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Evolution and species discrimination according to the *Leishmania* heat-shock protein 20 gene ☆

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ABSTRACT

The *Leishmania* genus comprises up to 35 species, of which 20 are responsible for human disease. However, the taxonomic status for many of them is under discussion. The small Heat Shock Proteins (sHSPs) are physiologically relevant, protecting cellular proteins from aggregation and maintaining cellular viability under intensive stress conditions. In *Leishmania*, a protein of this class was previously described, the 20-kDa heat-shock protein (HSP20), which is encoded by a single gene. In the present study, we used this target, alone or in combination with *hsp70* gene, to investigate the phylogenetic relationships among *Leishmania* species. Using a pair of degenerate primers it was possible amplifying a 370 bp fragment of the *hsp20* coding region in 39 strains of very different geographic origins, representing in total 16 *Leishmania* species (14 if *L. chagasi* and *L. archibaldi* are considered synonymous names of *L. infantum* and *L. donovani*, respectively). Nucleotide sequences were readily obtained by direct sequencing of the amplification products. Both phylogenetic trees and networks based on either *hsp20* sequences or combined datasets of *hsp20* and *hsp70* sequences were constructed. These phylogenic analyses supported the division of the *Leishmania* genus into nine species: *L.* (*L.*) *donovani*, *L.* (*L.*) *major*, *L.* (*L.*) *tropica*, *L.* (*L.*) *aethiopica*, *L.* (*L.*) *mexicana*, *L.* (*V.*) *lainsoni*, *L.* (*V.*) *naiffi*, *L.* (*V.*) *guyanensis* and *L.* (*V.*) *braziliensis*. Additionally, by network analysis, the subspecies *L.* (*L.*) *donovani infantum* and *L.* (*V.*) *braziliensis peruviana* were recognized within the *L.* (*L.*) *donovani* and *L.* (*V.*) *braziliensis* species, respectively. Therefore, *hsp*20 gene was found to be a suitable molecular marker for *Leishmania* typing and classification purposes. In addition, this study represents a solid contribution to the objective of establishing a more reliable taxonomy for the genus *Leishmania*.

1. Introduction

The leishmaniases are a complex of diseases caused by kinetoplastid flagellates of the genus *Leishmania*. Clinical manifestations range from simple self-healing cutaneous lesions (cutaneous leishmaniasis, CL) through metastasizing mucocutaneous forms (mucocutaneous leishmaniasis, MCL) to often lethal visceral disease (visceral leishmaniasis, VL). A recent epidemiological study recorded reports of endemic leishmaniasis transmission in a total of 98 countries and three territories on five continents. Furthermore, the global yearly incidence was estimated as 0.2–0.4 million cases of VL and 0.7–1.2 million of CL, while 12 million people worldwide are currently affected by the disease (Alvar et al., 2012). The genus *Leishmania* comprises some 35 species of morphologically similar kinetoplastid protozoa, among which 20 species are responsible for human disease. The outcome of *Leishmania* infection depends on biological and genetic traits of both the host and the infecting parasite species. Therefore, distinguishing between species is crucial for the correct diagnosis and prognosis of the disease as well as for making decisions regarding treatment and control measures (Schönian et al., 2010).

In order to design reliable diagnostic tools, it is crucial to agree on a clear definition of the taxa to be identified (Bañuls et al., 2002). The classification of *Leishmania* was initially based on ecobiological criteria such as vector, geographical distribution, tropism, antigenic properties and clinical manifestations and, later, on immunological and biochemical data (Pratt and David, 1981; Lainson and Shaw, 1987; Schönian et al., 2010). Hierarchical taxonomic schemes have been proposed using the categories of subgenera, species complexes, species, and subspecies. The two

☆ Note: Nucleotide sequence data reported in this paper are available in GenBank, EMBL and DDBJ databases under the accession numbers JX630109–JX630148.

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subgenera *L.* (*Leishmania*) and *L.* (*Viannia*) are separated on the basis of their location in the vector's intestine (Lainson and Shaw, 1987), and the species within the subgenera were established using isoenzyme analysis (Rioux et al., 1990). However, the validity of the other taxonomic categories is still debated, and the status of some species is being questioned (Bañuls et al., 2007; Schönian et al., 2010). It is therefore urgent to agree on the definition of species based on reliable and well-contrasted traits. The use of molecular sequencing techniques is providing a large amount of additional data for phylogenetic analyses, but in spite of this, evolutionary relationships remain to be solved (Hughes and Piontkivska, 2003). Different molecular markers have been used for phylogenetic studies in *Leishmania*: the internal transcribed spacer (ITS) 1 and 2 of the ribosomal DNA array (Cupolillo et al., 1995; Dávila and Momen, 2000; Berzunza-Cruz et al., 2002; Orlando et al., 2002; Kuhls et al., 2005; Spanakos et al., 2008; Sukmee et al., 2008; Villinski et al., 2008), a repetitive DNA sequence (Piarroux et al., 1995), the gene for DNA polymerase α catalytic subunit (POLA, Croan et al., 1997), the gene encoding the largest subunit of RNA polymerase II (RPOIILS, Croan and Ellis, 1996; Croan et al., 1997), the cytochrome oxidase II gene (COII, Ibrahim and Barker, 2001; Cao et al., 2011), the glycoprotein 63 gene (*gp*63, Mauricio et al., 2007), cysteine protease B genes (*cpb*, Hide et al., 2007), the mini-exon (Sukmee et al., 2008), 7SL RNA (Zelazny et al., 2005; Guan et al., 2012), the small subunit ribosomal RNA (SSU rRNA, Guan et al., 2012), and the cytochrome B gene (*cyt*B, Luyo-Acero et al., 2004; Asato et al., 2009). Recently, Boité et al. (2012) evaluated the phylogeny of *Leishmania* (*Viannia*) parasites based on multilocus sequence analysis using different housekeeping genes. However, most of these studies were aimed to analyse isolates/strains from one particular geographic region, and only a few authors have investigated phylogenetic relationships within the entire genus (Croan and Ellis, 1996; Croan et al., 1997; Dávila and Momen, 2000; Berzunza-Cruz et al., 2002; Luyo-Acero et al., 2004; Zelazny et al., 2005; Spanakos et al., 2008; Villinski et al., 2008; Asato et al., 2009; Guan et al., 2012).

