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Original Article

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

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Abstract

Introduction: Leishmaniasis, a neglected infectious disease affecting humans, domestic, and wild animals, is caused by 20 from 53 *Leishmania* genus species and transmitted by sandflies. *Leishmania* genus, belonging to the Trypanosomatidae family and Kinetoplastida order, are grouped into five subgroups according to the biogeographic and evolutive history of parasites and hosts, leading to incongruences and paraphyly. The GH18 *Leishmania* chitinase, which is encoded by a species-specific single-copy gene, conserved in the basal groups of trypanosomatids, and absent in the genus *Trypanosoma*, was evaluated as a phylogenetic marker and a diagnostic target.

Methods: Primers were designed to detect *Leishmania* in its host biological samples and obtain the chitinase sequence of species that are unavailable in public databanks. The GH18 chitinase gene and its genomic context were evaluated phylogenetically. A protocol was developed to discriminate *Leishmania* subgenera by adopting polymerase chain reaction (PCR) and restriction fragment length polymorphism and using *in silico* tools. The adopted PCR method for detecting a partial 953 bp GH18 chitinase-encoding gene represented high sensibility and specificity on DNA of isolated parasites and was used as negative controls, *Trypanosoma cruzi*, and DNA from *Leishmania* hosts.

Results: Preservation of the chitinase *locus* in the aquatic free-living protozoan *Bodo saltans* disclosed a primitive common origin. Based on the comparative analysis, the amino acid sequence of GH18 trypanosomatidae chitinase demonstrated its high similarity to that of chitinase from marine prokaryotes and protozoans. Phylogenetic reconstruction based on chitinase corroborated the Supercontinent Origins Theory for *Leishmania*.

Conclusion: The chitinase-encoding gene was effectively detected in biological samples and thus could be considered for differential molecular diagnosis among *Leishmania* clinical important species worldwide.

Keywords: Marine-derived chitinase, *Leishmania*, Molecular diagnosis, *Leishmania* evolution theory

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Introduction

Protozoan parasites of the *Leishmania* genus are the causative agents of multiple leishmaniasis manifestations that affect humans, domestic dogs, and wild animal hosts and are transmitted by the insect vectors of the Psychodidae Family (sandflies) of which *Phlebotomus* (Old World) and *Lutzomyia* genus (New World - the Americas) are the most important ones (1). The 53 *Leishmania* species are divided into five subgenera, including *Leishmania, Viannia, Sauroleishmania, Mundinia*, and *Paraleishmania*. Out of these five subgenera, 20 species, most of which are zoonotic, are implicated to cause human diseases (2).

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 has been estimated that up to 0.4 and 1.2 million cases of VL and TL annually occur in 98 endemic countries, respectively (2). Brazil predominantly accounts for the highest incidence in the Americas, where TL is widely spread and VL is expanding (3). VL has posed serious concerns for human health, causing over 50,000 deaths annually. According to reports, it is caused by *Leishmania donovani* and *Leishmania infantum* in India and Mediterranean

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countries, respectively (4) In the Americas, this disease is also caused by *L. infantum*, which has probably entered the region by Mediterranean colonizers carrying infected dogs (5). 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 (6). In South America, TL, which is mainly caused by the most prevalent *Leishmania* species, viz., *L. (Viannia) braziliensis*, *L. amazonensis*, and *L. mexicana*, is endemic. In spite of extensive efforts, a precise diagnostic test and effective treatment for leishmaniasis are still unavailable (7,8). 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 with data on biogeographic dispersion and evolution of their vertebrates and sandfly hosts. Three main theories have been proposed for *Leishmania* origin, including a Palearctic, Neotropical, a Neotropical/African, and multiple, independent origins (2). The most supported theory for the *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 (9), and is in agreement with biogeographic data, animals' host migration propositions. This hypothesis was supported by a comprehensive phylogenetic analysis using a large multi-gene dataset (over 200 000 informative sites) (9). An important caveat in the phylogenetic reconstruction of basal trypanosomatids, including *Leishmania* species, relies on our limited knowledge of their specie and genera-specific sequence diversity, partly due to the difficulties of in vitro culture isolation. Thus, this paper focused on exploring the Trypanosomatidae GH18 chitinase as a molecular marker to identify *Leishmania* species directly from biological specimens to differentially diagnose the clinically worldwide important species and conduct evolutionary studies of the phylogenetic relationships of *Leishmania*.

