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Original article

Analysis of ribosomal DNA internal transcribed spacer sequences of the *Leishmania donovani* complex

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Abstract

To understand phylogenetic relationships of species and strains within the *Leishmania donovani* complex, we have analyzed the ribosomal DNA internal transcribed spacer (ITS) sequences of 27 *Leishmania infantum*, 2 *Leishmania chagasi*, 18 *L. donovani* and 5 *Leishmania archibaldi* strains of different zymodemes and geographical origin. Eight ITS sequence types were found. All detected sequence variation within ITS1 and ITS2 was based on 12 polymorphic microsatellites. The *L. infantum* strains from the Mediterranean region, China and *L. chagasi* from the New World formed a phylogenetic group well separated from the second main group including all strains from East Africa and India. Within the latter group three distinct phylogenetic subgroups could be differentiated: (1) *L. donovani* (Sudan/Ethiopia, China) + *L. archibaldi* (Sudan), (2) *L. donovani* (Sudan/Ethiopia) + *L. infantum* (Sudan) + *L. archibaldi* (Sudan/Ethiopia), and (3) *L. donovani* (Kenya, India). These groups are not consistent with previous species definitions based on isoenzyme analyses, e.g. *L. infantum* is polyphyletic and *L. archibaldi* is not supported as a distinct species. Two groups of Indian strains could be differentiated, one of which has an identical sequence type to the strains from Kenya. Three main lineages of strains can thus be differentiated in East Africa: two quite distantly related groups of strains from Sudan/Ethiopia, and a third group including all strains from Kenya, which is more closely related to part of the Indian strains than to any of the Sudanese/Ethiopian groups. The ITS sequence analysis presented here supports the need for revision of the taxonomy of the *L. donovani* complex.

Keywords: *Leishmania infantum*; *Leishmania archibaldi*; *Leishmania donovani*; Molecular markers; Microsatellites; Ribosomal internal transcribed spacer; Taxonomy; Molecular systematics

1. Introduction

Leishmaniases are diseases caused by parasites belonging to the genus *Leishmania* Ross, 1903, a member of the order Kinetoplastida, family Trypanosomatidae. This genus can be divided into several species complexes, one of which is *Leishmania donovani* [1], the causative agent of visceral leishmaniasis (VL) or Kala-Azar, a serious disease which affects approximately half a million people each year [2], with a high rate of HIV co-infections.

The taxonomical status of *L. donovani* (*sensu lato*) is still debated. Some authors [3] believe that the term *L. donovani sensu lato* includes two distinct species: *L. donovani sensu stricto* and *L. infantum*. Another opinion is that *L. donovani* can be divided into two subspecies: *L. d. donovani* and *L. d. infantum* [4]. Lainson and Shaw [1] combined four taxa in the species complex *L. donovani*, namely *L. donovani* Laveran and Mesnil, 1903, *L. infantum* Nicolle, 1908, *L. archibaldi* Castellani and Chalmers, 1919 and *L. chagasi* Cunha and Chagas, 1937. *L. chagasi*, considered by many authors as a synonym for *L. infantum* [5–7], occurs exclusively in South America. The name *L. archibaldi* (*L. donovani* var. *archibaldi*) was originally proposed for East African strains, espe-

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cially for strains from Sudan. In 1936 *L. archibaldi* was synonymized with *L. donovani* [8]. The name *L. archibaldi* was re-introduced in 1990 [5] for a single zymodeme (MON-82), the taxonomical status of which remains, however, unresolved.

L. infantum/chagasi, *L. donovani* and *L. archibaldi* all cause VL—with some minor differences. *L. donovani sensu stricto* is the causative agent of the so-called Kala-Azar (VL) and post-Kala-Azar dermal leishmaniasis (PKDL) and also affects adults. The existence of a reservoir host is suspected for African strains, but VL caused by *L. donovani* is an anthroponotic disease in India. *L. infantum* causes VL and cutaneous leishmaniasis (CL), which was traditionally a childhood disease, however today adults form 80% of all infected patients [9]. Dogs are the main reservoir hosts for *L. infantum* and the incidence of canine viscerocutaneous leishmaniasis is high in all European Mediterranean countries [10,11], as well as in non-endemic areas due to tourism and the dog trade ([12], Steuber, unpublished). *Leishmania*/HIV coinfections are the main health problem in all endemic regions, where up to 70% of adult cases of VL are associated with HIV infection [13].

There are differences in the geographical distribution of the respective species/subspecies. *L. donovani* is endemic in three main regions: (1) India/Bangladesh/parts of Nepal; (2) China; (3) Sudan, Ethiopia, Kenya. *L. infantum/chagasi* is distributed over four areas: (1) the Mediterranean basin (southern Europe and northern Africa to Iran), (2) China, (3) Sudan, Ethiopia, (4) Central and South America.

To date, the gold standard for species and strain identification has been isoenzyme analysis. It has also been used to infer phylogenetic relationships within the genus [5,6,14–16]. A revised classification of the genus *Leishmania* was proposed in 1990 [5], introducing the term 'phenetic complex' for a subset of zymodemes. The authors discriminated between the *L. donovani* (including *L. archibaldi*) and the *L. infantum* (with the synonym *L. chagasi*) phenetic complexes. Isoenzyme typing has, however, several drawbacks: it is time-consuming, it requires cultivation of the parasites, post-translational modifications can influence the electrophoretic mobility, etc. To overcome these problems DNA-based methods have been used to identify and differentiate *Leishmania* parasites. A series of PCR-amplified DNA sequences have been used for diagnosis [17–21] and for phylogenetic [22–29] and epidemiological studies [15,20,30–35].