In a recent work, we carried out a comprehensive phylogenetic study based on sequence analysis of a 1380 bp fragment of the *hsp70* coding region, including for the first time 16 species commonly causing leishmaniasis and several strains per species (Fraga et al., 2010). As a result, eight monophyletic groups were clearly defined, four in each *Leishmania* subgenus (*Leishmania* and *Viannia*). These groups correspond to the following species: *L.* (*L.*) *donovani*, *L.* (*L.*) *major*, *L.* (*L.*) *tropica*, *L.* (*L.*) *mexicana*, *L.* (*V.*) *lainsoni*, *L.* (*V.*) *naiffi*, *L.* (*V.*) *guyanensis* and *L.* (*V.*) *braziliensis*. Using phylogenetic network analysis, some subspecies were further recognized: *L.* (*L.*) *donovani infantum*, *L.* (*V.*) *guyanensis panamensis*, and *L.* (*V.*) *braziliensis peruviana*. More recently, phylogenetic data based on the 3′-unstranslated region (UTR) of *hsp70-I* genes supported a separation between *L.* (*L.*) *tropica* and *L.* (*L.*) *aethiopica* (Requena et al., 2012), species that cannot be separated based on *hsp70* coding sequence evolutionary trees (Fraga et al., 2010).

In the search for additional molecular markers useful for improving the resolution of current phylogenetic classification, the *hsp20* gene was considered to be a plausible candidate. Proteins belonging to heat-shock protein (HSP) family play an important role in folding, assembly, intracellular localization, secretion, regulation, stabilization and degradation of proteins (Young et al., 2004). Given their functional relevance, most HSPs are highly conserved in sequence and function (Folgueira and Requena, 2007). However, there is an exception, the small heat-shock proteins (sHSPs): although they are widespread in both eukaryotic and prokaryotic organisms, they are poorly conserved in sequence (Fu et al., 2006). Nevertheless, in spite of this low amino acid sequence conservation, the tridimensional structure of the α-crystallin domain, which represents the signature motif of sHSPs, is well conserved. This domain is responsible for dimer formation, which is the basic functional unit of many sHSPs (Haslbeck et al., 2005). In addition, sHSPs contain amino- and carboxy-terminal extensions that are involved in modulating oligomerization, substrate binding and chaperone function (Sun and MacRae, 2005). In *Leishmania* and other trypanosomatids, only one member of the sHSPs was described, the 20-kDa heat-shock protein (HSP20) (Folgueira and Requena, 2007). Amino acid sequence alignment of HSP20s from *L. braziliensis*, *L.* (*L.*) *amazonensis*, *L.* (*L.*) *major* and *L.* (*L.*) *infantum* showed that this protein, even though conserved within the genus, has accumulated a significant number of amino acid substitutions in the different *Leishmania* species (Montalvo-Álvarez et al., 2008). In this study, we investigate the discriminatory value of the *hsp20* coding region for phylogenetic analysis; the results indicated that this molecular marker is useful for species discrimination within the *Leishmania* genus, reinforcing, and even improving, previous phylogenetic classifications. In addition, we combined *hsp20* and *hsp70* sequence data in phylogenetic analyses as a strategy to further strengthen the phylogenetic relationships within the *Leishmania* genus.

2. Material and methods

2.1. PCR amplification of hsp20

PCR primers were designed in conserved regions of the *hsp20* gene based on a multiple alignment of the previously characterized *hsp20* sequences: *L.* (*L.*) *amazonensis* (AM712297), *L.* (*V.*) *braziliensis* (XM_001566535), *L.* (*L.*) *infantum* (XM_001466741) and *L.* (*L.*) *major* (XM_842817) (see Table 1). Finally, two degenerate primers, named hsp20F and hsp20R, we designed to amplify a fragment of around 370 bp (Table 2).

PCR products were amplified from the strains listed in Table 1. The reaction mix (50 μL) contained 1× standard PCR buffer including 1.5 mM $MgCl_2$, 1× Q-buffer, 200 μM of each deoxynucleoside triphosphate, 0.5 U HotStarTaq Plus DNA polymerase (Qiagen, Hilden, Germany), 0.8 μM of each primer, and 10 ng of genomic DNA isolated from parasite culture. The thermal cycling parameters of the assay were: initial denaturation at 95 °C for 5 min; followed by 35 cycles consisting of 94 °C for 40 s, 58 °C for 1 min, and 72 °C for 1 min; and a final extension step of 8 min at 72 °C. Amplicons were analyzed on a 2% agarose gel, and afterwards sequenced.

2.2. DNA sequencing

Both strands of the *hsp20* PCR products were sequenced using the oligonucleotides hsp20F and hsp20R as primers. DNA sequencing was carried out in the facilities of the Servicio de Genómica (Parque Científico de Madrid-UAM), using the Big Dye Terminators v3.1 kit (Applied Biosystem).

2.3. Phylogenetic analysis

For all analyses, the priming sites were trimmed from both ends of all sequences. In addition to the sequences generated in this study, previously published sequences (see Table 1 for a complete list of sequences) were also included in the alignments, which were carried out with the software package MEGA (Molecular Evolutionary Genetic Analysis Version 5.05; Tamura et al., 2011). The sequence alignment was screened for detecting species-specific single nucleotide polymorphisms (SNPs) and indels. The MEGA software was also used to build phylogenetic trees with both, distance and character-based methods, and to analyze synonymous versus non-synonymous nucleotide substitutions.

Table 1
Leishmania strains analyzed in this study.

