TESTING AND VALIDATING PCR-RFLP OF HEAT-SHOCK PROTEIN 70 GENE FOR FURTHER USE AS A UNIVERSAL TOOL FOR *LEISHMANIA* IDENTIFICATION AND FOR REPLACING MLEE

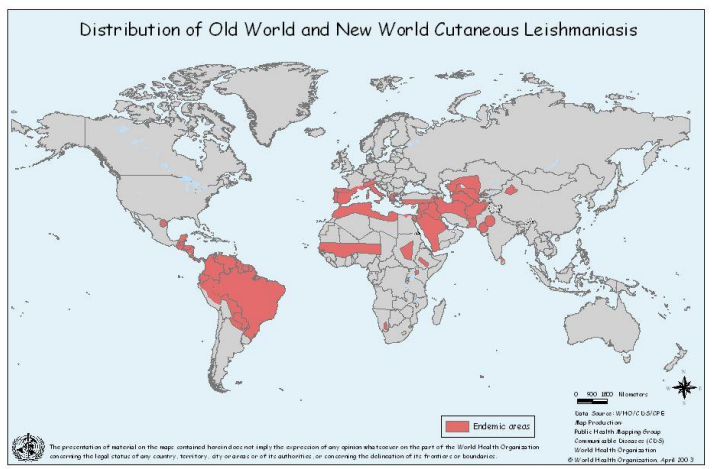
Laboratory of Research in Leishmaniasis

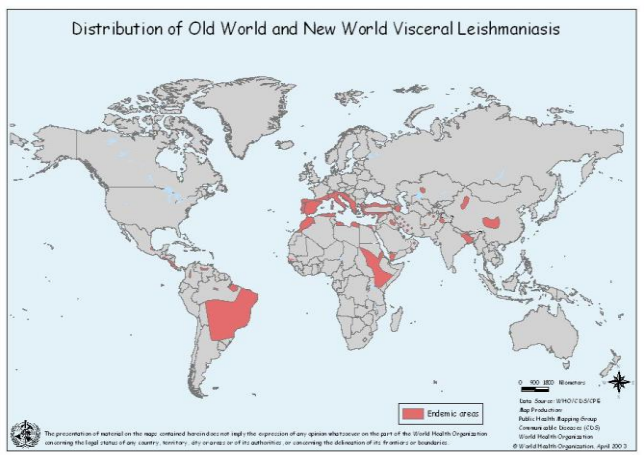
CLIOC – *Leishmania* Collection of Oswaldo Cruz Institute

Mariana Côrtes Boité
Taíse Salgado de Oliveira
Barbara Neves dos Santos
Graziele Cardoso da Graça
Elisa Cupolillo

Leishmania and Leishmaniasis

Leishmaniasis





- World-wide disease: 88 countries

-Incidence per year: 1-1.5 million new cases of CL and 500 000 new cases of VL

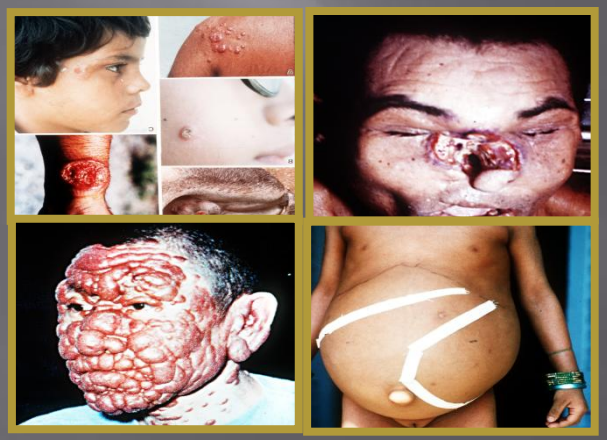
-Population at risk: 350 millions

- Risk factors: urbanization, migration,

http://gamapserver.who.int/mapLibrary/

Why Leishmania typing is needed?

http://www.brasilescola.com



Cutaneous;

Visceral;

Mucocutaneous;

Difuse;

Leishmania species

Extrinsic aspects

Why Leishmania typing is needed?

The use of biochemical and molecular methods increased our capacity to detected discreet differences among the *Leishmania* species and strains through diverse typing approaches. Such achievement, although positive, now raises the concern of which methodology to use from now on, and how to integrate the discoveries in a taxonomic way.

Regarding public health, now we ask how to gather all the methods available in order to provide an efficient and standardized diagnosis for leishmanisis.

There is an increasing demand for differential diagnosis to identify the infecting species, as prognosis of disease progression.

Why Leishmania typing is needed?

Subreino: Protozoa

Ordem: Kinetoplastida

Família: Tripanossomatidae

30 species
20 pathogenic

Gênero: *Crithidia* *Leptomonas* *Herpetomonas* *Blastocrithidia* ***Leishmania*** *Sauroleishmania* *Trypanosoma* *Phytomonas* *Endotrypanum*

Subgênero: *Leishmania* | *Viannia*

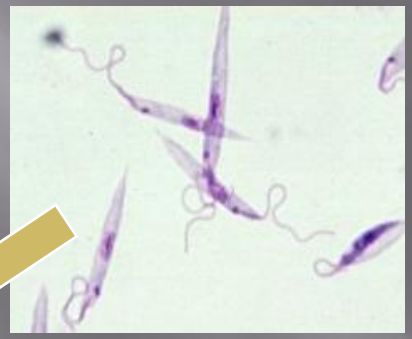
Complexo	Espécie
L. donovani	*L. donovani* *L. infantum* *L. archibaldi* *L. chagasi*
L. tropica	*L. killicki* *L. tropica*
L. major	*L. major*
L. aethiopica	*L. aethiopica*
L. mexicana	*L. amazonensis* *L. mexicana* *L. pifanoi* *L. venezuelensis* *L. forattinii* *L. garnhami*
L. braziliensis	*L. braziliensis* *L. peruviana*
L. naiffi	*L. naiffi*
L. guyanensis	*L. panamensis* *L. shawi* *L. guyanensis*
L. lainsoni	*L. lainsoni*

Paraleishmania

L. equatoriensis
L. colombiensis

Why Leishmania typing is needed?

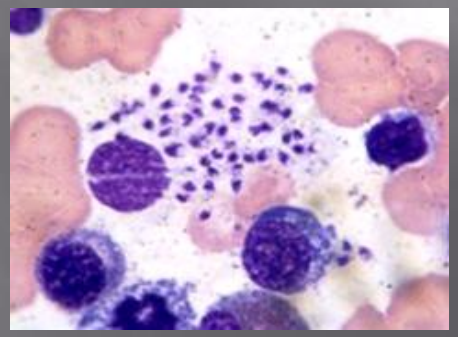
CLIOC



Promastigote;

Insect vector;

Culture



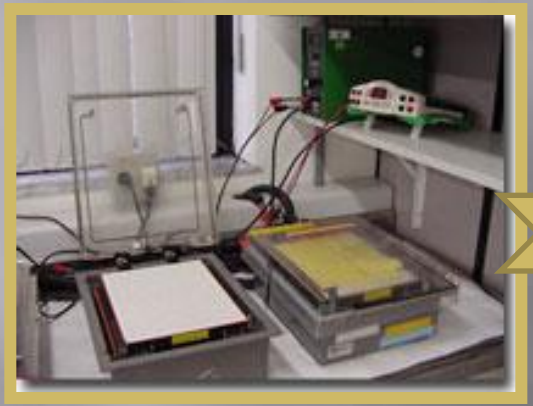
Amastigote;

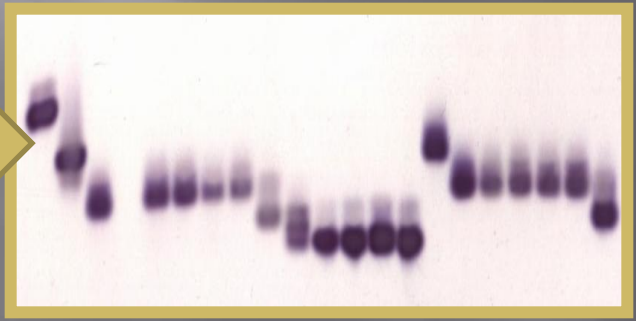
Host;

More difficult to maintain in culture;

Observed in biopsies

The gold standard method: Multilocus enzyme electrophoresis - MLEE





Drawbacks !

