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

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(*Received 31 August 2009; revised 6 November 2009 and 4 January 2010; accepted 4 January 2010; first published online 5 May 2010*)

SUMMARY

Introduction. Species typing in leishmaniasis gains importance in diagnostics, epidemiology, and clinical studies. A restriction fragment length polymorphism (RFLP) assay of PCR amplicons from a partial heat-shock protein 70 gene (*hsp*70) had been described for the New World, allowing identification of some species. **Methods.** Based on an initial *in silico* analysis of 51 *hsp*70 sequences, most of which we recently determined in the frame of a phylogenetic study, species-specific restriction sites were identified. These were tested by PCR-RFLP on 139 strains from 14 species, thereby documenting both inter- and intra-species variability. **Results.** Our assay could identify *Leishmania infantum*, *L. donovani*, *L. tropica*, *L. aethiopica*, *L. major*, *L. lainsoni*, *L. naiffi*, *L. braziliensis*, *L. peruviana*, *L. guyanensis*, and *L. panamensis* by applying 2 subsequent digests. *L. mexicana*, *L. amazonensis*, and *L. garnhami* did not generate species-specific restriction fragment patterns. **Conclusion.** Currently no assay is available for global *Leishmania* species discrimination. We present a universal PCR-RFLP method allowing identification of most medically relevant Old and New World *Leishmania* species on the basis of a single PCR, obviating the need to perform separate PCRs. The technique is simple to perform and can be implemented in all settings where PCR is available.

Key words: *Leishmania*, leishmaniasis, HSP70, species typing, differential diagnosis, restriction fragment length polymorphism, PCR-RFLP.

INTRODUCTION

Leishmaniasis is a parasitic disease endemic in 88 countries and caused by several species of *Leishmania* (WHO, 1999), transmitted by female sand flies. Host factors and parasite diversity underlie a considerable clinical pleomorphism in humans: cutaneous leishmaniasis (CL) (Bailey and Lockwood, 2007; Reithinger *et al*. 2007), diffuse cutaneous leishmaniasis (DCL) (Leon *et al*. 1992), the severely mutilating mucocutaneous leishmaniasis (MCL) (Passos *et al*. 1999), the lethal visceral leishmaniasis (VL) or kala azar (KA) (Berman, 2006), and post-kala azar dermal leishmaniasis (PKDL) (Zijlstra and El-Hassan, 2001). In addition, there is compelling evidence that not each infection results in a clinical phenotype, resulting in asymptomatic carriers of the parasite (Sassi *et al*. 1999; de Gouvêa Viana *et al*. 2008). Besides humans, also various domestic and sylvatic animals serve as hosts and reservoirs, e.g. dogs, wild rodents, and small marsupials (Lainson and Shaw, 1987).

Identification of the exact parasite species isolated from human and animal hosts, as well as from sand fly vectors, gains increasing importance for diagnostics, epidemiological surveillance, and clinical studies. Even though in the context of diagnosis the major concern is parasite detection, there is an increasing demand for differential diagnosis to identify the infecting species, as prognosis of disease progression (www.who.int/gb/ebwha/pdf_files/WHA60/A60_10-en.pdf) and the therapeutic approach can be species-dependent (Croft *et al*. 2002; Arévalo *et al*. 2007). This is most often relevant in travel clinics, where infections from different geographical origins are encountered, and the technology for species typing is readily available. Further, clinical studies conducted in areas where different species co-circulate require extensive documentation of the study material, for which species identification nowadays is a must.

The current gold standard for *Leishmania* species identification is multi-locus enzyme electrophoresis (Rioux *et al*. 1990; Bañuls *et al*. 2007). However, as this method requires mass cultivation of parasites

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doi:10.1017/S0031182010000089

and is cumbersome to perform, implementation is difficult and even impossible for most practical purposes. Instead, the polymerase chain reaction (PCR) offers alternative possibilities, allowing investigation of small-size cultures and even clinical samples, thereby obviating the need for parasite isolation. In addition, PCR is currently applied in clinical and research labs worldwide, which facilitates application of additional assays based on such technology. Many species identification tools use either a PCR that amplifies specific DNA fragments from one particular species or group (e.g. Hide and Bañuls, 2006; Marcussi *et al*. 2008; Berzunza-Cruz *et al*. 2009), or combine PCR with a post-PCR analysis step based on restriction fragment length polymorphisms (RFLPs). In the latter approach, the PCR-generated product is digested with a restriction endonuclease into fragments of a species-specific size, which can be detected on a conventional agarose gel. The resulting PCR-RFLP banding pattern is indicative for the *Leishmania* species present in the sample (Marfurt *et al*. 2003; Volpini *et al*. 2004; Rotureau *et al*. 2006; Gadisa *et al*. 2007; Reithinger and Dujardin, 2007).

