Evolutive History of *Leishmania* Genus and Differential Diagnosis of Clinical Important Species Based on A Unique Kinetoplastida Chitinase

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Research Article

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Abstract

Leishmaniasis, a neglected infectious disease affecting humans, domestic and wild animals, caused by 20 from 53 *Leishmania* genus species, is transmitted by sandflies. *Leishmania* genus, belonging to Trypanosomatide Family and Kinetoplastida Order, are grouped in five subgroups according to biogeographic and evolutive history of parasites and hosts, which has led to incongruences and paraphyly. The GH18 *Leishmania* chitinase, encoded by a specie-specific single copy gene, conserved in basal groups of trypanosomatids, and absent in the genus *Trypanosoma*, was evaluated as a phylogenetic marker and a diagnostic target. Primers were designed to detect *Leishmania* in its host biological samples and to obtain the chitinase sequence of species not available in public databanks. The GH18 chitinase gene and its genomic context was evaluated phylogenetically. A protocol to discriminate among *Leishmania* subgenera by PCR and restriction fragment length polymorphism (RFLP) was developed using *in silico* tools. A PCR method to detect a partial 953 bp GH18 quitinase encoding gene presented high sensibility and specificity on isolated parasites DNA and using as negative controls, *Trypanosoma cruzi*, and DNA from *Leishmania* hosts. Preservation of the chitinase *locus* in the aquatic free-living protozoan *Bodo saltans*, disclose a primitive common origin. GH18 trypanosomatide chitinase amino acid sequence comparative analysis revealed high similarity to chitinase from marine prokaryotes and protozoan. Phylogenetic reconstruction based on chitinase corroborates the Supercontinent Origins theory for *Leishmania*. The chitinase encoding gene was effectively detected in biological samples and for differential molecular diagnosis among *Leishmania* clinical important species worldwide.

Introduction

Protozoan parasites of the *Leishmania* genus are the causative agents of multiple leishmaniases manifestations that affect humans, domestic dogs, and wild animal hosts, all of them transmitted by insect vectors of the Psycodidae Family (sand flies) of which *Phlebotomus* (Old World) and *Lutzomyia* genus (New World - the Americas) are amongst the most important (Akhoundi et al., 2016b). The 53 *Leishmania* species are divided into five subgenera: *Leishmania, Viannia, Sauroleishmania, Mundinia* and *Paraleishmania*. Of these, 20 species are implicated to cause human disease, and most of them are zoonotic (Akhoundi et al., 2016b).

Leishmaniases can range from mild tegumentar ulcerations (tegumentary leishmaniasis, TL) to fatal visceral infection (visceral leishmaniasis, VL) depending on parasite species and host immunity conditions. It is estimated that up to 0.4 and 1.2 million cases of VL and TL, respectively, occur in 98 endemic countries each year (Alvar et al., 2012). Brazil predominantly accounts for the highest incidence in the Americas, where TL is widely spread and VL is expanding (Georgiadou, Makaritsis, and Dalekos, 2015). VL poses important concerns for human health, causing over 50,000 deaths annually, and in India and Mediterranean countries is caused by *L. (Leishmania) donovani* and *L. (Leishmania) infantum*, respectively (2017). In the Americas this disease is also caused by *L. infantum*, which probably entered the region by Mediterranean colonizers carrying infected dogs (Marcili et al., 2014). TL causes morbidity and disfiguring scars in various regions worldwide, but Afghanistan, Algeria, Colombia, Brazil, Iran, Syria,

Ethiopia, Sudan, Costa Rica and Peru together account for more than 75% of new cases (2017). In South America, TL mainly caused by the most prevalent *Leishmania* species, viz., *L. (Viannia) braziliensis*, *L. (Leishmania) amazonensis* and *L. (Leishmania) mexicana*, is endemic. In spite of much efforts, a precise diagnostic test and effective treatment for leishmaniasis are still unavailable (Scheufele, Giesey, and Delost, 2020). Thus, a detailed understanding of all aspects of specific biology and host-parasite relationships is important prior to facilitating the formulation of innovative and effective drugs and diagnostic tests for developing adequate prevention and control strategies.

