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Evolutionary Bioinformatics

The Comparative Genomics and Phylogenomics of *Leishmania amazonensis* Parasite

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ABSTRACT: Leishmaniasis is an infectious disease caused by *Leishmania* species. *Leishmania amazonensis* is a New World *Leishmania* species belonging to the Mexicana complex, which is able to cause all types of leishmaniasis infections. The *L. amazonensis* reference strain MHOM/BR/1973/M2269 was sequenced identifying 8,802 codifying sequences (CDS), most of them of hypothetical function. Comparative analysis using six *Leishmania* species showed a core set of 7,016 orthologs. *L. amazonensis* and *Leishmania mexicana* share the largest number of distinct orthologs, while *Leishmania braziliensis* presented the largest number of inparalogs. Additionally, phylogenomic analysis confirmed the taxonomic position for *L. amazonensis* within the "Mexicana complex", reinforcing understanding of the split of New and Old World *Leishmania*. Potential non-homologous isofunctional enzymes (NISE) were identified between *L. amazonensis* and *Homo sapiens* that could provide new drug targets for development.

KEYWORDS: *Leishmania amazonensis*, comparative genomics, phylogenomics

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Introduction

Leishmaniasis is an infectious disease caused by parasites of the genomes *Leishmania*. It has a worldwide impact with considerable morbidity and mortality rates, especially in the developing countries. The lack of a vaccine and effective treatments is of great concern, since most of the drugs available are toxic and usually lead to side effects.[1] The disease is distributed across 88 countries, and it is estimated that more than 12 million people are currently infected with *Leishmania*. Around 350 million people are living in endemic areas (poor rural and suburban zones),[2,3] and only a few countries (Afghanistan, Algeria, Brazil, India, Iran, Nepal, Peru, Saudi Arabia, Sudan, and Syria[2]) account for about 90% of the global cases. In Brazil, cutaneous leishmaniasis (CL) is endemic and

caused by at least six *Leishmania* species from the subgenus *Viannia* and *Leishmania*. The main agents of CL in the south of the Amazon basin are *Leishmania* (*Viannia*) *braziliensis* and *Leishmania amazonensis*, showing no differences in clinical manifestations.[4,5]

At least 20 *Leishmania* species are currently known to infect humans and can cause a variety of clinical manifestations depending on the species and the host immune response, ranging from cutaneous lesions to fatal visceral leishmaniasis (VL).[6–8] The most severe is VL, caused by the *Leishmania donovani* complex, in which the parasites affect mainly the liver and spleen, resulting in host immunesuppression, progressive fever, weight loss, and anemia.[2,9] In CL, the parasites cause localized long-term ulceration, inducting chronicity, latency, and, depending on the species, tendency to metastasize in the human host.[10] Mucocutaneous leishmaniasis (MCL), caused mainly by *L. braziliensis*, induces the destruction of nasopharyngeal tissue with hideous disfiguring lesions. Diffuse cutaneous leishmaniasis (DCL), caused by *L. amazonensis*, *Leishmania guyanensis*, and *Leishmania aethiopica*, is a long-lasting disease because of a deficient cellular-mediated immune response presenting a progressive primary lesion and multiple metastatic lesions.[11–13]

L. amazonensis is associated with a variety of clinical manifestations, CL, DCL (rare manifestation), MCL, VL, and post-kala-azar dermal leishmaniasis (PKDL), a systemic cutaneous form that occurs in some patients following VL treatment and apparent cure.[6] In Brazil, PH8 (IFLA/BR/67/PH8) is one of the most studied *L. amazonensis* strains as it is a component of Leishvaccine.[14] However, this strain was isolated from sand fly and an isolate from human disease was chosen. In this study, we present the genome of the *L. amazonensis* (MHOM/BR/71973/M2269), which was isolated from a single human cutaneous lesion.

Many advances have occurred during the last decade in the genomic area, mostly after the development of the high-throughput sequencing methods. Several trypanosomatids genomes have already been sequenced, among them are *Trypanosoma cruzi*, *Trypanosoma brucei*, *Leishmania major*, *Leishmania infantum*, *L. braziliensis*, and *Leishmania mexicana*.[15–21] *L. major* was the first *Leishmania* genome sequenced showing 32.8 Mbp size and 8,311 predicted protein-coding genes (codifying sequences (CDS)).[15] A comparative analysis using other three *Leishmania* species (*L. major*, *L. infantum*, and *L. braziliensis*) was carried out in 2007 and revealed a highly conserved genomic organization, in which the genomes display 8,300 genes in average of which more than 99% of the genes were highly syntenic. However, around 200 genes were differentially distributed among the three compared genomes, showing 47, 27, and 5 exclusive genes (species specific or unique) for *L. braziliensis*, *L. infantum*, and *L. major*, respectively.[18] The most recently sequenced *Leishmania* genome was *L. mexicana* published in 2011[21] and, during our sequencing effort, the *L. amazonensis* genome was described by Real and colleagues[22] in 2013.

Considering the "TriTryp" genomes (*T. cruzi*, *T. brucei*, and *L. major*), approximately 6,200 genes are conserved among the three species and 94% of these genes were syntenic.[23] Most of the species-specific genes are located in non-syntenic regions/chromosomes and consist of members of large surface antigen families.[24] Such observation may indicate that differences detected among these parasites' pathogenesis are likely. In this study, we performed a detailed comparative analysis of *L. amazonensis* M2269 genome with the *L. mexicana* U1103, *L. major* Friedlin, *L. infantum* JPCM5, *L. donovani* BPK282A, and *L. braziliensis* M2604 genomes retrieved from GenBank, identifying new features of this parasite genome and also performing a phylogenomic analysis of the genus.

Material and Methods

DNA preparation and sequencing. *L. amazonensis* reference strain MHOM/BR/1973/M2269, provided by Dr Paul Bates, was used in this study. Genomic DNA was extracted using a Qiagen QIAamp DNA Kit, according to the manufacturer's instructions. The extracted DNA was sequenced in a Solexa sequencer (Illumina) using paired-end reads of 50 + 50 bases.

Assembly, sequence analysis, and annotation. Genomic sequences of 66.869.406 reads were trimmed for platform-dependent systematic errors and then quality was evaluated using Phred (cutoff $Q = 26$).[25,26] High-quality reads (~28.52× genome coverage) were assembled using Velvet version 0.7.55 software[27] resulting in 10,721 contigs with mean contig length of 2,817 bp and N50 value of 6,946. De novo and reference genome assembly strategies were applied using the *L. mexicana* genome (GenBank Assembly ID: GCA_000234665.4 and RefSeq Assembly ID: GCF_000234665.1) and *L. major* genome (GenBank Assembly ID: GCA_000002725.2 and RefSeq Assembly ID: GCF_000002725.2). Assemblies were merged using in-house developed Perl scripts, and contigs were generated by comparing the assembled scaffolds and contigs with the *Leishmania* genomes available at the GenBank.

The multifasta files of the assembled *L. amazonensis* genome were submitted to STINGRAY pipeline (Wagner, et al., 2014)[28] (http://stingray.biowebdb.org), an improved version of the original GARSA[29] system, for semi-automatic annotation. The STINGRAY pipeline and a TblastX[30] approach were used by transferring the *L. mexicana*[21] annotation to *L. amazonensis*, which was further improved by the identification of conserved domains.

Protein families (Pfam) and domain identification. Pfam-A (v. 26.0)[31,32] and Hmmer 3.0[33] were used against the 8,802 predicted protein sets using hmmsearch program with an e-value 1e − 5 and other default parameters.

Gene ontology (GO) inference. The *L. amazonensis* proteins were also analyzed using GO.[34] Briefly, similarity analysis was performed with the STINGRAY pipeline,[29] using Blastp (v. 2.2.23)[30] against the GO database (go_20130223-seqdb.fasta), and then proteins were classified within one of three

GO categories, as follows: (i) biological process, (ii) molecular function, and (iii) cellular component.

Conserved domain identification. Conserved domains were identified using RpsBlast (v. 2.2.23)[30] analysis on the 8,802 proteins inferred in *L. amazonensis* against seven databases simultaneously (CDD.v3.10–Conserved Domain Database, COG.v1.0–Cluster of Orthologous Groups, KOG.v1.0–Cluster of Eukaryotic Orthologous Groups, Pfam.v26.0–Protein Family, PRK.v6.0, SMART.v6.0, and TIGR.v13.0) with an e-value 1e – 05.

Identification of orthologous and paralogous groups. The identification of orthologous proteins was performed using results generated by the OrthoMCL v.1.4 software.[35] Orthologous proteins shared by all six *Leishmania* species (*L. major*, *L. infantum*, *L. donovani*, *L. braziliensis*, *L. mexicana*, and *L. amazonensis*) were inferred. The protein function of those inferred orthologs was semi-automatically transferred from previously annotated *Leishmania* genomes. Inparalogous and recent paralogous proteins in *L. amazonensis* were also identified inside the output file generated by the OrthoMCL software.

The orthologous proteins shared among the different *Leishmania* species as well as the inparalogous proteins from *L. amazonensis* and other species were used to generate a Venn diagram using R software.[36]

Putative orphan proteins identification. To find putative orphan proteins, ie, not homologous to any protein in this study, a first list of protein identifiers was generated and used as input to OrthoMCL to build a second list with protein identifiers clustered by OrthoMCL. Then, these two lists (submitted versus clusterized) were compared using a script written in Ruby language to obtain the identifiers of the putative orphan proteins. Since these potential orphans are based on a universe of only six *Leishmania* genomes and to minimize possible misclassification, we performed a BlastP search (v. 2.2.28+)[30] with these putative orphan proteins against RefSeq database (r.56 18, 132, 578 sequences). These steps allowed us to identify proteins that were classified as putative orphans having similarity to prokaryotic or other eukaryotic (non-*Leishmania*) protein. Finally, proteins without any match to Refseq database were considered as orphan proteins in this study.

Phylogenomics. The phylogenomic tree was inferred based on the studies of Ocaña and Dávila,[37] and Ciccarelli and colleagues.[38] Thirty-one universal orthologous (UO) genes showing 1:1 orthologous relationships were used. These UO genes originally identified by Ciccarelli et al.[38] showed the following characteristics: (i) were present in all complete genomes available at GenBank until 2006 and (ii) were not involved in horizontal transfer. Since these 31 UO genes are directly connected to the protozoan genome available at RefSeq and ProtozoaDB,[39] they were mapped to the *L. major* proteins using both (a) the best blast hits (e-value 1e – 50) and (b) the manual verification of the annotation (the RefSeq annotation of the best hits needed to match the UO annotation). Once mapped, the *L. major* protein sequences corresponding to these 31 UO genes were searched in the orthologous groups identified in the six *Leishmania* species by OrthoMCL. We also mapped the 31 orthologs in 28 distinct protozoa species, as follows: *Angomonas deanei*, *Babesia bovis*, *Babesia equi*, *Babesia microti*, *Cryptosporidium muris*, *Dictyostelium discoideum*, *Entamoeba dispar*, *Entamoeba histolytica*, *Entamoeba invadens*, *Giardia lamblia*, *L. amazonensis*, *L. braziliensis*, *L. donovani*, *L. infantum*, *L. major*, *L. mexicana*, *Naegleria gruberi*, *Neospora caninum*, *Plasmodium berghei*, *Plasmodium cynomolgi*, *Plasmodium falciparum*, *Plasmodium knowlesi*, *Plasmodium vivax*, *Polysphondylium pallidum*, *Strigomonas culicis*, *Tetrahymena thermophila*, *Theileria annulata*, *Theileria orientalis*, *Theileria parva*, *Toxoplasma gondii*, *Trichomonas vaginalis*, *T. brucei*, *T. cruzi*, and *Trypanosoma vivax*. Finally, each of these 31 mapped orthologs were exported as multifasta files and aligned using Mafft v5.861,[40] using the default parameters.

A supermatrix tree was obtained using concatenated multiple alignments from entire protein sequences. The individual alignments were concatenated using an in-house perl script, resulting in a global supermatrix of 9,450 positions for the six species. The resulting supermatrix was used to generate the phylogenomic tree with MEGA 5,[41] inferred by Maximum Likelihood using 1,000 bootstrap replicates. We opted to use the JTT model in the single (concatenated) alignment, which was also the model adopted in the phylogenomics studies of Ciccarelli et al.[38] and Ocaña and Dávila.[37] Jones, Taylor and Thornton (JTT) model assumes that there are two classes of sites, one class being invariable and the other class being free to change.[42]

Intragenomic and intergenomic non-homologous isofunctional enzymes (NISE) identification. To identify in the genome of *L. amazonensis* possible cases of intra- and intergenomic NISE between this genome and the *Homo sapiens* genome, we applied methodologies previously described.[43–45] Briefly, protein sequences of enzymes with the same functional activity were downloaded and grouped according to its functional activity as determined by the classification from the International Union of Biochemistry and Molecular Biology, the Enzyme Commission (EC) number.[46] Protein sequences and functional classification were obtained from KEGG (Kyoto Encyclopedia of Genes and Genomes, version 58.1).[47] After grouping, we performed a step to confirm the functional activity assigned by KEGG. First, we removed sequences with less than 60 amino acids from the 8,802 *L. amazonensis* predicted proteins, since they may represent protein fragments, resulting in a data set of 8,575 predicted proteins. Then, the protein primary structures inside each protein functional group were compared in a pairwise, all-against-all manner, using Blastp. Functional activities were confirmed via the AnEnPi's module,[43] which classifies the enzymes in accordance to the EC number. This classification is obtained after parsing the results of Blastp, using the data set of predicted proteins from *L. amazonensis* as query and the groups

previously obtained as subjects. A restrictive e-value of 10^{-20} was used as a threshold[44,45,48] to include a primary structure in a group or cluster. Proteins were considered to be NISE if, inside each group of functionally related enzymes, they were allocated in different clusters after parsing the results from Blastp. Possible analogy cases were verified by the examination of their folding categories as classified by the SCOP (http://scop.mrc-lmb.cam.ac.uk/scop/)[49] and SUPERFAMILY databases (supfam.cs.bris.ac.uk).[50] Further refinement of confirmed NISEs was achieved by three-dimensional (3D) structure prediction of *L. amazonensis* proteins by homology modeling and structural comparison with their human analogous counterparts (see below).