In the New World, the gene encoding the heat-shock protein of 70 kDa (*hsp*70) was previously used as a target for PCR-RFLP using the restriction enzyme *Hae*III (García *et al*. 2004). This assay allowed discrimination of *L. lainsoni*, *L. amazonensis*, *L. infantum*, the *L. braziliensis*/*L. peruviana* complex, and the *L. guyanensis*/*L. panamensis* complex. In addition, it was shown to be applicable on clinical samples from Bolivian and Colombian patients (García *et al*. 2007*a*; Montalvo Alvarez *et al*. 2010). In the present study we extend the use of the *hsp*70 PCR-RFLP for identification of Old World and additional New World species, and improve resolution within the New World species complexes, as validated on DNA from reference strains that were recently incorporated in a broad phylogenetic study using the same target (Fraga *et al*. 2010). We advocate the use of this target and RFLP assays as a universal species typing tool, applicable across the entire *Leishmania* genus for most purposes.

MATERIALS AND METHODS

In silico *analysis of* hsp*70 coding sequences*

An alignment of 51 *Leishmania hsp*70 gene sequences representing 14 New and Old World human *Leishmania* species (Table 1 and Fig. 1) was used to identify restriction endonuclease recognition sites allowing discrimination of species that group together in the *Hae*III PCR-RFLP approach. Theoretical fragment sizes and gel patterns were determined with VectorNTI (Invitrogen, Carslbad, CA, USA), and the online tools NEBcutter (tools.neb.com/NEBcutter2) and DistinctiEnz (www.bioinformatics.org/~docreza/cgi-bin/restriction/DistinctiEnz.pl). The software package MEGA (Tamura *et al*. 2007; www.megasoftware.net) was used to analyse single nucleotide polymorphisms (SNPs), and to construct a maximum parsimony tree (Eck and Dayhoff, 1966; Fitch, 1971) from the sequence alignment as a general reference framework. Even though maximum parsimony does not necessarily reflect evolution most accurately, it was chosen because both this character method and restriction site analysis are SNP-based.

Strains and DNA

Table 1 lists the species and geographical origin of the 144 reference strains and isolates included in our study, 139 of which were tested in PCR-RFLP, while the 5 remaining ones were included only in the *in silico* analysis. The complete table with the 144 individual strains is available from the authors upon request. All DNAs were isolated from parasite cultures, and either parasites or DNA were obtained from the various institutes acknowledged at the end of this paper. As DNA was obtained from different sources, the species identification was done using a combination of targets, e.g. zymodeme typing, and analysis of ribosomal internal transcribed spacers and genes encoding cysteine proteinase B, glycoprotein 63 and heat-shock protein 70. Species determination was confirmed in an *hsp*70 sequence derived dendrogram for the strains and isolates shown in Fig. 1.

hsp*70 PCR*

Amplification was performed with the primers HSP70sen (5′-GACGGTGCCTGCCTACTTCA-A-3′) and HSP70ant (5′-CCGCCCATGCTCTGG-TACATC-3′) (Sigma Aldrich, www.sigmaaldrich.com) previously reported by García *et al*. (2004). PCR conditions were adapted as follows: the reaction mix (50 μl) contained 1× SilverStar *Taq* DNA polymerase buffer, 200 μM of each deoxynucleoside triphosphate, 1·5 mM $MgCl_2$, 1·5 U SilverStar Taq DNA polymerase (Eurogentec, Seraing, Belgium), 0·4 μM of each primer, 0·25% DMSO and 1 μl of genomic DNA, irrespective of the concentration. A negative control tube containing all the components except for DNA was always included, as well as a positive tube containing 10 ng of DNA from *L. braziliensis* strain MHOM/PE/91/LC2043. Thermal cycling was performed in either a PTC-0150 Mini-Cycler (MJ Research, Waltham, MA, USA) or a MyCycler™ Thermal Cycler (Bio-Rad, Foster City, CA, USA) using the following cycling parameters: (i) initial denaturation at 94 °C for 5 min; (ii) 33 cycles of 94 °C for 30 s, 61 °C for 1 min and 72 °C for 3 min; and (iii) a final extension step of