Efficient , fast and reproducible typing system

PCR based methods

(da Silva et al., 2010)

(Montalvo et al., 2010; Fraga et al., 2010)

RFLP of Hsp70

Heat Shock Protein (Hsp70)

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

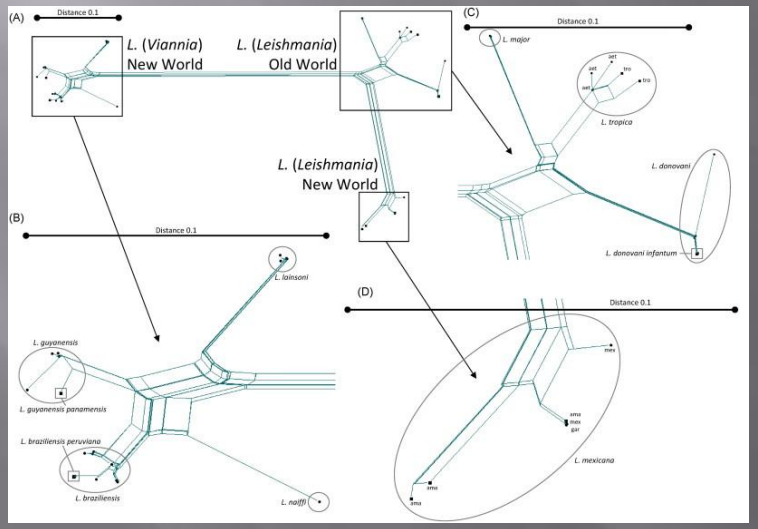
journal homepage: www.elsevier.com/locate/meegid

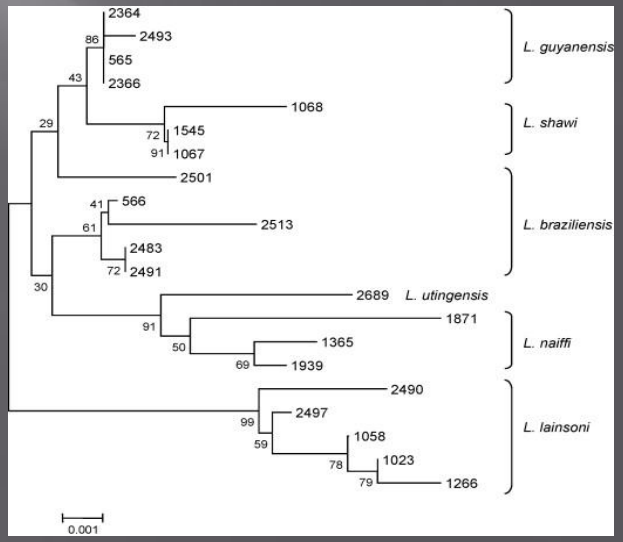
Phylogeny of *Leishmania* species based on the heat-shock protein 70 gene☆

Jorge Fraga [a], Ana Margarita Montalvo [a], Simonne De Doncker [b], Jean-Claude Dujardin [b], Gert Van der Auwera [b,*]

[a] Parasitology Department, Institute of Tropical Medicine Pedro Kouri, La Havana, Cuba
[b] Department of Parasitology, Institute of Tropical Medicine Antwerp, Nationalestraat 155, 2000 Antwerp, Belgium

Hsp70 gene sequencing to perform phylogenetic analysis





Da Silva et al., 2010

Heat Shock Protein (Hsp70)

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Sequence analysis and PCR-RFLP profiling of the *hsp70* gene as a valuable tool for identifying *Leishmania* species associated with human leishmaniasis in Brazil

Leonardo Alves da Silva [1], Cíntia dos Santos de Sousa [1], Grazielle Cardoso da Graça, Renato Porrozzi, Elisa Cupolillo *

Analysis of Hsp70 sequences of *Leishmania* species associated with leishmaniasis in Brazil;

Identification of restriction enzymes that could be used for PCR-RFLP;

The results were in agreement with MLEE results = replacement in Leishmania typing

Heat Shock Protein (Hsp70)

Contents lists available at ScienceDirect

Transactions of the Royal Society of Tropical Medicine and Hygiene

journal homepage: http://www.elsevier.com/locate/trstmh

Differentiation of *Leishmania (Viannia) panamensis and Leishmania (V.) guyanensis* using *Bcc*I for *hsp*70 PCR-RFLP

Ana Margarita Montalvo Alvarez[a,*], Jorge Fraga Nodarse[a], Ivón Montano Goodridge[a], Lianet Monzote Fidalgo[a], Marcel Marin[b], Gert Van Der Auwera[c], Jean-Claude Dujardin[c], Iván Darío Velez Bernal[b], Carlos Muskus[b]

[a] Instituto de Medicina Tropical Pedro Kourí, Apartado Postal 601, M
[b] Programa de Estudio y Control de Enfermedades Tropicales, Unive
[c] Institute of Tropical Medicine, Antwerp, Belgium

Heat-shock protein 70 PCR-RFLP: a universal simple tool for *Leishmania* species discrimination in the New and Old World

A. M. MONTALVO[1], J. FRAGA[1], L. MONZOTE[1], I. MONTANO[1], S. DE DONCKER[2], J. C. DUJARDIN[2,3] *and* G. VAN DER AUWERA[2*]

[1] *Instituto de Medicina Tropical Pedro Kourí, Departamento de Parasitología, La Habana, Cuba*
[2] *Institute of Tropical Medicine Antwerp, Antwerp, Belgium*
[3] *Department of Biomedical Sciences, University of Antwerp, Antwerp, Belgium*

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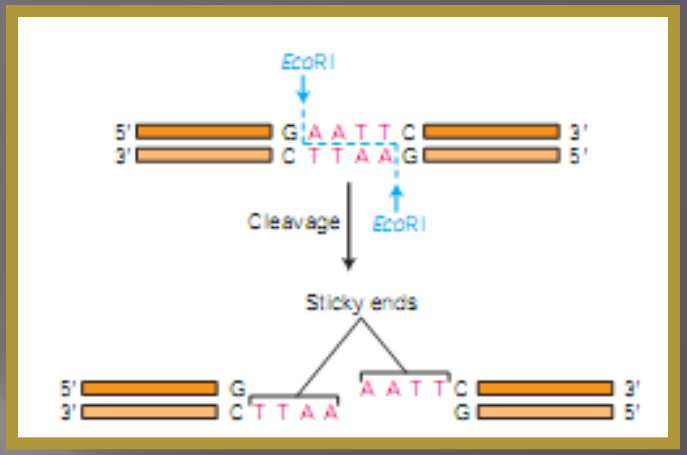
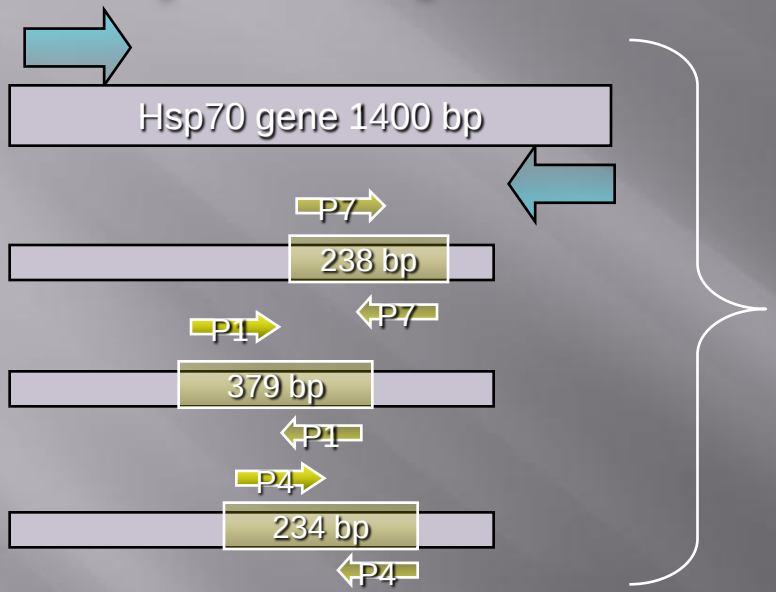
Hsp70 gene 1400 bp

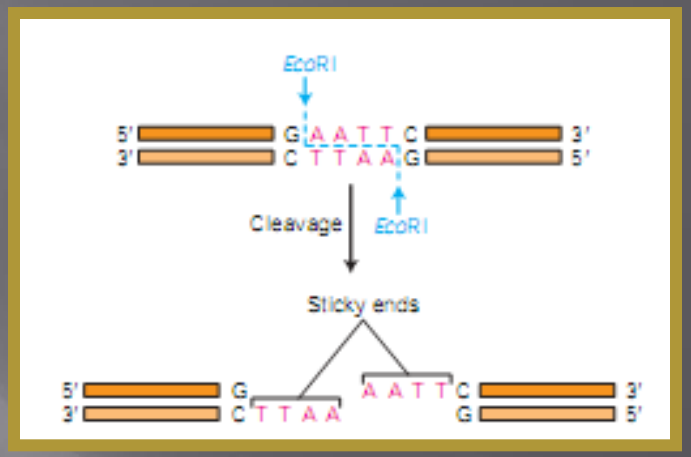
Restriction Fragment Length Polymorphisms - RFLP

In the RFLP approach the sample, or PCR product, is digested by restriction enzymes, and the fragments obtained are separated by their sizes in a electroforesis gel.