Species[a]	Strain name	Country	Accession Number *hsp20* sequences	Accession Number *hsp70* sequences[b]
L. aethiopica (n = 4)	MHOM/ET/89/GERE	Ethiopia	JX630109	FN395018
	NLB 107-08	Kenya	JX630110	FN395019
	MHOM/ET/83/169-83	Ethiopia	JX630111	FN395020
	MHOM/ET/72/L100	Ethiopia	JX630112	FN395021
L. tropica (n = 2)	MHOM/IN/79/DD7	India	JX630113	FN395025
	MHOM/KE/81/NLB_030B	Kenya	JX630114	FN395026
L. major (n = 4)	UQ_8	Sudan	JX630115	FN395022
	L137	Spain	JX630116	FN395023
	GITHURE	Kenya	JX630117	FN395024
	MHOM/IL/80/Friedlin	Israel	XM_842817[c]	XM_001684512[c]
L. donovani (n = 4)	MHOM/SD/–/1-S	Sudan	JX630118	FN395027
	MHOM/SD/82/GILANI	Sudan	JX630119	FN395029
	MHOM/NP/03/BPK282	Nepal	XM_003862626[c]	NA
	MHOM/SD/97/LEM3463[d]	Sudan	JX630120	FN395030
L. infantum (n = 6)	MHOM/MT/85/BUCK	Malta	JX630121	FN395031
	MHOM/ES/1988/LLM175	Spain	JX630122	NA
	MCAN/ES/98/LLM-877	Spain	XM_001466741[c]	XM_001470287[c]
	MCAN/BR/06/MAIKE[e]	Brazil	JX630123	FN395031
	MHOM/BR/07/WC[e]	Brazil	JX630124	FN395036
	MHOM/BR/07/ARL[e]	Brazil	JX630125	FN395037
L. mexicana	MHOM/GT/01/U1103	Guatemala	XM_003872264[c]	XM_003877072[c]
L. amazonensis (n = 3)	MHOM/PE/02/LH2312	Peru	JX630126	FN395038
	MHOM/BR/73/M2269	Brazil	JX630127	EU599091
	IFLA/BR/67/PH8	Brazil	AM712297[b]	NA
L. garnhami	MHOM/VE/76/JAP78	Venezuela	JX630129	EU599092
L. braziliensis (n = 7)	MHOM/BO/–/CUM180	Bolivia	JX630130	FN395039
	MHOM/PE/02/LH2182	Peru	JX630131	FN395040
	MHOM/BO/94/CUM29	Bolivia	JX630132	FN395041
	MHOM/PE/91/LC2177	Peru	JX630133	FN395042
	MHOM/BR/06/ICA	Brazil	JX630134	FN395043
	MHOM/BR/75/M2903	Brazil	JX630135	M87878
	MHOM/BR/75/M2904	Brazil	XM_001566535[c]	XM_001566275[c]
L. peruviana (n = 4)	MHOM/PE/03/LH2864	Peru	JX630136	FN395044
	MHOM/PE/03/LH2439	Peru	JX630137	FN395045
	MHOM/PE/90/LC468	Peru	JX630138	FN395046
	MHOM/PE/90/LCA08	Peru	JX630139	EU599089
L. guyanensis (n = 3)	MHOM/PE/02/LH2372	Peru	JX630140	FN395051
	MHOM/GF/85/LEM699	French Guiana	JX630141	FN395052
	MHOM/BR/07/029-ZAV	Brazil	JX630142	FN395053
L. panamensis	MCHO/PA/00/M4039	Panama	JX630143	FN395055
L. naiffi (n = 2)	MDAS/BR/78/M5210	Brazil	JX630144	FN395056
	MDAS/BR/79/M5533	Brazil	JX630145	FR872767
L. lainsoni (n = 3)	MHOM/BO/95/CUM71	Bolivia	JX630146	FN395047
	MHOM/PE/91/LC1581	Peru	JX630147	FN395048
	MHOM/PE/02/LH2344	Peru	JX630148	FN395049
T. cruzi	TINF/BR/63/CL Brener	Brazil	XM_816981[c]	XM_812645[c]
T. b. brucei	927/4 GUT at 10.1	Kenya	XM_838833[c]	XM_824101[c]

NA: sequence not available.
[a] Species as defined by MLEE typing. Whenever available, the full WHO code of the strain is provided.
[b] Sequences from strains retrieved from GenBank, the remaining ones were determined in this study.
[c] Accession numbers starting with XM are derived from a contemporary annotation of full genome sequences, and were also retrieved from GenBank database.
[d] Isolates formerly typed as *L. archibaldi*.
[e] Isolates formerly typed as *L. chagasi*.

Table 2
Primers used for *hsp20* gene PCR and sequencing.

Primer	Primer Sequence (5′–3′)[a]	Nucleotide position[b]
hsp20F	RGRGACTCGCTCAKCAACAGCG	21–43
hsp20R	CGTTGAAGSTGGCCTTGATTTTGCTG	365–391

[a] R = A or G; K = G or T; S = G or C
[b] The annealing position of the primers is given relative to GenBank accession entry AM712297 (*L.* (*L.*) *amazonensis* strain PH8).

The number of synonymous differences per synonymous site, and the number of nonsynonymous differences per non-synonymous site were averaged over all *Leishmania* sequence pairs, using the Nei–Gojobori method (Nei and Gojobori, 1986). Distances and characters from nucleotide sequences were estimated with the Kimura-2 parameter model (Kimura, 1980), and trees were built with the Neighbor-Joining (NJ) (Saitou and Nei, 1987), Minimum Evolution (ME) (Rzhetsky and Nei, 1992) and Maximum Parsimony (MP) (Eck and Dayhoff, 1966; Fitch, 1971) methods. Also we used the Maximum likelihood (ML) (Felsenstein, 1981) with appropriate substitution models (T92+I) chosen using the Akaike information criterion (AIE), as implemented in the MEGA 5.05 software. Distances from predicted amino acid sequences were determined with the *p*-distance model. As out-group, the *hsp20* sequences from two species of the genus *Trypanosoma*, another Trypanosomatidae genus, were used. The support of monophyletic groups was assessed by the bootstrap method (Felsenstein, 1985) with 2000 replicates. Additionally, phylogenetic networks were established with the SplitsTree4 software (Huson, 1998; Huson and Bryant, 2006), using the Kimura-2 parameter model (Kimura, 1980) and the Neighbor-Net method. Such networks depict alternative evolutionary paths supported by the data set.

Finally the *hsp20* and *hsp70* gene sequences (Table 1) were concatenated and analyzed using the MEGA 5.05 and SplitsTree4 programs as specified above.

3. Results

A fragment covering 370 bp of the *hsp20* coding region (468 bp in length) was successfully PCR-amplified and sequenced for 39 strains from 14 *Leishmania* species with different geographic origins (Table 1). Additionally, six *Leishmania hsp20* sequences present in the GenBank database were retrieved, amounting to a total of 45 sequences of different strains. For phylogenetic analyses, the *hsp20* sequences for *Trypanosoma brucei brucei* and *Trypanosoma cruzi* were retrieved from GenBank (Table 1).