Table 1. Strains and isolates analysed *in silico* or by *hsp*70 PCR-RFLP

Species[a]	Number[b]	Country of origin	*Hsp*70 sequence Accession numbers[c]
L. donovani	3[g]	Ethiopia	X52314[d]
	3	India	FN395028
	3	Kenya	
	8[g]	Sudan	FN395027, FN395029, FN395030
	2	Unknown	
L. infantum	7[g]	Brazil	FN395035, FN395036, FN395037
	2	China	
	5	France	
	2	Italy	
	1	Malta	FN395031, FN395033
	1	Portugal	FN395032
	8	Spain	XM_001470287[d]
L. aethiopica	16	Ethiopia	FN395021, FN395020, FN395018
	2	Kenya	FN395019
L. tropica	1	India	FN395025
	1	Iraq	
	1	Israel	
	3	Kenya	FN395026
	1	Namibia	
	3	Palestinian Territory	
	2	Sudan	
L. major	1	Burkina Faso	
	3	Israel	FN395023, XM_001684512[d]
	1	Kenya	FN395024
	1	Saudi Arabia	
	2	Sudan	FN395022
	1	Tunisia	
	1	Uzbekistan	
	5	Unknown	
L. amazonensis	1	Bolivia	
	3	Brazil	EU599090, L14604[e], L14605[e]
L. garnhami	1	Venezuela	EU599092
L. mexicana	1	Belize	EU599091
	1	Peru	FN395038
L. peruviana	12	Peru	FN395044, FN395046, EU599089, FN395045
L. braziliensis	3	Bolivia	FN395039, FN395041
	5	Brazil	FN395043, M87878[d], XM_001566275[d]
	5	Peru	FN395040, FN395042[f], EU599088[f]
L. naiffi	1	Brazil	FN395056
L. panamensis	2	Panama	EU599094, FN395055
L. guyanensis	3	Brazil	FN395053, EU599093
	1	French Guiana	FN395052
	5	Peru	FN395051
L. lainsoni	4	Bolivia	FN395047
	6	Peru	FN395049, FN395050, FN395048

[a] Species designations are according to current literature, even though the species status of some is a matter of debate. Species were determined as explained in the Materials and Methods section, and confirmed for the strains in Fig. 1. The species order is as in Figs 1 and 2, and Table 2.

[b] The number of strains and isolates used from each respective country and species includes those analysed *in silico* as well as those analysed in PCR-RFLP. The number of samples tested in PCR-RFLP is hence the total number listed here minus the strains only included in the *in silico* analysis (see also footnotes d, e, and f).

[c] Accession numbers of sequences of the respective species and origin included in the *in silico* analysis, whereby Accessions starting with XM are GenBank-specific references to contemporary annotations of full genome sequences. In Fig. 1 the strain or isolate name of each Accession is shown.

[d] Strains from which these sequences were derived were not tested by *hsp*70 PCR-RFLP.

[e] Both Accessions were determined from the same strain MHOM/BR/77/LTB0016/C1S1 (Bock and Langer, 1993).

[f] Both Accessions were determined from the same isolate MHOM/PE/91/LC2177, whereby EU599088 is from a parasite clone.

[g] One isolate from Ethiopia and 2 from Sudan were formerly typed as *L. archibaldi*, while the 7 from Brazil were typed as *L. chagasi*. These species are no longer recognized, and are respectively *L. donovani* and *L. infantum* (Lukeš *et al*. 2007).