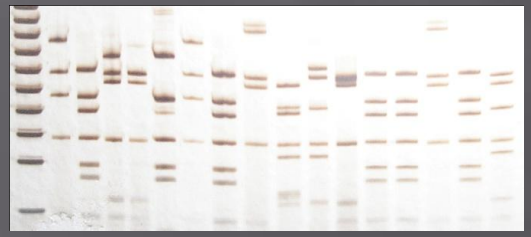
Restriction Fragment Length Polymorphisms - RFLP





Hae III *Sau 3AI*

Acrylamide 12,5%



RFLP Panel

Methodology

Parte superior do formulário

IOC/L0563 (MHOM/ET/1967/HU3) *L. (L.) donovani*

IOC/L0565 (MHOM/BR/1975/M4147) *L. (V.) guyanensis*

IOC/L0566 (MHOM/BR/1975/M2903) *L. (V.) braziliensis*

IOC/L0571 (MHOM/SU/1958/STRAIN OD) *L.(L.) tropica*

IOC/L0575 (IFLA/BR/1967/PH8) *L.(L.) amazonensis*

IOC/L0579 (MHOM/BR/1974/PP75) *L. (L.) chagasi*

IOC/L0581 (MHOM/SU/1973/5-ASKH) *L.(L.) major*

IOC/L0582 (MCOE/PA/1965/C8) *L. hertigi*

IOC/L0888 (MCHO/EC/1982/LSP1) *L. equatorensis*

IOC/L1023 (MHOM/BR/1981/M6426) *L. (V.) lainsoni*

IOC/L1245 (IGOM/PA/1985/E582.34) *L. colombiensis*

IOC/L1365 (MDAS/BR/1979/M5533) *L. (V.) naiffi*

IOC/L1545 (MCEB/BR/1984/M8408) *L. (V.) shawi*

IOC/L2272 (MHOM/ET/1967/L82;HV3;LV9) *L.(L.) donovani*

IOC/L2732 (MHOM/TN/1993/LV10) *L. (L.) infantum*

IOC/L2821 (MHOM/IL/1980/FRIEDLIN) *L. (L.) major*

IOC/L2906 (MHOM/BR/2002/LPC-RPV) *L. (L.) chagasi*

Parte inferior do formulário

17 reference strains representing 13 species of *Leishmania*

http://clioc.fiocruz.br/

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collections

CLIOC • *Leishmania* Collection

português

home | history | services | staff | catalogue | projects | protocols & manuals | contact

The *Leishmania* collection of the Oswaldo Cruz Institute (Coleção de *Leishmania* do Instituto Oswaldo Cruz), CLIOC, was created in 1980, with the support of the Oswaldo Cruz Institute, the Oswaldo Cruz Foundation and the World Health Organization.

CLIOC's mission is to act as a Biological Resource Center (as defined by the Organisation for Economic Cooperation and Development, OECD), dedicated to preservation, storage, distribution, taxonomic characterization, and identification of *Leishmania* and associated information, contributing to the scientific and technological development of the country. CLIOC attends public research and education institutions, industry in general, offering assistance and technical and scientific consultancy, Training and development of specific research projects. Its field of work is directly associated to collective health sectors of the country.

CLIOC is registered in the *World Federation for Culture Collections, WFCC* - WDCM 731 - and is recognized as a Depository Authority by the Ministry of the Environment [Fiel Depositária pelo Ministério do Meio Ambiente, MMA] (D.O.U. 05.04.2005).

The team of professionals and collaborators associated to CLIOC are qualified in parasitology, *Leishmania* systematics, with experience in molecular systematics, phylogeny, and in the use of culture independent methods to characterize *Leishmania* species associated to human diseases or that are found infecting other vertebrates and their vectors, phlebotomic insects.

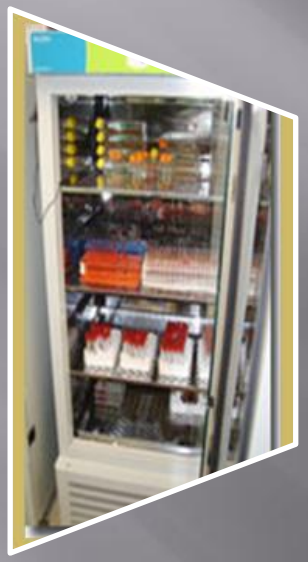
CLIOC's holdings are limited to the different species of the genera *Leishmania*, pathogen to humans or not. Genetically modified organisms are accepted.

CLIOC maintains an information system about *Leishmania* holdings deposited in its collection.