The aligned sequence of *Leishmania hsp20* genes was 319 bp (primers were trimmed from the sequences), except for *L.* (*V.*) *lainsoni* strains that have 316 bp. The *hsp20* sequences of *Leishmania* spp. were found to be GC rich (55.8–60.1%), with a sequence identity among *Leishmania* sequences between 82.2% and 100% (average 90.4%). The mean dissimilarity between the *Leishmania* and *Trypanosoma* species is 47.2%, which is three times as high as the highest intra-*Leishmania* values (17.8%). The nucleotide sequence variation is sufficient to discriminate *Leishmania* species: 88 nucleotide positions (27.6%) are polymorphic and 82 positions (25.7%) are parsimony informative. Table 3 shows those 25 SNPs and indel positions that were found to be discriminative for the studied *Leishmania* species. Species-specific SNPs were detected for *L.* (*L.*) *infantum*, *L.* (*L.*) *tropica*, *L.* (*L.*) *major*, *L.* (*L.*) *aethiopica*, *L.* (*L.*) *amazonensis*, *L.* (*V.*) *naiffi*, *L.* (*V.*) *lainsoni*, *L.* (*V.*) *guyanensis* and *L.* (*V.*) *peruviana*. Also 3 positions represent deletions specific for the *L.* (*V.*) *lainsoni* species. No species-specific SNPs were detected for *L.* (*V.*) *braziliensis*, *L.* (*L.*) *donovani*, *L.* (*L.*) *mexicana* and *L.* (*L.*) *garnhami*.

The analysis of deduced amino acids for the sequenced *hsp20* region (105 amino acids) revealed substitutions at 29 positions (27.6%), of which 28 sites (26.6%) were parsimony informative. The number of synonymous substitutions per synonymous site (*dS*) was 32.6%, the number of non-synonymous substitutions per non-synonymous site (*dN*) was 5.6%.

The Neighbor-Joining tree based on the *hsp20* sequence alignment (Fig. 1) shows that *L.* (*Leishmania*) and *L.* (*Viannia*) were separated into distinct monophyletic clades. Within the *L.* (*Leishmania*) subgenus, the Old and New World *Leishmania* species are located in two different branches of the tree. Within the New World, five species/complexes can be reliably recognized: the *L.* (*L.*)*mexicana* complex, *L.* (*V.*) *lainsoni*, *L.* (*V.*) *naiffi*, the *L.* (*V.*) *guyanensis* complex, and the *L.* (*V.*) *braziliensis* complex. In the Old World, *hsp20* based trees support a separation of *L.* (*L.*) *major*, the *L.* (*L.*) *donovani* complex, *L.* (*L.*) *aethiopica* and *L.* (*L.*) *tropica*. All these clusters were also observed using ML, ME and MP phylogenies (data not shown), indicating that the derived groups are robust and not dependent of the evolutionary method used. Trees based upon amino acid sequences are in agreement with the nucleotide-based phylogenies (data not shown).

Fig. 2 displays a phylogenetic network obtained from the same sequences as in Fig. 1, excluding the *Trypanosoma* sequences. This phylogenetic network shows a separation of *L.* (*L.*) *infantum* (bootstrap value 70%) and *L.* (*V.*) *peruviana* (bootstrap value 87.4%) as subgroups within the *L.* (*L.*) *donovani* and *L.* (*V.*) *braziliensis* complexes, respectively.

In order to appraise whether a combined analysis of *hsp20* and *hsp70* gene sequences would further improve the phylogenic relationships within the *Leishmania* genus, *hsp20* and *hsp70* sequences from 42 strains of different geographic origin, accounting for 14 different *Leishmania* species, were concatenated and analyzed as before. Sequence accession numbers for the sequences analyzed are shown in Table 1. The analysis, using NJ (Fig. 3), ML, MP and ME, showed a consistent separation of *Leishmania* genus into nine monophyletic clusters, corresponding to the *L.* (*L.*) *mexicana* complex, *L.* (*V.*) *lainsoni*, *L.* (*V.*) *naiffi*, the *L.* (*V.*) *guyanensis* complex, the *L.* (*V.*) *braziliensis* complex, *L.* (*L.*) *major*, the *L.* (*L.*) *donovani* complex, *L.* (*L.*) *aethiopica* and *L.* (*L.*) *tropica*. In addition, this analysis allowed a distinction of *L.* (*V.*) *peruviana* as a subgroup within the *L.* (*V.*) *braziliensis* complex. The phylogenetic network obtained from the same sequences as in Fig. 3, excluding the *Trypanosoma* sequences, showed a separation of *L.* (*L.*) *infantum* (bootstrap value 74.5%) and *L.* (*V.*) *peruviana* (bootstrap value 99%) as subgroups within the *L.* (*L.*) *donovani* and *L.* (*V.*) *braziliensis* complexes, respectively (data not show).

4. Discussion

Although sHSPs have been used for phylogenetic studies, and considered a good molecular marker (Kriehuber et al., 2010), this is the first time that the *hsp20* gene has been employed for estab-

Table 3
Single nucleotide polymorphism and indel sites on *hsp20* among 14 *Leishmania* species

Species[b]	Nucleotide at *hsp20* alignment positions[a]																								
	52 C	63 T	83 T	84 A	108 C	113 T	114 C	115 T	117 A	129 C	135 G	144 A	159 G	168 C	179 C	181 C	186 T	231 G	238 T	245 T	252 C	258 T	291 G	292 C	296 A
L. donovani (4)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L. infantum (6)	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L. tropica (2)	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L. major (4)	T	.	C	.	T	.	.	G	.	.	.	G	.	T	T	.	.	A	.	.	.	.	.	.	.
L. aethiopica (4)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	G	.	C	.	.	.	.	.	.
L. mexicana (1)	.	.	.	.	.	.	.	G	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.
L. amazonensis (3)	.	.	.	.	.	.	T	G	T	T	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.
L. garnhami (1)	.	.	.	.	.	.	T	G	.	T	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.
L. naiffi (2)	.	.	.	.	.	.	.	.	C	.	A	.	.	.	.	.	.	C	G	.	.	.	.	.	.
L. lainsoni (3)	.	C	.	G	.	–	–	–	C	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	T
L. guyanensis (3)	.	.	.	G	.	.	.	.	C	.	.	.	C[c]	.	.	.	.	.	G	C[c]	.	.	.	A[c]	.
L. panamensis (1)	.	.	.	G	.	.	.	.	C	.	.	.	C[c]	.	.	.	.	.	G	C[c]	.	.	.	A[c]	.
L. peruviana (4)	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	G	.	T	.	A	.	.
L. braziliensis (7)	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.