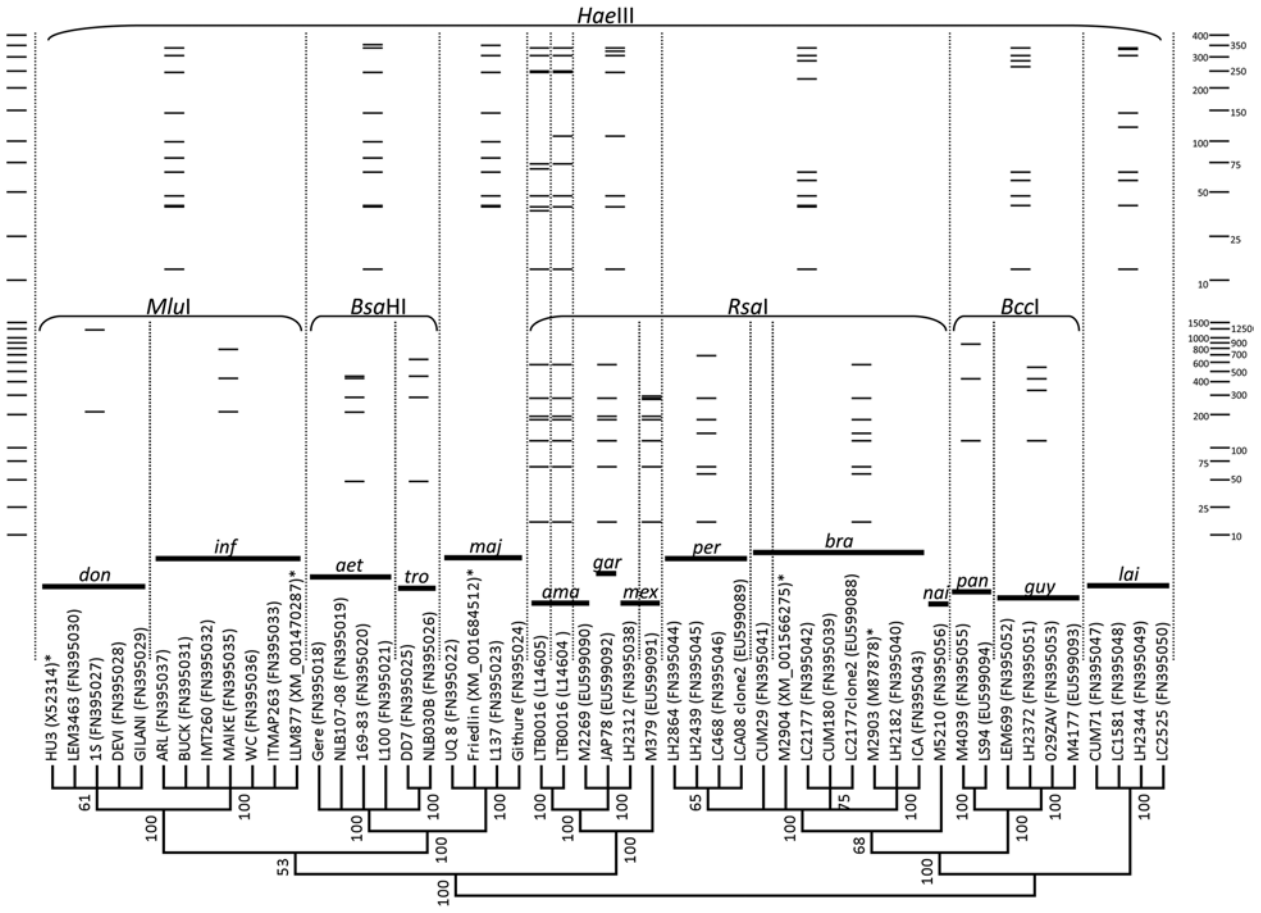

Fig. 1. *In silico* RFLP analysis of *hsp*70 sequences shown in the dendrogram at the bottom. The GenBank Accession numbers are in parentheses, whereby Accessions starting with XM are derived from a contemporary annotation of full genome sequences and are GenBank specific. The dendrogram is a consensus maximum parsimony tree, whereby the percentages of most parsimonious trees in which each cluster was found is shown at the internodes. As only the topology is represented, branch lengths have no meaning. Species are indicated with lines above the tree, and are abbreviated as follows from left to right: *don*, *Leishmania donovani* including *L. archibaldi* (see Table 1); *inf*, *L. infantum* including *L. chagasi* (see Table 1); *aet*, *L. aethiopica*; *tro*, *L. tropica*; *maj*, *L. major*; *ama*, *L.amazonensis*; *gar*, *L. garnhami*; *mex*, *L. mexicana*; *per*, *L. peruviana*; *bra*, *L. braziliensis*; *nai*, *L. naiffi*; *pan*, *L. panamensis*; *guy*, *L. guyanensis*; *lai*, *L. lainsoni*. The upper part displays the theoretically obtained fragments from digestion with *Hae*III, and the different patterns are separated by vertical lines grouping sequences that give identical fragments. The same size marker is depicted on both sides, and the length of each fragment is indicated on the right, as is also the case for the other digests shown below the *Hae*III results. Here, digests with different enzymes allowing further separation within the *Hae*III defined groups are simulated. Again, only different patterns are depicted, separated by vertical lines grouping the sequences resulting in the same pattern, except for 2 *L. amazonensis* sequences that differed with *Hae*III but were identical with *Rsa*I, and for which the pattern is repeated even though identical to, for instance, *L. garnhami*. The theoretical *Rsa*I pattern of CUM29 is not given because of a sequence ambiguity in the recognition site distinguishing *L. peruviana* from *L. braziliensis* (see Table 2). All strains and isolates included in this figure were also used for wet PCR-RFLP analysis, except those indicated with * following the Accession number in the dendrogram.