Material and Methods

Cultivation and DNA Extraction of Leishmania Reference Species

The *Leishmania* reference strains used in this study are presented in Table 1. These strains were obtained from the National *Leishmania* Typing Reference Laboratory, Leishmaniasis Research Laboratory, Oswaldo Cruz Institute, Oswaldo Cruz Foundation, and Ministry of Health. The *Leishmania* reference species were cultivated in M199 or LIT culture media. Exponentially growing cultures were centrifuged and frozen in 2-mL cryogenic tubes in liquid nitrogen after the addition of the 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 and Tissue Kit (QIAGEN®, Valencia, CA, USA) according to the manufacturer's instructions. The quality of DNA was determined by 1% agarose gel electrophoresis stained with UniSafe (Uniscience), and quantification was performed using the Low Mass Ladder (Thermo Fisher Scientific).

Differential Diagnosis of Leishmania Genus Based on Chitinase-Encoding Gene

To specifically amplify the chitinase-encoding gene from *Leishmania* spp., oligonucleotides were designed after multiple alignments of chitinase gene sequences available in public databanks (Table 1) using Clustal X (version 2.1). Sequence specificity and secondary structure medium temperature (Tm) were evaluated by primer-Blast (10) and Mfold (11), respectively. Temperature and polymerase chain reaction (PCR) cycling conditions for obtaining high specificity and sensitivity of the oligonucleotides were tested with Platinum Taq DNA Polymerase (Invitrogen), DNA from different *Leishmania* species, and DNA from humans, phlebotomines, dogs, cats, and *Trypanosoma cruzi* as negative controls. Several oligonucleotide sets were tested, and Lquit224F (5' GTTCMACTACGAGGCCTTCTTCAA3') and Lquit1182R (5' CAGATCATTATCCCAGACAAGTT 3'), which amplify a 953 bp fragment corresponding to the single copy chitinase-encoding gene, were selected due to their 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* unavailable in public databanks, was obtained through PCR using Platinum Taq DNA polymerase high fidelity (Invitrogen). The PCRs were conducted in a 9700 Perkin Elmer Termocycler, and the conditions included 94 °C for 3 minutes, followed by 40 cycles of 94 °C for 1 minute, 64 °C for 30 seconds, 72 °C for 45 seconds, and 72 °C 7 minutes. Chitinase amplicons were cloned into *pGEM-T* (Promega), transformed in *Escherichia coli* Mach T1, and the clones were selected by PCR miniscreen with oligonucleotides M13F and M13R. Two clones carrying the chitinase amplicon from each *Leishmania* species were sequenced by the 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 the public databank and the sequences

Table 1. Nucleotide Sequence Information

Species	Gene	Source	Observations
Angomonas deanei	Chitinase	ENA: EPY25377	
Blechomonas ayalai	Chitinase	TriTrypDB: 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

Note. 1. GenBank (39); ENA: European Nucleotide Archive (40); TriTrypDB (16): Kinetoplastid Genomics Resource.

generated in this study (Table 1) were subjected to alignment and selection for restriction enzymes sites using GeneQuest from Lasergene software (10) to discriminate among *Leishmania* subgenera employing PCR followed by restriction fragment length polymorphism.

Phylogenetic Analysis

To perform the phylogenetic reconstruction of trypanosomatids harboring the chitinase gene, a 953 bp of the encoding gene from *Leishmania* species and other reference trypanosomatids were obtained from GenBank and EMBL (Table 1). Multiple alignments of the gene were performed using Muscle, version 3.8.3 (12), and curated manually (13). Phylogenetic reconstruction was performed by the maximum likelihood method in PhyML (14), version 3.3.20180621 with the model GTR+I+G, selected through the Akaike's information criterion within jModelTest 2.1.10 program (15). The tree branch support indexes were determined by bootstrap resampling with 1000 replicates. Orthology was defined by sequence identity and genomic context conservation (synteny) using retrieved sequences from TriTrypDB (16) after searching for the chitinase gene.