[a] Position number, relative to GenBank accession entry AM712297 (*L.* (*L.*) *amazonensis* strain PH8), and most frequent nucleotide found among the sequences analyzed. Species-specific SNPs are underlined, conserved positions are indicated by a point, and the deleted positions in the *L. lainsoni hsp20* are indicated by a dash.
[b] In brackets: number of sequences corresponding to each *Leishmania* species
[c] These SNPs are shared by *L. guyanensis* and *L. panamensis*

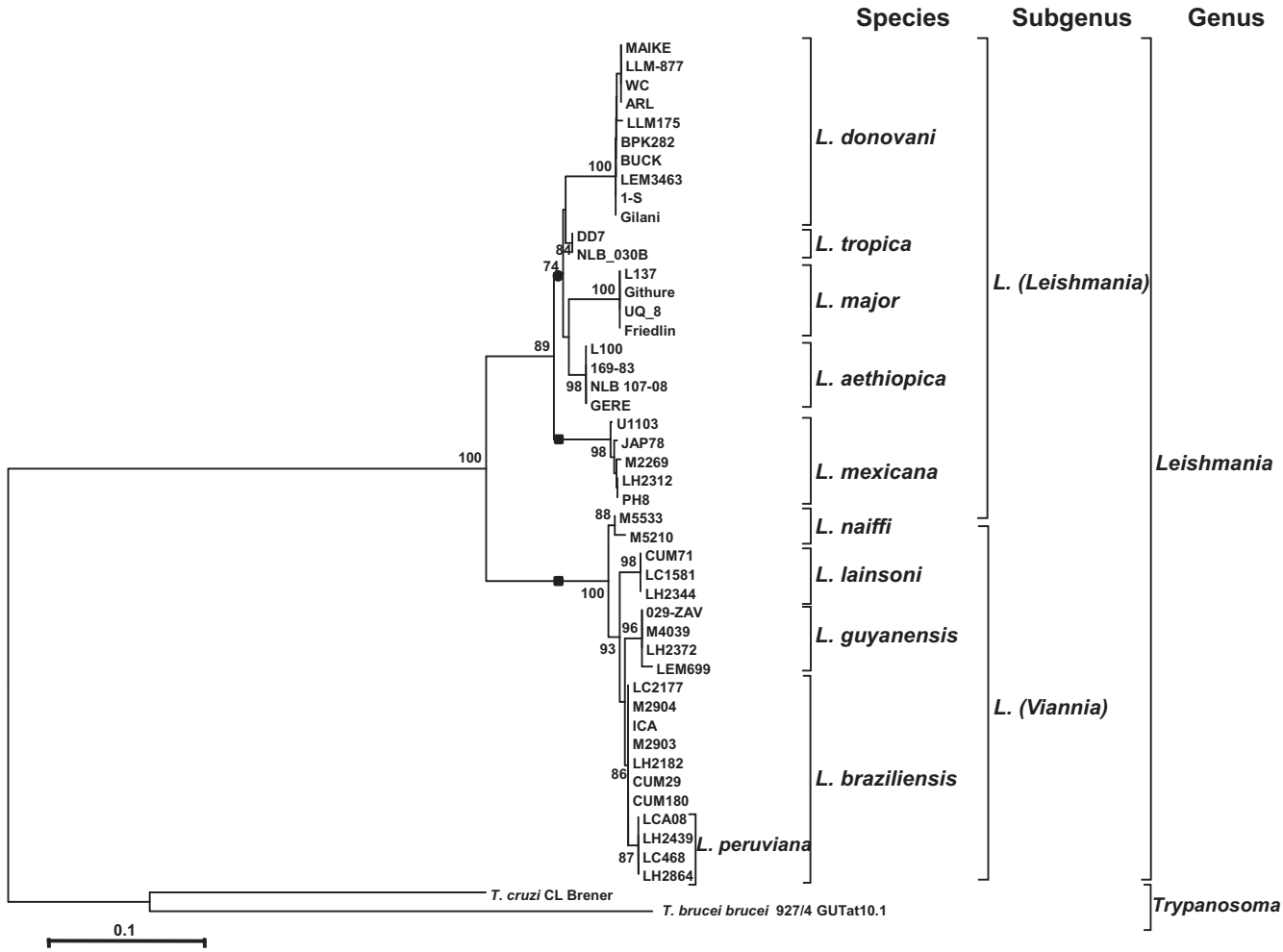

Fig. 1. Neighbor-Joining phylogeny of the *hsp20* nucleotide sequences from different strains belonging to14 *Leishmania* species. Distances were estimated using the Kimura-2 parameter model. Bootstrap support of the branches was inferred from 2000 replicates, and is given in percentages at the internodes when exceeding 70%. The tree is drawn to the scale at the bottom, expressed as distance per nucleotide. Supported monophyletic species and (sub) genera are depicted at the right, irrespective of the species classification presented in Table 1. Old World clusters are indicated by a dot on the branch leading to the cluster, while a block is used for New World groups (except for Brazilian *L.* (*L.*) *infantum/chagasi* strains that clustered with European strains). The tree was rooted with the corresponding sequences from *T. cruzi* and *T. brucei*.

lishing phylogenetic relationships within the *Leishmania* genus. In addition, the newly identified SNPs could be explored for *Leishmania* species typing, especially by PCR-RFLP and/or sequencing. Comparison of synonymous (silent, *dS*) and nonsynonymous (aminoacid changing, *dN*) nucleotide substitution rates in protein coding genes provides an important means for understanding molecular evolution. The ratio of *dN* to *dS* obtained for the *hsp20* sequences was 0.17; this value confirms a purifying selection in this gene due to functional constraints of the protein (Yang and Nielsen, 2000). However, as shown in this study, the evolutionary rate of this gene is large enough to support the use of *hsp20* coding sequences for analysis of the phylogenetic relationships within the *Leishmania* genus at the species, intra- and supra-species levels.

Our *hsp20* phylogenetic study included 45 strains from different geographic origin, belonging to 14 MLEE-defined *Leishmania* species that represent the most common causative agents of leishmaniasis in the New and Old World. As expected, *Leishmania hsp20* sequences were clearly distinct from the homologous sequences in *T. brucei* and *T. cruzi*. Also, our analyses agree with the division of mammalian species, proposed by Lainson and Shaw (1987), into the peripylarian *Viannia* and the suprapylarian *Leishmania* subgenera, both of which form distinct monophyletic clusters (Figs. 1 and 3). While *L.* (*Viannia*) is restricted to neotropical regions, *L. (Leishmania)* occurs both in the New (neotropical and southern neoarctic) and Old (palearctic, African and oriental) World (Kerr, 2000). This geographical dichotomy of the *Leishmania* subgenus (except for *L.* (*L.*) *infantum*) is also reflected in the phylogenetic trees (Figs. 1 and 3), which shows a separation between the New (*L.* (*L.*) *mexicana* complex) and Old World groups. Similar conclusions were drawn with phylogenetic studies based on sequences from *pol*A and *rpo*IILS (Croan et al., 1997), ITS rDNA (Dávila and Momen, 2000), 7SL RNA (Zelazny et al., 2005), *cyt*B (Luyo-Acero et al., 2004; Asato et al., 2009) and *hsp70* (Fraga et al., 2010).