Few authors have investigated phylogenetic relationships within the entire complex *L. donovani* using DNA-based methods such as sequence analysis [26,36], RFLP analysis [27–29,33], and SCAR (sequence-confirmed amplified region) analysis [37]. Most of these studies included only isolates of a single species, isolates of just one geographical region, or only single strains from the respective geographic regions. Some of these DNA-based studies led to conflicting results with regard to the three species recognized by isoenzyme analysis in Sudan and Ethiopia. Moreover, the taxonomical status of *L. archibaldi* is very controversial, as different authors use this name for geographically different sets of strains — for all strains originating from Sudan [38], or for all East African strains [39], or for all strains from East Africa excluding strains from Kenya and Somalia [1]. Three different zymodemes found for Sudanese strains have been linked with *L. archibaldi* [5,16].

Here we present a study of the *L. donovani* complex including strains of *L. donovani*, *L. infantum*, *L. chagasi* and *L. archibaldi* of different geographical origins. Sequence analysis of the ribosomal internal transcribed spacers ITS1/ITS2 has been applied to infer phylogenetic relationships between different groups of strains with emphasis on the relationship between the three known species found in Sudan and Ethiopia. The present paper is the most comprehensive study so far based on sequences of genetic markers that includes all species of the complex, all three known zymodemes of *L. archibaldi,* the three described zymodemes of Sudanese *L. infantum*, as well as several strains from each of the different geographical regions.

2. Materials and methods

2.1. Parasite DNA

Sources, designation and geographical origins of the *Leishmania* strains are listed in Table 1. All parasites were cultivated as described previously [23]. Strains were obtained from the following culture collections: Centre National de Référence des *Leishmania*, Montpellier, France; KIT (Royal Tropical Institute), Amsterdam, Netherlands; WHO's Jerusalem Reference Centre for Leishmaniases, Hebrew University—Hadassah Medical School, Jerusalem, Israel; Indian Institute of Chemical Biology, Calcutta, India; London School of Hygiene and Tropical Medicine, London, UK. Six *L. donovani* and two *L. archibaldi* strains were isolated from an endemic focus in eastern Sudan [30]. DNA was isolated using proteinase K—phenol/chloroform extraction [23], suspended in TE-buffer [40] and stored at 4 °C until use.

2.2. PCR amplification assays

ITS1 and ITS2 were amplified with primer pairs LITSR/L5.8S and L5.8SR/LITSV, respectively, and PCR conditions described elsewhere [31,32,41]. The amplification products were tested by electrophoresis in 1.2% agarose gels in 1 × TBE-buffer and visualized by staining with ethidium bromide [40].

2.3. DNA sequencing

Direct cycle sequencing of PCR-amplified fragments was performed either by the conventional manual method or by an automated sequencer A373 (Applied Biosystems). Prior to sequencing the amplified PCR fragments were purified

Table 1
Designation and characteristics of *Leishmania* strains used in this study

Taxa [a]	Lab code	WHO-code	Origin	Zymodeme	Pathology [b]	ITS sequence type	Accession number EMBL
L. infantum	INF-01 [c]	MHOM/TN/80/IPT1	Tunisia	MON-1	VL	A	AJ000289
	INF-39	MHOM/FR/78/LEM75	France	MON-1	VL	A	AJ634339
	INF-40	MHOM/FR/95/LPN114	France	MON-1	VL	A	AJ634340
	INF-41	MHOM/ES/93/PM1	Spain	MON-1	VL	A	AJ634341
	INF-42	MHOM/FR/97/LSL29	France	MON-1	CL	A	AJ634342
	INF-43	MHOM/ES/86/BCN16	Spain	MON-1	CL	A	AJ634343
	INF-44	MHOM/PT/00/IMT260	Portugal	MON-1	CL	A	AJ634344
	INF-02	MHOM/CN/54/Peking	China	MON-1	VL	A	AJ634345
	INF-03	MHOM/FR/62/LRC-L47	France	n.d.	VL	B	AJ000288
	INF-35	MCAN/FR/87/RM1	France	MON-108	CanVL	A	AJ634346
	INF-37	MHOM/ES/88/LLM175	Spain	MON-198	VL	A	AJ634347
	INF-45	MHOM/FR/96/LEM3249	France	MON-29	CL	A	AJ634348
	INF-46	MHOM/ES/91/LEM2298	Spain	MON-183	VL	A	AJ634349
	INF-48	MHOM/MT/85/BUCK	Malta	MON-78	CL	A	AJ634350
	INF-47	MHOM/FR/80/LEM189	France	MON-11	CL	A	AJ634351
	INF-32	MCAN/ES/86/LEM935	Spain	MON-77	CanVL	B/A	AJ634355
	INF-55	MHOM/ES/92/LLM373	Spain	MON-199	VL	A	AJ634352
	INF-56	MHOM/IT/94/ISS1036	Italy	MON-228	VL	A	AJ634353
	INF-57	MHOM/IT/93/ISS800	Italy	MON-188	VL	A	AJ634354
	INF-05	MHOM/CN/78/D2	China	LON-49	VL	A	AJ000303
	INF-38	MHOM/SD/97/LEM3472	Sudan	MON-267	PKDL	F	AJ634370
	INF-33	MHOM/SD/62/3S	Sudan	MON-81	VL	E	AJ634361
	INF-58	MHOM/SD/93/452BM	Sudan	MON-30	PKDL	F	AJ634371
	INF-59	MHOM/SD/82/GILANI	Sudan	MON-30	VL	E/F	AJ634369
	INF-60	MHOM/SD/93/45-UMK	Sudan	MON-30	VL	E	AJ634362
	INF-61	MHOM/SD/93/762L	Sudan	MON-30	VL	E	AJ634363
	INF-62	MHOM/SD/93/597-2	Sudan	MON-30	PKDL	E	AJ634364
L. chagasi	CHA-01 [c]	MHOM/BR/74/PP75	Brazil	MON-1	VL	A	AJ000304
	CHA-03	MHOM/BR/85/M9702	Brazil	n.d.	VL	A	AJ000306
L. donovani	DON-11	MHOM/CN/00/Wangjie1	China	MON-35	VL	C	AJ000294
	DON-38	MHOM/ET/00/HUSSEN	Ethiopia	MON-31	VL	D/E	AJ634360
	DON-06	MHOM/SD/75/LV139	Sudan	n.d.	CL	E	AJ000291
	DON-07	MHOM/SD/62/LRC-L61	Sudan	n.d.	not known	E	AJ634365
	DON-08	MHOM/SD/68/1S	Sudan	n.d.	VL	E	AJ000293
	DON-17	MHOM/SD/93/9S	Sudan	MON-18	VL	F	AJ634372
	DON-24	MHOM/ET/67/HU3	Ethiopia	MON-18	VL	F	AJ634373
	DON-23	MHOM/SD/93/338	Sudan	MON-18	PKDL	E/F	AJ634368
	DON-49	MCAN/SD/00/LEM3946	Sudan	MON-274	CanVL	D	AJ634356
	DON-02	MHOM/KE/83/NLB189	Kenya	MON-37	PKDL	G	AJ634374
	DON-03	MHOM/KE/84/NLB218	Kenya	n.d.	PKDL	G	AJ000296
	DON-04	MHOM/KE/85/NLB323	Kenya	MON-37	VL	G	AJ000297
	DON-01 [c]	MHOM/IN/80/DD8	India	MON-2	VL	H	AJ000292
	DON-39	MHOM/IN/00/DEVI	India	MON-2	VL	H	AJ634376
	DON-40	MHOM/IN/96/THAK35	India	MON-2	not known	H	AJ634377
	DON-45	MHOM/IN/01/BHU20140	India	n.d.	VL	H	AJ634378
	DON-10	MHOM/IN/71/LRC-L51a	India	n.d.	VL	G	AJ000290
	DON-48	MHOM/IN/54/SC23	India	MON-38	VL	G	AJ634375
L. archibaldi	ARC-21	MHOM/SD/93/35-band	Sudan	MON-82	VL	E	AJ634366
	ARC-43	MHOM/ET/72/GEBRE1	Ethiopia	MON-82	VL	E	AJ634367
	ARC-13	MHOM/SD/93/GE	Sudan	MON-82	VL	D	AJ634357
	ARC-46	MHOM/SD/97/LEM3429	Sudan	MON-257	VL	D	AJ634358
	ARC-47	MHOM/SD/97/LEM3463	Sudan	MON-258	VL	D	AJ634359