Homology modeling of *L. amazonensis* proteins and comparative structural analysis with human proteins. Modeling of the 3D structure of the selected NISEs of *L. amazonensis* was performed by the satisfaction of spatial restraints method implemented in the program Modeller 9v10.[51] Global pairwise sequence alignment between the target *L. amazonensis* sequences and the respective templates was performed with the needle (Needleman–Wunsch) program within the EMBOSS v.6.3.1 package.[52] The models were constructed using as templates the atomic coordinates of PDB IDs listed in Supplementary Table S2 for each of the selected analogy cases. Ten models were generated for each protein target sequence, and the model with the most favorable DOPE-score and the lowest Modeller objective function value was subjected to external assessment of the stereochemical and overall structural quality within the Structural Analysis and Verification Server (SAVES v.4) (http://services.mbi.ucla.edu/SAVES/). All models selected for further analysis had at least 95% of residues in the most favorable and additionally allowed regions of Ramachandran plots along with other reasonable stereochemical quality parameters. Inspection of molecular structures and other structural analysis was performed by SYBYL X–1.3 software (Tripos L.P., St. Louis, MO).

***L. amazonensis* genome functional categorization.** To briefly know the genome content of *L. amazonensis*, we performed a functional categorization through similarity analysis using Blast and RpsBlast programs against the database of orthologous genes in prokaryotes (COG/NCBI) and eukaryotic orthologous genes (KOG/NCBI),[53,54] which are classified in functional categories (ftp://ftp.ncbi.nih.gov/pub/COG/COG/fun.txt). To infer to which functional category each protein belongs, a cutoff e-value of 1e − 5 was used in both programs and databases. Plots of the functional categories were created with R software.

L. amazonensis proteome was also characterized by Pfam (v. 26.0)[32] and by CDD (v 3.10) through RpsBlast. A further analysis was performed using in-house perl scripts to identify (i) which genes were identified only by Pfam with Hmmer 3, (ii) which ones were identified only by "Conserved Domains" (CDD) and (iii) which ones were characterized by both of them (Pfam and CDD).

Leishmania core proteome identification. The *Leishmania* spp. core proteins (LCP) were identified and analyzed among the orthologous groups and defined as orthologous proteins shared by all the six *Leishmania* species studied. To find the LCP, the OrthoMCL results were analyzed, and only orthologs shared by the "*6 taxa*" were chosen. LCP functions were accessed through annotation provided with the sequences.

***L. amazonensis database*.** The contigs generated from the assembly of sequencing reads, and the genes and proteins found from these contigs are all available for public consulting in the STINGRAY pipeline (http://stingray.biowebdb.org). Furthermore, *L. amazonensis* contigs were submitted to GenBank under BioProject ID PRJNA221875, locus_tag prefix Q771.

L. amazonensis RNA Interference (RNAi) Machinery.

Identification of RNAi genes in *L. amazonensis* genome was performed through Blast analysis using as query RNAi genes from *Leishmania* spp. and *T. brucei* genes annotated as participants of the RNAi pathway in GeneDB database[55] (www.genedb.org). The genes related to RNAi machinery in *L. amazonensis* were then submitted to a phylogenetic analysis using MEGA5,[41] and a tree was inferred by Neighbor-Joining using 1,000 bootstrap replicates.

Synteny Analysis: *L. mexicana* Versus *L. amazonensis*

The synteny analysis was performed using the ABACAS[56] (v. 1.3.1) pipeline, and the results were visualized with ACT (Artemis Comparison Tool),[57] v. 12.0.0. The following steps were carried out to accomplish the analysis: (i) the 34 *L. mexicana* chromosomes were concatenated in a single fasta file; (ii) the multifasta of the 8,552 putative *L. amazonensis* CDS was compared to that of the *L. mexicana* genome (chromosome) using ABACAS; and (iii) the *L. amazonenesis* genome on the ABACAS output had the CDS reordered, and the resulting comparison was visualized on ACT.

Results

Sequencing, assembly, and genome characteristics. The *L. amazonensis* genome assembly was obtained via a reference-guided approach where the obtained contigs were aligned against the reference *L. mexicana* genome. The assembly resulted in 10,305 contigs, with approximately 59% GC content. The smallest and largest detected contigs had 96 and 141,211 bases, respectively, with a mean of 2,879 bp and median of 853 bp (Table 1). *L. amazonensis* genome presented 8,802 protein-coding genes after analysis with TblastX against *L. mexicana* and Refseq databases. The largest coding region had 19,872 bp and the smallest only 66 bp with median and mean of 1,637 bp and 1,209 bp, respectively (Fig. 1). The GC content for coding regions was 61.1%. Of these 8,802 proteins, 5,554 were putative proteins, and 887 were not clusterized by OrthoMCL and then were

Table 1. Summary of the *Leishmania amazonensis* assembly and genome.

Contigs	10,305
Sum of consensus sequences length	29,670,588 bases
Number of scaffolds >1 K nt	4827 (46.8%)
Number of scaffolds >10 K nt	732 (7.1%)
Number of scaffolds >100 K nt	2 (0.02%)
Coding genes: CDS	8,802
Chromosome	34
%GC content: Contigs/CDS	59%/(61.125%)
Size: Contigs/CDS	
Max (bases)	141,211/(19,872)
Min (bases)	96/(66)
Mean (bases)	2,879/(1,637)
Median (bases)	853/(1,209)
N50 scaffold length	8,346
CDS ontology	
Molecular function	4,065
Biological process	4,007
Cellular component	4,054
Protein families (PFAM)	3,075
Conserved domains (CDD)	6,144
Annotated as "Hypothetic protein"	5,554
Putative orphans (OrthoMCL)	887

analyzed with Blast against Refseq database with e-value 1e – 5, resulting in 14 proteins classified as orphans in this study (Table 2). Furthermore, while some genes were found occurring in single copy, such as ribosomal protein S2 (rpS2) and ribosomal protein L7, other genes exhibited multiple copies, such as ATP-binding cassette (ABC) transporter (50 copies) and calpains (44 copies). Nonetheless, 63% of the CDS were annotated as proteins with hypothetical function (Table 3).

Functional Analysis of *L. amazonensis* Proteins

Taking into consideration the GO classification, the most frequent molecular functions of *L. amazonensis* proteins were protein binding (9% or 1,153/12,328), nucleotide binding (8% or 947/12,328), metal ion binding (5% or 653/12,328), receptor activity (4% or 473/12,328), DNA binding (4% or 468/12,328), signal transducers activity (4% or 453/12,328), and binding (4% or 451/12,328) (Fig. 2A). The most representative functions related to biological process were signal transduction (3% or 308/11,799); transmembrane transport (3% or 306/11,799); regulation of transcription, DNA dependent (2% or 251/11,799); and transport (2% or 192/11,799) (Fig. 2B).The last GO category, Cellular Component, had most frequent components related to: cytoplasm (12% or 1,441/12,111), membrane (10% or 1,168/12,111), nucleus (7% or 856/12,111), intracellular (7% or 810/12,111), and plasma membrane (6% or 694/12,111) (Fig. 2C). The most abundant protein-coding genes detected in the *L. amazonensis* genome were ABC transporter, kinesin, ATP-dependent RNA helicase, heat shock proteins (HSPs), protein kinase, dynein heavy chain, calpains, and amastin surface glycoprotein

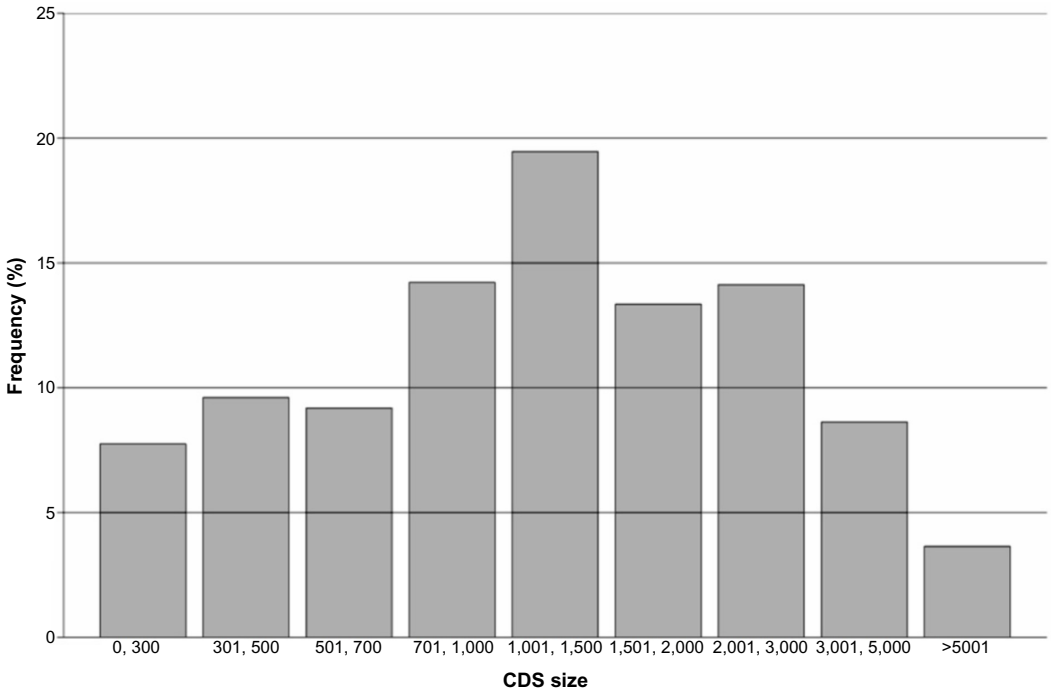


Figure 1. Average size (bases) from putative CDS identified in *L. amazonensis* genome.

Table 2. Resume table of most abundant and single copy genes/domains found in *Leishmania amazonensis* genome analysis.

MOST ABUNDANT GENES/DOMAINS	SINGLE COPY GENES/DOMAINS
ABC transporter	rpS2
Amastin surface glycoprotein	rpS5
ATP-dependent RNA helicase	rpS8
Calpains	rpS10
Dynein heavy chain	rpS12
Heat Shock Proteins (HSPs)	rpL7
Kinesin	rpL12
Protein kinase	rpL13
WD40	rpL19
Chaperone DNAJ	rpL23

Notes: Most abundant genes/domains found in the initial *Leishmania amazonensis* genome analysis. Genes/domains found in single copy during the analysis. 40S ribomosomal proteins (rpS) and 60S ribosomal proteins (rpL).

(Table 3). Pfam and conserved domain (CDD) analyses were performed to identify the families/domains present in the 8,802 putative proteins. Of these, 3,075 proteins were assigned to the family level using Pfam, representing a total of 1,004 different families in the *L. amazonensis* genome. The largest family assigned by Pfam was kinase, which contains 69 entries for Pkinase_Tyr and 64 for Pkinase, accounting for about 2% of the total families detected (Figs. 3 and 4). The families TPR, zf-C3Hc4_2, DnaJ, RRM_1, AAA22, Helicase C, URR1, URR6, and AAA25 range in size from 58 to 36 proteins, and 617 families were represented

Table 3. List of orphans proteins found in *Leishmania amazonensis* with their respective identification, description and length (aa).

IDENTIFICATION	DESCRIPTION	LENGTH
LAJMNGS001H06.b.195	Unspecified product	98
LAJMNGS002H09.b.421	Unspecified product	150
LAJMNGS005H02.b.1027	Hypothetical protein, conserved	79
LAJMNGS006F03.b.1178	Hypothetical protein, unknown function	771
LAJMNGS018E09.b.3196	Carboxypeptidase, putative	325
LAJMNGS018H07.b.3264	Hypothetical protein	951
LAJMNGS027A04.b.4532	Hypothetical protein	167
LAJMNGS030G04.b.5103	Unspecified product	48
LAJMNGS031F02.b.5255	Unspecified product	212
LAJMNGS038C10.b.6191	Unspecified product	37
LAJMNGS038E01.b.6205	Unspecified product	94
LAJMNGS051A11.b.7995	Hypothetical protein	139
NODE_5216_1	Hypothetical protein, Unknown function	68
NODE_20256_1	Unspecified product	81

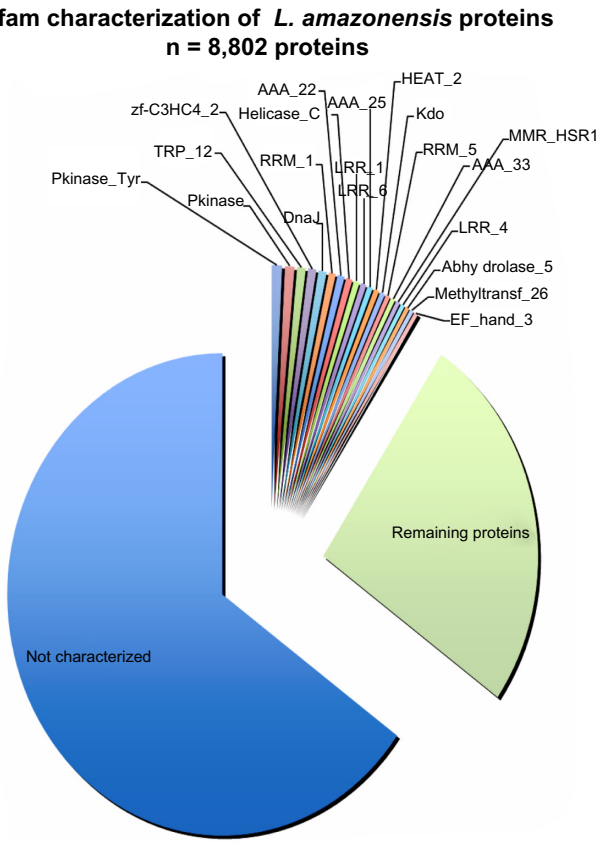


Figure 2. Proteins families identification generated by PFAM database.
Notes: Only 20 most abundant families were represented in the figure. Remaining families are grouped into green square and not characterized proteins are in blue.

by a single protein (Fig. 3). The RpsBlast analysis, used to find the CDDs, characterized 6,144 domains (Fig. 5), in which approximately 1,800 were found in single copies. The domains most frequently found in *L. amazonensis* proteins were SMC_prok_B (chromosome segregation protein SMC) with 131 hits, PHA03247 (large tegument protein UL36) with 126 copies, and PRK07003 (DNA polymerase III subunits gamma and tau) with 113 copies. Altogether, the fact that more than 60% (5,554/8,802) of the proteins were annotated as hypothetical, 6,144 domains were found using CDD, and 1,004 different families were identified by Pfam highlights the great and unknown diversity of *Leishmania* spp. functionality. The combination of Pfam and CDD results (Fig. 4) showed 2,483 proteins simultaneously assigned to some Pfam family and CDD, with Pkinase_Tyr being the most frequent family found that has some CDD associated. Nevertheless, nearly 5,500 *L. amazonensis* proteins were not functionally annotated or were not assigned to any protein family, which is consistent with other *Leishmania* genomes. The functional analysis of *L. amazonensis* according to KOG and COG categories confirmed the specificity of its proteins, since R category (general function prediction only) was the most abundant category found (Fig. 6).