As shown in Fig. 1, the phylogenetic analysis based on the *hsp20* sequence allowed the separation of the mammalian-infecting *Leishmania* species, analyzed in this work, into nine monophyletic clusters. Some of these clusters correspond to species as defined by standard MLEE (Table 1), but often they group several species. In recent times, the usefulness for taxonomic purposes of MLEE is under dispute, and the results of this work further support the idea that MLEE-species definition should be left aside and definitely replaced for a classification based exclusively on genetic markers (da Silva et al., 2010; Van der Auwera et al., 2011; Requena et al., 2012). On the other hand, the phylogenies based on *hsp20* sequences and/or the combined analysis of *hsp20* and *hsp70* se-

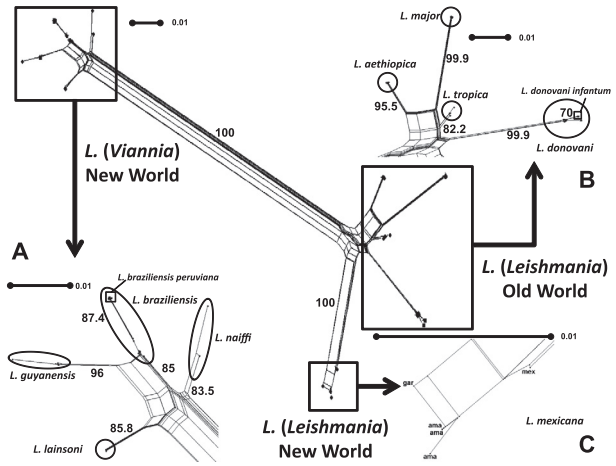

Fig. 2. Complete phylogenetic network of the *Leishmania hsp20* sequences. Networks were constructed using the Neighbor-Net algorithm (Bryant and Moulton, 2004), excluding all conserved sites. The Kimura 2-parameter model for nucleotides was used, calculating the fraction of differences between each pair of sequences. Each of the three panels (A–C) is drawn to the scale indicated. (A) *L.* (*Viannia*) subgenus sequences separate into four species. Squares are used to indicate the position of subspecies. (B) Old World sequences of the *L. (Leishmania)* subgenus are separated into four species. Within *L.*(*L.*) *donovani*, the square shows the location of *L.*(*L.*) *donovani infantum* strains. (C) Phylogenetic relationships into the New World *L. (Leishmania)* subgenus. The strains belonging to *L.*(*L.*) *garnhami*, *L.*(*L.*) *mexicana*, and *L.*(*L.*) *amazonensis* (listed in Table 1), are denoted by gar, mex, and ama, respectively.

quences reinforced previous conclusions, but, interestingly, allowed the discrimination of *L.* (*L.*) *tropica* and *L.* (*L.*) *aethiopica*, species which cannot be distinguished through evolutionary analysis based on *hsp70* coding sequences alone (Fraga et al., 2010). Recently, using the 3′-UTR of *hsp70-I* genes as molecular marker, this separation was also strongly supported with a bootstrap value of 99% (Requena et al., 2012). Evidence of this separation was previously obtained from phylogenetic studies based on non-coding sequences like ITS of the ribosomal DNA array and 7SL RNA gene (Dávila and Momen, 2000; Berzunza-Cruz et al., 2002; Zelazny et al., 2005; Spanakos et al., 2008; Villinski et al., 2008), but both species formed a single cluster when coding sequences for *gp*63 or *cyt*B were used (Croan et al., 1997; Luyo-Acero et al., 2004; Mauricio et al., 2007; Asato et al., 2009). These two species are certainly very close, they share rock hyrax (*Procavia capensis*) as reservoir (Ashford, 2000) and they can parasitize the same *Phlebotomus* species (Gebre-Michael et al., 2004). However, these species have different geographical distribution, whereas *L.* (*L.*) *tropica* has a wide distribution (Southern Europe, Africa and Middle East), *L.* (*L.*) *aethiopica* is restricted to the highlands of Ethiopia and Kenya (Ashford, 2000). Additionally, infection of *L.* (*L.*) *aethiopica* in man results in a spectrum of diseases ranging from simple self-healing CL to diffuse cutaneous and mucocutaneous forms (Ashford, 2000; Bañuls et al., 2007). Thus, these ecological and clinicopathological aspects support the idea, based on molecular data, that *L.* (*L.*) *tropica* and *L.* (*L.*) *aethiopica* are distinct groups that may be considered as two separated species. Nevertheless, additional studies including a large number of strains are needed.