Internet | Modo Protegido: Desativado

Methodology

Strains retrieved from cryobank

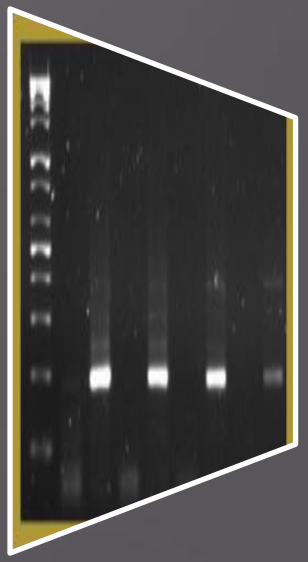


Culture

Characterization by MLEE

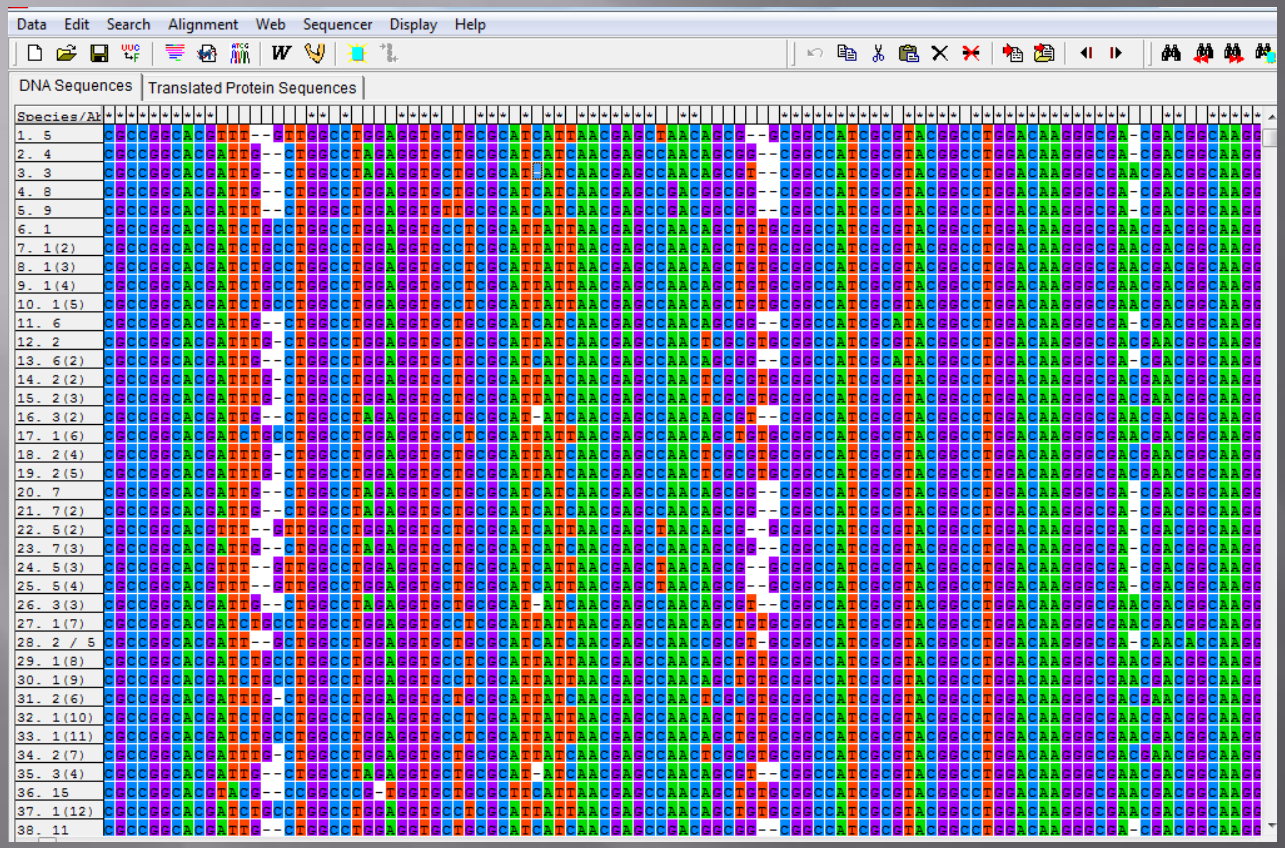
DNA extraction

PCR

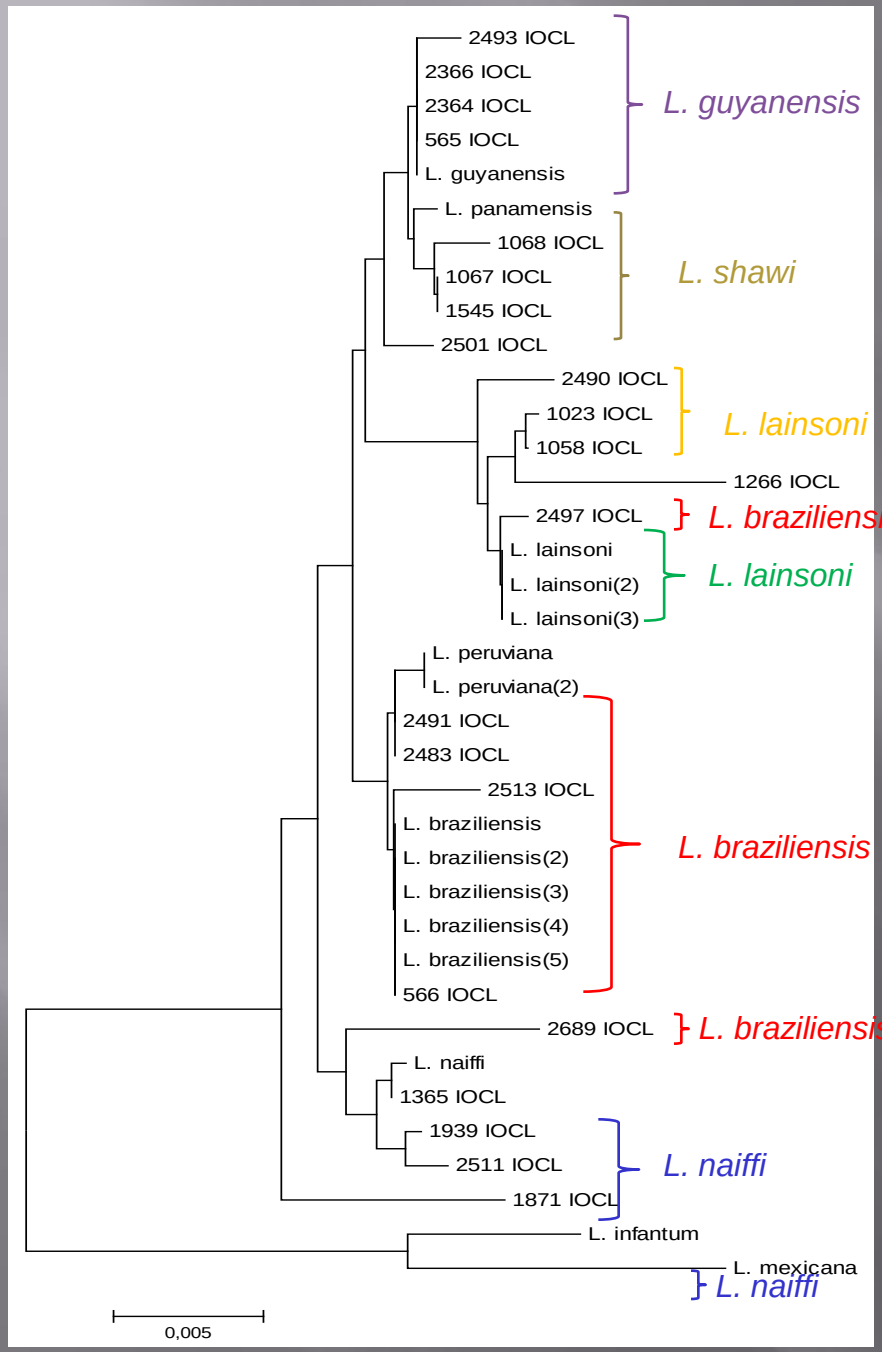


Agarose gel electrophoresis

Hsp70 gene 1400 bp



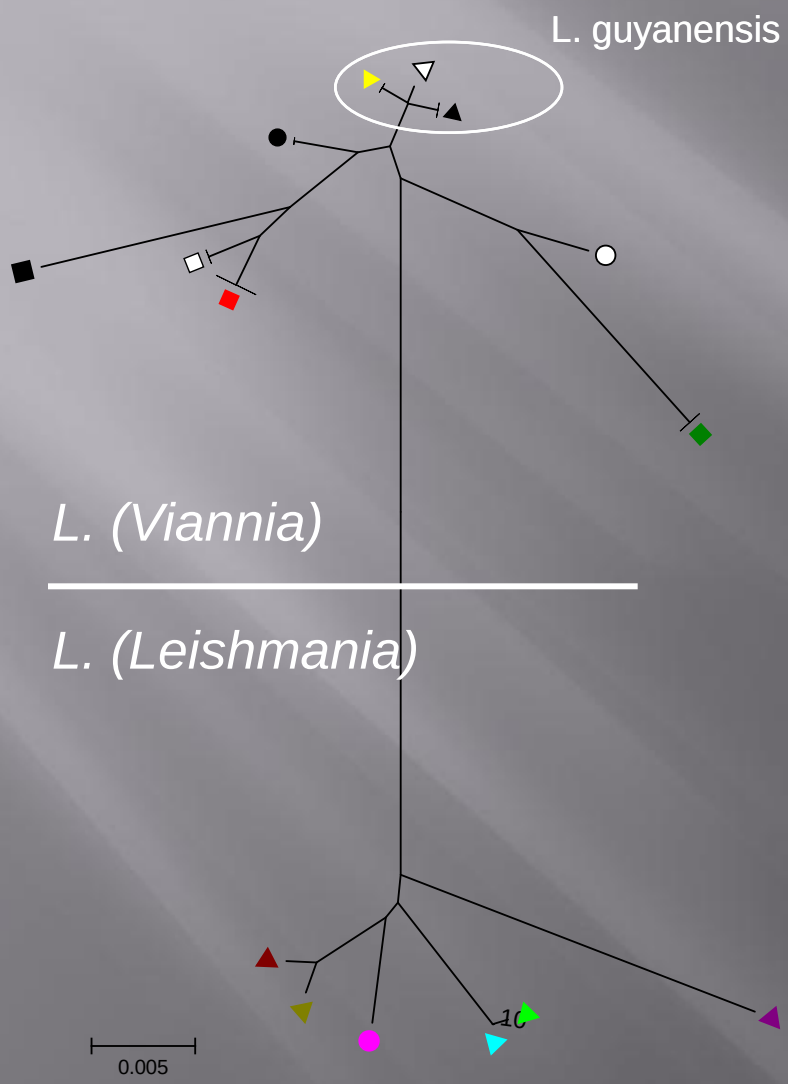
40 Hsp70 DNA sequences available in Genbank (http://www.ncbi.nlm.nih.gov/) representing 14 different *Leishmania* species were aligned using MEGA software



Hsp70 gene 1400 bp

NJ tree obtained after the alignment of Hsp70 of sequences available in Genbank

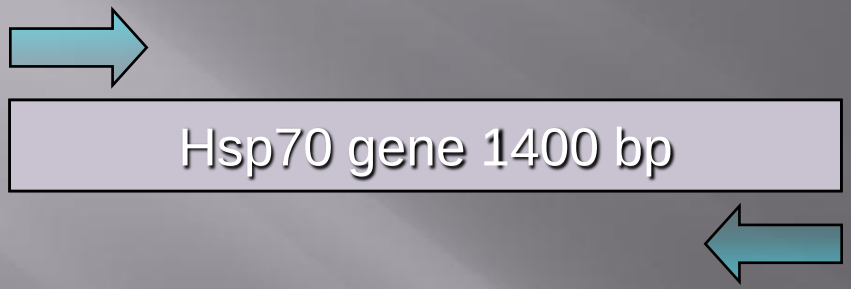
Heat Shock Protein (Hsp70)



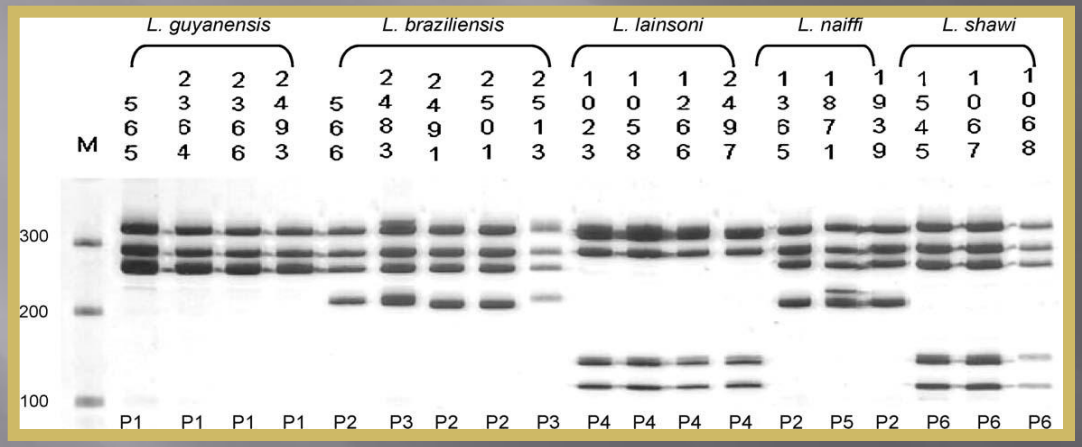
Hsp70 gene 1400 bp

NJ tree obtained after the alignment of Hsp70 of sequences available in Genbank

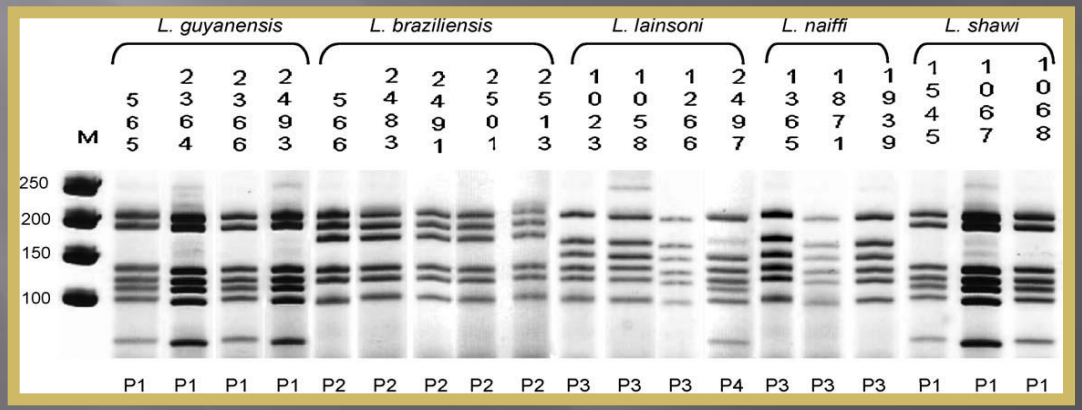
Heat Shock Protein (Hsp70)