The evolutionary origins of *Leishmania* parasites and their genetic relationships can be investigated using phylogenetic reconstructions associated to data on biogeographic dispersion and evolution of their vertebrates and sand flies hosts. Three main theories have been proposed for *Leishmania* origin: 1) a Palearctic, Neotropical; 2) a Neotropical/ African; 3) multiple, independent origins (Akhoundi et al., 2016b).The most supported theory for *Leishmania* origin corresponds to the Supercontinent hypothesis, a variation to the Multiple Origins hypothesis, which denotes the independent evolution of the *Viannia* and *Leishmania* subgenera during the separation of South America from Africa. The Supercontinent hypothesis places the origin of *Leishmania* on Gondwana, emerging from monoxenous parasites (Harkins et al., 2016) and is in agreement with biogeographic data, animals' host migration propositions and was supported by a comprehensive phylogenetic analysis using a large multi-gene dataset (over 200,000 informative sites) (Harkins et al., 2016). An important caveat in phylogenetic reconstruction of basal trypanosomatids, including *Leishmania s*pecies, relies on our limited knowledge of their specie and genera specific sequence diversity, partly due to the difficulties of in vitro culture isolation. Thus, in this paper the Trypanosomatidae GH18 chitinase is explored as a molecular marker to identify *Leishmania* species directly from biological specimens, to differentially diagnose the clinically worldwide important species, and to conduct evolutionary studies of the *Leishmania* phylogenetic relationships.

Material And Methods

Cultivation and DNA extraction of *Leishmania* reference species.

The *Leishmania* reference strains used in this study are identified in Table 1 and were obtained from the National *Leishmania* Typing Reference Laboratory, Leishmaniasis Research Laboratory, Oswaldo Cruz Institute, Oswaldo Cruz Foundation, Ministry of Health. Cultivation of the *Leishmania* reference species were performed in M199 or LIT culture media. Exponentially growing cultures were centrifuged and frozen in 2 mL cryogenic tubes in liquid nitrogen after addition of culture medium with 20% DMSO and 10% fetal bovine serum. An aliquot of 2 mL of the culture of each *Leishmania* species was subjected to DNA extraction using the *DNeasy® Blood & Tissue Kit* (QIAGEN®, Valencia, CA, USA) according to manufacturer instructions. Quality of DNA was determined by 1% agarose gel electrophoresis stained with Unisafe (Uniscience) and quantification was performed using the *Low Mass Ladder* (ThermoFisher Scientific).

Differential diagnosis of *Leishmania* genus based on chitinase-encoding gene

To amplify specifically the chitinase-encoding gene from *Leishmania* spp., oligonucleotides were designed after multiple alignment of chitinase gene sequences available in public databanks (Table 1), using *Clustal X* v.2.1. Sequence specificity and secondary structure medium temperature (Tm) were evaluated by *primer-Blast* (Ye et al., 2012) and *Mfold* (Zuker, 2003), respectively. Temperature and PCR cycling conditions to obtain high specificity and sensitivity of the oligonucleotides were tested with *Platinum Taq DNA Polymerase (Invitrogen)*, DNA from different *Leishmania* species and DNA from human, phlebotomines, dogs, cats, and *Trypanosoma cruzi* as negative controls. Several oligonucleotides sets were tested and the Lquit224F (5' GTTCMACTACGAGGCCTTCTTCAA3') and Lquit1182R (5' CAGATCATTATCCCAGACAAGTT 3'), which amplify a 953 bp fragment corresponding to the single copy chitinase-encoding gene was selected due to its sensitivity and specificity to detect *Leishmania* species. Using Lquit224F and Lquit 1182R the partial chitinase gene sequence from the species *L. guyanensis, L. shawi, L. lainsoni, L. naiffi* and *L. amazonensis* not available in public databank, was obtained through PCR using Platinum Taq DNA polymerase High Fidelity (Invitrogen). The PCR reactions were conducted in a 9700 Perkin Elmer Termocycler and conditions used were 94^0C for 3 min followed by 40 cycles of 94^0C for 1 min; 64^0C for 30 s; 72^0C for 45 s; and 72^0C 7 min. Chitinase amplicons were cloned into *pGEM-T* (Promega), transformed in *Escherichia coli* Mach T1; and the clones were selected by PCR miniscreen with the oligonucleotides M13F and M13R. Two clones carrying the chitinase amplicon from each *Leishmania* species were sequenced by Sanger method using *BigDye 3.1 Terminator Cycle Sequencing Kit* (Perkin Elmer) in an automatic sequencer ABI 310 (Applied Biosystems).

All available chitinase 953 bp fragment sequences deposited in public databank and the sequences generated in this study (Table 1) were subjected to alignment and selection for restriction enzymes sites, using the software GeneQuest from Lasergene (Burland, 2000), to discriminate among *Leishmania* subgenera employing PCR followed by restriction fragment length polymorphism (PCR-RFLP).

Phylogenetic analysis

To perform the phylogenetic reconstruction of trypanosomatids harboring the chitinase gene, a 953 bp of the encoding gene from *Leishmania* species and others reference trypanosomatids were obtained from GenBank and EMBL (Table 1). Gene multiple alignment was carried out using Muscle version 3.8.3 (Edgar, 2004) and manually curated (Okonechnikov et al., 2012). Phylogenetic reconstruction was performed using the maximum likelihood method in PhyML (Guindon et al., 2010) version 3.3.20180621 with the model GTR+I+G, selected through the Akaike's information criterion within jModelTest 2.1.10 program (Darriba et al., 2012). The tree branch support indexes were determined by bootstrap resampling with 1,000 replicates. Orthology was defined by sequence identity and genomic context conservation (synteny) using retrieved sequences from TriTrypDB (Aslett et al., 2010), after search for the chitinase gene.