Ethics Statement

The source of DNA from humans, used as controls in PCR diagnostic reactions, was described in another study of our research group (19) and obtained from the patients of Marilia, an endemic visceral leishmaniasis locality that is 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 samples from phlebotomines were obtained from insects collected in Marilia, localized in the Brazilian São Paulo State, with authorization by the Biodiversity Authorization and Information System, of the Chico Mendes Institute for Biodiversity Conservation, Brazilian Ministry of Environment, 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

To evaluate the phylogenetic relationship of the GH18 family chitinase in Kinetoplastida, initially, chitinase amino acid and nucleotide sequences were retrieved from multiple trypanosomatids that were available in public databanks, and comparative analysis was performed using BLAST (20). The results revealed that chitinase sequences are highly conserved within the *Leishmania* genus, with amino acid identity ranging from 78% to 100%. Further, a similar GH18 *Leishmania* chitinase sequence was identified in the basal trypanosomatids *Leptomonas*, *Strigomonas*, and *Angomonas* with the identity of 60%, 40%, and 35%, respectively, compared with *Leishmania* species. Our genomic searches also indicated that parasites from the *Trypanosoma* genus lack this sequence. Furthermore, a protein with the identity of 32% with the *Leishmania* genus chitinase was present in *Bodo saltans*, the free-living aquatic protozoa from the Order Kinetoplastida and belonging to the Family Bodonidae, which is commonly used as the external group of Trypanosomatidae in phylogenetic studies. This suggests an early origin of this sequence and the occurrence of a homologous GH18 chitinase in an ancient Kinetoplastida lineage (Figure 1A).

Using the TriTrypDB genomic resource tools, the genomic contexts of the chitinase-encoding gene from all available Kinetoplastida sequences, including *B. saltans*, *Leptomonas, Angomonas, Strigomonas*, and *Leishmania*, were shown to be conserved, further strengthening the hypothesis of a common origin. In all included organisms in the analysis, the GH18 chitinase appears as a single-copy gene (Figure 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 were used for phylogenetic reconstruction using the maximum likelihood method (Figure 1A). The phylogenetic reconstruction of *Leishmania*, based on the GH18 chitinase-encoding gene, corroborated the Supercontinent Origin of the *Leishmania* genus (9) with high bootstrap support, showing a clear separation between *Leishmania* spp., *Leishmania* (*Viannia*) spp., and *Leishmania* (*Paraleishmania*) spp.; in addition, the single *Leishmania* (*Sauroleishmania*) representative (*L. tarentolae*) appeared as a sister clade of *Leishmania* species (Figure 1A).

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

Conventional PCR Associated With Restriction Length Polymorphism Differentiated Leishmania Subgenera of

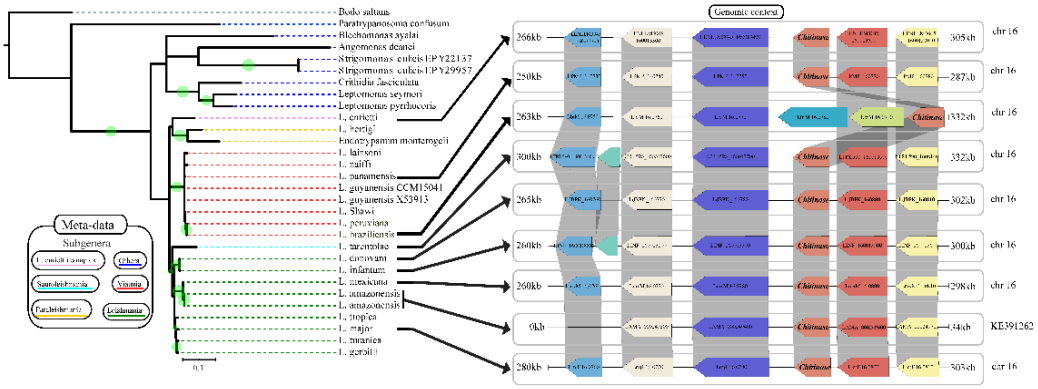

Figure 1. Maximum Likelihood Phylogenetic Tree and Genomic Context of the Trypanosomatid Chitinase GH18 Encoding Gene: (A) Maximum Likelihood Phylogenetic Tree Representing the Evolutionary History of the Chitinase From Basal Trypanosomatid Representatives. *Note*. Bootstrap support values (1000 replicates) 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 demonstrate sequence conservation (nucleotide identity ≥90%) among the genomic contexts.