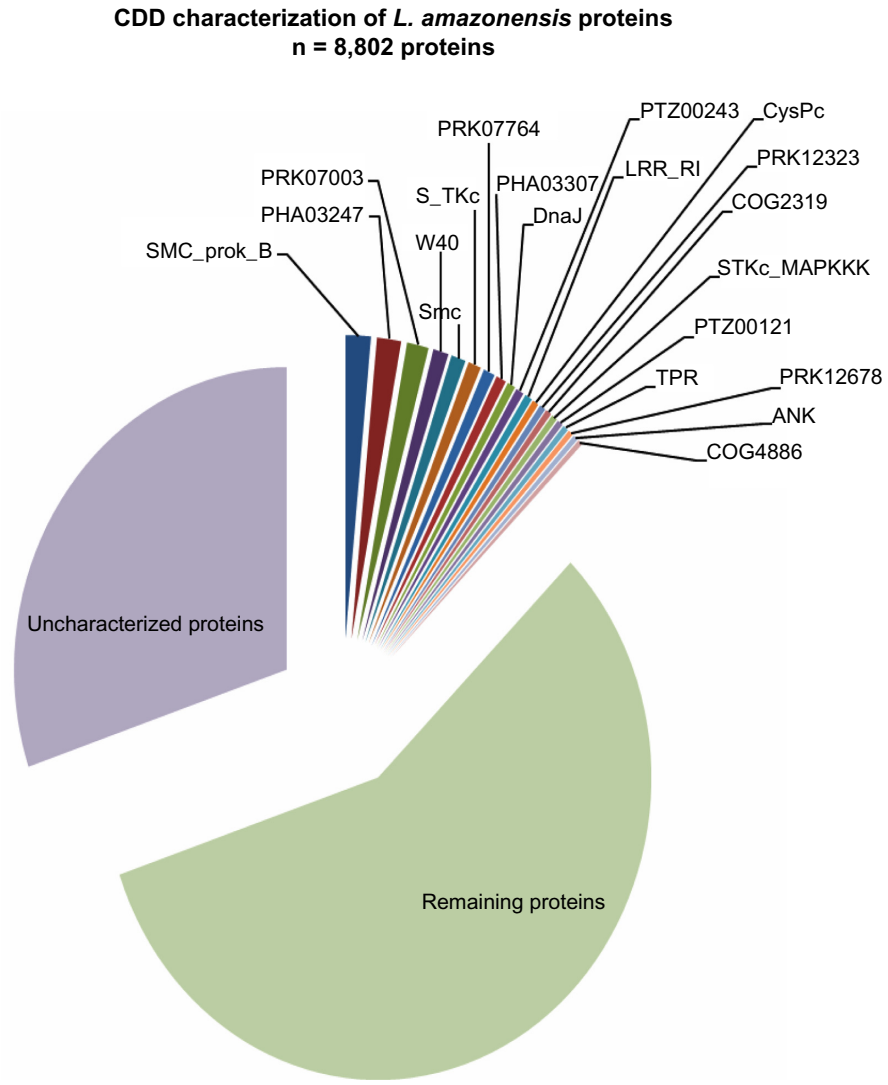
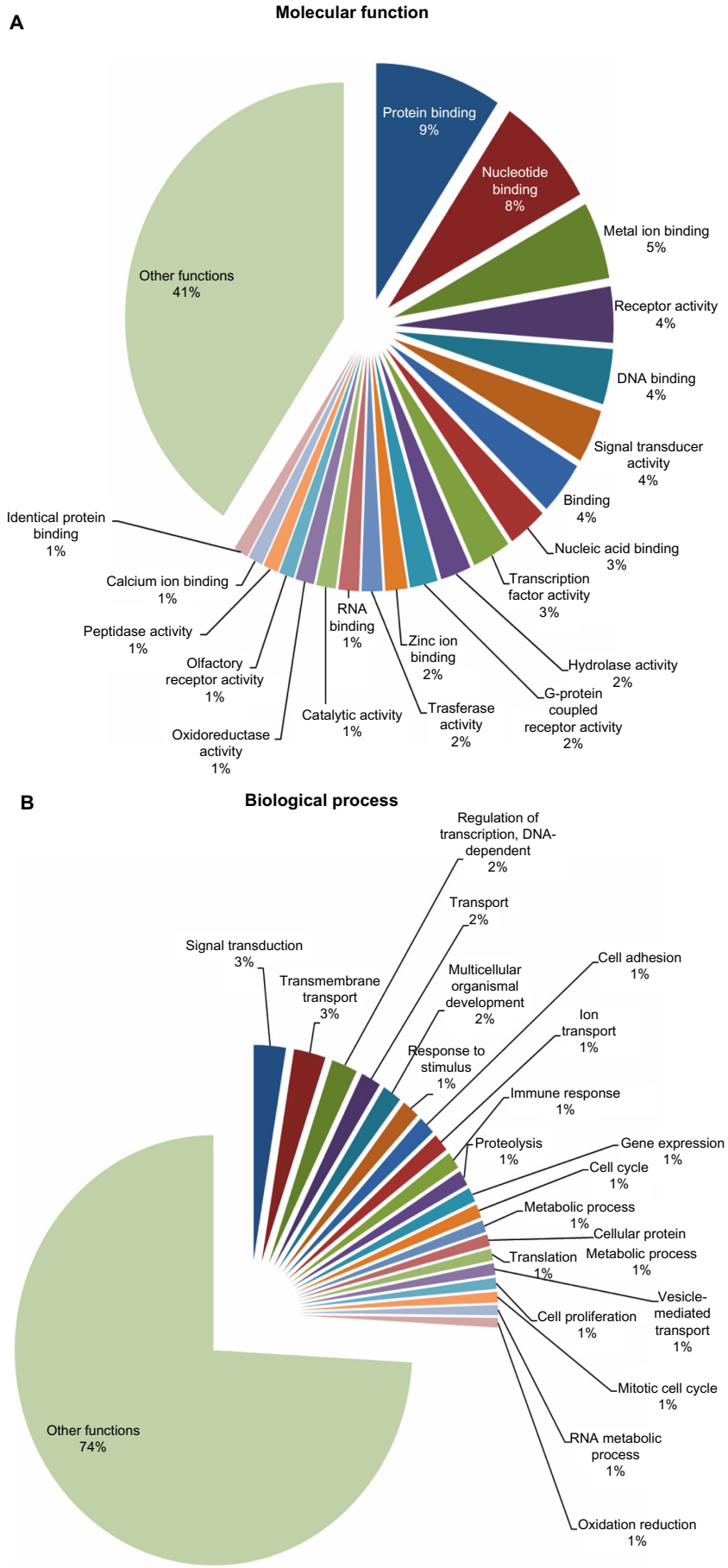


Figure 3. Conserved domains identification generated by RpsBlast with CDD database.
Notes: Only 20 most domains were represented in the legend. Remaining families are grouped into green square and uncharacterized proteins are in purple.

Comparative Analysis

A comparative analysis to identify orthologous proteins among the six different *Leishmania* genomes was performed using OrthoMCL. Most of the *L. amazonensis* proteins revealed to be orthologs were observed on all genomes evaluated, consisting of the *Leishmania* spp. core genome. A total of 7,016 (79.7%) orthologous groups were found among *L. amazonensis*, *L. donovani*, *L. mexicana*, *L. infantum*, *L. braziliensis*, and *L. major* (Fig. 7) (Supplementary File, Table S1). Within LCP, approximately 4,800 (68.4%) orthologs were annotated as hypothetical proteins; however, among those who have a defined function, we found proteins such as amastin, calpain-like cysteine peptidase, 40S ribosomal protein S16, RNA helicase, protein kinase, dynein heavy chain, activated protein kinase c receptor (LACK), ABC transporter, tuzin, and DNA primase large subunit. Considering genes shared between two *Leishmania* species, we found 18 orthologous protein groups between *L. amazonensis* and *L. mexicana*, which are closely related and belong to the *L. mexicana* complex (Table 4 and Supplementary File). Within these 18 orthologous groups, 7 proteins had an identified function (kinetoplast-associated protein, 3-hydroxyisobutyryl-coenzyme a hydrolase-like protein, viscerotropic Leishmaniasis antigen, ribosomal protein L1a, amastin, viscerotropic Leishmaniasis antigen, and flagellar calcium-binding protein) and 12 were classified with hypothetical function. The comparison of the most distant species inside *Leishmania* genus, *L.* (*L.*) *amazonesis* versus *L.* (*V.*) *braziliensis*, showed that nine proteins were exclusive and shared by both, among which four had known function: heat shock 70-related protein 1, beta tubulin, tyrosine/dopa decarboxylase, and oxidoreductase (Table 4). When inparalogous proteins were evaluated in *L. amazonensis*, one paralog was found: triacylglycerol lipase-like protein (Table 4).

A phylogenomic analysis was performed based on 31 UO genes to confirm that *Leishmania* species are closely related, mainly regarding the *Leishmania* and the *Vianna* subgenus. Figure 6 shows the relationship between the species from *L. mexicana* (*L. amazonensis* and *L. mexicana*) and *L. donovani*


A
Molecular function
Protein binding 9%
Nucleotide binding 8%
Metal ion binding 5%
Receptor activity 4%
DNA binding 4%
Signal transducer activity 4%
Binding 4%
Nucleic acid binding 3%
Transcription factor activity 3%
Hydrolase activity 2%
G-protein coupled receptor activity 2%
Trasferase activity 2%
Zinc ion binding 2%
RNA binding 1%
Catalytic activity 1%
Oxidoreductase activity 1%
Olfactory receptor activity 1%
Peptidase activity 1%
Calcium ion binding 1%
Identical protein binding 1%
Other functions 41%
B
Biological process
Regulation of transcription, DNA-dependent 2%
Transport 2%
Signal transduction 3%
Transmembrane transport 3%
Multicellular organismal development 2%
Cell adhesion 1%
Ion transport 1%
Response to stimulus 1%
Immune response 1%
Proteolysis 1%
Gene expression 1%
Cell cycle 1%
Metabolic process 1%
Cellular protein Metabolic process 1%
Translation 1%
Vesicle-mediated transport 1%
Cell proliferation 1%
Mitotic cell cycle 1%
RNA metabolic process 1%
Oxidation reduction 1%
Other functions 74%

(*Continued*)

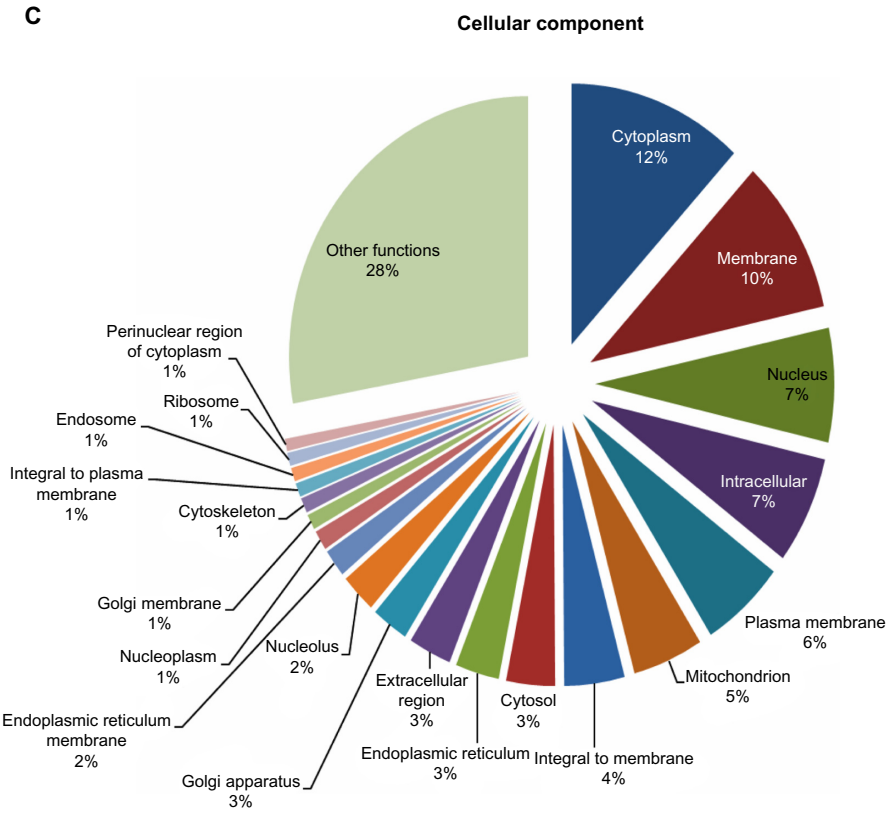


Figure 4. Gene ontology results to protein characterization in level of molecular function (**A**), Biological process (**B**) and cellular component (**C**). Only the 20 most abundant characteristics were listed here.