Ethics statement

Source of DNA from human, used as controls in PCR diagnostic reactions was described in other study of our research group (Suzuki et al., 2016) and were obtained from patients of Marilia city, an endemic visceral leishmaniasis locality, localized in São Paulo, Brazil. The protocol of human samples, which were anonymized, was approved by Marilia Medical School Human Experimental Ethical Committee (CAAE 50128015.5.0000.5413). DNA from cats and DNA and sera of dogs collected in São Luis Island in the Brazilian State of Maranhão were gently donated by Dr. Andrea Pereira da Costa from Universidade Estadual do Maranhão, Brazil. DNA from phlebotomines were obtained from insects collected in Marilia city, localized in the Brazilian São Paulo State, with authorization by the Biodiversity Authorization and Information System (SISBIO), of the Chico Mendes Institute for Biodiversity Conservation (ICMBio), Brazilian Ministry of Environment (MMA), under number 64603-1 (10/18/2018).

Results

Chitinase genetic locus conservation among Kinetoplastida supports its ancient origin and corroborate the Supercontinent Origin hypothesis for Leishmania genus

In order to evaluate the phylogenetic relationship of the GH18 family chitinase in Kinetoplastida, we initially retrieved chitinase amino acid and nucleotide sequences from multiple trypanosomatids that were available in public databanks, and comparative analysis was performed using BLAST (Altschul et al., 1990). These results revealed that chitinase sequences are highly conserved within the *Leishmania* genus, with amino acid identity ranging from 78–100%. Also, a similar GH18 *Leishmania* chitinase sequence was identified in the basal trypanosomatids *Leptomonas*, *Strigomonas* and *Angomonas*, with identity of 60%, 40% and 35%, respectively, compared with *Leishmania* species. Our genomic searches also indicated that parasites from the *Trypanosoma* genus lacks this sequence. A protein with identity of 32% with the *Leishmania* genus chitinase was also present in *Bodo saltans*, the free living acquatic protozoa from the Order Kinetoplastida and belonging to the Family Bodonidae, which is commonly used as external group of Trypanosomatidae in phylogenetic studies, suggesting an early origin of this sequence and the occurrence of a homologous GH18 chitinase in an ancient Kinetoplastida lineage (Fig. 1A).

Using the TrytripDB genomic resources tools, the genomic contexts of chitinase-encoding gene from all available Kinetoplastida sequences, including *B. saltans*, *Leptomonas, Angomonas, Strigomonas* and *Leishmania*, showed to be conserved, further strengthening the hypothesis of a common origin. In all organisms included in the analysis, the GH18 chitinase appears as a single-copy gene (Fig. 1B).

A partial 953 bp chitinase encoding gene fragment and the corresponding amino acid sequence from trypanosomatids available in genomic databanks and generated in this study was used for phylogenetic reconstruction using the maximum likelihood method (Fig. 1A). The phylogenetic reconstruction of *Leishmania* based on GH18 chitinase encoding gene corroborated the Supercontinent Origin of the *Leishmania* genus (Harkins et al., 2016) with high bootstrap support, showing a clear separation between

L. (*Leishmania*) spp, *L.* (*Viannia*) spp. and *L.* (*Paraleishmania*) spp., with the single *L.* (*Sauroleishmania*) representative, *L. tarentolae*, appearing as a sister clade of *L.* (*Leishmania*) species (Fig. 1A).

Moreover, the BLAST analysis of the chitinase amino acid sequence from *Bodo saltans* showed identity of 38% with the chitinase of the marine microorganisms *Perkinsus marinus* and *Micromonas pusilla*. This suggests a possible marine environment origin in the trypanosomatids.

Conventional PCR associated to restriction length polymorphism differentiated Leishmania **subgenera of Old and New World.**

Next, we aimed to evaluate whether the chitinase-encoding sequence could be used as a molecular marker to differentiate *Leishmania* subgenera. For this, we initially performed *in silico* analyses of publicly available *Leishmania* chitinases deposited in GenBank, classified in the glycosil-hydrolase 18 (GH18) family (Table 1), localized in chromosome 16, and encoded by a single-copy gene, indicated high inter-subgenera identity in all important putative domains and post-translational modifications (Cabral et al., 2020).