[a] Strain identification according to isoenzyme analysis.
[b] VL, Visceral leishmaniasis; CL, Cutaneous leishmaniasis; PKDL, Post kala-azar dermal leishmaniasis; CanVL, Canine VL.
[c] WHO reference strain.

using the QIAquick PCR purification kit (QIAGEN, Chatsworth, CA) according to the manufacturer's protocol. Purified double-stranded PCR fragments were sequenced using the Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit (USB, Cleveland, USA). Cycle sequencing was performed according to the manufacturer's protocol using

a Trioblock Cycler (Biometra). Sequencing reactions were run on 8% polyacrylamide/TBE-urea gels. Finally, gels were fixed, dried and exposed to Kodak SB film. Automated sequencing reactions were carried out with a *Taq* DyeDeoxy™ Terminator Cycle Sequencing Kit (Applied Biosystems) applying PCR conditions recommended by the manufacturer. Samples were then loaded on a 6% polyacrylamide gel attached to the sequencer. The ITS1 region (~320 bp) was sequenced using primers LITSR and L5.8S, and ITS2 (740 bp) using internal primers LIS2MV and LIS2MR [32] in addition to primers L5.8SR and LITSV.

2.4. Data analysis

Sequences were aligned using the multiple alignment program CLUSTAL V and manually adjusted (alignments are available upon request). Their phylogenetic analysis was performed using maximum parsimony as implemented by the PAUP (Phylogenetic Analysis Using Parsimony) computer program, Version 4.0b [42]. Robustness of the internal branches was tested by bootstrap analysis [43] from 1000 bootstrap replications using the heuristic search option and retaining groups compatible with the 50% majority rule consensus tree.

Calculation of base pair differences was carried out by a pairwise comparison of the strains from alignments. Variation (V) of the compared sequences was calculated as $V = n_d/n$, where n_d is the number of different base pairs and n is the total number of base pair positions compared [44].

Microsatellites were analyzed separately using the program MICROSAT [45]. Microsatellite genetic distances were calculated for the numbers of repeats within a locus using the measure D_{AS}, which is based on the proportion of shared alleles [46] and calculates multilocus pairwise distance measurements as $1 - s/n$, where n is the number of loci compared and s is the total number of shared alleles at all loci. The neighbor-joining tree of the distance matrix was constructed in PAUP, Version 4.0b [42].

3. Results

The ITS1 and ITS2 were sequenced for 52 strains of *L. infantum*/*L. chagasi*, *L. donovani* and *L. archibaldi*. ITS1 varied in size from 232 to 238 bp and ITS2 from 602 to 615 bp for strains of the *L. donovani* complex. Four and eight polymorphic microsatellites were detected, respectively, in the ITS1 and ITS2 regions within the *L. donovani* complex (Table 2). Remarkably, polymorphism of ITS1 and ITS2 was based exclusively on variable repeats of these microsatellites. A single nucleotide exchange was found for just one strain (DON-11 from China). Sequence variation (V) determined by the polymorphic microsatellites was very low within the *L. donovani* complex, 0–2.9% for ITS1, and 0–2.3% for ITS2.

Eight distinct sequence types (A–H) were identified from the combined ITS1 and ITS2 sequences (Table 1, Fig. 1). Polymorphism of the sequences based on different microsatellite repeat numbers are shown in Table 2. Four strains (Table 1) had two simultaneous sequence types, with one being dominant, that was used for parsimony analysis. In microsatellite analysis both alleles were considered.