Hae III

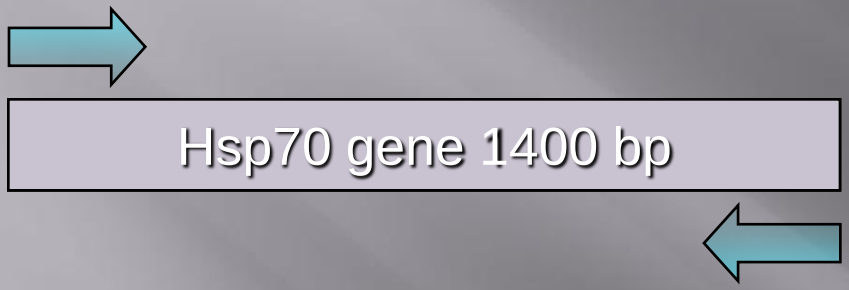


Mbo I

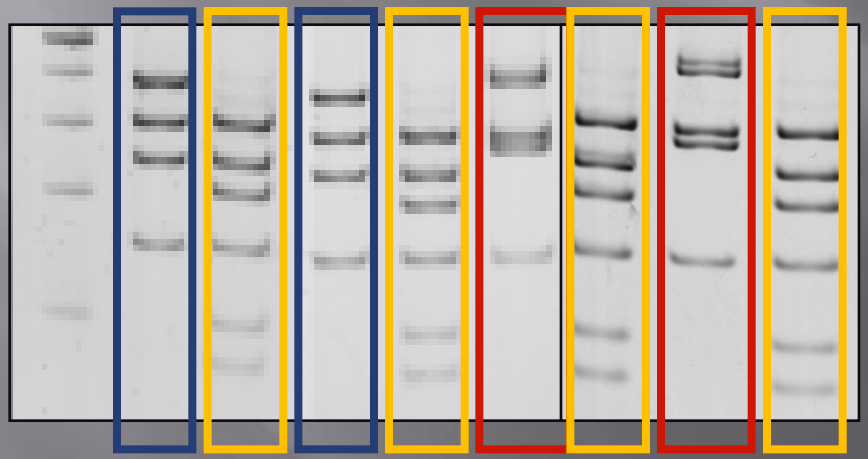


The two restriction enzymes allowed to distinguish between five *Leishmania* species of *L. (Viannia)* subgenus

Heat Shock Protein (Hsp70)



Hae III



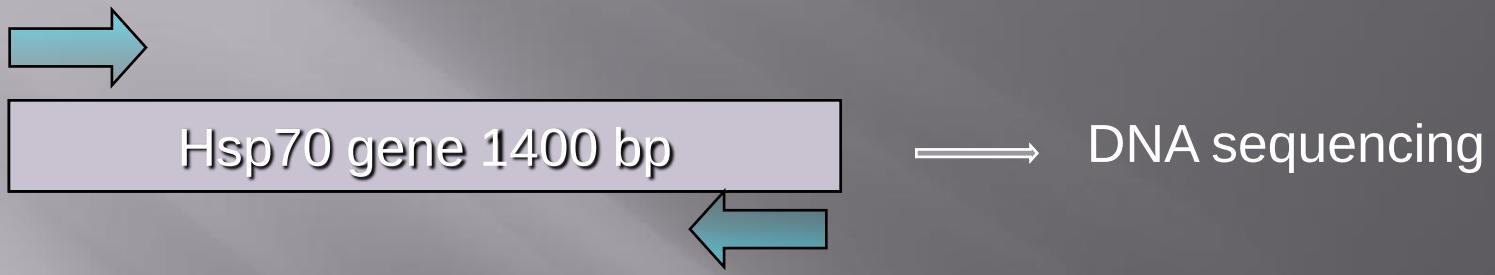
6% acrylamide gel, silver stained

L. mexicana,
L. amazonensis

L. donovani,
L. infantum syn. chagasi

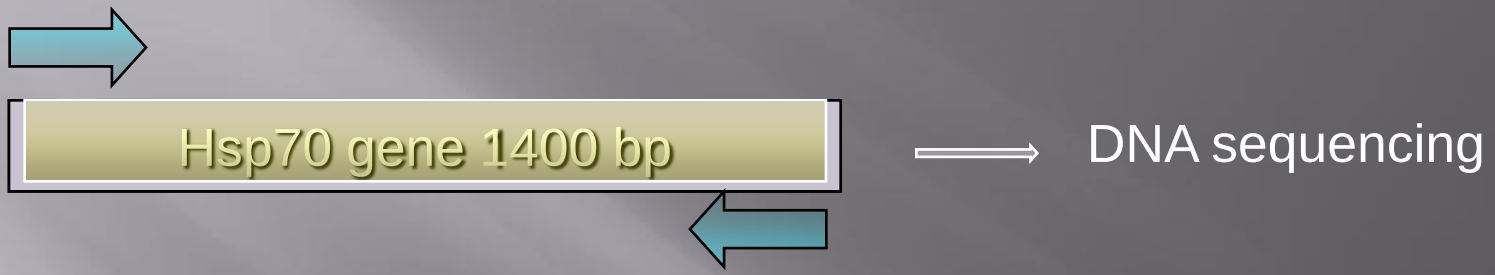
L. major

Polymorphic sites



Is it possible to use the same approach to type isolates directly from clinical material?

Polymorphic sites



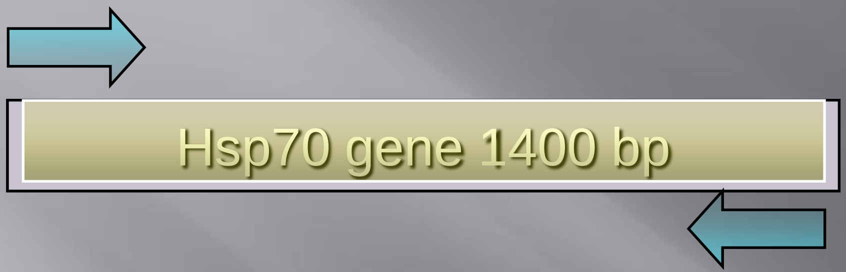
Amastigotes;

Smaller number of parasite cells;

More sensitive PCR;