10 min at 72 °C. Analysis on a 1·2% agarose gel was used to verify the amplified product length of 1422 bp.

RFLP analysis

*hsp*70 PCR products were ethanol precipitated and reconstituted in 10 μl of water. Digests were performed on 5 μl of amplicon solution in 10 μl of the optimal buffer provided by the manufacturers, using 1U *Hae*III (MBI Fermentas, St Leon-Rot, Germany), 1U *Bcc*I (New England Biolabs, Ipswich, MA, USA), 5U *Rsa*I (MBI Fermentas), 5U *Mlu*I (MBI Fermentas), or 5U *Bsa*HI (MBI Fermentas). Reactions were incubated overnight at 37 °C and completely analysed by electrophoresis in a 3% small fragment agarose gel (Gentaur, Brussels, Belgium), running at $\pm$3·5 V/cm for 2·5 h. The Gene Ruler^{TM} 100 bp DNA Ladder (MBI Fermentas) was used as a size reference marker. Each PCR-RFLP experiment

was repeated at least once to ensure repeatability of the results.

RESULTS

The *in silico* analysis divided the analysed *Leishmania* sequences (Table 1) into 9 different patterns on the basis of digestion with *Hae*III, as shown in Fig. 1. In this figure, also the relationships between the analysed sequences are depicted to provide a general reference framework. Two species, i.e. *L. lainsoni* and *L. major*, and 4 species complexes could be identified on the basis of this digest. The 3 *L. amazonensis* sequences all have a different restriction pattern, one of which is shared with the other sequences of the complex, i.e. *L. mexicana* and *L. garnhami*. No other intra-species diversity was observed at the level of restriction digests. Table 2 provides an overview of the nucleotide polymorphisms that underlie the *Hae*III restriction patterns. All sequences generating identical fragments also have identical nucleotides at the restriction endonuclease recognition sites, except for *L. naiffi*, which shares the pattern with *L. braziliensis* and *L. peruviana* (Fig. 1). In the latter two, the absence of the *Hae*III recognition sequence at position 1276 is caused by a T, while this is an A in *L. naiffi*. In order to further resolve the *Hae*III groups, additional restriction recognition sites were identified. These are shown in Fig. 1, with the corresponding nucleotide polymorphisms in Table 2. *Mlu*I separates *L. donovani* from *L. infantum*, *Bsa*HI identifies *L. tropica* and *L. aethiopica*, while *Bcc*I discriminates *L. panamensis* from *L. guyanensis*. *Rsa*I distinguishes *L. peruviana* from *L. braziliensis* and *L. naiffi*, but the latter two cannot be separated. With *Rsa*I, *L. mexicana* M379 can be separated from the other *L. mexicana* sequence LH2312, which shares the pattern with *L. amazonensis* and *L. garnhami*. The *in silico* pattern of *L. braziliensis* CUM29 with *Rsa*I could not be determined because of an unresolved sequence ambiguity (Table 2).

Following *in silico* analysis, the identified enzymes were used to perform PCR-RFLP on the strains and isolates indicated in Table 1. Selected results are depicted in Fig. 2, and all strains belonging to the same species showed an identical pattern except for *L. mexicana* M379 when digested with *Rsa*I, and CUM29 showing an *L. peruviana* pattern (not shown). There was excellent agreement between the *in silico* and the wet analyses, except for *L. naiffi* and *L. amazonensis* strain LTB0016 with *Hae*III. Even though the *hsp*70 sequence was determined from the same DNA isolate of strain M5210 as used for PCR-RFLP, the latter assay showed additional *L. naiffi* *Hae*III fragments, allowing discrimination from the *L. braziliensis* – *L. peruviana* group. LTB0016 on the other hand was identical to the other isolates of the *L. mexicana* species complex (i.e. *L. mexicana*, *L. garnhami*, and *L. amazonensis*), allowing separation of the complex from other species.

DISCUSSION

We successfully applied *hsp*70 PCR-RFLP with different restriction endonucleases to identify *Leishmania* species from the New and Old World. Using this approach we were able to discriminate *L. donovani*, *L. infantum*, *L. aethiopica*, *L. tropica*, *L. major*, *L. braziliensis*, *L. peruviana*, *L. guyanensis*, *L. panamensis*, *L. naiffi*, and *L. lainsoni*. No diversity within these species was observed for the strains and isolates tested despite their various geographical origins, which supports the universal validity of our method. For some species, however, variability might not be adequately reflected due to sampling limitations, notably for *L. naiffi*, *L. panamensis*, and the *L. mexicana* complex, also circulating in regions from where we had no samples available.