Old and New World

Next, the study evaluated whether the chitinase-encoding sequence could be used as a molecular marker to differentiate *Leishmania* subgenera. For this purpose, we initially performed the *in silico* analyses of publicly available *Leishmania* chitinases deposited in GenBank, classified into the glycoside-hydrolase 18 (GH18) family (Table 1), localized in chromosome 16, and encoded by a single-copy gene. The results indicated high inter-subgenera identity in all important putative domains and post-translational modifications (21).

The trypanosomatide chitinase genomic sequence alignment was employed to select short sequences and specificity to amplify the corresponding gene from the *Leishmania* genus species. Lquit224F and Lquit1182R primers were found effective for molecular diagnosis after the PCR analysis of several oligonucleotide sets on *Leishmania*, *Trypanosoma*, *Lutzomyia*, human, dog, and cat genomic DNA. The PCR with these oligonucleotides generated a 953 bp fragment of the *Leishmania* chitinase-encoding gene, leading to the detection of less than 100 fg of DNA from the *Leishmania* species of subgenus *Leishmania* (*L. amazonensis, L. mexicana, L. infantum*, and *L. donovani*) and *Viannia* (*L. shawi*) (Figure 2). Considering the genome size of the *Leishmania* species of approximately 35 Mb with a variation in the order of 10 (22), 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.

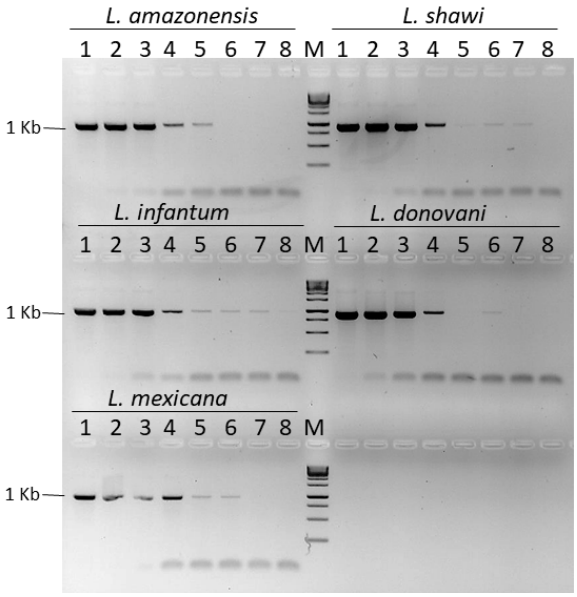

Figure 2. Sensitivity Test of the 953 bp *Leishmania* Chitinase-Encoding Gene Fragment Amplification by PCR. *Note*. PCR: Polymerase chain reaction. 2% agarose gel electrophoresis stained with UniSafe containing the PCR products after PCR with the oligonucleotides L224_fow e L1182_rev on genomic DNA of *Leishmania* genus species; **M.** 1Kb ladder (GeneO'ruler); **1-8** different concentrations of genomic DNA: 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, and 1 fg, respectively.

The oligonucleotides L224_fow and L1182_rev were used to amplify and sequence the chitinase gene 953 bp fragment from *L. guyanensis, L. lainsoni, L. naiffi*, and *L. shawi* species, all belonging to the *Viannia* subgenus, and from *L. hertigi,* which is grouped within the *Paraleishmania.* Of note, sequences for these representatives were unavailable 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 98-99% of identity among *L. braziliensis, L. panamensis*, and *L. peruviana*. However, the identity of the chitinase fragment from *L. hertigi* ranged from 79% to 83% of identity to the same *Viannia* subgenus species.

After the restriction analysis of the 953 bp chitinase fragment, enzyme P*st I* was effective for differential diagnosis of Old and New World *Leishmania* species from *Leishmania*, *Viannia*, and *Mundinia* subgenera (Figure 3). The separation between Old World *Leishmania* subgenus parasite species causing VL and TL, as well as between *Sauroleishmania* and *Viannia* subgenera was obtained with D*de I* restriction analysis (Figure S1 and Table S1, see online Supplementary file 1).