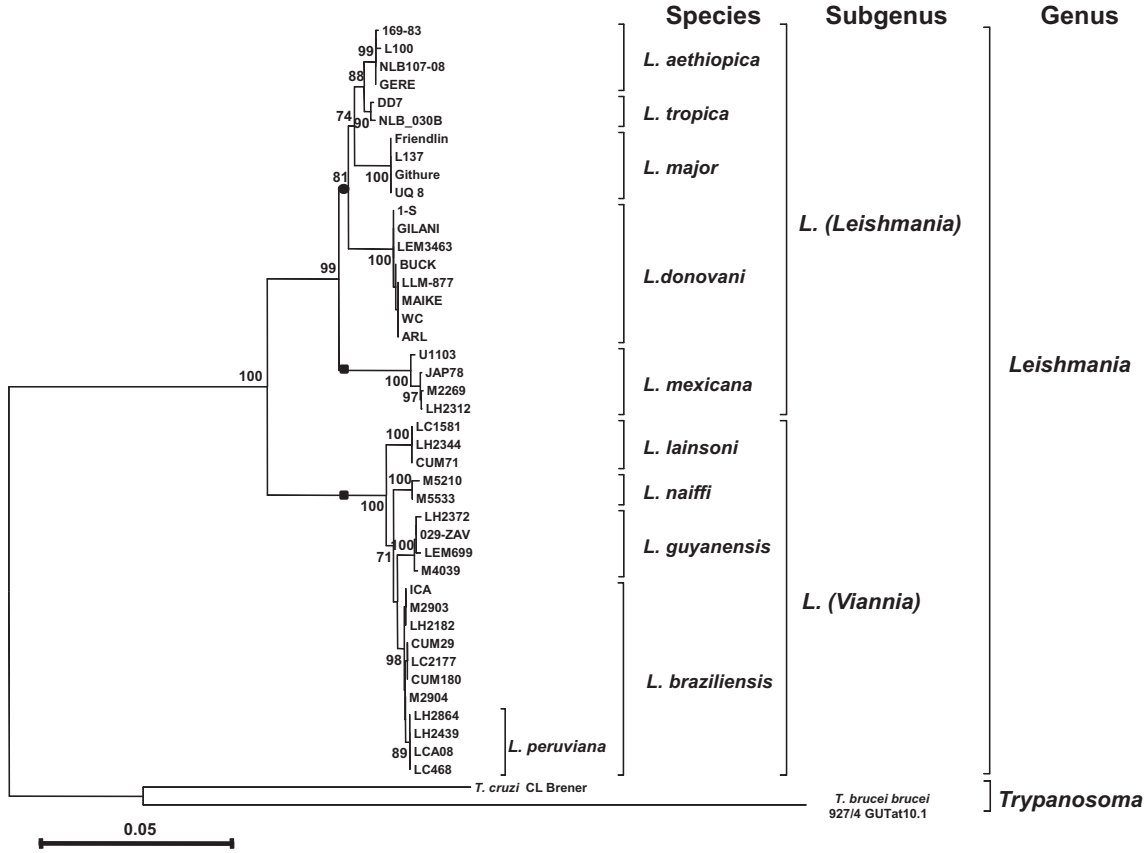

Fig. 3. Neighbor-Joining phylogeny based on concatenated *hsp20* and *hsp70* sequences. Distances were estimated using the Kimura-2 parameter model. Bootstrap support (expressed as percentage) of the branches was inferred from 2000 replicates, and it is shown at the internodes when exceeding 70%. The tree is drawn to the scale at the bottom, expressed as distance per nucleotide. Supported monophyletic species and (sub) genera are depicted at the right, irrespective of the species classification presented in Table 1. Old World clusters are indicated by a dot on the branch leading to the cluster, while a block is used for New World groups (except for Brazilian *L.* (*L.*) *infantum/chagasi* strains that clustered with European strains).

Within the *L.* (*V.*) *braziliensis* species (or complex), *L.* (*V.*) *peruviana* strains form a separate subgroup (Figs. 1 and 3), and, therefore, we suggest the status of subspecies for this subgroup. The separation of *L. (V.) peruviana* is also strongly supported by the phylogenetic network analysis (Fig. 2). In the literature, there is some controversy regarding whether *L.* (*V.*) *braziliensis* and *L.* (*V.*) *peruviana* should be considered either a sole species (Grimaldi et al., 1987; Arana et al., 1990), a heterogeneous species, with *L.* (*V.*) *peruviana* being a subspecies of *L.* (*V.*) *braziliensis* or two distinct species (Dujardin et al., 1993, 1998; Victoir et al., 1998; Bañuls et al., 2000; García et al., 2005). Both species have been separated by MLEE (Bañuls et al., 2000), molecular karyotyping (Dujardin et al., 1993, 1995), RAPD (Bañuls et al., 1999, 2000), MLST (Tsukayama et al., 2009), *gp*63 PCR-RFLP (Victoir et al., 1998) and *cyt*B PCR-RFLP (García et al., 2005). Although the *hsp70* phylogeny does not support a statistically significant separation between *L.* (*V.*) *peruviana* and *L.* (*V.*) *braziliensis*, network analysis separated both groups of strains and, therefore, a subspecies status for *L.* (*V.*) *peruviana* was suggested (Fraga et al., 2010). More recently, Odiwuor et al. (2012), after analyzing the *L.* (*V.*) *braziliensis* complex by amplified fragment length polymorphisms (AFLP) and *hsp70* sequencing, have also suggested that *L.* (*V.*) *peruviana* should be considered as a subgroup of the *L.* (*V.*) *braziliensis* species. Remarkably, in the study by Odiwuor et al. (2012), some strains of *L.* (*V.*) *braziliensis* were found to be very different from the others (and from the *L.* (*V.*) *peruviana* strains); it would be interesting to analyze the phylogeny of this atypical group of strains based on *hsp20* sequences. In an outstanding work, Boité et al. (2012) have analyzed the taxonomy of the *Viannia* subgenus by multilocus sequence typing based on sequences from glucose-6-phosphate dehydrogenase (G6PD), 6-phosphogluconate dehydrogenase (6PGD), mannose phosphate isomerase (MPI) and isocitrate dehydrogenase (ICD) genes. In this comprehensive study, according to maximum parsimony-based haplotype network analysis, *L. peruviana* sequences clustered with the most frequent haplotype of *L. braziliensis*. Our network analysis positioned *L.* (*V.*) *peruviana* as a subgroup within the *L.* (*V.*) *braziliensis* strains, even though they form a single node (Fig. 2). Furthermore, in the phylogenetic trees based on either the *hsp20* alone (Fig. 1) or both markers (Fig. 3), the *L.* (*V.*) *peruviana* strains appeared separated from the *L.* (*V.*) *braziliensis* ones with bootstrap values of 87 and 89, respectively. As proposed previously (Bañuls et al., 2000), our data would sustain the idea that *L.* (*V.*) *b. braziliensis* and *L.* (*V.*) *b. peruviana* are distinct monophyletic clades; however, given the evolutionary proximity denoted in these analyses, following the suggestion by Fraga et al. (2010), we favor the idea of grouping both clades within the same species, *L.* (*V.*) *braziliensis*, which would be composed of two subspecies: *L.* (*V.*) *b. braziliensis* and *L.* (*V.*) *b. peruviana*.