The trypanosomatide chitinase genomic sequence alignment was used to select short sequences and specificity to amplify the corresponding gene from *Leishmania* genus species. After PCR analysis of several oligonucleotides sets on *Leishmania*, *Trypanosoma*, *Lutzomyia*, human, dog, and cat genomic DNA, the Lquit224F and Lquit1182R primers were found effective for molecular diagnosis. PCR with these oligonucleotides generated a 953 bp fragment of the *Leishmania* chitinase encoding gene and it detected less than 100 fg of DNA from *Leishmania* species of subgenus *Leishmania* (*L. amazonensis, L. mexicana, L. infantum and L. donovani*) and *Viannia* (*L. shawi*) (Fig. 2). Considering the genome size of *Leishmania* species of approximately 35 Mb with a variation in the order of 10 (Schonian, 2017), sensitivity tests with the developed molecular diagnostic method revealed the detection of up to a single parasite with approximately 100 fg. The highest sensitivities were obtained with *L. infantum* and *L. shawi* genomic DNA.

The oligonucleotides L224_fow and L1182_rev were used to amplify and sequence the chitinase gene 953 bp fragment from the species *L. guyanensis, L. lainsoni, L. naiffi* and *L. shawi*, all belonging to the *Viannia* subgenus, and from *L. hertigi*, which is grouped within the *Paraleishmania.* Of note, sequences for these representatives were not available in public genomic databanks. The obtained sequences were deposited in GenBank under accession numbers MN520614 to MN520618, and the chitinase fragment from the *Viannia* subgenus species presented 98 to 99% of identity among *L. braziliensis, L. panamensis* and *L. peruviana*, while the identity of the chitinase fragment from *L. hertigi* ranged from 79 to 83% of identity to the same *Viannia* subgenus species.

After restriction analysis of the 953 bp chitinase fragment, the enzyme P*st I* was effective for differential diagnosis of Old and New World *Leishmania* species from *Leishmania*, *Viannia* and *Mundinia* subgenera (Fig. 3). Separation between Old World *Leishmania* subgenus parasite species that cause VL and TL and

between *Sauroleishmania* and *Viannia* subgenera were obtained with D*de I* restriction analysis (Online resource 1 and 2).

Discussion

Chitinases catalyze the β-1,4-glycoside bond hydrolysis reaction of N-acetylglucosamine residues present in chitins and chitodextrins (Cohen-Kupiec and Chet, 1998). Amino acid sequence similarity analysis indicated that these enzymes cluster in the GH18 and GH19 glycosyl hydrolase families. Chitinase and N-acetylglucosaminidase activities in *Leishmania* were initially found in promastigote supernatant cultures of *L. (Leishmania) major*. Apparently these enzymes were not secreted through the sand fly gut (Schlein, Jacobson, and Shlomai, 1991), thereby indicating chitinolytic action secreted by a specific parasite (Rogers, Chance, and Bates, 2002). The activity of both enzymes was observed in *L. donovani, L. infantum, L. braziliensis, Leptomonas seymouri, Crithidia fasciculata* and *Trypanosoma lewisi*. The molecular approach led to the identification and biochemical characterization of the gene encoding a GH18 chitinase from *L. donovani* (Ld Cht1). This sequence was found to be well distributed within the *Leishmania* genus (*L. major, L. infantum, L. donovani* and *L. braziliensis*) (Shakarian et al., 2010).

Homologous episomal overexpression of chitinase in both amastigotes and promastigotes of *L. mexicana* revealed an increase in vector transmission rate and increased pathogenicity in the vertebrate host, thereby indicating that chitinase plays an important role in parasite development, survival and transmission in mammalian hosts (Joshi et al., 2005; Rogers et al., 2008). However, the presence and role of this protein in human blood and tissues of leishmaniasis patients remain unknown. Given the importance of chitinase, its conservation across the *Leishmania* genus, species-specific amino acid and nucleotide sequence expression in all parasite developmental stages, in this work we geared our focus towards the study of chitinase-encoding gene as a molecular phylogenetic marker.

The genomic locus of GH18 chitinase encoding gene is conserved among basal trypanosomatids, including *B. saltans* and is absent in the *Trypanosoma* genus and also it was not found in genomic sequences of parasites from genus *Phytomonas*. In addition, amino acid sequence comparison among GH18 chitinases from trypanosomatids using public genome database revealed 35% identity of GH18 chitinases from marine protozoa and bacteria to the corresponding *B. saltans* ortholog. These results strongly suggest that the GH18 chitinase from the Kinetoplastida derived from a common marine ancestor, harboring the primitive enzyme. The phylogenetic reconstruction of basal trypanosomatids, based on the GH18 chitinase, corroborated the most accepted theory for *Leishmania* origin, the Supercontinent hypothesis (Harkins et al., 2016), which was based on a multigene analysis, with more than 200,000 nucleotides as informative sites. Thus, the GH18 chitinase, present in basal trypanosomatids, can be used as a molecular marker to identify unknown microorganisms, related to *Leishmania* genus, contributing to investigate the diversity and the evolutive history of this group.