Discussion

Chitinases catalyze the β-1,4-glycoside bond hydrolysis reaction of N-acetylglucosamine residues present in chitins and chitodextrins (23). Amino acid sequence similarity analysis indicated that these enzymes are clustered into GH18 and GH19 glycosyl hydrolase families. Chitinase and N-acetylglucosaminidase activities in *Leishmania* were initially found in the promastigote supernatant cultures of *L. major*. Apparently, these enzymes were not secreted through the sandfly gut (24), thereby indicating the chitinolytic action secreted by a specific parasite (25). 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*) (26).

Homologous episomal overexpression of chitinase in the amastigotes and promastigotes of *L. mexicana* revealed an increase in the vector transmission rate and increased pathogenicity in the vertebrate host, highlighting that chitinase plays an important role in parasite development, survival, and transmission in mammalian hosts (27,28). 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-

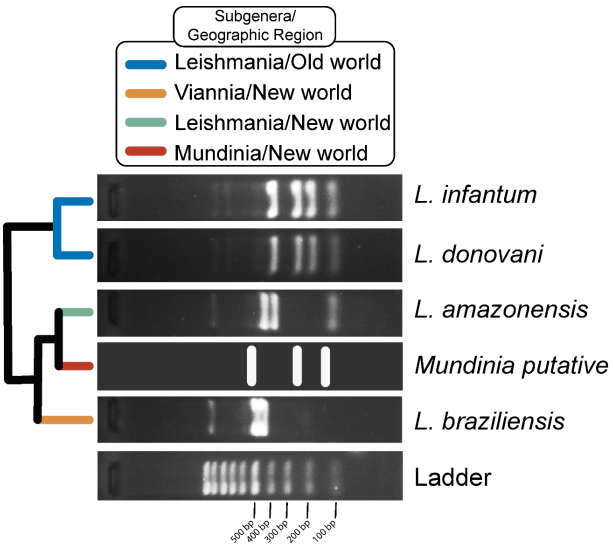

Figure 3. Differential Diagnosis of *Leishmania* Species by the Amplification of a 953 DNA Fragment Corresponding to the Chitinase-Encoding Gene by PCR and Restriction Length Size Polymorphism Using P*stI*. *Note*. PCR: Polymerase chain reaction. Subgenera and corresponding geographic distribution are 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 P*stI* restriction fragments of the 953 bp *Leishmania* chitinase-encoding gene PCR fragment. **M** – 100 bp *ladder* (GeneO'ruler).

specific amino acid, and nucleotide sequence expression in all parasite developmental stages, this study focused on investigating the chitinase-encoding gene as a molecular phylogenetic marker.

The genomic locus of the GH18 chitinase-encoding gene is conserved among basal trypanosomatids, including *B. saltans*, while it is absent in the *Trypanosoma* genus; it was not found in the genomic sequences of parasites from the *Phytomonas* genus. In addition, amino acid sequence comparison among GH18 chitinases from trypanosomatids using a 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 was 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 the *Leishmania* origin, the supercontinent hypothesis (9), 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 for identifying unknown microorganisms related to the *Leishmania* genus, contributing to the investigation of the diversity and the evolutive history of this group.

The phylogenetic position of subgenus *Sauroleishmania* according to the supercontinent hypothesis represents the switch of its *Leishmania* ancestors from mammalian to reptilian hosts (29). Considering the 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 similarities to the basal groups of trypanosomatids could be found in fish and amphibians. Given the conservation of the chitinase-encoding gene in *Leishmania*, the diagnostic method developed in this work can be applied to directly investigate this hypothesis on biological samples, circumventing the isolation difficulties of unknown *Leishmania*-related parasites.