L. chagasi could not be distinguished from *L. infantum*, but it has since long been established that these are the same species (Cupolillo *et al.* 1994). Equally so, *L. archibaldi* is no longer recognized as a species (Lukeš *et al.* 2007), which makes its identification obsolete. In practice, it will also be difficult to separate *L. major* from *L. donovani*, as a 351 bp *Hae*III fragment of *L. major* cannot be distinguished from the corresponding 338 and 13 bp fragments of *L. donovani*, the latter being undetectable on agarose gel. As for *L. braziliensis* isolate CUM29, the sequence shows an ambiguity at *Rsa*I site 115 but in PCR-RFLP it generated an *L. peruviana* pattern, disagreeing with its classification shown in Fig. 1 and the fact that it was isolated from a mucocutaneous lesion. The additional bands seen in PCR-RFLP of *L. naiffi* as compared to its *in silico* result, and allowing its distinction from the *L. braziliensis* – *L. peruviana* group, although reproducible, can currently not be explained. Here, intra-species variation needs further study as we included only one strain.

PCR-RFLP showed a *Hae*III pattern specific for the *L. mexicana* species complex, even though we were not able to resolve the separate species *L. mexicana*, *L. garnhami*, and *L. amazonensis*. However, *in silico* the 2 sequences from strain LTB0016 showed a different pattern as compared to the remaining sequences of the complex, which could be explained by sequencing errors in Accession numbers L14604 and L14605. Indeed, as these are respectively derived from a cDNA and corresponding genomic clone (Bock and Langer, 1993), they would be expected to be identical, which is not the case at position 37. In addition, the deviation from all other aligned sequences at position 1171 (GGCC instead of GCGC) could be caused by a misinterpreted GC inversion, which given the sequencing technology would not be uncommon due to

Table 2. SNPs affecting restriction endonuclease recognition sites

Enzyme and recognition sequence	*Hae*III (GGCC)										*Rsa*I (GTAC)	*Rsa*I (GTAC)	*Bcc*I (CCATC)	*Bsa*HI (GRCGYC)	*Mlu*I (ACGCGT)
	SNPs in the different sequence groups for the enzymes listed above[a]														
Recognition sites with SNPs[b]	37	65	119	703	743	790	1156	1171	1196	1276	115	390	1080	763	429
L. donovani (5)[c]	...A	....	....	....	....	....	..A.	.CG.	....	....					C
L. infantum (7)[d]	...A	....	....	....	....	....	..A.	.CG.	....	....					.....
L. aethiopica (4)	...A	....	....	....	....	A...	..A.	.CG.	....	....				.....[f]	
L. tropica (2)	...A	....	....	....	....	A...	..A.	.CG.	....	....				...A..[f]	
L. major (4)	...A	....	..T.	....	....	....	..A.	.CG.	....	....					
L. amazonensis LTB0016 (L14605)[e]	....	..G.	....	....	....	....	..A.	....	..T.	.T.G		.C..			
L. amazonensis LTB0016 (L14604)[e]	...A	..G.	....	....	....	....	..A.	....	..T.	.T.G		.C..			
L. amazonensis M2269	...A	..G.	....	....	....	....	..A.	.CG.	..T.	.T.G		.C..			
L. garhnami JAP78	...A	..G.	....	....	....	....	..A.	.CG.	..T.	.T.G		.C..			
L. mexicana LH2312	...A	..G.	....	....	....	....	..A.	.CG.	..T.	.T.G		.C..			
L. mexicana M379	...A	..G.	....	....	....	....	..A.	.CG.	..T.	.T.G		....			
L. peruviana (4)	...G	....	....	...G	....	....	....	.CG.	....	.T..	A...				
L. braziliensis CUM29	...G	....	....	...G	....	....	....	.CG.	....	.T..	R...[g]				
L. braziliensis (7)	...G	....	....	...G	....	....	....	.CG.	....	.T..	....				
L. naiffi M5210	...G	....	....	...G	....	....	....	.CG.	....	.A..					
L. panamensis (2)	...G	....	....	...G	....	....	....	.CG.	..T.	.A..			.T...		
L. guyanensis (4)	...G	....	....	...G	....	....	....	.CG.	..T.	.A..			.....		
L. lainsoni (4)	...G	....	....	...G	..T.	....	....	.CG.	.CT.	....					
Additional recognition sites[b] common to all strains for which the SNPs are shown					106 457 1097						685 865 1147 1213 1348 1405[h]	115 685 865 1147 1213 1405[h]	108	47 334 973	1209

[a] The sequences and groups are listed in the same order as in Fig. 1, where also the GenBank Accession numbers are given. When more than 1 sequence is present in a group, the number of sequences is indicated between brackets in which case the separate entries can be seen in Fig. 1. Only the differences from the enzymes' recognition sequences are shown, identical nucleotides are represented by dots (see also footnote f).