The phylogenetic position of subgenus *Sauroleishmania* according to the Supercontinent hypothesis indicates the switch of its *Leishmania* ancestors from mammalian to reptilian hosts (Akhoundi et al.,

2016a). In considering a probable marine environment emergence of the trypanosomatid GH18 chitinase, it is possible to explore that the *Sauroleishmania* subgenus could diverge from an ancestor before the rise of mammals, during the transition of animals from marine to the terrestrial environment. In this case, parasites with similarity to basal groups of trypanosomatids could be found in fish and amphibians. Considering the conservation of the chitinase-encoding gene in *Leishmania*, the diagnostic method developed in this work can be used to investigate this hypothesis directly on biological samples, circumventing the isolation difficulties of unknown *Leishmania* related parasites.

Nucleic acid detection techniques in samples from people and/or animals infected with *Leishmania*, such as PCR, are used for detection and identification of the parasite since the 1980's. PCR include amplification of fragments of the gene encoding the small ribosomal RNA subunit (SSU rDNA,(van Eys et al., 1992), the transcribed internal ribosomal DNA spacer (ITS) (Schonian et al., 2003), sequences corresponding to kinetoplast (kDNA) (Cortes et al., 2004), mini-exon (Paiva et al., 2004), the gene encoding the heat shock protein HSP70, among others (da Silva et al., 2010). In spite of the high sensitivity of PCR and, depending on the molecular target, high specificity, it is more used in epidemiological studies than as a routine diagnostic method, and the gold standard method to diagnose *Leishmania* is the observation of the parasite by microscopic analysis (Thakur, Joshi, and Kaur, 2020). In addition, to achieve high sensitivity in the methodologies evaluated so far, PCR complementation with other techniques including nested PCR and hybridization is required. For identification of *Leishmania* species, the methodologies include restriction fragment size analysis of PCR products obtained, and as most of gene targets have multiple copies, interpretation of the results increases the difficulty of using these techniques in the clinical routine (Rogers et al., 2011; Ubeda et al., 2008). In addition, false positives are possible due to contamination with other post-PCR amplified samples or DNA fragments and cross-reaction with other pathogens, including *Trypanosoma* (Degrave et al., 1994; Viol et al., 2012).

The differential diagnosis showed in this study, based on the detection of the GH18 chitinase gene, presents advantages over other molecular methods, since it employs a single copy gene, absent in *Trypanosoma* genus, enabling specific detection of *Leishmania* parasites. Also, the sensitivity of the method, regarding the large size of the amplified fragment supports post-PCR analysis after a single PCR reaction performed directly from biological samples. Restriction analysis of the 953 bp *Leishmania* chitinase PCR fragment with P*stI* permitted the identification of medically important species in Latin America where three different *Leishmania* subgenera circulates in animal reservoirs, human and sand flies (Fig. 2). Given the specificity of the *Leishmania* chitinase-encoding gene, the molecular diagnostic method can also be used to identify isolated parasites from biological samples, with high specificity, by restriction analysis and/or sequencing (Suzuki et al., 2016). Also, using the restriction enzyme D*de I* on the 953 bp chitinase PCR fragment, it is possible to differentiate *L. major* from all others Old World *Leishmania* subgenus species, which is of clinical importance in Oriental TL endemic countries (Hijjawi et al., 2016) (Online resource 1 and 2).

Leishmania chitinase is present in basal groups of trypanosomatids genera, probably derived from an ancestor living in a marine environment, and unique in the human pathogen group. To the best of our

knowledge, there are no *Leishmania* chitinase or homologous proteins described with a molecular structure associated to biochemical characterization. Considering the biological importance and the specificity of this protein to the *Leishmania* genus, molecular studies to define its biochemical function are warranted. Additionally, the diagnostic method described in this work enables detection of basal groups of trypanosomatids, directly from biological sources, helping in the identification of unknown species which may contribute to the Kinetoplastida evolutive history.

Declarations

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Conflicts of interest/Competing interests

The authors have no conflicts of interest to declare that are relevant to the content of this article.

Availability of data and material

All data and materials as well as software application or custom code support their published claims and comply with field standards.

Code availability

Not applicable.

Authors' contributions

Conception, hypothesis, and design: Aline Diniz Cabral, Felipe Trovalim Jordão, Márcia Aparecida Sperança; all authors participated in data acquisition, analysis, read the manuscript and approved the final version.