(*L. donovani* and *L. infantum*) complexes and also other 28 protozoa species. Even though these orthologous genes are very close, the differences among groups could still be observed in the generated dendrogram (Fig. 8). This result was supported by an alignment using one of the UOs (DNA-directed RNA polymerase; Fig. 9), where *L. amazonensis* and *L. mexicana* have very similar sequences, while *L. braziliensis* has the most divergent sequence, indeed presenting gaps in the multiple alignment. *L. braziliensis*, which belongs to *Viannia* subgenus, was in fact positioned in a different clade, underlining their differences and reflecting the divergence observed in the alignment (Fig. 9). At the same *Leishmania* clade, another five Kinetoplastida species are found, reflecting the monophyletic nature of this genus.

NISE

After the initial clustering of 4,215 ECs available in KEGG with AnEnPi, 412 ECs present in more than one cluster were detected. This group of 412 ECs was parsed for *L. amazonensis* sequences allocated in different clusters with the same enzymatic activity. Using this procedure, it was possible to identify 25 potential cases of NISEs when *L. amazonensis* was compared to *H. sapiens* (termed "intergenomic NISE"). In addition, 31 potential cases of NISEs were identified when *L. amazonensis* protein sequences (termed "intragenomic NISE") were searched. The presence of NISEs was detected in five of the six main EC classes, such as Oxidoreductases (EC 1), Transferases (EC 2), Hydrolases (EC 3), Lyases (EC 4), and Isomerases (EC 5), but no cases of functional analogy on Ligases (EC 6) were found (Supplementary File, Tables S2–S4).

Complementary analyses based on the SUPERFAMILY database at potential NISEs excluded few cases, where the status of "Predicted NISE" was given to cases with no significant hits on the SUPERFAMILY database, since although the sequences were allocated in different clusters, we could not confirm the structural differences (Supplementary File, Tables S2 and S3). Among the 25 potential intergenomic NISEs, 14 cases were confirmed and 1 case was considered as predicted NISE. Among the 31 potential intragenomic NISEs, 15 cases were confirmed and 1 was considered as predicted NISE (partially demonstrated on Tables 5 and 6 and completely on Supplementary File, Tables S2 and S3). It is important to emphasize that the approach considered here is very restrictive since only NISEs under the same EC that had different folds (not sharing any type of fold) were taken into account.

Further structural characterization was performed using three confirmed intergenomic NISE cases that showed clear homology (above 30% sequence identity) with a protein with known 3D structure deposited in PDB and that had a solved structure for its human analogous counterpart: LAJMNGS050H11.b.7960 (EC1.1.1.2 – putative

Table 4. Identification of orthologous groups between *L. amazonensis* and *Leishmania* species and inparalogous from *L. amazonensis* only characterized orthologs are listed.

ORTHOMCL	*L. AMAZONENSIS* ACCESSION	PFAM ANNOTATION	CDD ANNOTATION	PROTEIN DESCRIPTION	L. AM	L. ME	L. DO	L. BR
ORTHOMCL7819	LAJMNGS015A07.b.2588 LAJMNGS029B11.b.4872			Triacylglycerol lipase	X			
ORTHOMCL7785	NODE_9861_1 gi|401424225			Kinetoplast-associated protein	X	X		
ORTHOMCL7789	NODE_20602_1 gi|401430272		pfam07344, Amastin	Unspecified product	X	X		
ORTHOMCL7794	NODE_11369_4 gi|401427459		pfam13766, ECH_C, 2-enoyl-CoA Hydratase	3-hydroxyisobutyryl-coenzyme a hydrolase	X	X		
ORTHOMCL7802	LAJMNGS046H11.b.7373 gi|401414833			Viscerotropic *Leishmania*sis antigen,	X	X		
ORTHOMCL7803	LAJMNGS046G07.b.7351 gi|401418572		cd00051, EFh, EF-hand, calcium binding motif	Flagellar calcium-binding protein, putative	X	X		
ORTHOMCL7808	LAJMNGS033H06.b.5610 gi|401428307		PTZ00201, amastin surface glycoprotein	Amastin-like protein	X	X		
ORTHOMCL7813	LAJMNGS029D07.b.4914 gi|401427209	PF13415.1Kelch_3	pfam01344, Kelch_1	Hypothetical protein	X	X		
ORTHOMCL7814	LAJMNGS029D03.b.4903 gi|401430342	PF00806.14PUF	cd07920, Pumilio	Unspecified product	X	X		
ORTHOMCL7822	LAJMNGS010A05.b.1767 gi|401415906		PTZ00428, 60S ribosomal protein L4	Ribosomal protein L1a, putative	X	X		
ORTHOMCL7717	NODE_33600_1 gi|398019921 gi|398019923			Amino acid permease	X		X	
ORTHOMCL7788	NODE_21871_1 gi|398010889		PTZ00201, amastin	Amastin-like protein	X		X	
ORTHOMCL7790	NODE_20189_1 gi|398010239		cd03213, ABCG_EPDR	ATP-binding cassette protein subfamily G, member 1	X		X	
ORTHOMCL7792	NODE_12712_1 gi|398023645		PTZ00263, protein kinase A	Protein kinase A catalytic subunit isoform 2	X		X	
ORTHOMCL7795	NODE_10493_4 gi|398023914			Phosphoglycan beta 1,3 galactosyltransferase 4	X		X	
ORTHOMCL7800	LAJMNGS047C07.b.7405 gi|398019480		COG1788, Acyl CoA: acetate/3-ketoacid	Succinyl-coa:3-ketoacid-coenzyme a transferase- like protein	X		X	
ORTHOMCL7801	LAJMNGS047B12.b.7397 gi|398015472		PTZ00243, ABC transporter	Multidrug resistance protein, putative,p-glycoprotein, putative, ABC transporter	X		X	
ORTHOMCL7809	LAJMNGS033B09.b.5509 gi|398014545			Calpain-like cysteine peptidase	X		X	
ORTHOMCL7811	LAJMNGS030F01.b.5087 gi|398010628			Vacuolar-type Ca2 -ATPase, putative	X		X	
ORTHOMCL7823	LAJMNGS008G11.b.1562 gi|398015632		COG1621, SacC, Beta-fructosidases	Beta-fructosidase, invertase,sucrose hydrolase	X		X	

ORTHOMCL7719	NODE_11708_1 gi|154341831 gi|154341835	PTZ00186, heat shock 70 kDa	Heat shock 70-related protein 1, mitochondrial precursor, putative	X	X
ORTHOMCL7793	NODE_1256_4 gi|154343852	PLN00220, tubulin beta chain	Beta tubulin	X	X
ORTHOMCL7798	LAJMNGS050C12.b.7841 gi|389602223	PLN02880, tyrosine decarboxylase	Tyrosine/dopa decarboxylase	X	X
ORTHOMCL7815	LAJMNGS027C01.b.4566 gi|154334177	pfam00201, UDP-glucoronosyl and glucosyl transferase	Hypothetical protein, conserved	X	X
ORTHOMCL7818	LAJMNGS015D10.b.2693 gi|154331940	PTZ00261, acyltransferase	Unspecified product	X	X
ORTHOMCL7825	LAJMNGS008E08.b.1511 gi|154344054	PF00107.21ADH_zinc_N	cd08250, Mgc45594_like	Oxidoreductase-like protein	X	X

NADP-dependentalcoholdehydrogenase"), LAJMNGS010C07.b.1806 (EC 1.3.1.34 – "putative 2,4-dienoyl-coa reductase FADH1"), and LAJMNGS034G09.b.5743 (EC 5.3.3.2 – "putative isopentenyl-diphosphate delta-isomerase"). The comparison of the 3D models for *L. amazonensis* proteins with the experimental structures of the respective human isofunctional enzymes confirmed the distinct folds adopted by the proteins and allowed the detailed characterization of the differences in catalytic sites employed by each analog (Fig. 10).

Finally, a search for the intergenomic NISE detected in this study was performed against drug target databases such as TDR targets, TTD, and DrugBank, verifying that some of these NISE are already under study as potential drug targets against other pathogens. The complete list containing such targets and the pathogens is in Supplementary File (Supplementary Table S5).

RNAi Pathway in *L. amazonensis*

Some RNAi pathway-related genes are present in *L. amazonensis* (Table 7). Dicer seems to be missing in trypanosomatids that lack a functional RNAi pathway. We were unable to detect Dicer in *L. amazonensis* genome or any sequence bearing the characteristic Rnc (dsRNA-specific ribonuclease) domain. However, the presence of a possible functional Dicer homolog with very divergent sequence is not definitely discarded, and more studies need to be carried out. Nine DEAD/H box RNA helicase and two ribonuclease III genes with putative relationship to RNAi pathway were identified in *L. amazonensis* (Table 7). Although Dicer was not identified, some Dicer-related genes were characterized. Four ERI sequences were identified in *L. amazonensis* genome data set (LAJMNGS009D01.b.1653, LAJMNGS023D01.b.3956, LAJMNGS034E11.b.5717, and LAJMNGS035F02.b.5853) (Table 7). Two genes of the RNA-induced silencing complex (RISC; a major effector complex of the RNAi pathway) were also identified: tudor and piwi (argonaute family) (Table 7). The *L. amazonensis* argonaute-like gene identified (LlPWI1) is phylogenetically related to TbPWI1, which is not involved in RNAi. The full sequence of the LlPWI1 gene in *L. amazonensis* and its orthologs was submitted for phylogenetic analysis (Fig. 11). The neighbor-joining tree clearly distinguishes two functionally different forms of argonaute family proteins based on *T. brucei* TbAGO1 and TbPWI1. Only *Leishmania* from subgenus *Viannia* (*L. braziliensis* and *L. guyanensis*) are related to TbAGO1, while the *Leishmania* species from subgenus *Leishmania* (*L. mexicana*, *L. major*, *L. donovani*, *L. infantum*, and *L. amazonensis*) falls into TbPWI1 group. Besides *T. brucei*, only *L. braziliensis* possesses the two forms of argonaute family genes (ACI22628 and XP_001564757), which are related to TbAGO1 and TbPWI1, respectively.

Synteny Analysis Between *L. mexicana* and *L. amazonensis*

The results of the synteny analysis between the *L. mexicana* and *L. amazonensis* genomes (Fig. 12) showed no synteny

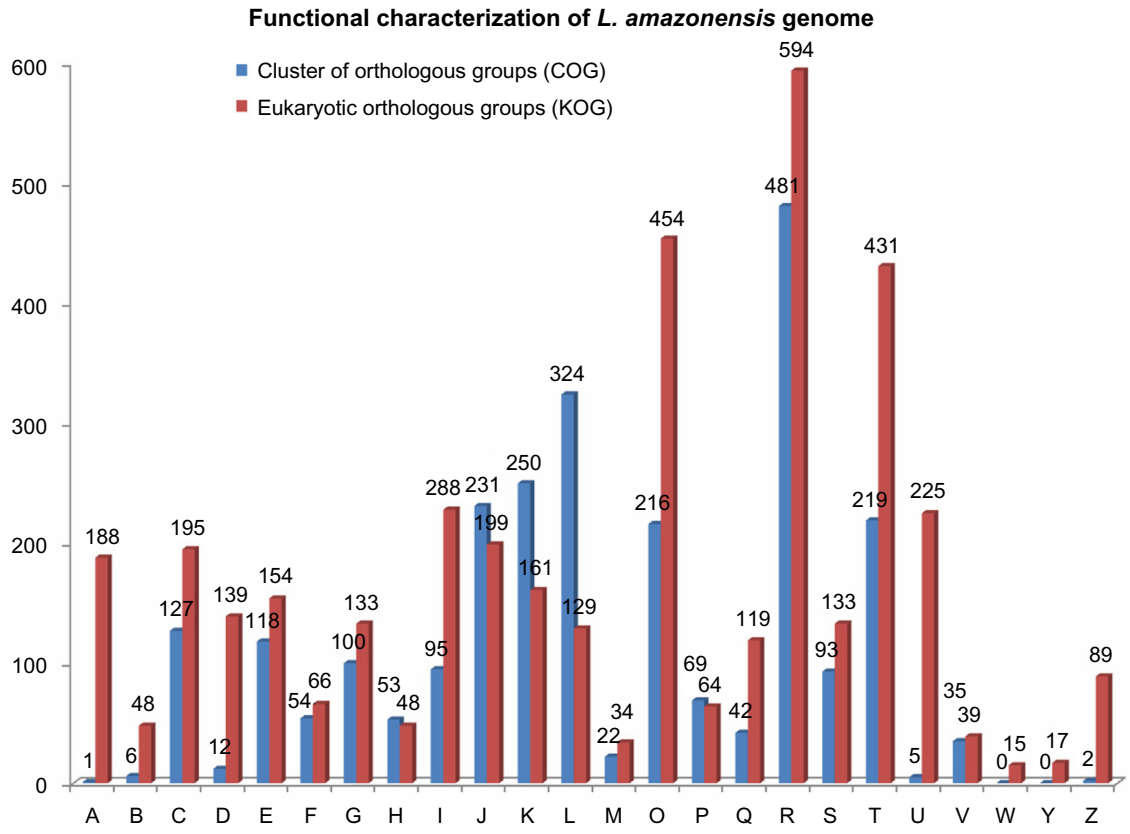


Figure 5. Functional category by KOG and COG for *Leishmania amazonensis* proteins INFORMATION STORAGE AND PROCESSING: [**J**] Translation, ribosomal structure and biogenesis, [**A**] RNA processing and modification, [**K**] Transcription, [**L**] Replication, recombination and repair, [**B**] Chromatin structure and dynamics. CELLULAR PROCESSES AND SIGNALING: [**D**] Cell cycle control, cell division, chromosome partitioning, [**Y**] Nuclear structure, [**V**] Defense mechanisms, [**T**] Signal transduction mechanisms, [**M**] Cell wall/membrane/envelope biogenesis, [**N**] Cell motility, [**Z**] Cytoskeleton, [**W**] Extracellular structures, [**U**] Intracellular trafficking, secretion, and vesicular transport, [**O**] Posttranslational modification, protein turnover, chaperones. METABOLISM: [**C**] Energy production and conversion,[**G**] Carbohydrate transport and metabolism, [**E**] Amino acid transport and metabolism, [**F**] Nucleotide transport and metabolism, [**H**] Coenzyme transport and metabolism, [**I**] Lipid transport and metabolism, [**P**] Inorganic ion transport and metabolism, [**Q**] Secondary metabolites biosynthesis, transport and catabolism POORLY CHARACTERIZED: [**R**] General function prediction only, [**S**] Function unknown.