[b] The recognition site number refers to the first nucleotide position of the recognition sequence in an alignment used for RFLP analysis, i.e. containing the strain sequences and the *hsp*70 PCR primers.

[c] Includes *L. archibaldi* LEM3463, which is no longer recognized as a separate species (Lukeš *et al*. 2007).

[d] Includes the 3 *L. chagasi* strains MAIKE, WC, ARL. *L. chagasi* is the synonym for *L. infantum* of the New World (Cupolillo *et al*. 1994; Lukeš *et al*. 2007).

[e] Two different sequences of this strain are listed in the GenBank sequence data base, the Accession numbers of which are indicated between brackets (see also Table 1).

[f] The SNPs are indicated as dots when compatible with the *Bsa*HI recognition sequence ambiguities, whereby R = G or A and Y = C or T.

[g] R represents a sequence ambiguity, i.e. purine (A or G).

[h] This recognition site is present in PCR primer HSP70ant.

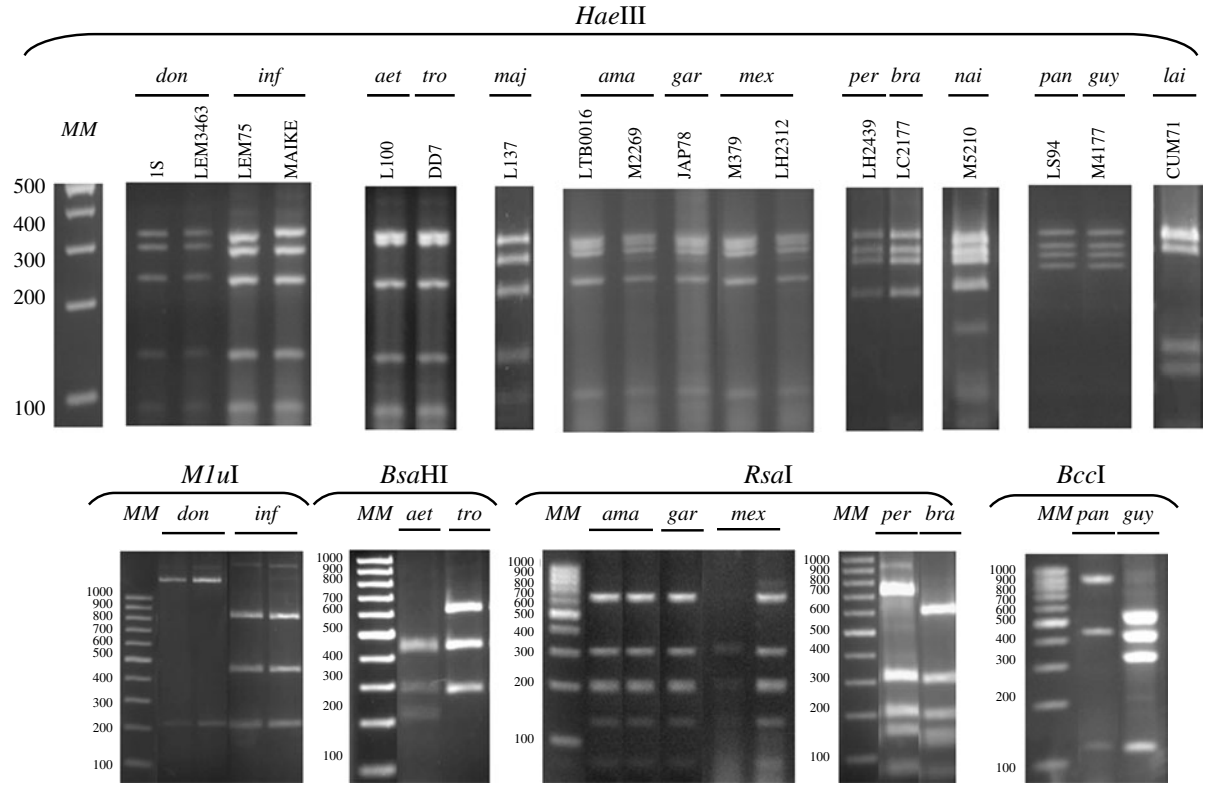

Fig. 2. Overview of all PCR-RFLP patterns obtained with the restriction endonucleases indicated. A selection of isolates and strains listed in Table 1 are depicted, all of which, except LEM75, are also included in Fig. 1, using the same species order and abbreviations. The examples shown are representative for each entry in Table 1 according to enzyme and species, except for CUM29 generating a *Leishmania peruviana* instead of an *L. braziliensis* pattern (not depicted). *L. mexicana* M379 shows an isolate-specific pattern for *Rsa*I. The order in which the isolates and strains are shown is identical in the upper and lower panel. The Gene Ruler^{TM} 100 bp DNA Ladder size marker (MM) is depicted on the left hand side for each enzyme, with an indication of fragment sizes.