Ethics approval

Source of DNA from human, used as controls in PCR diagnostic reactions was described in other study of our research group (Suzuki et al., 2016). The protocol of human samples, which were anonymized, was approved by Marilia Medical School Human Experimental Ethical Committee (CAAE 50128015.5.0000.5413). DNA from cats and DNA and sera of dogs were gently donated by Dr. Andrea Pereira da Costa from Universidade Estadual do Maranhão, Brazil. DNA from phlebotomines were obtained from insects collected with authorization by the Biodiversity Authorization and Information

System (SISBIO), of the Chico Mendes Institute for Biodiversity Conservation (ICMBio), Brazilian Ministry of Environment (MMA), under number 64603-1 (10/18/2018).

Consent to participate

Not applicable.

Consent for publication

Not applicable.

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Tables

Table 1. Nucleotide sequences information

Species	Gene	Source	Observations
Angomonas deanei	chitinase	ENA[2]:EPY25377	
Blechomonas ayalai	chitinase	Tritrypdb[3]:rna_Baya_138_0020-1	B08-376
Bodo saltans	chitinase	Genbank CYKH01001162	*Locus* sequence
C. fasciculata	chitinase	Tritrypdb::CFAC1_120016000	strain Cf-Cl
Endotrypanum monterogeii	chitinase	Tritrypdb:EMOLV88_160012400.1	strain LV88
Fimbriiglobus ruber	chitinase	Genbank OWK46432.1	Bacterium from Order *Planctomycetales*, associated to Crustacea
Homo sapiens	chitinase	Genbank AAG10644.1	Chitotriosidase, Macrofago, CH1; GH18
L. amazonensis	chitinase	Genbank: MG869127	Strain IOCL 0575
L. braziliensis	chitinase	Genbank LS997615	MHOM/BR/75/M2904
L. donovani	chitinase	Genbank CP019523	Strain MHOM/IN/1983/AG83
L. enrietti	chitinase	Tritrypdb:LENLEM3045_160013500.1	Strain LEM 3045
L. gerbilli	chitinase	Tritrypdb:LGELEM452_160013100.1	Strain LEM452
L. guyanensis	chitnase	ENA:CCM15041	
L. infantum	chitinase	Genbank:FR796448	Strain JPCM5
L. major	chitinase	Genbank:FR796412.1	Strain Friedlin
L. Mexicana	chitinase	Genbank:AY572789	
L. panamensis	chitinase	Genbank CP009385	Strain MHOM/PA/94/PSC-1
L. peruviana	chitinase	Genbank LN609244	PAB 4377
L. tarantolae	Chitinase	Tritrypdb:LtaP16.0770	Parrot-TarII
L. tropica	Chitinase	TritrypdbLTRL590_160013800.1	Strain L590
L. turanica	Chitinase	Tritrypdb:LTULEM423_160013000.1	Strain LEM 423
Leptomonas pyrrhocoris	chitinase	Tritrypdb:rna_LpyrH10_15_0870	H10
Leptomonas seymori	chitinase	Tritrypdb:PCLsey_0068_0030	ATCC 30220

Lutzomyia longipalpis	chitinase	Genbank AAN71763.1	GH18 chitinase
Micromonas pusilla	chitinase	Genbank XP_003063458.1	Marine photosynthetic eukaryotic microorganism
Paratrypanosoma confusum	chitinase	Tritrypdb:PCON_0062580	Isolate cul13
Perkinsus marinus	chitinase	Genbank XM_002788039.1	Eukaryotic microorganism, pathogen of oysters
Phlebotomus papatasi	chitinase	Genbank AAV49322.1	GH18 chitinase
Planktomyces sp	chitinase	Genbank OAI56776.1	Bacterium from marine environment
Strigomonas culicis	chitinase	ENA:EPY22137	522 amino acids
Strigomonas culicis	chitinase	ENA:EPY29957	311 amino acids

1.Genbank(Benson et al., 2004); 2. ENA: European Nucleotide Archive(Archive) ; 3. TritrypDB(Aslett et al., 2010) : Kinetoplastid Genomics Resource.