Two sequence types (A and B) were characteristic for *L. infantum* from the Mediterranean region and China, and for *L. chagasi* the synonymous species from the New World. Most strains (90.9%) were type A, one strain had the slightly different type B, and one strain had both A and B. The seven *L. infantum* strains from Sudan (MON-30, MON-81, and MON-267) had sequences different from those of Mediterranean, Chinese and New World strains.

Six sequence types were found for *L. donovani* strains (C–H) and two for *L. archibaldi* (D, E). Sequence type C was unique to a strain of *L. donovani* from China (DON-11, MON-35). Types D, E, and F were found for all strains originating from Sudan and Ethiopia, regardless of whether they had been typed by isoenzyme analysis as *L. donovani*, *L. archibaldi* or *L. infantum*. Moreover, there was no strict correlation between the zymodemes MON-30, 81, 267, 18, 31, 274, 82, 257 and 258 found in these strains and sequence types D–F. All *L. infantum* strains from Sudan and Ethiopia (MON-30, 81, 267) belonged to type E or F, and were thus completely different from the Mediterranean and Chinese strains.

Sequence type G was typical for *L. donovani* strains from Kenya (MON-37), as well as for two strains from India (MON-38 and one strain of unknown zymodeme). All three MON-2 strains and one strain of undefined zymodeme from India belonged to sequence type H.

Two types of analyses have been performed for tree inference. The entire ITS sequences have been used for parsimony analysis (Fig. 2A). The repeat numbers for each microsatellite have been used for distance-based analysis, applying the D_{as} measure, which is usually used for microsatellites (Fig. 2B). Both trees are based on microsatellite polymorphisms.

Two major clades (I and II) can be recognized for the *L. donovani* complex in the maximum parsimony tree based on the combined ITS1 and ITS2 sequences (Fig. 2A). Clade I includes all *L. infantum*/*L. chagasi* strains from the Mediterranean region, China and South America, but not *L. infantum* from East Africa. The second major clade (II) contains all *L. donovani* and *L. archibaldi* strains as well as all *L. infantum* strains from East Africa. This clade can be subdivided into two general subclades. Subclade IIa includes the majority of strains from Sudan and Ethiopia (*L. donovani* MON-18 and MON-31, *L. archibaldi* MON-82, and *L. infantum* MON-30, MON-81 and MON-267), as well as all *L. donovani* strains from Kenya and India. Subclade IIb contains two of the *L. donovani* strains (DON-38- MON-31, DON-49 -MON-274) and three of the five *L. archibaldi* strains (MON-82, MON-257, MON-258) from Sudan/Ethiopia. The single strain from China (MON-35) has a basal position in this subclade. Subclade IIa splits into IIa1 and IIa2. Subclade IIa1 contains only

Table 2
Microsatellite repeat numbers found for strains of the *L. donovani* complex belonging to different zymodemes

Species	Zymodeme	Number of strains	0rigin	Sequence type	ITS1 poly (C)	ITS1 poly (A)	ITS1 poly (TA)	ITS1 poly (A)	ITS2 poly (AT)	ITS2 poly (TA)	ITS2 poly (G)	ITS2 poly (G)	ITS2 poly (TGG)	ITS2 poly (GT)	ITS2 poly (AT)	ITS2 poly (C)
L. infantum	MON-1	8	France, Spain, China Portugal, Tunisia	A	3	6	4	8	5	5	7	6	1	6	5	5
	LON-49 [a]	1	China	A	3	6	4	8	5	5	7	6	1	6	5	5
	MON-11	1	France	A	3	6	4	8	5	5	7	6	1	6	5	5
	MON-29	1	France	A	3	6	4	8	5	5	7	6	1	6	5	5
	MON-77	1	Spain	A/B	3	6	4	8	5	5	9/7	6	1	6	5	5
	MON-78	1	Malta	A	3	6	4	8	5	5	7	6	1	6	5	5
	MON-108	1	France	A	3	6	4	8	5	5	7	6	1	6	5	5
	MON-183	1	Spain	A	3	6	4	8	5	5	7	6	1	6	5	5
	MON-188	1	Italy	A	3	6	4	8	5	5	7	6	1	6	5	5
	MON-198	1	Spain	A	3	6	4	8	5	5	7	6	1	6	5	5
	MON-199	1	Spain	A	3	6	4	8	5	5	7	6	1	6	5	5
	MON-228	1	Italy	A	3	6	4	8	5	5	7	6	1	6	5	5
	n.d.	1	France	B	3	6	4	8	5	5	9	6	1	6	5	5
	MON-267 [b]	1	Sudan	F	2	9	5	7	5	5	5	7	1	6	5	6
	MON-81 [b]	1	Sudan	E	2	8	6	8	5	5	5	7	1	6	5	6
	MON-30 [b]	5	Sudan	E, F	2	8, 9	6, 5	8, 7	5	5	5	7	1	6	5	6
L. chagasi	MON-1	1	Brazil	A	3	6	4	8	5	5	7	6	1	6	5	5
	n.d.	1	Brazil	A	3	6	4	8	5	5	7	6	1	6	5	5
L. donovani	MON-2	3	India	H	2	8	5	7	7	5	2+T+2	6	1	6	5	6
	MON-38	1	India	G	2	8	5	7	5	5	2+T+2	6	2	6	5	6
	n.d.	2	India	G, H	2	8	5	7	5, 7	5	2+T+2	6	2, 1	6	5	6
	MON-37	3	Kenya	G	2	8	5	7	5	5	2+T+2	6	2	6	5	6
	MON-18	3	Sudan, Ethiopia	E, F	2	8, 9	5, 6	7, 8	5	5	5	7	1	6	5	6
	MON-274	1	Sudan	D	3	6	4	7	5	4	5	6	1	5	3	5
	MON-31	1	Ethiopia	D/E	3	6	4	7	5	4/5	5	6/7	1	5/6	3/5	5/6
	n.d.	3	Sudan	E	2	8	6	8	5	5	5	7	1	6	5	6
	MON-35	1	China	C	3	6	4	7	5	4	5	6	1	6	3	6
L. archibaldi	MON-82	3	Sudan, Ethiopia	D, E	2, 3	8, 6	6, 4	8, 7	5	4, 5	5	6, 7	1	5, 6	3, 5	5, 6
	MON-257	1	Sudan	D	3	6	4	7	5	4	5	6	1	5	3	5
	MON-258	1	Sudan	D	3	6	4	7	5	4	5	6	1	5	3	5
Total				**8 types**	**2 alleles**	**3 alleles**	**3 alleles**	**3 alleles**	**2 alleles**	**2 alleles**	**4 alleles**	**2 alleles**	**2 alleles**	**2 alleles**	**2 alleles**	**2 alleles**

Where numbers are separated by a dash and zymodemes are labeled by bold letters, two microsatellite fragments differing in size were found for single strains.