breaks or inversions. The red line connecting the *L. mexicana* (upper) and *L. amazonensis* sequences (bottom) represents a good match (more than 92% of identity), and 99.87% of the *L. amazonensis* sequences (8,541/8,552) have a good match with the *L. mexicana* sequence. Only three *L. amazonensis* sequences (succinyl-coa:3-ketoacid-coenzyme a transferase-like protein and two unspecified products) did not match with *L. mexicana* chromosome sequence.

Discussion

The present assembly of the *L. amazonensis* genome resulted in 29,670,588 bases, consisting of 8,802 putative CDS with a GC content of 59% for the contigs and 61.12% for the CDS, while Real and colleagues[22] found a genome size of 29.6 Mb consisting of 8,168 putative genes with a GC content of 58.5% for the genome and 61% for the CDS. Thus, our results are similar and complementary to those obtained by Real and colleagues.[22]

L. amazonensis contains multiple copies of different genes that encode proteins such as ABC transporter and calpain-like cysteine peptidase (Table 2). Fifty copies of ABC transporter were annotated in *L. amazonensis*. This large number of copies is expected because the superfamily of ABC transporters is one of the largest families of proteins found in eukaryotes,[58,59] and these genes are important in Leishmania because they are involved in drug resistance, infectivity, and are related to treatment failure.[59–61] In *L. major* and *L. infantum*, 42 ABC transporter genes were described; *T. cruzi* and *T. brucei* have 28 and 22 copies, respectively.[59,60] Besides the 50 ABC copies annotated by us in *L. amazonensis*, we observed 33 copies in *L. mexicana*. Possibly, some ABC transporter genes in *L. amazonensis* may be incomplete and the number overestimated because of the presence of the same gene on multiple contigs. Fourty-four calpains were found in *L. amazonensis*. Mottram and colleagues[62] found 27 in *L. major*, and Ersfeld and colleagues[63] found 24 copies in *T. cruzi* and 18 copies in *T. brucei*. Calpains are involved in the remodeling of cytoskeletal or membrane attachments and have been found mostly in invertebrates and lower eukaryotes. The importance of cytoskeleton remodeling during *Leishmania* spp. differentiation may explain the high number of Calpain genes in these parasites.[62,63]

Table 5. Intergenomic NISEs, their official enzyme names, sequences IDs, Uniprot IDs for human sequences, PDB structures and the identity for each sequence.

EC	ENZYME NAME (OFFICIAL)	ORGANISM	SEQUENCES IDs (*)	UNIPROT ACCESS	PDB [BEST HIT] (**)	IDENTITY [PDB]
1.1.1.2	Alcohol dehydrogenase (NADP(+))	*L. amazonensis*	LAJMNGS050H11.b.7960	N/A	1UUF	160/332 (48%)
		H. sapiens	hsa:10327	P14550	2ALR	Structure solved
1.3.1.34	2,4-dienoyl-CoA reductase (NADPH)	*L. amazonensis*	LAJMNGS010C07.b.1806	N/A	1PS9	294/730 (40%)
			LAJMNGS024B09.b.4107	N/A		198/658 (30%)
		H. sapiens	hsa:1666	Q16698	1W6U	Structure solved
			hsa:26063	Q9NUI1	4FC6	Structure solved
1.3.1.74	2-alkenal reductase	*L. amazonensis*	LAJMNGS036G08.b.6014	N/A	4GBY	139/482 (29%)
		H. sapiens	hsa:22949	Q14914	1ZSV (+)	Structure solved
2.7.4.2	Phosphomevalonate kinase	*L. amazonensis*	LAJMNGS005E09.b.95	N/A	N/A	N/A
		H. sapiens	hsa:10654	Q15126	3CH4	Structure solved
				Q6FGV9		
3.1.11.2 (Predicted NISE)	Exodeoxyribonuclease III	*L. amazonensis*	LAJMNGS001G08.b.166	N/A	N/A	N/A
		H. sapiens	hsa:5810	O60671	3G65 (+)	Structure solved
			hsa:5883	Q99638	3GGR (+)	Structure solved
			hsa:11219	Q9BQ50	1Y97	Structure solved
			hsa:11277	Q9NSU2	3U6F	178/304 (59%)
				Q5TZT0		
5.3.3.2	Isopentenyl-diphosphate Delta-isomerase	*L. amazonensis*	LAJMNGS034G09.b.5743	N/A	2ZRU	118/352 (34%)
		H. sapiens	hsa:91734	Q9BXS1	2PNY	Structure solved
			hsa:3422	Q13907	2ICJ	Structure solved

Notes: (*) The sequences IDs from *H. sapiens* are from KEGG database. (**) The (+) signal on "PDB [Best hit]" column represent that there are more structures solved for this sequence.

Calpain is essential for the parasite and has a great potential for drug target. It was demonstrated that MDL 28170, a calpain inhibitor, showed a high antileishmanial activity against *L. amazonensis*.[64] The knowledge of these calpain sequences may help future studies on drug design.

Other interesting genes found were tuzins and amastins. Eight tuzin copies were found in the *L. amazonensis* genome with a moderate diversity. For comparison, *L. mexicana* and *L. tarentolae* have 4 copies,[65] *L. infantum* 6, and *L. major* the highest diversity with 28 copies.[18] Among the tuzin copies in *L. amazonensis*, one copy forms an ortholog group only with *L. mexicana* (Table 4), given the fact that *L. amazonensis* and *L. mexicana* belong to the same taxonomic complex.[66,67] Amastins belong to a large family of surface proteins unique

Table 6. Intragenomic NISEs, their official enzyme names, sequences IDs, PDB structures identified and the identity for each sequence.

EC	ENZYME NAME (OFFICIAL)	ORIGINAL ANNOTATION	SEQUENCES IDs: *L. AMAZONENSIS* (*)	PDB [BEST HIT]	IDENTITY [PDB]
4.2.1.1	Carbonate dehydratase	carbonic anhydrase-like protein	LAJMNGS019E05.b.3366	4G7A	53/164 (32%)
	Carbonate dehydratase	carbonic anhydrase family protein, putative	LAJMNGS035D05.b.5816	1I6O	97/229 (42%)
4.2.99.18	DNA-(apurinic or apyrimidinic site) lyase	endonuclease III, putative	LAJMNGS002A05.b.218 (2)	1P59	66/194 (34%)
	DNA-(apurinic or apyrimidinic site) lyase	endonuclease/exonuclease protein-like protein	LAJMNGS041H02.b.6678 (2)	2ISI	37/106 (35%)
5.4.2.1	Phosphoglycerate mutase	phosphoglycerate mutase protein, putative	LAJMNGS013E01.b.2299	4IJ5	45/152 (30%)
	Phosphoglycerate mutase	2,3-bisphosphoglycerate-independent phosphoglycerate mutase,2,3-bisphosphoglycerate-independentphosphoglyceratemutase	LAJMNGS025H05.b.4375 (2)	3IGY	497/552 (90%)

Notes: (*) The numbers between parenthesis on "Sequences IDs: *L. amazonensis*" column, represent the number of copies of this enzyme.

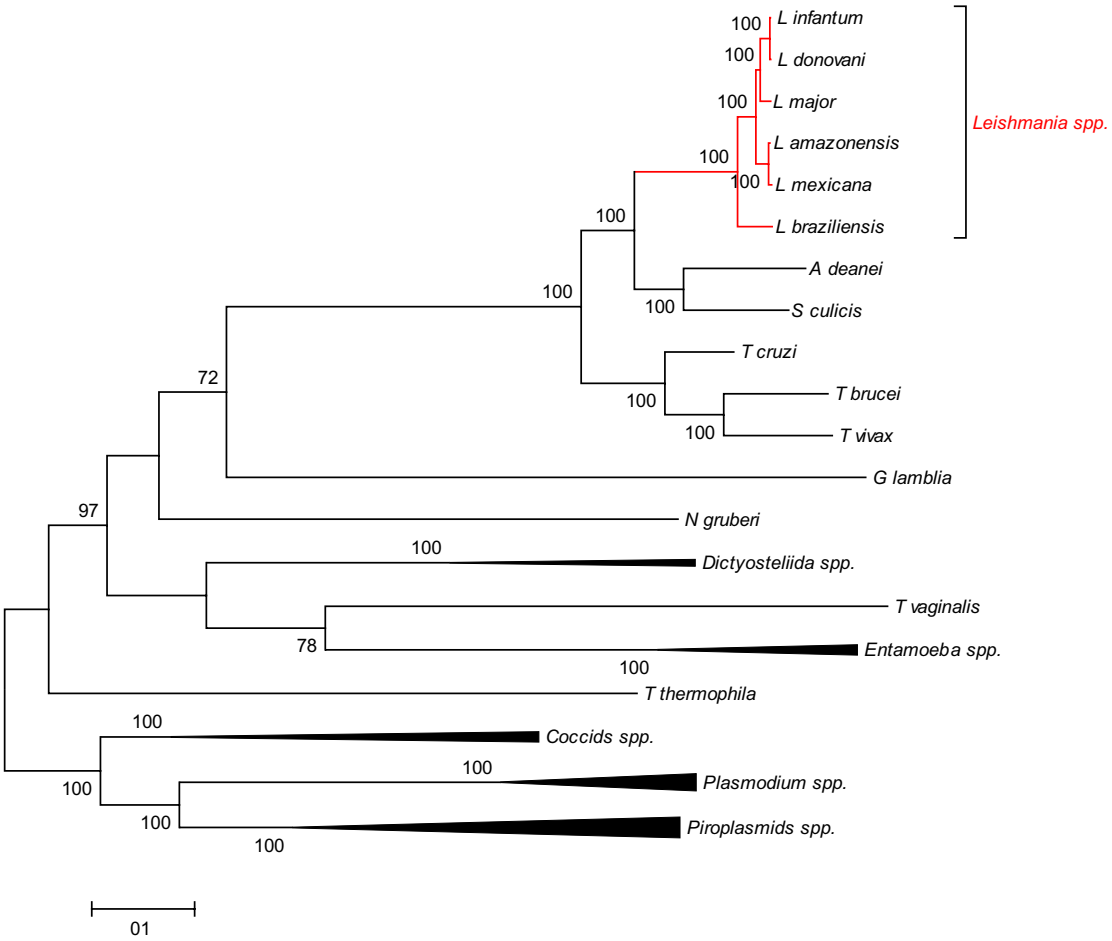


Figure 6. Phylogenomics analysis tree for all six *Leishmania* species (in red) and for other 28 protozoa species, inferred by Maximum Likehood with 1,000 boostrap replicates, based on thirty-one universal orthologous (UO) genes. Name and legend of the 34 species: *Angomonas deanei* (A deanei), *Strigomonas culicis* (S culicis), *Leishmania amazonensis* (L amazonensis), *Leishmania braziliensis* (L braziliensis), *Leishmania donovani* (L donovani), *Leishmania infantum* (L infantum), *Leishmania major* (L major), *Leishmania mexicana* (L mexicana), *Trypanosoma brucei* (T brucei), *Trypanosoma cruzi* (T cruzi), *Trypanosoma vivax* (T vivax), *Giardia lamblia* (G lamblia), *Naegleria gruberi* (N gruberi), Dictyosteliida spp.: *Dictyostelium discoideum* and *Polysphondylium pallidum. Trichomonas vaginalis* (T vaginalis), Entamoeba spp.: *Entamoeba dispar, Entamoeba histolytica* and *Entamoeba invadens*. *Tetrahymena thermophila* (T thermophila), *Plasmodium spp.*: *Plasmodium berghei, Plasmodium cynomolgi, Plasmodium falciparum, Plasmodium knowlesi* and *Plasmodium vivax*. *Coccids spp.*: *Cryptosporidium muris, Neospora caninum* and *Toxoplasma gondii*. Piroplasmids spp.: *Babesia bovis*, *Babesia equi*, *Babesia microti, Theileria annulata, Theileria orientalis and Theileria parva*.

to kinetoplastids, which are expressed specifically in the amastigote stage of the parasite.[68] Among the 14 amastin copies found in *L. amazonensis*, 10 of them are found in 8 orthologous genes shared with the other 5 analyzed species (*L. braziliensis*, *L. infantum*, *L. major*, *L. mexicana*, and *L. donovani*), while only 1 copy is shared between *Leishmania* subgenera. *L. mexicana* shares two amastin genes exclusively with *L. amazonensis*, which has a total of 28 genes of this family. These results are expected because amastin family has four subfamilies, among which we found some copies more conserved and other more divergent, explaining the fact that we found some copies shared among LCP, or only within the mexicana complex, that may be more specific subfamilies.[68,69] Other studies have demonstrated that amastin satisfies some antigenic criteria and is used for epitopic analysis,[70] suggesting the use as relevant biomarker for the VL serodiagnosis.[71] Despite the fact that *L. amazonensis* and *L. mexicana* belong to the same complex,[66] they show differentiated epidemiology. It is interesting to note the presence of an amastin that could be used as a marker for the VL, shared only by these two species. It is known that *L. amazonensis* rarely causes VL[72], while *L. mexicana* can visceralize.[73] Rogers and collaborators[21] found a unique gene in *L. mexicana* that encodes a protein of unknown function that contains a predicted kelch actin binding domain (Pfam: PF01344). In our work, we found a hypothetical protein shared only by *L. amazonensis* and *L. mexicana*, which also contains a predicted kelch actin binding domain, with the same Pfam (PF01344) mapped, reinforcing the proximity between these two species, since these proteins were not found in the remaining four *Leishmania* species analyzed in this study.