Figures

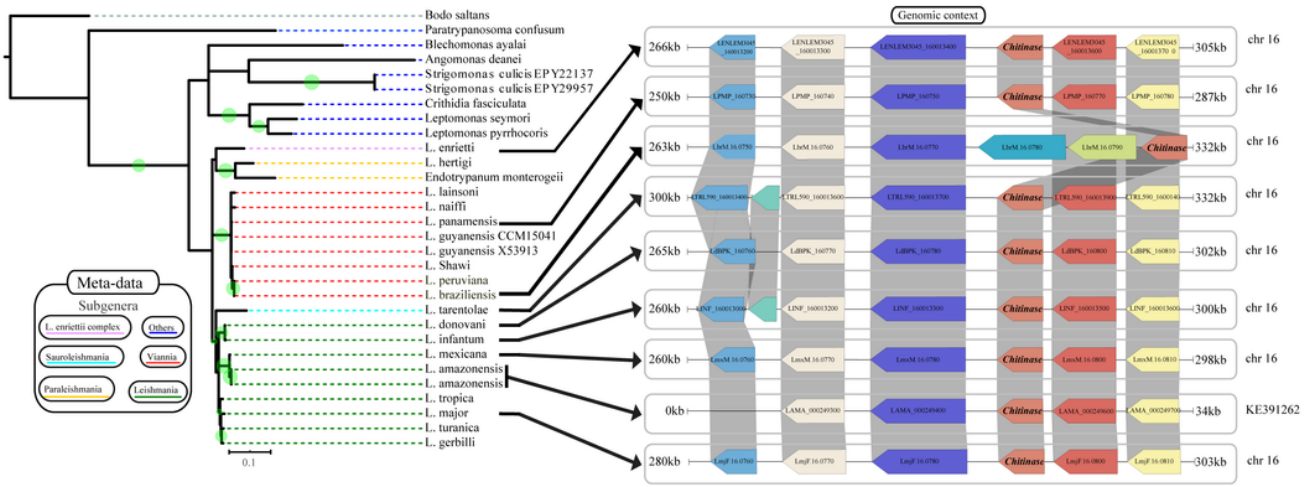

Figure 1

Maximum likelihood phylogenetic tree and genomic context of the trypanosomatid chitinase GH18 encoding gene. A) maximum likelihood phylogenetic tree representing the evolutionary history of the chitinase from basal trypanosomatid representatives. Bootstrap support values (1,000 replicates) greater than 80% are represented by a green circle in the branches of the tree. Colored dashes indicate the subgenus for each species according to the legend. Each gene is depicted as an arrow (where direction reflects gene orientation), and the connecting gray segments indicate sequence conservation (nucleotide identity ≥90%) among the genomic contexts.

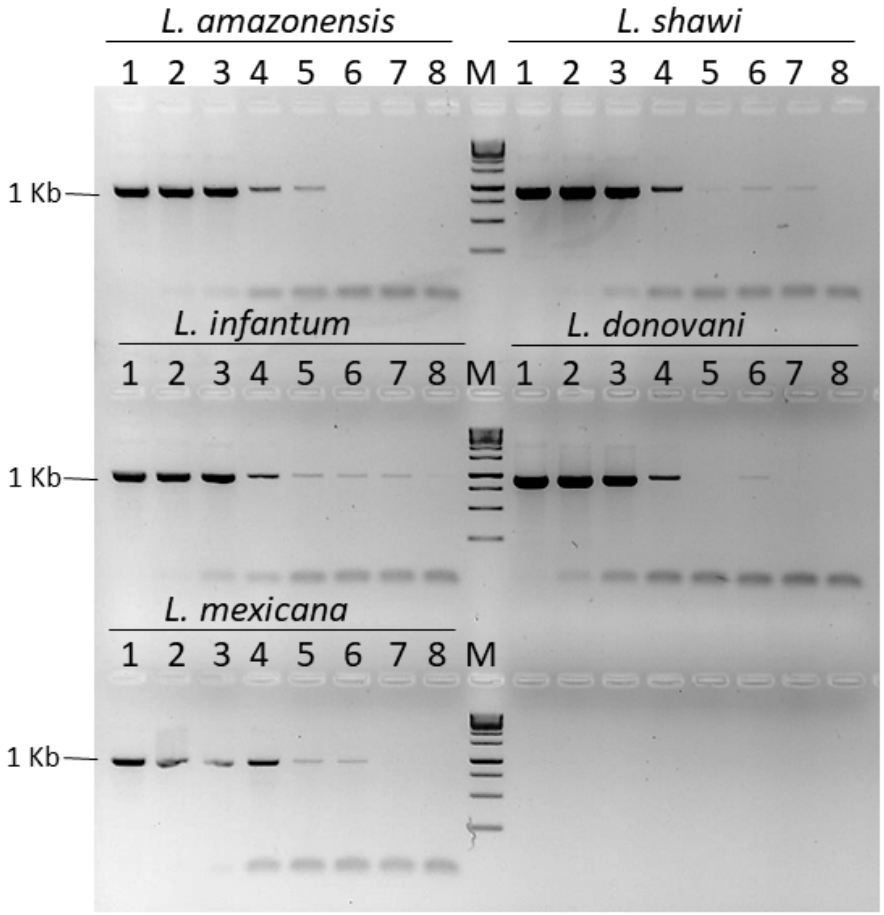

Figure 2

Sensitivity test of the 953 bp Leishmania chitinase encoding gene fragment amplification by PCR. 2% agarose gel electrophoresis stained with UniSafe containing the PCR products after PCR with the oligonucleotides L224_fow e L1182_rev on genomic DNA of Leishmania genus species; M. 1Kb ladder (GeneO'ruler); 1-8 different concentrations of genomic DNA: 10ng, 1ng, 100pg, 10pg, 1pg, 100fg, 10fg and 1fg, respectively.