compression gel artifacts in the GC-rich region where this site is situated. With *Rsa*I, only *L. mexicana* strain M379 was different from the other isolates of the complex, which might reflect its origin from Central (Belize) as opposed to South America. Hence, also *Rsa*I does not allow identification of the 3 species, but given the fact that these are also not supported as monophyletic entities in *hsp*70 phylogenies (Asato *et al*. 2009; Fraga *et al*. 2010) it is not surprising that we failed to find a restriction enzyme that could separate them unequivocally.

Currently, many species-identification assays are homemade and therefore difficult to compare, often using several different PCRs and targets to reach a conclusion (e.g. Schönian *et al*. 2003; Quispe-Tintaya *et al*. 2004). As we have shown that *hsp*70 PCR-RFLP is universally applicable for *Leishmania* species identification, it opens doors to the design of a standardized and democratic tool for species identification in many settings where the use of PCR and agarose gels are feasible, at the same time obviating the need for different PCRs, targets and primer sets. Even though from a scientific point of view comparison of different targets to study phylogeny and define species is still preferred, for practical reasons a pragmatic approach using one reference target can be beneficial if widely used by the scientific community. The added advantage is that universal use of one assay allows quality control and building of an extensive global data base, which in turn can serve to develop both high- and low-tech alternative techniques, such as sequencing or real-time PCR (Gangneux *et al*. 2003; Wortmann *et al*. 2005; Foulet *et al*. 2007).

In this study we have not explored the use of our system on other than cultured parasite material, to which end it would be necessary to assess the detection limit of the *hsp*70 PCR in various kinds of samples containing various *Leishmania* species, which was beyond the scope of the current evaluation. However, several studies already demonstrated the applicability of the *hsp*70 PCR-RFLP in different locations and settings, such as for the analysis of clinical samples and insect tissues (García *et al*. 2004, 2007*a*,*b*; Montalvo Alvarez *et al*. 2010; Pérez *et al*. 2007). This can further advance species differentiation in travel clinics, where the origin of infection is often unclear. In other settings, the use of a standardized and easily applicable reference test would facilitate comparisons across different

studies, and interpretation of global epidemiological data.

In conclusion, with this paper we further build on an existing *hsp*70 PCR-RFLP technology for *Leishmania* species identification. We advocate future exploration and application of this target to further validate its use in particular, specific settings. This will aid in attaining the final goal, which is a universal, accurate, and democratic tool for *Leishmania* species identification to the benefit of the people affected by the disease.

ACKNOWLEDGEMENTS

The authors would like to thank all colleagues and institutes that kindly donated the *Leishmania* reference strains or DNA, amongst whom J. Arévalo (Instituto de Medicina Tropical Alexander von Humboldt, Lima, Peru); L. García (Centro Universitario de Medicina Tropical, Cochabamba, Bolivia); E. Cupolillo (Instituto Oswaldo Cruz, Rio de Janeiro, Brazil); G. Schönian (Institut für Mikrobiologie und Hygiene, Berlin, Germany); I. Mauricio, V. Yardley, and D. Evans (London School of Hygiene and Tropical Medicine, London, UK); P. Desjeux (Instituto Boliviano de Biologia de Altura, La Paz, Bolivia); J.-P. Dedet and J.A. Rioux (Centre National de Référence des *Leishmania*, Montpellier, France); J.J. Shaw (University of São Paulo, São Paulo, Brazil); G. Schoone and A. El Harith (Royal Tropical Institute, Amsterdam, The Netherlands); I. D. Vélez and C. Muskus (Programa de Estudio y Control de Enfermedades Tropicales, Universidad de Antioquia, Medellín, Colombia); and the Leishepinet consortium (EU contract INCO-CT2005-015407). Jean-Pierre Wenseleers (Institute of Tropical Medicine, Antwerp, Belgium) is acknowledged for his help with the figures.

FINANCIAL SUPPORT

This study was funded by the second and third framework program of the Belgian Development Cooperation with ITMA and IPK, Cuba.

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