L. braziliensis presented the highest number of paralogous genes (15), similar to the results of Peacock and colleagues[18] and Rogers and colleagues.[21] Genes related to telomerase

Table 7. RNAi pathway related sequences in *L. amazonensis*.

Piwi-AGO
LAJMNGS037G03.b.6124
Tudor
LAJMNGS051A10.b.7989
ERI-1
LAJMNGS009D01.b.1653
LAJMNGS023D01.b.3956
LAJMNGS034E11.b.5717
LAJMNGS035F02.b.5853
DEAD-Box RNA helicase
LAJMNGS002E10.b.336
LAJMNGS005H09.b.1043
LAJMNGS016A07.b.2785
LAJMNGS018H11.b.3270
LAJMNGS021E12.b.3675
LAJMNGS024C05.b.4124
LAJMNGS042E06.b.6755
LAJMNGS045F10.b.7200
LAJMNGS046A05.b.7244
Ribonuclease III/Dicer
LAJMNGS020H09.b.3587
LAJMNGS021A10.b.3613

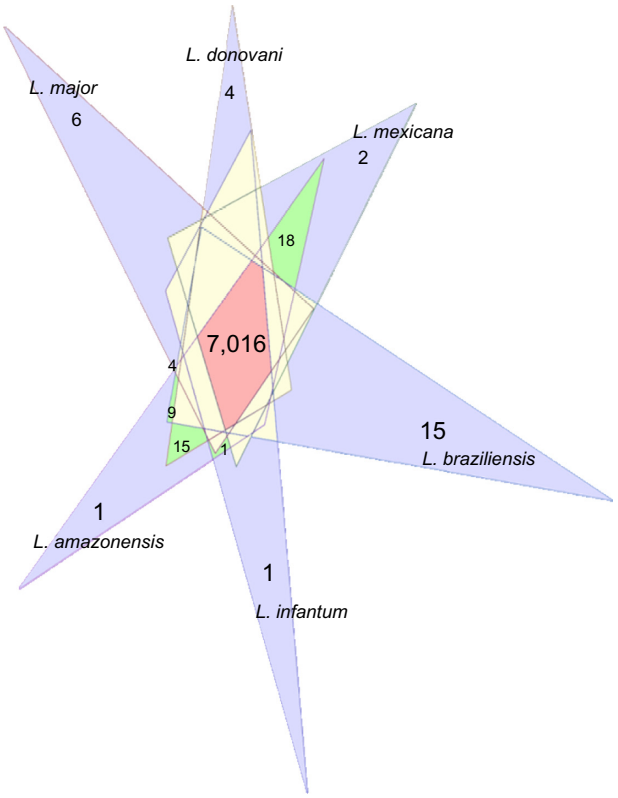


Figure 7. Comparative analysis of species Leishmania using orthologous and paralogous protein groups generated by OrthoMCL. The colors represent the number of protein shared between the species. blue (intern paralogous into specie); green orthologous groups between 2 species (*L. amazonensis and L. mexicana*: 18; *L. amazonensis* and *L. donovani*: 15; *L. amazonensis* and *L. braziliensis*: 9; *L. amazonensis* and *L. major*: 4; *L. amazonensis* and *L. infantum*: 1); and red: 7026 orthologous groups shared between all six *Leishmania* species. Orthologous groups shared between 3, 4 and 5 species are yellow.

activity and transposons were found such as TATE DNA transposon, SLACS-like gene retrotransposon element, which we know are unique compared with the other five species of *Leishmania* examined, including the recently sequenced *L. amazonensis* presented in our study. Another notable difference is that *L. braziliensis* contains a functional putative RNAi pathway, absent in *L. major*, *L. tarentolae*,[65] and *L. amazonensis* (Fig. 11). We also found some highly divergent copies of surface protein in *L. braziliensis*, not shared with the other *Leishmania* species analyzed, such as GP63, amastin, and surface antigen-like protein, corroborating previous studies.[18,21] It is known that GP63 protein is involved in *Leishmania* virulence,[74] and its function is host cell binding, conferring parasite protection from complement-mediated lysis.[18] Interestingly, some studies showed that GP63 is under positive selection,[75,76] and this incentive for changes may contribute to the functional variations of GP63 protease. It has been also described that GP63 is encoded by repeated gene cluster that seems to be enlarged fourfold in *L. braziliensis* compared with the Old World *Leishmania*.[18,75] *L. braziliensis* has 39 genes encoding GP63, while in *L. amazonensis* and *L. mexicana* only 7 genes were found. Curiously, even adding the previously published proteome of *L. donovani*[77] and the newly generated *L. amazonensis*, unique genes in *L. braziliensis* remained, although *L. amazonensis* and *L. braziliensis* have a similar geographical distribution. Only the distribution is similar in these species, once they have different vectors, have different clinical manifestations, and belong to different subgenera.[78] This corroborates the similarity results between studies, besides being the most divergent species in these studies.[77,79] We found one highly divergent inparalog gene in *L. infantum*, amastin. Rogers and colleagues[21] found 19 highly divergent inparalogs. Our study corroborated the presence of amastin, which has a unique highly divergent subfamily of the genus *Leishmania*. Furthermore, some amastin sites were found to be under positive selection,[69] which may explain the presence of this single paralog in *L. infantum*. Comparatively, we found a smaller number of *L. infantum* paralogs than those in previous studies.[18,69] These two studies compared *L. infantum* with *L. major*; however, the average amino acid identity between *L. infantum* and *L. major* is 92%,[18] and Downing et al.[77] show that *L. infantum* and *L. donovani* species are much closer than *L. infantum*/*L. major*, belonging to the same complex (*L. infantum*/*L. donovani* or Donovani complex).[80] Another study carried out with HSP70[81] demonstrated that these two species (*L. infantum*/*L. donovani*) are phylogenetically close, with *L. braziliensis* being more

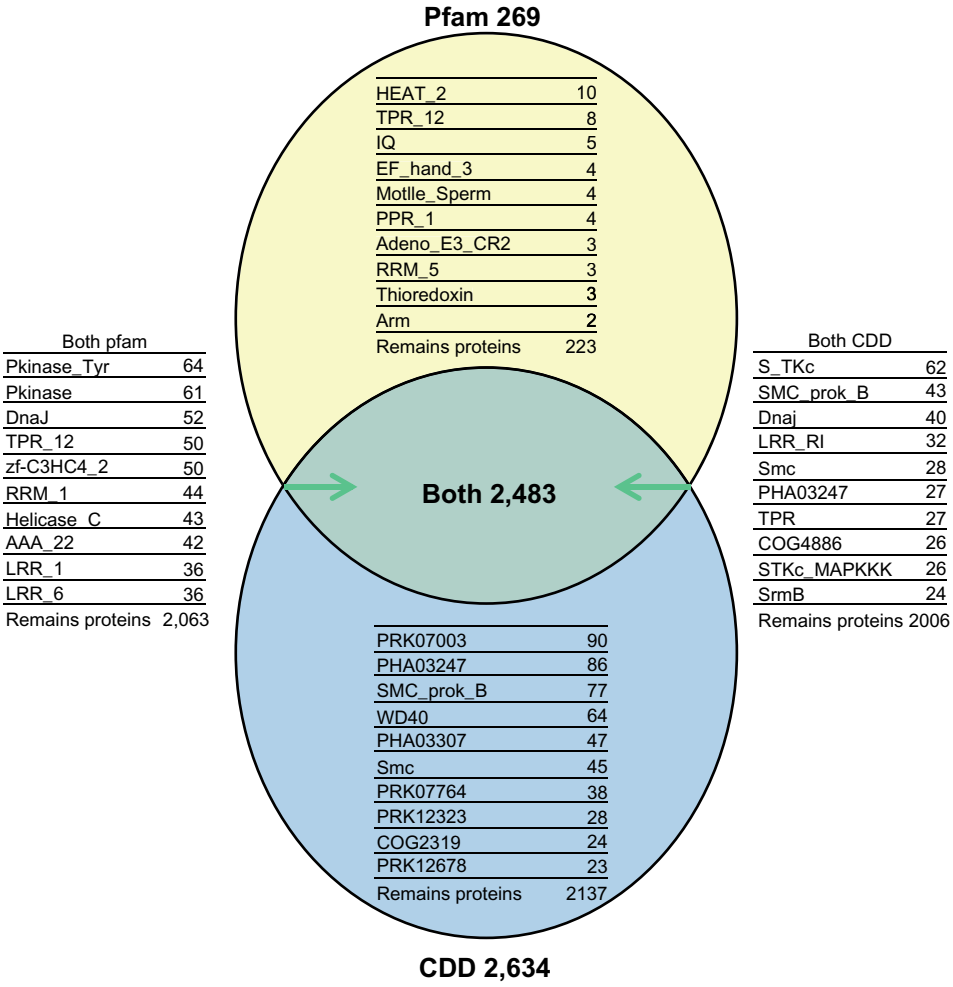


Figure 8. In green area, a total of 2,483 *L. amazonensis* proteins identified by both Conserved Domains Database (RpsBlast-CDD) and Protein Families Database (HMMER-PFam). In lateral tables we visualize most frequents Families (Pfam) and Domains (CDD). 269 proteins were identified only by Protein Families Database, and inside yellow area we show 10 most frequent families found by Pfam. A total of 2,634 proteins were identified only by Conserved Domains Database (CDD), and in blue area the 10 most common domains assigned by CDD in *L. amazonensis*.

divergent inside the genus. Since no comparative studies were carried out between *L. infantum* and *L. donovani* with the aim of identifying unique genes, the number of unique *L. infantum* genes (26) may be underestimated.[18] However, in our study the analysis of these two closely related species showed that they share 25 orthologs, and only one highly divergent paralog, amastin like, was found in *L. infantum*. The fact that *L. infantum* belongs to the same complex of *L. donovani* and they share 7,619 orthologous groups (93.5% of the *L. infantum* proteome), while with *L. braziliensis* it shares 7,401 (90.8% of its proteome) orthologs, could explain this scenario. Similarly, in *L. amazonensis*, only one inparalog found is probably because it belongs to the same *L. mexicana* complex and is very close to this species. For example, *L. amazonensis* and *L. mexicana* share several orthologous, 7,380 in total (85.8%), whereas *L. amazonensis* and *L. braziliensis* share 7,162 groups (83.3% of *L. amazonensis* proteome). As expected, we noted that the closer species have greater number of genes shared by them. This is especially true when comparing species within the same complex that shows the higher number of shared genes. Some large gene families present in *L. amazonensis* may have only one conserved domain in common. The remaining of their sequences is so divergent that subfamilies or classes are identified. As an example, we can mention amastin, which is found in all the six *Leishmania* species analyzed that present the signature C-[IVLYF]-[TS]-[LF]-[WF]-G-X-[KRQ]-X-[DENT]-C; however, some amastin genes are so divergent that they can be classified into four subfamilies or classes: α, β, γ, and δ.[69]

Phylogenomics

Mauricio et al.[80] used the gene mspC3 as a marker to reconstruct a phylogeny of species of *Leishmania* subgenus, and the results were very close to the ones found in this study, keeping *L. infantum* and *L. donovani* in the same branch, with *L. major* and *L. mexicana* more distant, which was expected since these species, *L. infantum* and *L. donovani*, belong to the same complex.[82] However, in this study of Mauricio and colleagues,[80] it was not possible to observe the separation of the subgenus *Leishmania* in New and Old World species. Differently, Simpson and

