0420
3	96	1	LbrM.03.ncRNA3	
4	125	5	LbrM.04.0210 LbrM.04.0220 LbrM.04.0230	LbrM.04.0950 LbrM.04.1020
5	129	4	LbrM.05.0380 LbrM.05.0390 LbrM.05.0990	LbrM.05.snoRNA1
6	130	10	LbrM.06.0100 LbrM.06.0110 LbrM.06.0200 LbrM.06.0330 LbrM.06.0590 LbrM.06.0680 LbrM.06.0940 LbrM.06.1170 LbrM.06.ncRNA1	LbrM.06.1280
7	129	3	LbrM.07.0150 LbrM.07.0155 LbrM.07.1060	
8	97	4	LbrM.08.0030 LbrM.08.0500 LbrM.08.0630 LbrM.08.0670	
9	169	5	LbrM.09.0770 LbrM.09.1070	LbrM.09.0070 LbrM.09.0170 LbrM.09.tRNA3
10	156	6	LbrM.10.0250 LbrM.10.0620	LbrM.10.0970 LbrM.10.1070

				LbrM.10.1080 LbrM.10.1090
11	123	1		LbrM.11.0910
12	94	1	LbrM.12.0710	
13	158	2	LbrM.13.0190 LbrM.13.0200	
14	169	11	LbrM.14.0010 LbrM.14.0030 LbrM.14.0060 LbrM.14.0200 LbrM.14.0220 LbrM.14.0250 LbrM.14.0660 LbrM.14.1110 LbrM.14.1120 LbrM.14.1300 LbrM.14.1310	
15	164	4	LbrM.15.0540 LbrM.15.1080 LbrM.15.1280 LbrM.15.1421	
16	174	2	LbrM.16.1510 LbrM.16.1520	
17	166	4	LbrM.17.0090 LbrM.17.0110 LbrM.17.1440 LbrM.17.1530	
18	171	5		LbrM.18.0010 LbrM.18.0450 LbrM.18.0460 LbrM.18.0470 LbrM.18.1720
19	192	7	LbrM.19.0120 LbrM.19.1180 LbrM.19.1730	LbrM.19.0340 LbrM.19.0350 LbrM.19.0360 LbrM.19.0950
20.1	451	17	LbrM.20.0620 LbrM.20.0780 LbrM.20.4290 LbrM.20.4340	LbrM.20.1.snoRNA2 LbrM.20.1.tRNA3 LbrM.20.1.tRNA4 LbrM.20.1.tRNA8 LbrM.20.1090 LbrM.20.1100 LbrM.20.1460 LbrM.20.2870

				LbrM.20.2960 LbrM.20.3230 LbrM.20.3900 LbrM.20.3950 LbrM.20.4000
20.2	181	2	LbrM.20.5550	LbrM.20.5590
21	212	5	LbrM.21.0180 LbrM.21.0620 LbrM.21.2020 LbrM.21.tRNA1	LbrM.21.1820
22	163	5	LbrM.22.0520 LbrM.22.1330 LbrM.22.1380 LbrM.22.ncRNA2	LbrM.22.0040
23	217	8	LbrM.23.1120 LbrM.23.1620	LbrM.23.0330 LbrM.23.0670 LbrM.23.1610 LbrM.23.tRNA10 LbrM.23.tRNA6 LbrM.23.tRNA8
24	248	2	LbrM.24.1990	LbrM.24.1590
25	203	7	LbrM.25.0340 LbrM.25.0410 LbrM.25.0900 LbrM.25.1010 LbrM.25.1290	LbrM.25.0590 LbrM.25.0610
26	278	6	LbrM.26.2060 LbrM.26.snoRNA13 LbrM.26.snoRNA14 LbrM.26.snoRNA6	LbrM.26.0890 LbrM.26.2610
27	286	12	LbrM.27.0260 LbrM.27.2330 LbrM.27.2751 LbrM.27.2810 LbrM.27.2830	LbrM.27.0070 LbrM.27.0760 LbrM.27.0830 LbrM.27.1040 LbrM.27.1730 LbrM.27.1950 LbrM.27.2260
28	328	6	LbrM.28.2090 LbrM.28.2100 LbrM.28.2760 LbrM.28.3240	LbrM.28.0220 LbrM.28.2980
29	291	4	LbrM.29.1010 LbrM.29.2700 LbrM.29.2720	LbrM.29.2430