[a] The putative MON zymodeme equivalent to LON-49 is MON-1.

[b] A group (MON-30, MON-81, MON-267) which is defined as *L. infantum* by isoenzyme analysis.

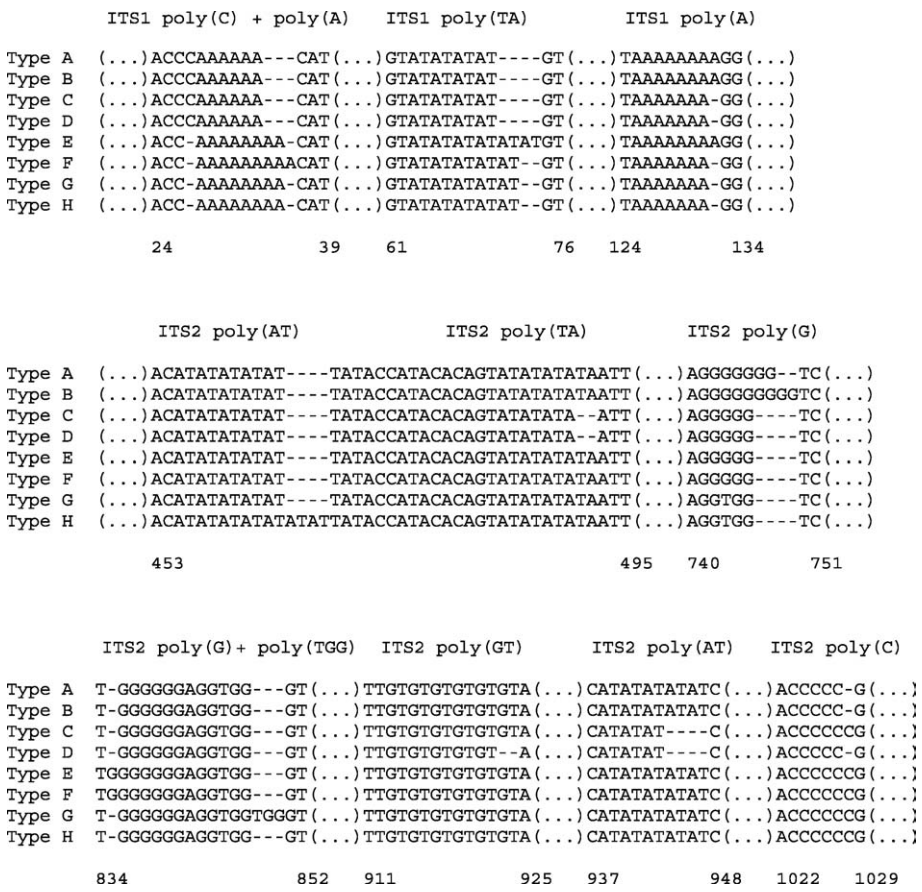

Fig. 1. Partial alignment of the eight ITS sequence types found for strains of the *L. donovani* complex. Differences between the sequence types are based solely on polymorphisms of the 12 microsatellites. Four polymorphic microsatellites have been found in ITS1 and eight in ITS2.

strains from Sudan and Ethiopia, whereas subclade IIa2 comprised of all the strains from India and Kenya. Two distinct groups were detected for strains from India ("India 1" with all MON-2 strains and "India 2" with MON-38). The strains from Kenya (MON-37) and the "India 2" group are members of the same subclade. (Fig. 2). The parsimony tree for ITS sequences points to the existence of three major groups of strains from East Africa. Subclade IIa1 includes the majority of strains from Sudan and Ethiopia analyzed (including *L. donovani, L. archibaldi* and *L. infantum*) ("Sudan 1") and shows further sub-division. A second group is part of subclade IIa2 with all *L. donovani* strains from Kenya. Clade IIb with *L. donovani* and *L. archibaldi* strains from Sudan/Ethiopia ("Sudan 2") represents the third most distantly related group. When the parsimony tree is rooted with *L. major* and *L. tropica* as outgroup species (data not shown), this latter group branches independently from the main clades of *L. donovani, L. archibaldi* and *L. infantum* strains (clade IIa) and of Mediterranean *L. infantum* strains (clade I).

Microsatellite variation within ITS has been analyzed separately with MICROSAT. D_{AS} distances have been used for the construction of an unrooted neighbor-joining tree (Fig. 2B). This tree is nearly identical to the sequence based parsimony tree.

4. Discussion

Increasingly, genotypic analyses have suggested that the isoenzyme-based taxonomy may not be adequate, but most have been based on a restricted number of strains. This study analyzes sequence variation in the ribosomal internal transcribed spacers in strains of the *L. donovani* complex of different geographical origins and with a diverse range of zymodemes.

The *L. donovani* complex had the lowest degree of ITS sequence polymorphism when compared with other *Leishmania* complexes ([47], own unpublished data). We found almost no differences in the ITS sequences within *L. infantum* strains (excluding the African strains) with only two sequence types, whereas *L. donovani* strains were more heterogeneous with six sequence types.