Within the *L.* (*L.*) *donovani* complex, four species have been described: *L.* (*L.*) *donovani*, *L.* (*L.*) *archibaldi*, *L.* (*L.*) *infantum,* and *L.* (*L.*) *chagasi* (Bañuls et al., 2007). All of them cause mainly visceral leishmaniasis in tropical and sub-tropical regions, even though asymptomatic infections are common (Rijal et al., 2010). However, the separation of these four species is not supported by most of the phylogenetic studies based on sequence analysis (Piarroux et al., 1995; Croan et al., 1997; Dávila and Momen, 2000; Berzunza-Cruz et al., 2002; Kuhls et al., 2005; Luyo-Acero et al., 2004; Mauricio et al., 2007; Asato et al., 2009; Fraga et al., 2010; Cao et al., 2011). According to our data, based either on *hsp20* sequences or on a combination of *hsp20* and *hsp70* sequences, *L.* (*L.*) *archibaldi* is indistinguishable from *L.* (*L.*) *donovani*. In fact, many authors have proposed to synonymize *L.* (*L.*) *archibaldi* with *L.* (*L.*) *donovani* (Kuhls et al., 2005), and we followed this recommendation (see Table 1). Also, *L.* (*L.*)*infantum* and *L.* (*L.*) *chagasi* are indistinguishable from each other (Figs. 1–3). A similar conclusion arose after phylogenetic studies based on RAPD, microsatellites or DNA sequences; thus, there is a general agreement that *L.* (*L.*) *chagasi* and *L.* (*L.*) *infantum* are synonym names for the same *Leishmania* species. This is consistent with the theory of the introduction of *L.* (*L.*) *infantum* in the New World from a European population which had crossed the Atlantic Ocean since the XVIth century via infected dogs (Rioux et al., 1990; Cupolillo et al., 1994; Mauricio et al., 2000; Lukeš et al., 2007; Leblois et al., 2011; Kuhls et al., 2011). Thus, currently, within the *L.* (*L.*) *donovani* complex, two species are recognized: *L.* (*L.*) *donovani*, which is distributed in Asian and East Africa, and *L.* (*L.*) *infantum* that is found in the Mediterranean basin and the American continent (Lukeš et al., 2007; Schönian et al., 2010). Apart from MLEE typing, support for this separation has been obtained by microsatellite and multilocus sequence typing (MLST) of the *L.* (*L.*) *donovani* complex (Mauricio et al., 2006; Kuhls et al., 2007). However, our phylogenetic trees based on sequence analysis do not show a clear separation between *L.* (*L.*) *donovani* and *L.* (*L.*) *infantum* strains (Figs. 1 and 3). However, phylogenetic network analyses using either *hsp70* (Fraga et al., 2010) or *hsp20* sequences (Fig. 2) show a separation between *L.* (*L.*) *infantum* and *L.* (*L.*) *donovani* strains. Thus, we propose considering *L.* (*L.*) *donovani* as a species composed by two subspecies (*L.* (*L.*) *d. donovani* and *L.* (*L.*) *d. infantum*). The SNP analysis (Table 3) showed the existence of a species-specific polymorphism at position 135 in the *hsp20* gene of *L.* (*L.*) *infantum*; the presence of this SNP in *L.* (*L.*) *infantum* and its absence from *L.* (*L.*) *donovani* merit further analysis using a larger number of strains, as this polymorphism might be important for the differentiation of both species/subspecies.

Other species that could not be discriminated by our phylogenetic analysis were *L. mexicana*, *L. garnhami* and *L. amazonensis*; accordingly they were grouped as *L. mexicana* in the trees (Figs. 1 and 3) and network (Fig. 2). Also, *L. guyanensis* and *L. panamensis* strains formed a monophyletic group that was referred as *L. guyanensis* in the Figs. 1–3. Finally, other species exist, such as *L.* (*L.*) *pifanoi*, *L.* (*L.*) *aristidesi, L.* (*L.*) *venezuelensis, L.* (*L.*) *foratinii* and *L.* (*V.*) *shawi*, that also should be studied in future phylogenetic analyses based on *hsp20* sequences (and in combination with other molecular markers) in order to define their species or subspecies status inside of the *Leishmania* genus.

Unraveling the phylogenetic relationships that underlie the origin of contemporary taxa requires the study of independent genes that display different evolutionary constraints (Phillipe, 1998), as the history of a gene might be different from that of the species in which it resides. In this regard, as it was demonstrated for the *hsp70* gene (Fraga et al., 2010; da Silva et al., 2010), *hsp20* represents a suitable molecular probe for *Leishmania* typing, complementing and reinforcing phylogenetic studies based on *hsp70* coding sequences. Nevertheless, it should be noted the existence of a difference in the tree topology between the phylogeny based on *hsp20* alone (Fig. 1) and that obtained with concatenated *hsp20+hsp70* sequences (Fig. 3). In the *hsp20* phylogeny, *L. donovani* and *L. tropica* are deriving from the same branch, even though both species constitute clearly different monophyletic lines. In contrast, the phylogenic trees based on *hsp70* (Fraga et al., 2010) or a combination of *hsp20+hsp70* (Fig. 3) shows *L. tropica* and *L. donovani* in different branches. Nevertheless, there are other phylogenetic studies that show a clustering of *L. tropica* and *L. donovani* in a branch different to that of *L. major* (Mauricio et al., 2007). These findings emphasize the need for using several molecular markers when evolutionarily relationships have to be inferred. Thus, increasing the number of both suitable molecular markers and analyzed strains should be a forthcoming objective to establish a definitive and non-ambiguous taxonomy for the genus *Leishmania* (Schönian et al., 2010). Knowing the evolutionary history of the *Leishmania* genus not only helps to build a reliable and objective taxonomic classification system, but also allows extrapolation of parasite-linked biological features to closely related strains. Reduc-

tion, simplification and delineation are crucial to attain a *Leishmania* taxonomy with the highest possible degree of practical relevance (Van der Auwera et al., 2011).

5. Conclusions

The *Leishmania hsp20* gene is a suitable molecular marker for both typing and phylogenetic purposes, having clear advantages over many other molecular markers: (i) a single pair of oligonucleotides suffices to PCR amplify of *hsp20* from any of the *Leishmania* species; (ii) the relatively small size of the amplicon (around 370 bp) allows obtaining the complete sequence in a single reaction.

On the basis of the molecular taxonomy, recently proposed by Fraga et al. (2010), and taking into account the results found in this work, we propose grouping the *Leishmania* strains analyzed in this study into the following nine species: *L.* (*L.*) *donovani*, *L.* (*L.*) *major*, *L.* (*L.*) *tropica*, *L.* (*L.*) *aethiopica*, *L.* (*L.*) *mexicana*, *L.* (*V.*) *lainsoni*, *L.* (*V.*) *naiffi*, *L.* (*V.*) *guyanensis* and *L.* (*V.*) *braziliensis*.

Thus, this classification represents a framework to build a robust taxonomy, showing the evolutionarily relationships existing among the different species and redefining the status for some of them.

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