30	383	9	LbrM.30.0440 LbrM.30.1491 LbrM.30.1720 LbrM.30.3640 LbrM.30.3760	LbrM.30.0450 LbrM.30.1790 LbrM.30.2420 LbrM.30.2970
31	376	14	LbrM.31.0570 LbrM.31.0920 LbrM.31.1660 LbrM.31.1670 LbrM.31.1800 LbrM.31.1840 LbrM.31.2220	LbrM.31.0010 LbrM.31.0080 LbrM.31.1210 LbrM.31.1260 LbrM.31.2940 LbrM.31.3270 LbrM.31.tRNA4
32	428	4	LbrM.32.2500 LbrM.32.3860	LbrM.32.1660 LbrM.32.1960
33	367	8	LbrM.33.1880 LbrM.33.2100	LbrM.33.0020 LbrM.33.0920 LbrM.33.0950 LbrM.33.0990 LbrM.33.1570 LbrM.33.ncRNA3
34	545	9	LbrM.34.0050 LbrM.34.0530 LbrM.34.0540	LbrM.34.1110 LbrM.34.1790 LbrM.34.2130 LbrM.34.3430 LbrM.34.3440 LbrM.34.3470
35	761	11	LbrM.35.3760 LbrM.35.7170 LbrM.35.snoRNA2 LbrM.35.snoRNA5 LbrM.35.snoRNA6	LbrM.35.0660 LbrM.35.1010 LbrM.35.1790 LbrM.35.1960 LbrM.35.snRNA1 LbrM.35.snoRNA4

Table S3. Genes resulted by the DNA sequence of *Leishmania braziliensis* under 30°C with a significative increase or decrease in copy number variation compared with the control temperature of 26°C.

Chromosome	**# Total genes**	**# Genes with CNVs**	**Gene IDs with increase CNVs**	**Gene IDs with decrease CNVs**
1	78	4	LbrM.01.0170 LbrM.01.0230	

			LbrM.01.0270 LbrM.01.0640	
2	68	7	LbrM.02.0140 LbrM.02.0160 LbrM.02.0220 LbrM.02.0290	LbrM.02.0300 LbrM.02.0420 LbrM.02.0550
3	96	4	LbrM.03.0360 LbrM.03.0660 LbrM.03.0930 LbrM.03.ncRNA3	
4	125	6	LbrM.04.0210 LbrM.04.0220 LbrM.04.0230	LbrM.04.0200 LbrM.04.0750 LbrM.04.0950
5	129	5	LbrM.05.0380 LbrM.05.0390 LbrM.05.snRNA1 LbrM.05.snoRNA3	LbrM.05.snoRNA1
6	130	3	LbrM.06.0110	LbrM.06.0040 LbrM.06.0720
7	129	4	LbrM.07.0155 LbrM.07.1060 LbrM.07.1080	LbrM.07.0550
8	97	2	LbrM.08.0050 LbrM.08.0670	
9	169	9	LbrM.09.0010 LbrM.09.0180 LbrM.09.0190 LbrM.09.1070 LbrM.09.1220	LbrM.09.0070 LbrM.09.0170 LbrM.09.0650 LbrM.09.tRNA3
10	156	2	LbrM.10.0530	LbrM.10.1080
11	123	2		LbrM.11.tRNA1 LbrM.11.tRNA2
12	94	0		
13	158	3	LbrM.13.0190 LbrM.13.0740	LbrM.13.0200
14	169	5	LbrM.14.1110 LbrM.14.1300 LbrM.14.1310	LbrM.14.0390 LbrM.14.1190
15	164	4	LbrM.15.0450 LbrM.15.0730 LbrM.15.1080 LbrM.15.1280	