All 52 strains of the *L. donovani* complex analyzed in this study belonged to the following major phylogenetic groups (Fig. 2): (i) clade I ("true" *L. infantum*) which is comprised of all zoonotic strains from the Mediterranean area, China, and South America, but not those from East Africa, and (ii) clade II which consists of zoonotic and anthroponotic strains from East Africa and Asia, regardless of whether they were typed by MLEE as *L. donovani*, *L. infantum* or *L. archibaldi*. Clade IIb represents a distinct group from Sudan and

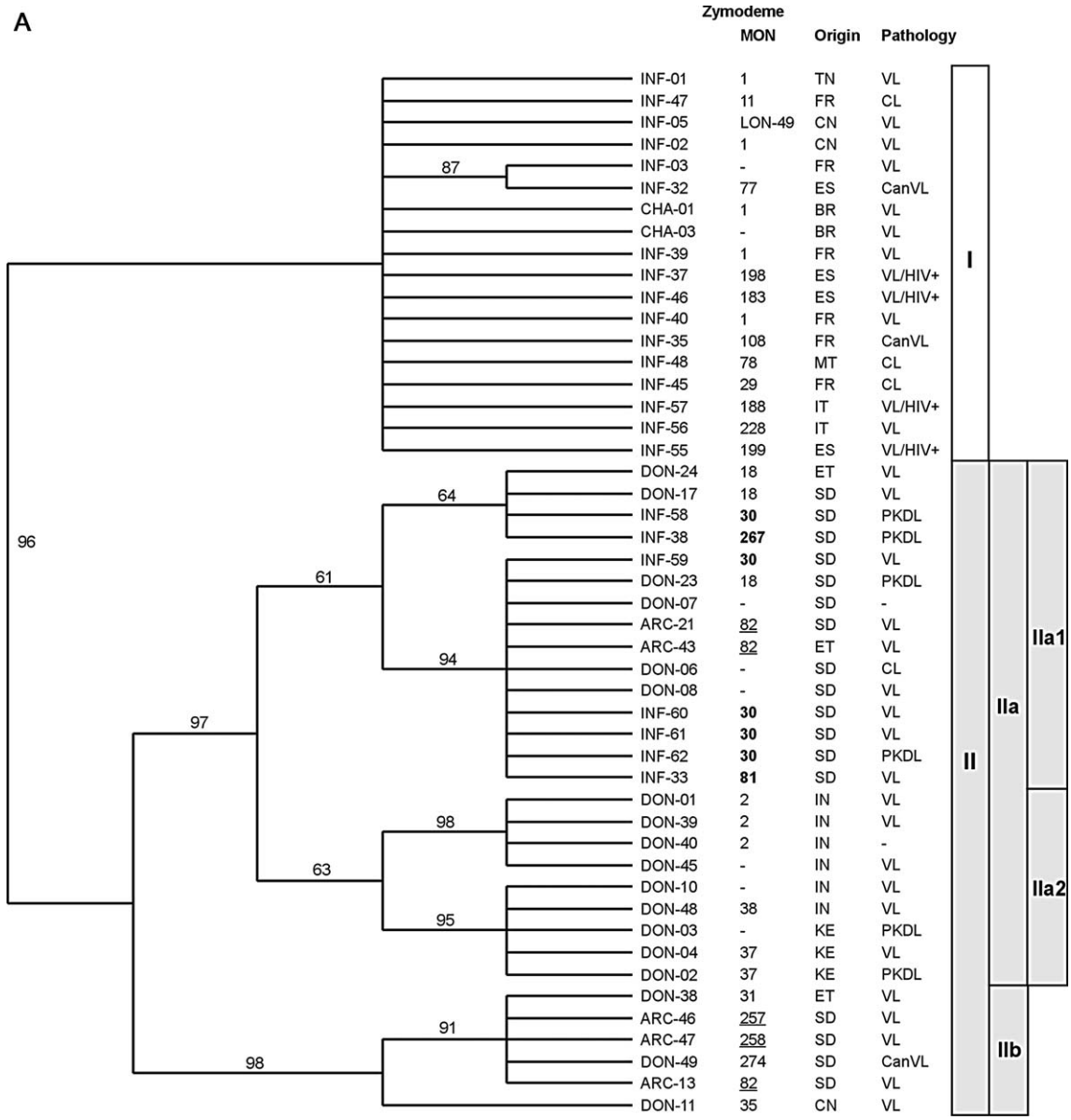

Fig. 2A. Phylogenetic relationships among strains of the *L. donovani* complex inferred by parsimony analysis of the nucleotide sequences of the ITS1 and ITS2 regions. Sequence variation is based only on microsatellite polymorphisms. The cladogram (34 steps, CI = 0.941, HI = 0.059, RI = 0.993) is the single most parsimonious tree found by heuristic search. The numbers above the branches indicate the percentages with which a given branch is supported in 1000 bootstrap replications. Strain names are based on isoenzyme identification. Bold zymodemes: zymodemes assigned to *L. infantum* strains from Sudan/Ethiopia, underlined zymodemes: zymodemes assigned to *L. archibaldi.* ITS sequences place all strains from Sudan/Ethiopia, regardless whether they were typed by isoenzyme analysis as *L. infantum*, *L. donovani* or *L. archibaldi*, into the same two East African groups. Abbreviations of origins: TN, Tunisia; FR, France; ES, Spain; CN, China; BR, Brazil; MT, Malta; IT, Italy; SD, Sudan; ET, Ethiopia; KE, Kenya; IN, India.

Ethiopia ("Sudan 2") with strains of *L. archibaldi* (MON-82, 257, 258) and *L. donovani* (MON-31, 274), whereas clade IIa1 ("Sudan 1") is comprised of the majority of Sudanese/Ethiopian strains (*L. donovani* MON-18; *L. infantum* MON-30, 81, 267; *L. archibaldi* MON-82), and IIa2 the Kenyan and Indian strains of *L. donovani.* In summary, there are three distinct groups of strains in East Africa: two groups from Sudan/Ethiopia and one group from Kenya. One of the Sudanese groups is more closely related to the Indian/Kenyan strains than to the other group from Sudan. The only Chinese *L. donovani* strain (MON-35) studied herein had a unique ITS microsatellite pattern, and was most closely related to the strains from Sudan/Ethiopia in clade IIb. Apart from the East African *L. infantum* strains, the same two major clades (*L. infantum* clade and *L. donovani* clade) appear in the trees based on isoenzyme and genotype analyses. Inconsistent data were also found for the strains of clade IIb and the status of *L. archibaldi*.