Nucleic acid detection techniques (e.g., PCR) in samples from people and/or animals infected with *Leishmania* have been used for the detection of the parasite since the 1980s. The PCR, among others, includes the amplification of the fragments of the gene encoding the small ribosomal RNA subunit, namely, SSU rDNA (30), the transcribed internal ribosomal DNA spacer (29), sequences corresponding to the kinetoplast (kDNA) (32), mini-exon (33), and the gene encoding the heat shock protein HSP70 (34). In spite of the high sensitivity of the PCR and, depending on the molecular target, high specificity, it is further employed in epidemiological studies rather than as a routine diagnostic method, and the observation of the parasite by microscopic analysis is considered the gold standard method for diagnosing *Leishmania* (35). In addition, to achieve high sensitivity in the methodologies evaluated so far, PCR complementation with other techniques, including nested PCR and hybridization, is necessary. For the identification of *Leishmania* species, the methodologies include the restriction fragment size analysis of the obtained PCR products. Given that most gene targets have multiple copies, the interpretation of the results increases the difficulty of using these techniques in the clinical routine (36,37). Moreover, false positives are possible due to contamination with other post-PCR amplified samples or DNA fragments and cross-reaction with other pathogens, including *Trypanosoma* (38,39).

The differential diagnosis 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 and is absent in the *Trypanosoma* genus, enabling specific detection of *Leishmania* parasites. Additionally, the sensitivity of the method, regarding the large size of the amplified fragment, supports the post-PCR analysis after a single PCR reaction performed directly from biological samples. The 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 circulate in animal reservoirs, humans, and sandflies (Figure 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 (19). Furthermore, using the restriction enzyme D*de I* on the 953 bp chitinase PCR fragment, it is possible to differentiate *L. major* from all other Old World *Leishmania* subgenus species, which are of clinical importance in oriental TL endemic countries (40) (see online Supplementary file 1).

Leishmania chitinase is present in the basal groups of trypanosomatids genera, probably derived from an ancestor living in a marine environment and is 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 with biochemical characterization. Considering the biological importance and the specificity of this protein to the *Leishmania* genus, molecular studies are warranted to define its biochemical function. Additionally, the diagnostic method described in this work enables the detection of the basal groups of trypanosomatids, directly from biological sources, helping in the identification of unknown species which may contribute to the Kinetoplastida evolutive history.

Acknowledgments

DNA from cats as well as DNA and sera from dogs were kindly donated by Dr. Andrea Pereira da Costa from the Universidade Estadual do Maranhão, Maranhão State, Brazil. This study was funded by Fundação de Amparo a Pesquisa do Estado de São Paulo (Grant numbers 2016/14514-4 and 2012/20221-9; fellowships 2013/26096-4 and 2018/05133-2), as well as CAPES and UFABC. LGA is the recipient of a Master's scholarship from Fundação de Amparo à Pesquisa do Estado da Bahia (FAPESB; protocol BOL0159/2019).

Availability of Data and Material

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

Conflict of Interests

The authors have no conflict of interests to declare that are relevant to the content of this article.

Authors' Contributions

Conception, hypothesis, and design: ADC, FTJ, and MAS; all authors participated in acquiring and analyzing data, read the manuscript, and approved the final version.

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Supplementary files

Supplementary file 1 contains Table S1 and Figure S1.