16	174	5	LbrM.16.1370 LbrM.16.1510 LbrM.16.1520	LbrM.16.0470 LbrM.16.0790
17	166	3	LbrM.17.0090 LbrM.17.0980	LbrM.17.1620
18	171	6		LbrM.18.0450 LbrM.18.0460 LbrM.18.0470 LbrM.18.0490 LbrM.18.1150 LbrM.18.1720
19	192	4	LbrM.19.1170 LbrM.19.1180 LbrM.19.1730	LbrM.19.0950
20.1	451	17	LbrM.20.0620 LbrM.20.0780 LbrM.20.1050 LbrM.20.1070 LbrM.20.1080 LbrM.20.1630 LbrM.20.2370 LbrM.20.2410 LbrM.20.4290	LbrM.20.0650 LbrM.20.0930 LbrM.20.0950 LbrM.20.1.snoRNA2 LbrM.20.1.tRNA10 LbrM.20.1.tRNA4 LbrM.20.1.tRNA8 LbrM.20.1.tRNA9
20.2	181	3	LbrM.20.5550	LbrM.20.5530 LbrM.20.5590
21	212	3	LbrM.21.0440 LbrM.21.0620	LbrM.21.0610
22	163	5	LbrM.22.0520 LbrM.22.1270 LbrM.22.1410 LbrM.22.1550 LbrM.22.ncRNA2	
23	217	11		LbrM.23.0621 LbrM.23.0670 LbrM.23.1000 LbrM.23.1110 LbrM.23.1130 LbrM.23.1830 LbrM.23.rRNA1 LbrM.23.tRNA10 LbrM.23.tRNA3 LbrM.23.tRNA6 LbrM.23.tRNA8
24	248	3	LbrM.24.0200 LbrM.24.1990	LbrM.24.1590

25	203	5	LbrM.25.0320 LbrM.25.0340 LbrM.25.1010 LbrM.25.1690	LbrM.25.0610
26	278	9	LbrM.26.0020 LbrM.26.0180 LbrM.26.1070 LbrM.26.snoRNA11 LbrM.26.snoRNA13 LbrM.26.snoRNA14 LbrM.26.snoRNA6 LbrM.26.snoRNA7	LbrM.26.snoRNA2
27	286	12	LbrM.27.0140 LbrM.27.0460 LbrM.27.0850 LbrM.27.2330 LbrM.27.2751 LbrM.27.2810 LbrM.27.2830	LbrM.27.0760 LbrM.27.1730 LbrM.27.1920 LbrM.27.2360 LbrM.27.2450
28	328	7	LbrM.28.0650 LbrM.28.2100 LbrM.28.2760 LbrM.28.3240	LbrM.28.1030 LbrM.28.1080 LbrM.28.2970
29	291	6	LbrM.29.2860	LbrM.29.0750 LbrM.29.1370 LbrM.29.1690 LbrM.29.2340 LbrM.29.2900
30	383	10	LbrM.30.0440 LbrM.30.1491 LbrM.30.2620 LbrM.30.3230 LbrM.30.3640 LbrM.30.3760 LbrM.30.tRNA1	LbrM.30.0450 LbrM.30.1790 LbrM.30.2970
31	376	27	LbrM.31.0280 LbrM.31.0290 LbrM.31.0380 LbrM.31.0430 LbrM.31.0530 LbrM.31.0920 LbrM.31.1430 LbrM.31.1670 LbrM.31.1840 LbrM.31.2190 LbrM.31.2260	LbrM.31.0080 LbrM.31.1250 LbrM.31.1380 LbrM.31.1520 LbrM.31.2001 LbrM.31.2120 LbrM.31.2250 LbrM.31.3640 LbrM.31.tRNA4

			LbrM.31.2740 LbrM.31.3290 LbrM.31.3330 LbrM.31.3370 LbrM.31.3420 LbrM.31.3620 LbrM.31.3630	
32	428	3	LbrM.32.2200 LbrM.32.2500 LbrM.32.4000	
33	367	9	LbrM.33.0340 LbrM.33.0930 LbrM.33.0960	LbrM.33.0330 LbrM.33.0920 LbrM.33.0950 LbrM.33.0990 LbrM.33.1010 LbrM.33.ncRNA3
34	545	11	LbrM.34.0170 LbrM.34.0530 LbrM.34.0540 LbrM.34.1670 LbrM.34.2470 LbrM.34.snoRNA1	LbrM.34.1100 LbrM.34.1110 LbrM.34.1790 LbrM.34.2160 LbrM.34.3350
35	761	19	LbrM.35.0120 LbrM.35.3760 LbrM.35.5260 LbrM.35.6650 LbrM.35.7170 LbrM.35.snoRNA2 LbrM.35.snoRNA6	LbrM.35.1140 LbrM.35.1570 LbrM.35.1960 LbrM.35.2730 LbrM.35.2800 LbrM.35.3820 LbrM.35.5990 LbrM.35.snRNA1 LbrM.35.snoRNA4 LbrM.35.snoRNA5 LbrM.35.tRNA1 LbrM.35.tRNA6