The existence of different phylogenetic groups is supported by some differences in biology and clinical behavior in strains of the *L. donovani* complex. VL in the Mediterranean area and in South America is zoonotic, whereas infections by Indian *L. donovani* are anthroponotic. Sudanese *L. donovani* has been considered, at least in part, to be zoonotic

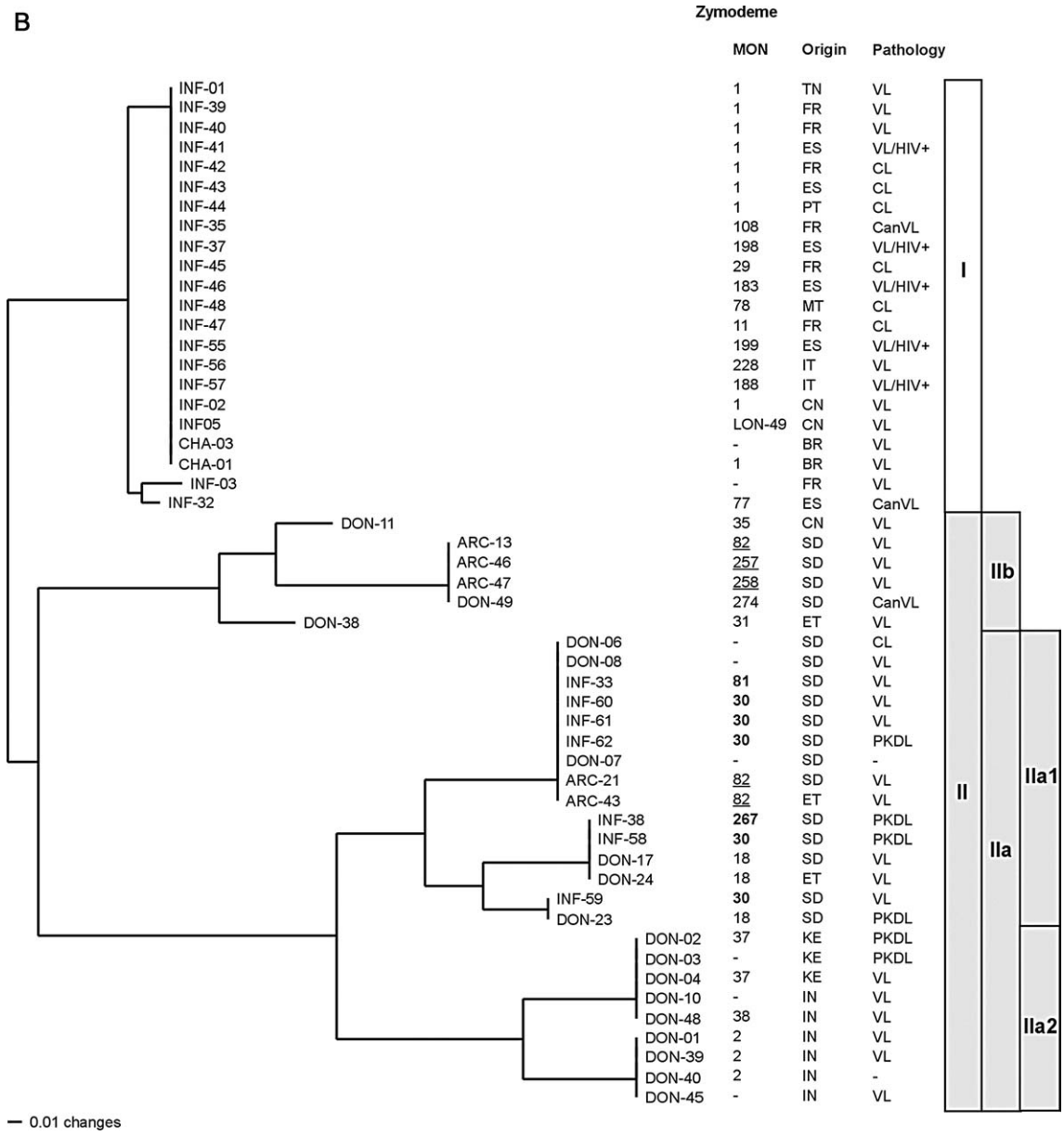

Fig. 2B. Neighbor-joining tree (unrooted) derived from distances (D_{AS}- proportion of shared alleles) calculated on the basis of the 12 microsatellites of ITS1 and ITS2. Strain names are based on isoenzyme identification. Bold zymodemes: zymodemes assigned to *L. infantum* strains from Sudan/Ethiopia, underlined zymodemes: zymodemes assigned to *L. archibaldi.* Microsatellite markers place all strains from Sudan/Ethiopia, regardless whether they were typed by isoenzyme analysis as *L. infantum*, *L. donovani* or *L. archibaldi*, into the same two East African groups.

[4]. All but two (MON-258, MON-276) of the Sudanese zymodemes were found in dogs [48,49]. We did not detect any correlation between zoonotic zymodemes or canine strains and the phylogenetic groups identified here.

The clear separation of Indian and Sudanese strains found here is in agreement with some observed clinical differences. PKDL develops in 10–20% of Indian patients usually 2–3 years after Kala-Azar treatment, in Sudan in 56% of the KA patients within weeks or a few months of treatment [50,51]. Spontaneous cure is the rule for PKDL in Sudan, but has not been reported in India, where all cases have to be treated. The range of PKDL in India is 6 months–32 years, but in Sudan 0–13 months [52]. The reported interval of 30 years for PKDL development in Kenya [52], a range atypical for African but common for Indian PKDL, is consistent with the close phylogenetic relationship between the Kenyan and the Indian *L. donovani* strains detected in this study.

