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

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**Introduction:** Leishmaniases, 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* subgroup by adopting polymerase chain reaction (PCR) and restriction fragment length polymorphism and using *in silico* tools. The adopted PCR method for determining 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 trypanosomatid 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

Received: April 7, 2021, Accepted: July 23, 2021, ePublished: October 1, 2021

**Introduction**

Protozoan parasites of the *Leishmania* genus are the causative agents of multiple leishmaniases 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 leishmaniases, TL) to fatal visceral infection ( visceral leishmaniases, 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
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 formulating 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, and 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 diagnostically 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 (‘GTTCMACTACGAGGCCTTCTTCAA3’) and Lquit1182R (‘CAGATCATTATCCCAGACAAGTT 5’), 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 Lquit1182R, 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 for 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...
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

<table>
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**Note.** 1. GenBank (39); ENA: European Nucleotide Archive (40); TriTrypDB (16): Kinetoplastid Genomics Resource.
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*, *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 intersubgenus identity in all important putative domains and post-translational modifications (21).

The trypanosomatid 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.

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 *Fst 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 Dde 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-acetylgalcosamine 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-acetylgalcosaminidase 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*, *Crithidium 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-
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), minixen (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 *PstI* 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 Dde 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 a 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.

Funding
This work 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) by CAPES and UFABC. LGA is the recipient of a Master’s scholarship from Fundação de Amparo a Pesquisa do Estado da Bahia (FAPESB; protocol BOL0159/2019).

Supplementary files
Supplementary file 1 contains Table S1 and Figure S1.

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