References

1. Parvizi P, Akhoundi M, Mirzaei H. Distribution, fauna and seasonal variation of sandflies, simultaneous detection of nuclear internal transcribed spacer ribosomal DNA gene of *Leishmania major* in *Rhombomys opimus* and *Phlebotomus papatasi*, in Natanz district in central part of Iran. Iran Biomed J. 2012;16:113-20. doi:10.6091/ibj.1038.2012
2. Akhoundi M, Kuhls K, Cannet A, et al. A historical overview of the classification, evolution, and dispersion of *Leishmania* parasites and sandflies. PLoS Negl Trop Dis. 2016;10:e0004349. doi:10.1371/journal.pntd.0004349
3. Georgiadou SP, Makaritsis KP, Dalekos GN. Leishmaniasis revisited: Current aspects on epidemiology, diagnosis and treatment. J Transl Int Med. 2015;3:43-50. doi:10.1515/jtim-2015-0002
4. Alvar J, Velez ID, Bern C, et al. Leishmaniasis worldwide and global estimates of its incidence. PLoS One. 2012;7:e35671. doi:10.1371/journal.pone.0035671
5. Marcili A, Speranca MA, da Costa AP, et al. Phylogenetic relationships of Leishmania species based on trypanosomatid barcode (SSU rDNA) and gGAPDH genes:Taxonomic revision of Leishmania (L.) infantum chagasi in South America. Infect Genet Evol. 2014;25:44-51. doi:10.1016/j.meegid.2014.04.001
6. Global leishmaniasis update, 2006-2015:a turning point in leishmaniasis surveillance. Wkly Epidemiol Rec 2017;92:557-65.
7. Scheufele CJ, Giesey RL, Delost GR. The global, regional, and national burden of leishmaniasis:An ecological analysis from the Global Burden of Disease Study 1990-2017. J Am Acad Dermatol. 2021;84(4):1203-1205. doi:10.1016/j.jaad.2020.08.043
8. Garedaghi Y, Firouzivand Y, Khan Ahmadi B, Zarei A, Salehizadeh E. The effect of Monomycin and Gentamycin Sulfate on Growth of Promastigotes of Leishmania under In Vitro Conditions. International Journal of Medical Parasitology & Epidemiology Sciences. 2021;2(1);16-18. doi:10.34172/ijmpes.2021.04
9. Harkins KM, Schwartz RS, Cartwright RA, Stone AC. Phylogenomic reconstruction supports supercontinent origins for Leishmania. Infect Genet Evol. 2016;38:101-9. doi:10.1016/j.meegid.2015.11.030
10. Burland TG. DNASTAR's Lasergene sequence analysis software. Methods Mol Biol. 2000;132:71-91. doi:10.1385/1-59259-192-2:71
11. Zuker M. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. 2003;31:3406-15. doi:10.1093/nar/gkg595
12. Edgar RC. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics. 2004;5:113. doi:10.1186/1471-2105-5-113
13. Okonechnikov K, Golosova O, Fursov M, team U. Unipro UGENE: a unified bioinformatics toolkit. Bioinformatics. 2012;28:1166-7. doi:10.1093/bioinformatics/bts091
14. Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst Biol. 2010;59:307-21. doi:10.1093/sysbio/syq010
15. Darriba D, Taboada GL, Doallo R, Posada D. jModelTest 2: more models, new heuristics and parallel computing. Nat Methods. 2012;9:772. doi:10.1038/nmeth.2109
16. Aslett M, Aurrecoechea C, Berriman M, et al. TriTrypDB:a functional genomic resource for the Trypanosomatidae. Nucleic Acids Res. 2010;38:D457-62. doi:10.1093/nar/gkp851
17. Benson DA, Karsch-Mizrachi I, Lipman DJ, Ostell J, Wheeler DL. GenBank: update. Nucleic Acids Res. 2004;32:D23-6.