Shorter PCR fragments

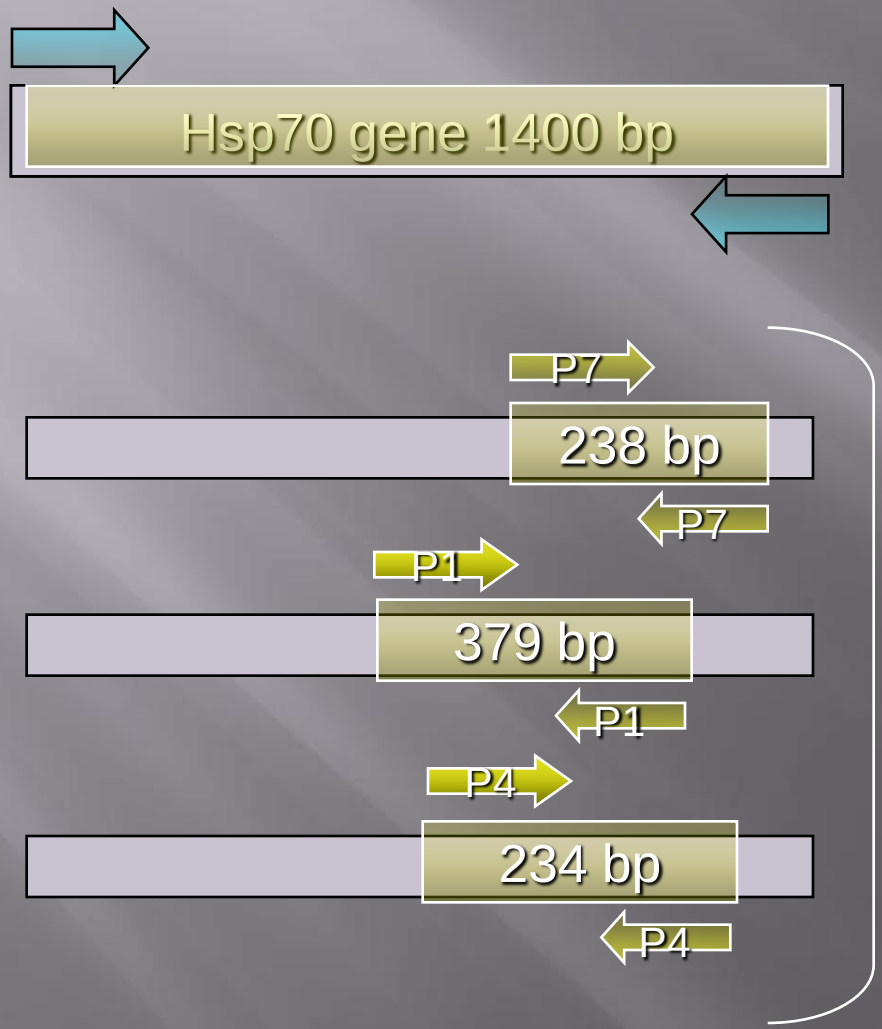
Polymorphic sites



DNA sequencing

```
                                                                          1111111111 1111111111 1111111111 1111111111
[                                              112222 2233444456 6677779999 0011111111 1111222222 2222222222 3333333333
[                                   1112345555 5679010258 9901016781 6901341389 4711122334 5677011114 5556667799 0123334555
[                                   1344921457 8114271846 1298592128 3627218649 3215678190 1323902382 3585781968 1536781057
#L._naiffi_{L_naiffi}               ATGGCCAAGG GCGGGGTTGG TTATCATAGT GGCTCTTAGC TATTCCACAT GGGCGTGCAG GAACTCCGGA GTACGCGGGA
#L._panamensis                      ...A...... .....C..C. ..GC..C..C .......... .......... ...TA..... ..GA...... .....T....
#L._guyanensis_{L_guyanensis}       ...A...... .....C..C. ..GC..C..C .......... .......... ...TA..... ..GA...A.. .....T....
#L._infantum_{L_infantum}           ......GG.. ......CCCA GGG.TCCG.C C..CGCCGA. GGCATGCAGC CA..C..GTC AG.AAAA..G .AGAAG...G
#L._mexicana                        ..T...GG.. ......CCCA GGG.TCCG.C C..CGCCGA. GGCATGCAGC CA.TC..GTC AT.AAAA..G .AGAAG..AG
#L._braziliensis_{L_braziliesis}    .......... .....C.... ..GC..C..C .......... .......... ......T... .TGA...A.. ..........
#L._braziliensis(2)_{L_braziliesis} .......... .....C.... ..GC..C..C .......... .......... ......T... .TGA...A.. ..........
#L._braziliensis(3)_{L_braziliesis} .......... .....C.... ..GC..C..C .......... .......... ......T... .TGA...A.. ..........
#L._braziliensis(4)_{L_braziliesis} .......... .....C.... ..GC..C..C .......... .......... ......T... .TGA...A.. ..........
#L._braziliensis(5)_{L_braziliesis} .......... .....C.... ..GC..C..C .......... .......... ......T... .TGA...A.. ..........
#L._peruviana                       .......... ..A..C.... ..GC..C.AC .......... .......... ......T... .TGA...... ..........
#L._lainsoni_{L_lainsoni}           .......C.. .......... ..G..CCC.C ..T....... .......... ..CTA.T... AGGA...A.. A....T....
#L._peruviana(2)                    .......... ..A..C.... ..GC..C.AC .......... .......... ......T... .TGA...... ..........
#L._lainsoni(2)_{L_lainsoni}        .......C.. .......... ..G..CCC.C ..T....... .......... ..CTA.T... AGGA...A.. A....T....
#L._lainsoni(3)_{L_lainsoni}        .......C.. .......... ..G..CCC.C ..T....... .......... ..CTA.T... AGGA...A.. A....T....
#565_IOCL_{L_guyanensis}            ...A...... .....C..C. ..GC..C..C .......... .......... ...TA..... ..GA...A.. .....T....
#566_IOCL_{L_braziliesis}           .......... T....C.... ..GC..C..C .......... .......... ......T... .TGA...A.. .......T..
#1023_IOCL_{L_lainsoni}             .......C.. .......... ..G..CCC.C ..T....... .......... ..CTACT.T. AG.A...A.. A....T....
#1058_IOCL                          ..-.-..C.. -......... ..G..CCC.C ..T....... .......... ..CTACT.T. AG.A...A.. .....T.T..
#1067_IOCL                          ...A...... T....C..C. ..GC..C..C .A........ .......... ...TA..... .GGA...... .....T.T..
#1068_IOCL                          ---A.....C -T..CC..C. ..GC..C..C .A........ .......... ...TA..... .GGA....A. .....T.---
#1365_IOCL_{L_naiffi}               .......... .......... .......... .......... .......... .......... .......... ..........
#1545_IOCL                          ...A...... .....C..C. ..GC..C..C .A........ .......... ...TA..... .GGA...... .....T....
#1871_IOCL                          ..-.....CT -......... ......C..C .......... ..C.....GC ....C...T. ..G....T.. ......A...
#1939_IOCL                          T.-..T.... -......... .......... .........T .......... .......... ..G....... ..........
#2364_IOCL                          ...A...... .....C..C. ..GC..C..C .......... .......... ...TA..... ..GA...A.. .....T.T..
#2483_IOCL                          ..-....... -....C.... ..GC..C..C .......... .......... ......T... .TGA...... .......T..
#2490_IOCL                          ..-....C.. T..A.C.... ..G..CCC.C ..T....... .......... ..CTA.T... AGGA...A.. .....T.T..
#2491_IOCL                          .......... .....C.... ..GC..C..C .......... .......... ......T... .TGA...... ........A.
#2493_IOCL                          ...A...... T....C..C. ..GC..C..C .......... .......... ...TA..... ..GA...A.. .....T.T..
#2497_IOCL                          .......C.T .......... ..G..CCC.C ..T....... .......... ..CTA.T... AGGA...A.. A....T.T..
#2501_IOCL                          .......... .....C.... ..GC..C..C .......... .......... ...TA..... ..GA...A.. .....T.T..
#2513_IOCL                          .........C .T..CC.... ..GC..C..C .......... .......... ......T... .TGA...AA. .......---
#1266_IOCL                          ..T.T..TCC ...A.C.... ..G..CCC.C ..T....... .......... ..CTACT.T. AG.A...A.. A....T.T..
#2366_IOCL                          ...A-..... T....C..C. ..GC..C..C .......... .......... ...TA..... ..GA...A.. .....T.T..
#2511_IOCL                          .CT.TT...T .......... .......... .........T .......... .......... ..G....... ..........
#2689_IOCL                          TC...T...T .....C.... ......C..C A......... .......... .......... ..G......- ......AT..
```

Polymorphic sites

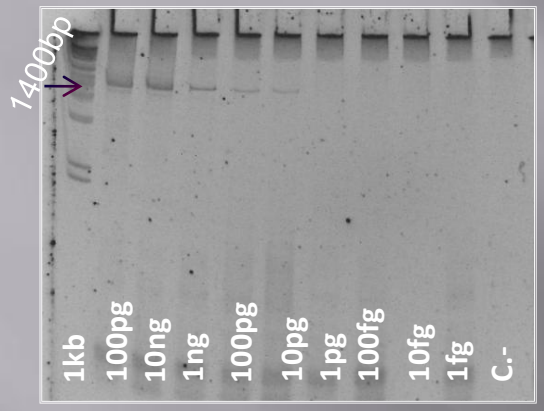


DNA sequencing

Design of primers amplifying shorter fragments, aiming to improve PCR sensitivity for further application on direct diagnosis; but still containing restriction sites to distinguish *Leishmania* species by RFLP

Results

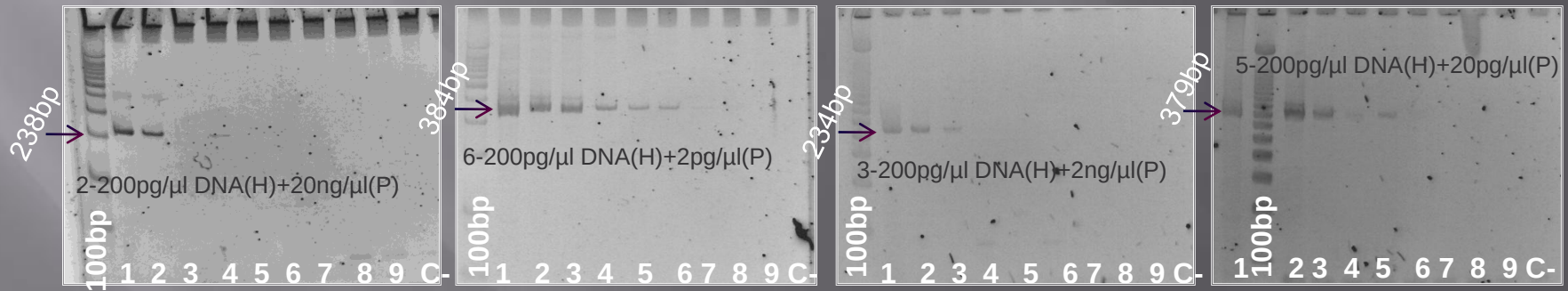
L-566 *L.braziliensis*



IOC-L 565 *L.guyanensis*
IOC-L 566 *L.braziliensis*
IOC-L 575 *L.amazonensis*
IOC-L 1023 *L.lainsoni*
IOC-L 1365 *L.naiffi*
IOC-L 1545 *L.shawi*

Objective:
To develop and standardize molecular methods for tegumentar leishmaniasis diagnosis, including direct *Leishmania* identification.