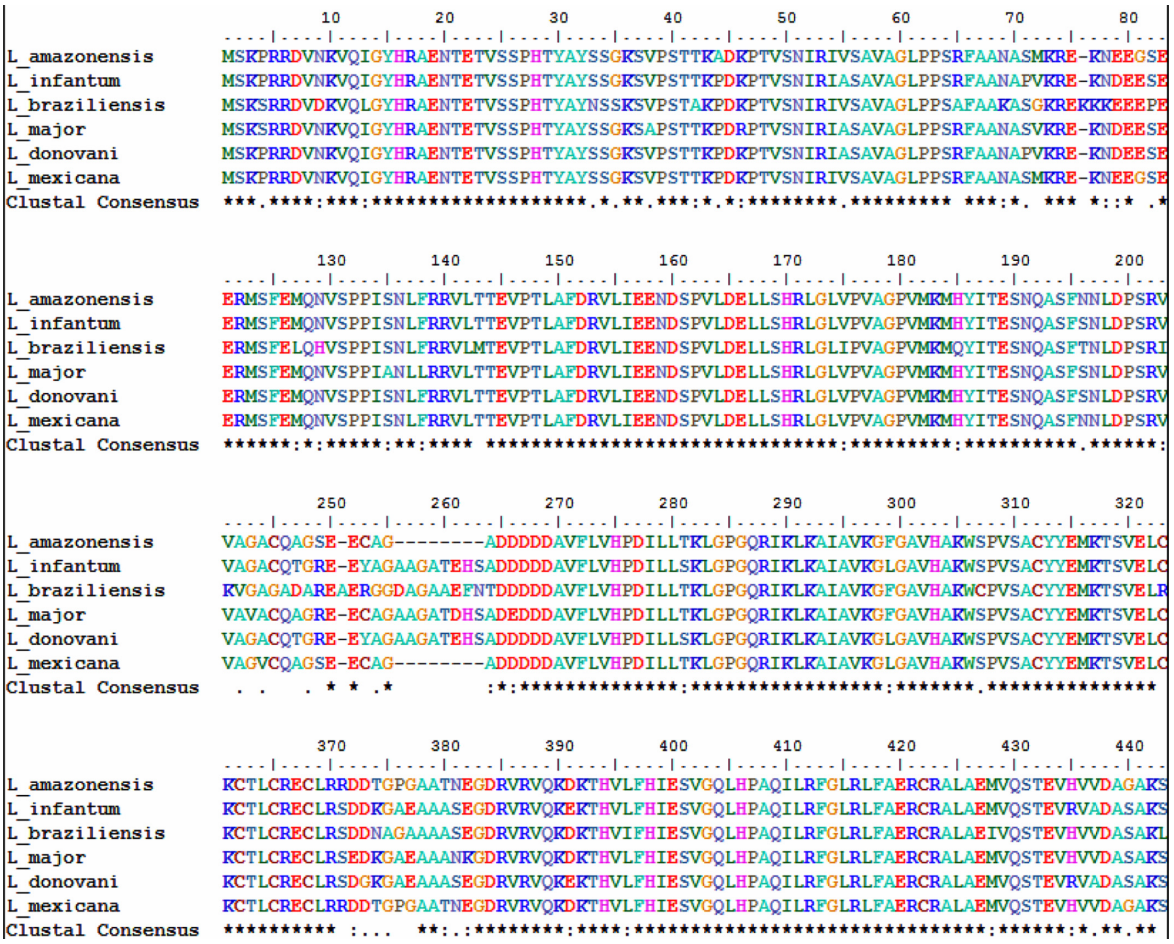


Figure 9. Alignment of DNA-directed RNA polymerase, alpha subunit, sequences between the 6 *Leishmania* species. We color-coded the sites with identical residues with the same color, and used asterisks to indicate the conserved residues in all species.

colleagues[81] achieved a reasonable and consistent separation of the subgenera *Viannia* and *Leishmania* using HSP70 genes, as well as the division of the Old and New World *Leishmania* species inside *Leishmania* subgenus. Mauricio and colleagues[75] using the GP63 gene, which is a multicopy gene under positive selection,[75,76] achieved a good separation between the subgenus and could identify those species originating from the New or Old World, although depending on which copy of the gene is used for classification, the results may be distinct. *L. donovani* complex formation (*L. donovani* and *L. infantum*) was constant in the majority of studies, as well as the formation of *L. mexicana* complex, besides the correct separation of subgenus, and within the *Leishmania* subgenus the separation between Old and New World species.[77,80,81,83–85] Fraga and colleagues[85] used the HSP20 gene to separate the Old/New World *Leishmania* subgenus, with a bootstrap of 89. However, when HSP20 and HSP70 genes were concatenated, the bootstrap support value of this separation improved up to 99 and 100 to support the division of the subgenus. This example demonstrates the advantage of concatenating genes to infer phylogenomic-based species trees. Our approach of species tree by using 31 UO genes and phylogenomic-based approach was robust showing a bootstrap support of 100 for the Kinetoplastida clade. This analysis showed the expected separation of this genus for all six species analyzed: *L.* (*V.*) *braziliensis* as outgroup, and *L. (L.) infantum* and *L.* (*L.*) *donovani* very close, reflecting the complex formed by them. As expected, this complex is closer to *L.* (*L.*) *major* recapitulating phylogeny of the Old Word species inside *Leishmania* subgenus. Nevertheless, *L. mexicana* and *L. amazonensis* are placed together on the same clade, reflecting the Mexicana complex, corroborating classical phylogeny. It should be noted that the bootstrap values were higher than that observed in other works (bootstrap value 100), although the taxonomic position of these species remained mostly the same.[75,77,80,81,84,85] Although the *L. amazonensis* taxonomic position is already known, the phylogenomic species tree obtained using 31 UO genes proved to be a good approach for robust species tree inference using multiple genes, and also a good option to avoid the bias of extrapolating single-gene phylogenies. Another interesting point was that our phylogenomic tree recapitulated the Kinetoplastida monophily and its correct separation.[81]

Intergenomic and Intragenomic NISE as Possible Drug Targets

This work identified a set of NISE (also known as analogous enzymes) between *L. amazonensis* and *H. sapiens* (Supple-

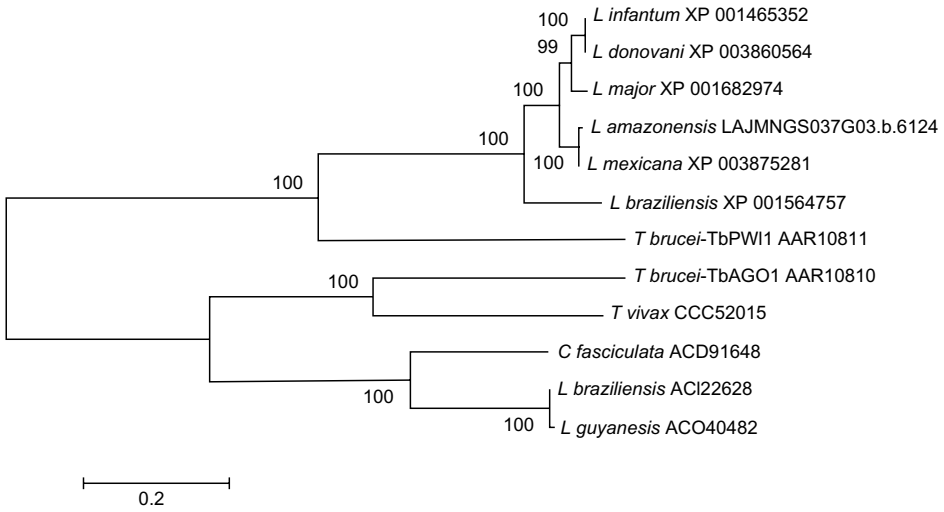


Figure 10. Phylogenetic relationship among argonaute-like genes in Trypanosomatids, constructed by Neighbor-Joinning with 1,000 bootstrap replicates.

mentary Table S2), and inside the proteome of *L. amazonensis* (Supplementary Table S3). Such enzymes display the same functional activity, but are unrelated from an evolutionary point of view, since no significant similarity is found either between their primary sequences or between their tertiary structures, which indicates different ancestries. These structural differences found between NISE could be exploited for the design of drugs that would be active against the parasite's enzyme, but with no effect to the host's enzyme. NISE may therefore represent a rather unexploited gene reservoir for the identification of potential drug targets. In fact, some drug targets found in study, such as trypanothione-disulfide reductase, present analogy between the enzyme of *L. donovani* and the enzyme of *H. sapiens*.[82,86]

Among the list of intergenomic NISE identified in this work (Tables 5 and S2), a few interesting cases are worth to be mentioned, such as phosphomevalonate kinase (EC: 2.7.4.2), exodeoxyribonuclease III (EC: 3.1.11.2), and isopentenyl-diphosphate delta-isomerase (EC: 5.3.3.2). Exodeoxyribonuclease III participates in DNA repair,[87] a very important activity for the survival of the organism. Actually, exodeoxyribonuclease III has already been proposed as a drug target candidate against TriTryps[45] and cancer.[88] The two other enzymes are involved in the isoprenoid biosynthetic pathway, a chemically diverse pathway responsible for the production of a very large number of natural metabolites such as sterols, carotenoids, dolichols, and ubiquinones, and some important classes of prenylated proteins, such as phosphomevalonate kinase, which is involved in the biosynthesis of isopentenyl diphosphate (IPP), the building block of all isoprenoids, while IPP isomerase is a key enzyme that catalyzes an essential activation step in isoprenoid biosynthesis by isomerization of the carbon–carbon double bond of IPP to create its electrophilic allylic isomer dimethylallyl diphosphate (DMAPP). Inhibition of this pathway offers potential for the development of antibiotics against bacteria[89] and *P. falciparum*.[90] Relevant information about other enzymatic activities is scarce, particularly when considering trypanosomatids. An example is 2-alkenal reductase (EC: 1.3.1.74). A defensive role has been shown for this enzyme in some plants, apparently by protecting them from oxidative stress by catalyzing the reduction of reactive carbonyls,[91,92] but no information about its biological role has been found for trypanosomatids.

On the other hand, the identification of NISEs inside *L. amazonensis* proteome (intragenomic NISE) could provide new insights about alternative biochemical pathways and the meaning of functional redundancy inside a genome. Among the NISEs inside *L. amazonensis* proteome (Tables 6 and S3), carbonate dehydratase (EC 4.2.1.1), DNA-(apurinic or apyrimidinic site) lyase (EC 4.2.99.18), and phosphoglycerate mutase (PGAM) (EC 5.4.2.1) could be proposed as potential drug targets. Carbonate dehydratase catalyzes the interconversion of CO_2 and HCO_3^-. This enzymatic function is present in animals, plants, yeast, archaea, bacteria, and parasites.[93] Studies have proposed this enzyme as a candidate drug target in *P. falciparum* since the inhibition of this enzyme affects the pathway of pyrimidine biosynthesis.[93,94] DNA-(apurinic or apyrimidinic site) lyase is involved in the repair of abasic sites caused by oxidative stress and external agents (chemical or physical), with spontaneous hydrolysis resulting in purine or pyrimidine loss.[95] PGAM catalyzes the interconversion of 2-phosphoglycerate (2PG) and 3-phosphoglycerate (3PG) in the glycolytic and gluconeogenic pathways. PGAM was structurally characterized in *L. mexicana*, and has been proposed as a possible drug target, since the enzymatic form in the parasite is structurally different from the host and has different properties,[96] an earlier example of analogy found by an experimental approach.

An integrative approach will be employed in the future to obtain a broader understanding of the biological role of the intergenomic and intragenomic NISE detected in this work.

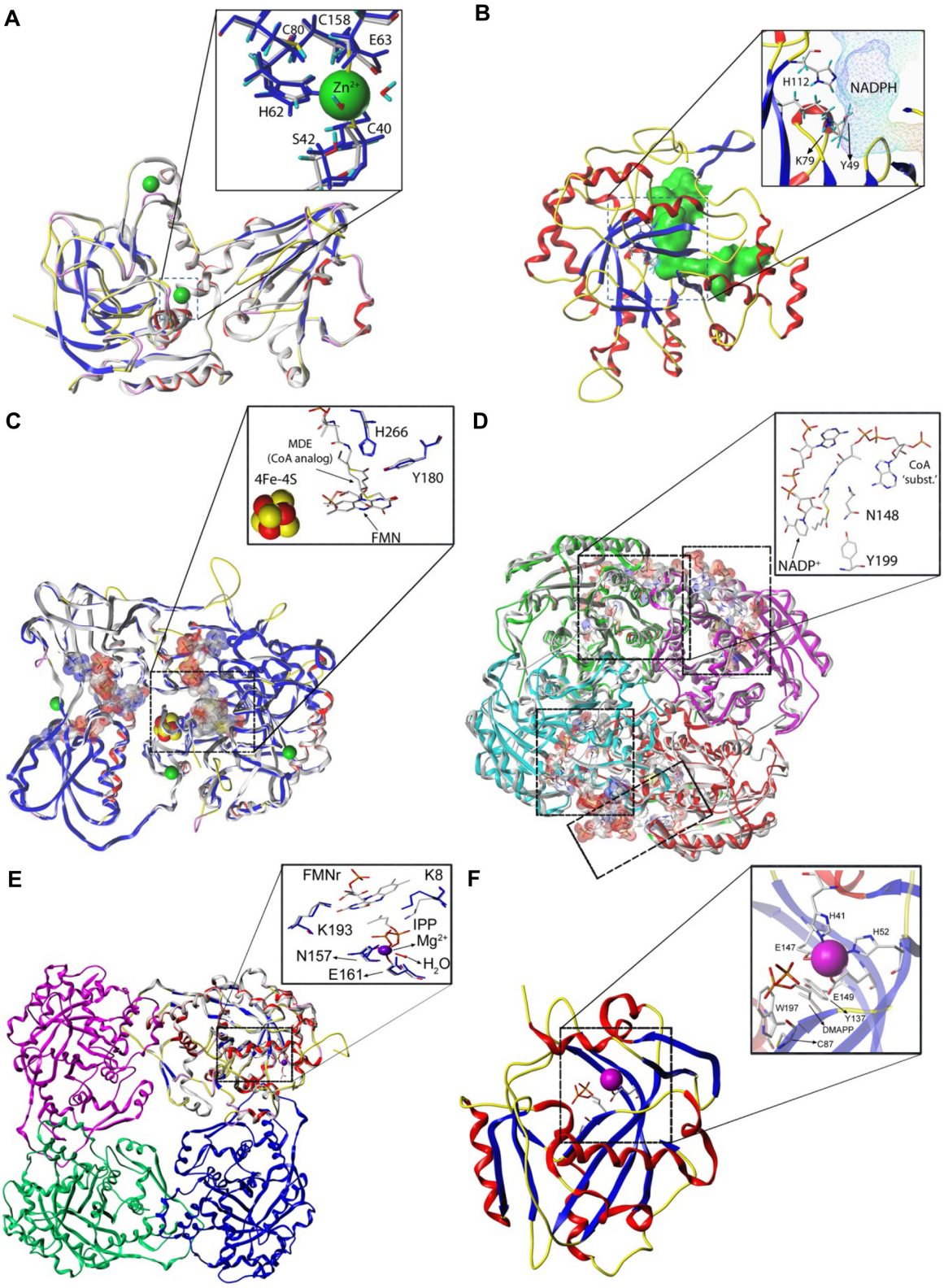


Figure 11. Structural comparison of selected intergenomic NISE cases between *L. amazonensis* and Human. Top panel (EC 1.1.1.2): A LAJMNGS050H11.b.7960 "putative NADP-dependent alcohol dehydrogenase" from *L. amazonensis* (**A**) and human Aldehyde reductase (PDB 2ALR) (**B**). Middle panel (EC 1.3.1.34): LAJMNGS010C07.b.1806 "putative 2,4-dienoyl-coa reductase FADH1" from *L. amazonensis* (**C**) and human mitochondrial 2,4-dienoyl-CoA reductase (PDB 1W6U) (**D**). Bottom panel (EC 5.3.3.2): LAJMNGS034G09.b.5743 "putative isopentenyl-diphosphate delta-isomerase" from *L. amazonensis* (**E**) and human Isopentenyl-diphosphate Delta-isomerase (PDB 2ICK) (**F**). Models for all proteins are presented as ribbons. Parasite proteins are colored by secondary structure and presented superposed on their templates (gray ribbons) used in homology modeling . Human analogs are colored by secondary structure, except for 1 W6U, which is colored by chain and presented superposed on the peroxisomal isoform (PDB) shown as gray ribbons. The insets show details of the proposed catalytic residues and co-factors for each analogous enzyme. Residues colored blue belong to the parasite enzymes while residues from human analogs are color-coded by atom type.