doi:10.1093/nar/gkh045
18. Archive EN. EMBL-EBI. [cited 2020]. Available from: http://www.ebi.ac.uk/ena/data/view/.
19. Suzuki RB, Cabral AD, Martins LP, Speranca MA. A highly sensitive Leishmania infantum chagasi isolation method from bone marrow and peripheral blood of adults and children. J Infect Dev Ctries. 2016;10:1275-7. doi:10.3855/jidc.8022
20. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990;215:403-10. doi:10.1016/S0022-2836(05)80360-2
21. Cabral AD, Garcia FB, Suzuki RB, et al. Dataset on recombinant expression of an ancient chitinase gene from different species of *Leishmania* parasites in bacteria and in Spodoptera frugiperda cells using baculovirus. Data Brief. 2020;32:106259. doi:10.1016/j.dib.2020.106259
22. Schonian G. Genetics and evolution of *Leishmania* parasites. Infect Genet Evol. 2017;50:93-94. doi:10.1016/j.meegid.2017.03.016
23. Cohen-Kupiec R, Chet I. The molecular biology of chitin digestion. Curr Opin Biotechnol. 1998;9:270-277. doi:10.1016/s0958-1669(98)80058-x
24. Schlein Y, Jacobson RL, Shlomai J. Chitinase secreted by Leishmania functions in the sandfly vector. Proc Biol Sci. 1991;245:121-126. doi:10.1098/rspb.1991.0097
25. Rogers ME, Chance ML, Bates PA. The role of promastigote secretory gel in the origin and transmission of the infective stage of *Leishmania mexicana* by the sandfly Lutzomyia longipalpis. Parasitology 2002;124:495-507. doi:10.1017/s0031182002001439
26. Shakarian AM, McGugan GC, Joshi MB, *et al*. Identification, characterization, and expression of a unique secretory lipase from the human pathogen *Leishmania donovani*. Mol Cell Biochem. 2010;341:17-31. doi:10.1007/s11010-010-0433-6
27. Joshi MB, Rogers ME, Shakarian AM, et al. Molecular characterization, expression, and in vivo analysis of LmexCht1: the chitinase of the human pathogen, *Leishmania mexicana*. J Biol Chem. 2005;280:3847-61. doi:10.1074/jbc.M412299200
28. Rogers ME, Hajmova M, Joshi MB, et al. Leishmania chitinase facilitates colonization of sand fly vectors and enhances transmission to mice. Cell Microbiol. 2008;10:1363-72. doi:10.1111/j.1462-5822.2008.01132.x
29. Akhoundi M, Cannet A, Arab MK, Marty P, Delaunay P. An old lady with Pediculosis pubis on the head hair. J Eur Acad Dermatol Venereol. 2016;30:885-7. doi:10.1111/jdv.13050
30. van Eys GJ, Schoone GJ, Kroon NC, Ebeling SB. Sequence analysis of small subunit ribosomal RNA genes and its use for detection and identification of *Leishmania* parasites. Mol Biochem Parasitol. 1992;51:133-42. doi:10.1016/0166-6851(92)90208-2
31. Schonian G, Nasereddin A, Dinse N, et al. PCR diagnosis and characterization of Leishmania in local and imported clinical samples. Diagn Microbiol Infect Dis. 2003;47:349-58. doi:10.1016/s0732-8893(03)00093-2
32. Cortes S, Rolao N, Ramada J, Campino L. PCR as a rapid and sensitive tool in the diagnosis of human and canine leishmaniasis using *Leishmania donovani* s.l.-specific kinetoplastid primers. Trans R Soc Trop Med Hyg. 2004;98:12-7. doi:10.1016/s0035-9203(03)00002-6
33. Paiva BR, Passos LN, Falqueto A, Malafronte Rdos S, Andrade HF Jr. Single step polymerase chain reaction (PCR) for the diagnosis of the *Leishmania* (*Viannia*) subgenus. Rev Inst Med Trop Sao Paulo. 2004;46:335-8. doi:10.1590/s0036-46652004000600007
34. da Silva LA, de Sousa Cdos S, da Graca GC, Porrozzi R, Cupolillo E. Sequence analysis and PCR-RFLP profiling of the hsp70 gene as a valuable tool for identifying *Leishmania* species associated with human leishmaniasis in Brazil. Infect Genet Evol. 2010;10:77-83. doi:10.1016/j.meegid.2009.11.001
35. Thakur S, Joshi J, Kaur S. Leishmaniasis diagnosis:an update on the use of parasitological, immunological and molecular methods. J Parasit Dis. 2020;44(2):253-272. doi:10.1007/s12639-020-01212-w
36. Rogers MB, Hilley JD, Dickens NJ, et al. Chromosome and gene copy number variation allow major structural change between species and strains of *Leishmania*. Genome Res. 2011;21:2129-42. doi:10.1101/gr.122945.111
37. Ubeda JM, Legare D, Raymond F, et al. Modulation of gene expression in drug resistant *Leishmania* is associated with gene amplification, gene deletion and chromosome aneuploidy. Genome Biol. 2008;9:R115. doi:10.1186/gb-2008-9-7-r115
38. Degrave W, Fernandes O, Campbell D, Bozza M, Lopes U. Use of molecular probes and PCR for detection and typing of *Leishmania*--a mini-review. Mem Inst Oswaldo Cruz. 1994;89:463-9. doi:10.1590/s0074-02761994000300032
39. Viol MA, Lima VM, Aquino MC, et al. Detection of cross infections by *Leishmania* spp. and *Trypanosoma* spp. in dogs using indirect immunoenzyme assay, indirect fluorescent antibody test and polymerase chain reaction. Parasitol Res. 2012;111:1607-13. doi:10.1007/s00436-012-2999-2
40. Hijjawi N, Kanani KA, Rasheed M, Atoum M, Abdel-Dayem M, Irhimeh MR. Molecular diagnosis and identification of *Leishmania* species in Jordan from saved dry samples. Biomed Res Int. 2016;2016:6871739. doi:10.1155/2016/6871739