Hsp70

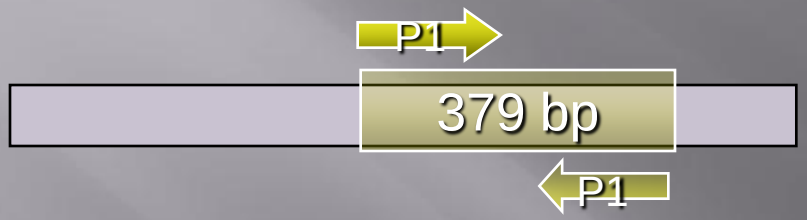


P1 P1* P4 P7

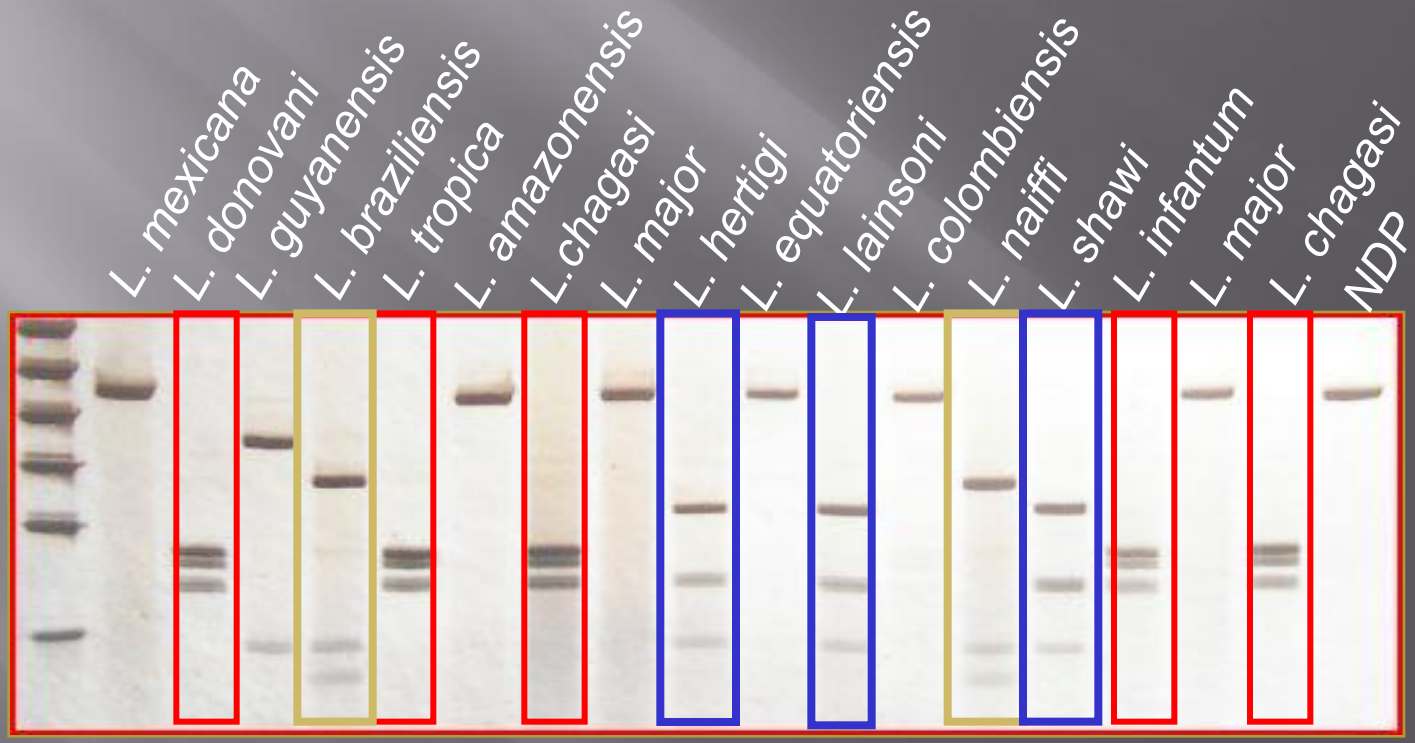
DNA humano+promastigota

Unpublished; da Graça et al.

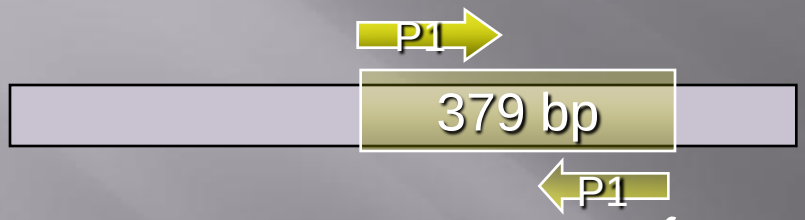
Results



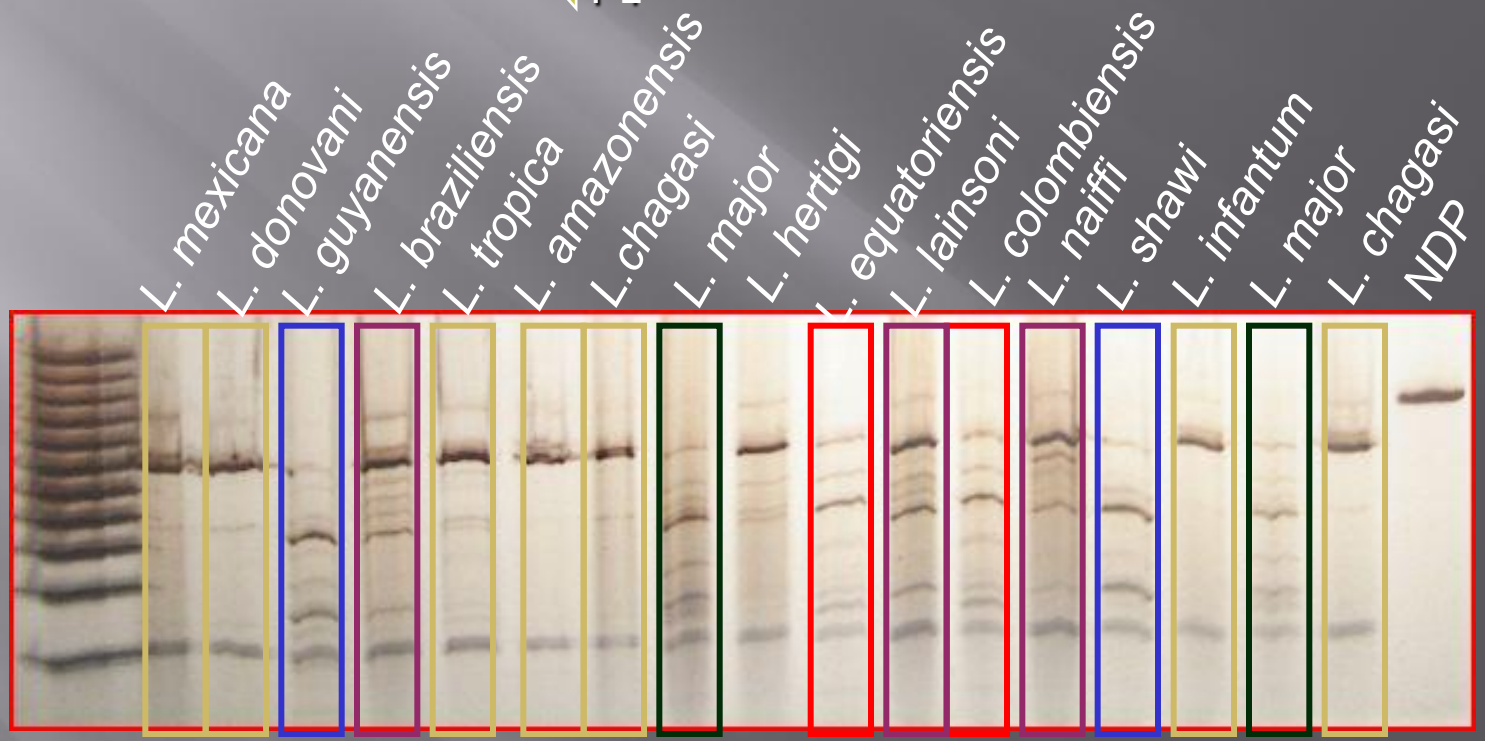
GenePhor gel, silver stained
enzyme ***Hae* III**