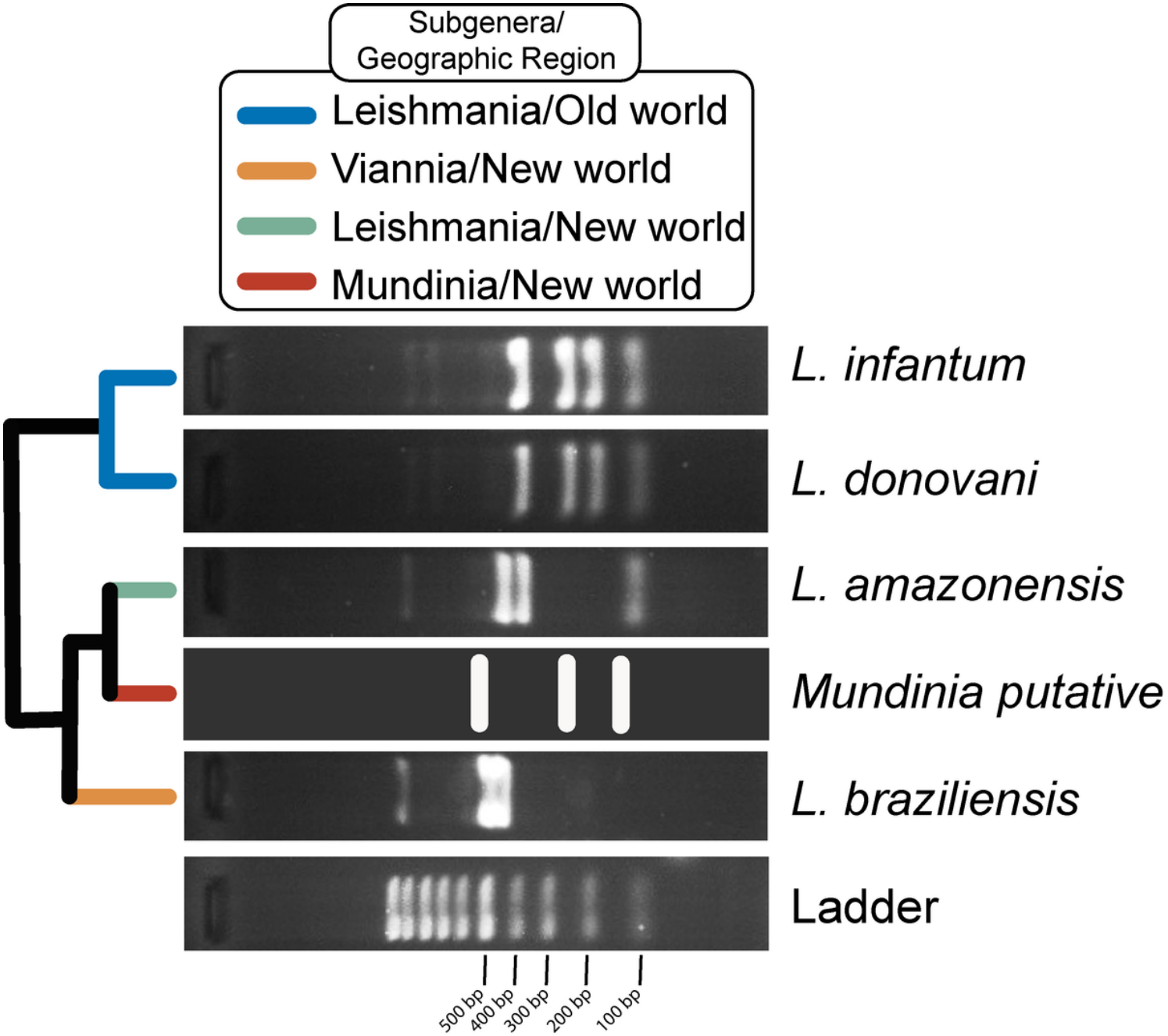

Figure 3

Differential diagnosis of Leishmania species by amplification of a 953 DNA fragment corresponding to chitinase encoding gene by PCR and restriction length size polymorphism using PstI. Subgenera and corresponding geographic distribution are resumed in the upper square represented by colored lines. The

size of the restriction fragments is shown in a 3% agarose gel electrophoresis stained with UniSafe containing the PstI restriction fragments of the 953 bp Leishmania chitinase encoding gene PCR fragment. M – 100 bp ladder (GeneO'ruler)

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