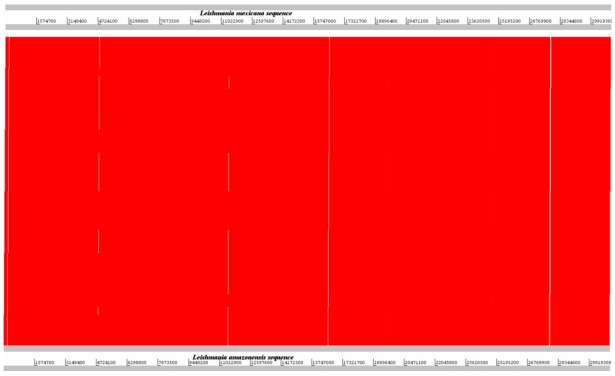


Figure 12. Comparison result between *L. mexicana* and *L. amazonensis*. Synteny map of *L. mexicana* (top) compared to *L. amazonensis* (bottom). The red lines connect the sequences and are proportional to sequence identity, the darker the more similar are the sequences. The scale and numbers represents nucleotide position on the genome/chromosome.

RNAi Machinery

One of the first organisms where functional RNAi pathway was described was *T. brucei*.[97] Since then, several trypanosomatids were subject to RNAi characterization through direct analysis or genome sequencing.[18,98,99] RNA silencing pathways play critical roles in gene regulation, virus infection, and transposon control. RNAi is mediated by small interfering RNAs (siRNAs), which are liberated from double-stranded (ds) RNA precursors by Dicer and guide the RISC to degenerate sequence-specific mRNA targets. Phylogenetic analysis suggests the presence of the RNAi pathway in the last common ancestor of eukaryotes with putative important role in defense responses against genomic parasites such as transposable elements and viruses.[100]

RNAi pathway-related genes present in different trypanosomatids[99] were used to identify orthologous genes in *L. amazonensis* genome (Table 7). A key step of RNAi pathway is the Dicer activity, which converts dsRNA into siRNA. Dicer was identified in *T. brucei* (Tb927.8.2370) and a protein with a similar architecture domain, bearing the two RNAse III-like domains, and was characterized in *L. braziliensis* (LbrM23_V2.0390). Such proteins seem to be missing in trypanosomatids that lack a functional RNAi pathway, such as *T. cruzi* and *L. major*.[101] Genomic analysis of *L. infantum*, *L. braziliensis*, and *L. major* has demonstrated the presence of Dicer only in *L. braziliensis* and, otherwise, shows synteny for the other *Leishmania* species.[18] We were unable to detect Dicer in *L. amazonensis* genome or in any sequence bearing the characteristic Rnc (dsRNA-specific ribonuclease) domain of *L. braziliensis* putative Dicer gene. Since Dicer activity might be performed by a combination of different proteins bearing typical RNAi domains such as DEAD-box RNA helicase and Ribonuclease III,[18] such domains were subject of analysis in *L. amazonensis* genome data set. Nine DEAD/H box RNA helicase and two Ribonuclease III were identified in *L. amazonensis,* with putative relationship to RNAi pathway (Table 7). Although Dicer was not identified, some Dicer-related genes were characterized. ERI proteins are another important components of RNAi pathway involved in the formation of the ERI/DICER complex.[102] We were able to identify four ERI sequences in *L. amazonensis* genome data set (LAJMNGS009D01.b.1653, LAJMNGS023D01.b.3956, LAJMNGS034E11.b.5717, and LAJMNGS035F02.b.5853) (Table 7). Two genes of the RISC (a major effector complex of the RNAi pathway) were also identified: tudor and piwi (argonaute family) (Table 7). Several argonaute family genes have been described in trypanosomatids. In *T. brucei*, two argonaute-like genes were identified (TbAGO1 and TbPWI1). Both forms are expressed in the procyclic culture stages but only TbAGO1 is involved in RNAi.[103] Previous data have demonstrated the presence of RNAi key genes argonaute and/or Dicer in *Leishmania* subgenus *Viannia* (*L. braziliensis*, *L. guyanensis*, and *L. panamensis*) but not in the subgenus *Leishmania* (*L. mexicana*, *L. major*, and *L. donovani*).[99]

Here we describe the first evidence through genomic analysis of RNAi pathway absence in *L. amazonensis*. So far, experimental evidences pointed out the absence of a functional RNAi pathway in the whole subgenus *Leishmania*,[18,99] corroborated by the analysis of *L. amazonensis* genome data set. Several arguments have been elegantly raised by Lye et al.[99] in an attempt to understand this phenomena; they describe the viral infections, genome plasticity, and phenotype selection as the major players of RNAi lost event. The identified sequences related to RNAi pathway in *L. amazonensis* might reflect the remains of an erstwhile ancient functional RNAi pathway. Hypothetically, the remaining functional genes might be present because of an association with different pathways required for parasite survival. It might be the case of ERI sequences where its dual role in rRNA processing and RNAi[104] might have prevented its loss.

Comparative genome analysis shows that, most likely, the last common eukaryote possesses two copies of argonaute-related genes, suggesting the presence of two distinct silencing machineries. The argonaute-like proteins had possibly been involved in transcriptional regulation by targeting RNAm in cytoplasm, while piwi-like proteins would act in nucleus targeting transposons.[105] In contrast to most eukaryotes, in which the argonaute duplication followed by functional diversification is common,[105] trypanosomatids have no more than one copy of each argonaute-like genes (ago and piwi) per species. Indeed, trypanosomatids with functional RNAi (*T. brucei*, *L. braziliensis*, *L. guyanensis*) have both genes; however, species with non functional RNAi pathway (*T. cruzi*, *L. amazonensis*, *L. major*, *L. mexicana*) possesses only the piwi version of the argonaute family.[18,99] The main difference in the protein domain architecture between the two argonaute families is the lack of a PAZ domain in piwi-like proteins.[105] The PAZ domain consists of two subdomains, with a oligonucleotide/oligosaccharide binding region which is responsible for 3′ ends ssRNA recognition typically found in 3′ overhangs of the siRNAs.[105] In early work on RNAi characterization in

trypanosomatids, two argonaute-like genes were identified in *T. brucei* termed TbAGO1 and TbPWI1.[103] After functional analysis, the authors showed that TbAGO1, but not TbPWI1, was involved in RNAi. *L. amazonensis* does not have the ago-like gene, and the piwi-gene (LaPWI1) is homologous to TbPWI1, with orthologs group in subgenus *Leishmania*. Recently, Padmanabhan et al.[106] identified putative functions for piwi-like gene in *L. infantum* and *L. major*. Similar to *T. brucei*, *Leishmania* piwi-like protein is neither related to RNAi pathway nor to siRNA biogenesis. Piwi-like gene is expressed in both parasite forms, but piwi mutation affects the amastigote infection delaying the pathology and increasing apoptosis susceptibility. The authors raised the hypothesis about piwi-like protein role: located in the parasite single mitochondrion, it might act as an apoptotic sensor.[106]

The absence of post-transcriptional control of the RNAi might help to explain also the differences observed among the *Leishmania* and *Viannia* subgenera related with pathogenicity in mammalian host, insect vector relationship, and distinct surface glycocalyx structure.[107,108]

Synteny Analysis

Comparisons of the *L. mexicana* and *L. amazonensis* genomes revealed that more than 99.5% of the genes were syntenic, as expected, since these two species were very close and belong to the same complex.[33,79,81,84] In fact, previous studies have described that the closer the species, the higher the degree of synteny between their genomes.[18,23,65] For instance, the work of El-Sayed et al.[23] showed that 94% of the genes (6,200 genes) that are conserved among the TriTryps are also syntenic, in spite of the fact that these three species are not as close as *Leishmania* spp. In another study, the analysis of the three related *Leishmania* species (*L. major*, *L. infantum* and *L. braziliensis*) revealed that more than 99% of the genes are syntenic among these species.[18] Likewise, the comparison of *L. tarentolae* to the three sequenced pathogenic *Leishmania* species[18] showed that these four species are highly syntenic.[65]

Conclusions

The *L. amazonensis* genome assembly resulted in approximately 29 million base pairs. The smallest contig had 96 bases and the largest 141,211 bases. The annotation resulted in 8,802 CDS, where the largest coding regions had 19,872 bases and the smallest only 66 bases, with a median and mean value of 1,637 and 1,209 bp, respectively. Of these *L. amazonensis* CDS, 63.1% (5,554/8,802) were annotated as "hypothetic protein" and 79.71% (7,016/8,802) were grouped into *Leishmania* spp. core proteome. Our work is the first to propose a *Leishmania* spp. core proteome using the six sequenced human-pathogenic *Leishmania*. Generally, the following housekeeping proteins were found within *Leishmania* spp. core proteome (LCP): 40S ribosomal protein S16, RNA helicase, protein kinase, dynein heavy chain, activated protein kinase c receptor (LACK), ABC transporter, calpain-like cysteine peptidase, and DNA primase. These LCP genes can be potentially explored as molecular markers, either for diagnosis or for the genotyping of *Leishmania* populations. Furthermore, some genes related to membrane surface were found: GP63, amastin, and tuzin. *L. amazonensis* and *L. mexicana* showed the largest number of specific shared orthologs (18), most of them without a defined function. However, divergent amastin-like protein and viscerotropic leishmaniasis antigen were found as an ortholog only between these two species, and it may be possible to use these as a complex marker. The specific *L. amazonensis*/*L. mexicana* orthologs are potential specific "mexicana complex" markers, since they are unique to these species. The orphans genes found can possibly be explored as markers for species-specific diagnosis, once they are uniquely present in these species. Our original phylogenomic tree confirmed the position of *L. amazonensis* as closer to *L. mexicana* and belonging to the New World *Leishmania* subgenus. In addition, RNAi pathway in *L. amazonensis* is likely to be not functional since key genes are missing in its genome. Finally, we present new information regarding the NISE search in *L. amazonesis* genome. The NISE search resulted in 25 potential analogous between *L. amazonensis* and *H. sapiens*. Also, 31 potential analogous were found in *L. amazonensis* protein sequences. Five out of the six main EC classes showed potential NISEs: Oxidoreductases (EC 1), Transferases (EC 2), Hydrolases (EC 3), Lyases (EC 4), and Isomerases (EC 5). These NISE findings are new and represent potential drug targets because analogous proteins perform the same function using different proteins and 3D structures. In other words, an analogous protein in *L. amazonensis* can be silenced without affecting the host.

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Author Contributions

Generated and validated the genome sequence: NJD, JCM. Assembled the genome: JR. Analyzed the data: DAT, GLN, RJ, JL, ASRD, MRG, LMP, DRL, PHS, AP, FPS. Wrote the first draft of the manuscript: DAT, GLN. Contributed to the writing of the manuscript: MRG, LMP, AP, HLMG, CMP, ECG, FPS. Jointly developed the structure and arguments for the paper: DAT, HLMG, ABM, FPS, AMRD. Made critical revisions and approved final version: DAT, GLN, RJ, JL, ASRD, MRG, LMP, DRL, PHS, HLMG, ABM, JR, AP, FPS, CMP, ECG, AMRD, NJD, JCM. All authors reviewed and approved the final manuscript.

Supplementary Material

Table S1. OrthoMCL identifier for each LCP ortholog, identifier for protein into LCP Ortholog, protein function

and species name for each sequence. Complete table of the orthologs shared only between *L. amazonensis* and the other five *Leishmania* species.

Table S2. Intergenomic NISEs, original annotation and oficial enzime names, fold and superfamily classification based on SUPERFAMILY database and the function of each enzyme.

Table S3. Intragenomic NISEs, original annotation and oficial enzime names, folds and superfamilies classification based on SUPERFAMILY database and the function of each enzyme.

Table S4. Total of enzimatic activities with NISE cases detected by our methodology.

Table S5. Intergenomic NISE as potential drug target searched among three drug target databases (TDR target, TTD, DrugBank).

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