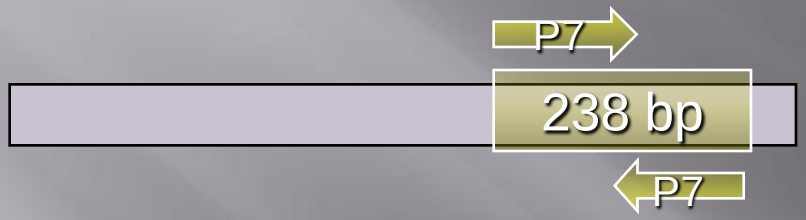
Results



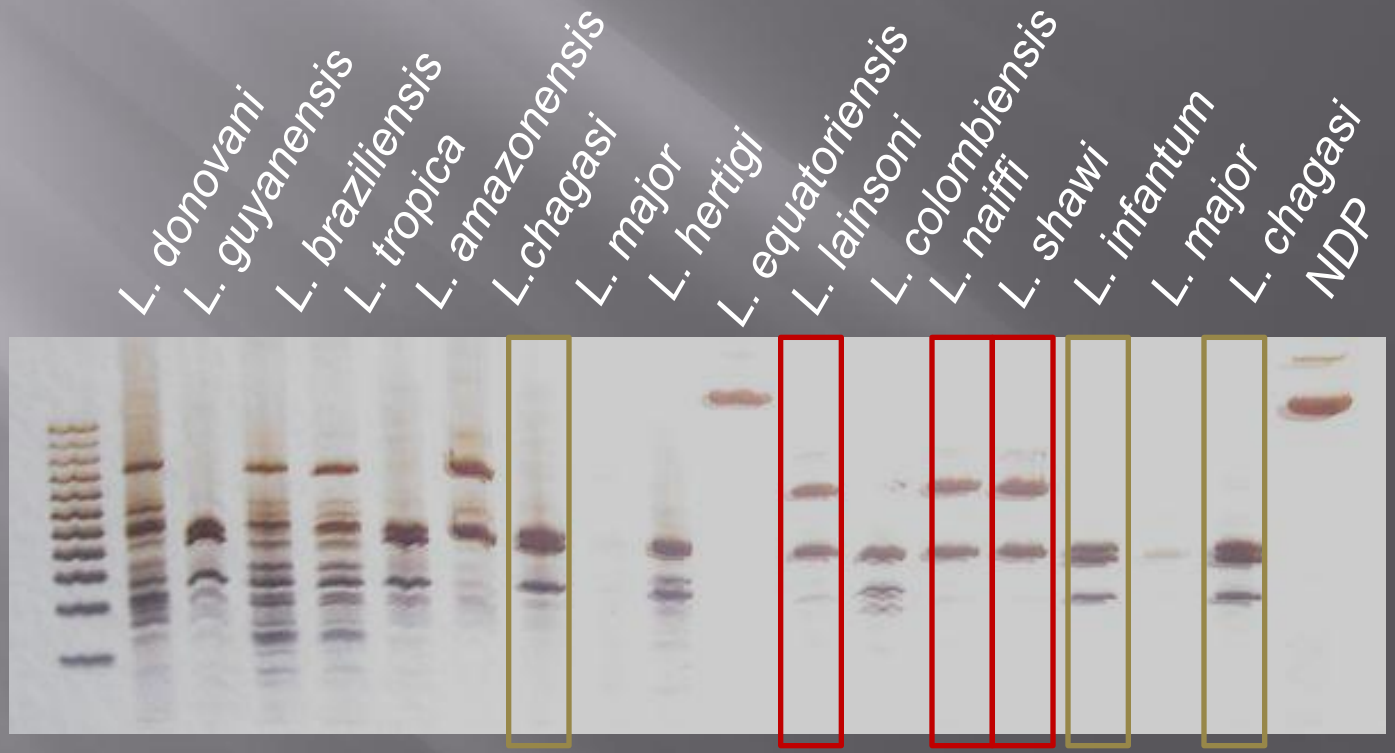
GenePhor gel, silver stained
enzyme ***Sau 3***



Results



GenePhor gel, silver stained
enzyme ***Sau 3***



Conclusions

- PCR-RFLP analysis of hsp70 has the potential to replace MLEE for *Leishmania* typing
- It thus present potential for direct diagnosis
- It is already used as an additional tool for *Leishmania* strain typing and characterization at the CLIOC routine.

Testing and Validating PCR-RFLP of Heat-shock Protein 70 Gene for Further Use as a Universal Tool for Leishmania Identification and for Replacing MLEE

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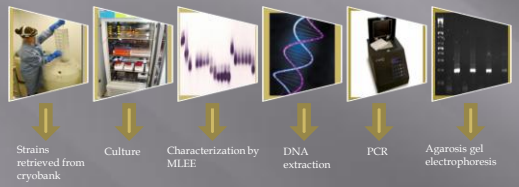
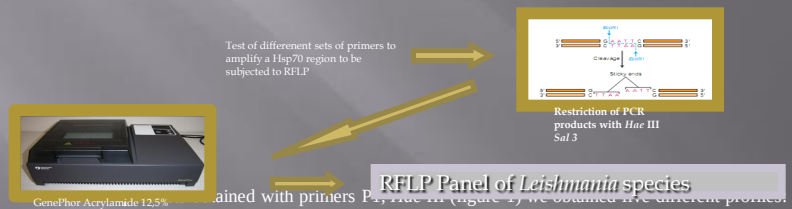
In an attempt to overcome the limitations of multilocus enzyme electrophoresis (MLEE) - gold standard tool for *Leishmania* identification - PCR-based methods have been developed and employed. Studies performed by our group (da Silva et al., 2010) and colleagues (Montalvo et al., 2010) have demonstrated that the target hsp70 differentiates many New and Old World *Leishmania* species through PCR-RFLP and DNA sequencing. These findings suggest that such approach might represent a universal and accurate tool for *Leishmania* species identification. Based on that, we aim to validate hsp70 PCR-RFLP as a substitute for MLEE for *Leishmania* typing.

To construct a hsp70 PCR-RFLP panel we have used 17 reference strains, listed below, representing *Leishmania* species of the New and Old World, from CLIOC – Oswaldo Cruz Institute Collection. The methodology followed the flux as follows:



One pair of primers and PCR conditions were the same from a previous study (da Silva et al., 2010). However, for the present project we included two more pair of primers to be tested, which amplify smaller products. The DNA was properly amplified for each set of primers and the fragments observed in 1% agarose gels to check PCR effiency. Hsp70 PCR products were digested with *Hae*III and *Sau* 3. Products were subjected to 12.5% acrylamide gel electrophoresis (Genephor) and also to conventional acrylamide 6% gel and then silver stained.



ained with primers P1, Hae III (figure 1) we obtained five different profiles: one only observed just in *L. guyanensis*, one for *L. donovani*, *L tropica* and *L. infantum syn. chagasi*, one for *L. braziliensis* and *L. naiffi*; one for *L. shawi*, *L. lainsoni* and *L. hertigi*. The other species did not present any restriction site for the enzyme (same profile from the non digested produtc (NDP). In figure 2 we also observed five profiles, and in this case *L. mexicana*, *L. tropica* and *L. donovani* complexes could not be distinguished, but *L. major* complex, as well as the Paraleishmania, could be differentiated for the other species. For the L. (Viannia) species this combination was not able to differentiate *L. guyanensis* and *L. shawi*, as well as *L. braziliesis*, *L. lainsoni* and *L. naiffi*.

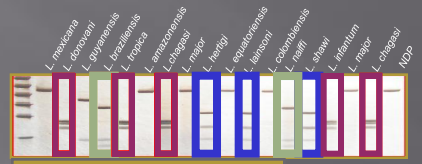


Figure 1: P1 PCR products, enzyme *Hae* III

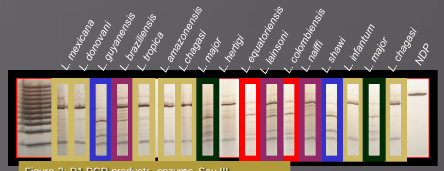


Figure 2: P1 PCR producrt, enzyme *Sau* III

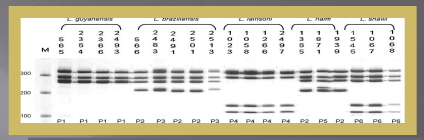


Figure 3: Previous results with PCR product 1400bp and *Hae* III

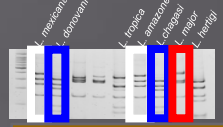
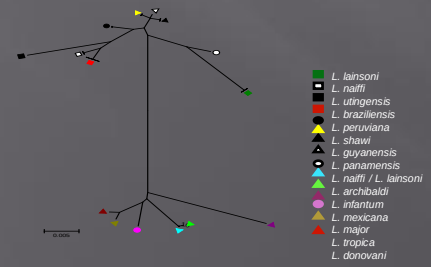


Figure 4: PCR product 1400bp and *Hae*III in a 6% acrylamide gel

Using the same set of primers from a previous study of our group (figure 3) we could reinforce the potential of Hsp70 marker as a target to differentiate New and Old Wolrd *Leishmania* species (figure4). In the first study, species of *L. (Viannia)* could be differentiated. Now we aim to increase the number of species studied, therefore species from subgenera *L. (Leishmania)* were included. The figure 4 shows a conventional 6% acrylamide gel, which was prepared in order to test the viability of its use for the present purpose, since Genephor® in a more expensive methodology. *L donovani*, *L. mexicana* and *L. major* complexes could be differentiated. However species from the same complex could not be distinguished.



40 Hsp70 DNA sequences available in Genbank (http://www.ncbi.nlm.nih.gov/) of 14 different *Leishmania* species were aligned in MEGA software and a Neighbor Joining tree was obtained. The tree shows that the target Hsp70 presents an interesting degree of polymorphism between species, which allows clear separation of subgenera and species.

Studies performed targeting Hsp70 found it as good single locus way to distinguish between *Leishmania* species. However, more tests must be applied in order to construct a final panel to be compared between laboratories and to extend the usefulness of this approach to, for instance, direct diagnosis of the disease.

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