Comparison of the current isoenzyme based taxonomy with the identified phylogenetic groups based on DNA sequences as shown in Fig. 2 leads to the following conclusions.

4.1. L. donovani

In this study all strains of *L. donovani* were grouped in clade II. Within this clade the strains are distributed between all three subclades. Strains from China, Sudan and Ethiopia were assigned to clades IIb (MON-35, MON-31, MON-274) and IIa1 (MON-18) together with strains from the same area

that were typed as *L. infantum* (MON-30, MON-81, MON-267) or *L. archibaldi* (MON-82, MON-257, MON-258) by isoenzyme analysis. Group IIa2 was comprised of strains from India and Kenya. Strains from India could be further subdivided into two subgroups: one of MON-2 strains, including the reference strain DD8; and a second consisting of the MON-38 strain and a strain of unknown zymodeme. The latter subgroup contained also all the strains from Kenya (MON-37).

It had already suggested in 1987 [1] that *L. donovani* in Kenya might be different from the Sudanese parasites. That Kenyan strains are only distantly related to strains from Sudan and more closely related to strains from India was later supported by RFLP analyses of *gp63* intergenic regions [28], by analyses of the mitochondrial cytochrome oxidase II gene sequence of one strain from each origin [26], by SCAR analyses of several strains per origin [37], and by analysis of tubuline spacer sequences and other microsatellite data (unpublished data).

The close relationship between strains from India and Kenya could be explained by a possible re-introduction of *L. donovani* strains from India to Kenya or vice versa through trade relationships.

4.2. L. archibaldi/L. infantum from Sudan/Ethiopia

Strains from Sudan and Ethiopia had been classified as *L. donovani*, *L. infantum* or *L. archibaldi* based on their profiles of GOT_1 and GOT_2 in isoenzyme typing [5,16]. Strains with GOT profiles of 100, 110 or 113 were considered as *L. infantum*, *L. archibaldi* and *L. donovani,* respectively. The position of the *L. archibaldi* MON-82 was not stable in zymodeme trees [5]. In phenograms it clustered with either *L. donovani* or *L. infantum* phenetic complexes, whereas in the cladogram it appeared as a distinct intermediate branch. Nevertheless *L. archibaldi* was included in the *L. donovani* phenetic complex [5,53]. In 2001 two new zymodemes (MON-257, MON-258) were assigned to *L. archibaldi* [16] and were investigated here by genetic markers for the first time. Our data suggest that *L. archibaldi* is polyphyletic and do not support its definition as a separate taxon. We propose that zymodemes MON-82, MON-257 and MON-258 should instead be considered as *L. donovani*, as had been previously suggested for MON-82 using other molecular methods [26,28–30,37].

All three East African *L. infantum* zymodemes were here found in the clade including most of the strains of *L. donovani* from Sudan and Ethiopia (clade IIa1: MON-30, MON-267, MON-81, MON-18, and MON-82). Zymodeme MON-30 had already been shown by several DNA-based methods to cluster with *L. donovani* strains from Sudan, and was thus considered as *L. donovani* [28–30,32,37]. The Mediterranean and the Sudanese *L. infantum* strains differ in the MDH profile, 104 for the Mediterranean strains and 112 for the Sudanese strains. There is thus increasing evidence to consider all Sudanese/Ethiopian zymodemes with MDH phenotype 112 as *L. donovani*. An important epidemiological consequence is that PKDL cases caused by *L. infantum* MON-30 and MON-267 in Sudan [49] were in fact caused by *L. donovani*.

4.3. L. infantum/L. chagasi

Our results indicate the existence of a clearly separated phylogenetic group (clade I), which includes all *L. infantum/L. chagasi* strains from the Mediterranean basin, China and South America. The strongest concordance between zymodemes and genotypes was found for this group.

In this study, it was not possible to differentiate between Mediterranean and Chinese *L. infantum* and South American *L. chagasi*. This is in agreement with previous studies based on isoenzymes [5,6] and combined RAPD analyses and RFLP analyses of the *gp63* intergenic region [7,27,28]. Authors that advocate for a separation into two distinct species [1,54] base their argument on minor phenotypic and genetic differences and often on only a single strain from each group. The lack of considerable sequence diversity in all sequences analyzed in this study supports the hypothesis that *L. chagasi* was only recently introduced into the Americas [55].

Our analysis of the ITS sequences emphasizes the necessity of a re-evaluation of the taxonomy of the *L. donovani* complex, in particular all Sudanese and Ethiopian zymodemes should be classified as *L. donovani*, and *L. archibaldi* should be abandoned as a species name. A close relationship between Sudanese zymodemes has already been assumed [56]. The author argued that the three zymodemes MON-30, MON-18 and MON-82 belong to a single population and that there may be no biological justification for regarding them as different species or subspecies. In the zymodeme based cladogram [16] the three zymodemes of *L. archibaldi* (MON-82, MON-257 and MON-258) were located at the base of the tree, phylogenetically closely related to the *L. infantum* MON-30, MON-81 and MON-267 zymodemes, and *L. donovani* MON-18. These authors concluded that because of the ancestral positions of all Sudanese strains, Sudan might be the possible origin of visceral leishmaniasis. Recently, another author [57] proposed to maintain the taxon *L. archibaldi* and to apply it to all parasites from Sudan. However, ITS sequence data suggest the existence of two different lineages of *L. donovani* strains (clades IIa1 and IIb) in Sudan/Ethiopia. They are more distantly related to each other than one of them is to the Indian strains and, therefore, cannot be treated as the same taxon. This means that if the taxon *L. archibaldi* is to be maintained, it cannot simply be applied to all parasites in Sudan (and Ethiopia). The significance of the two Sudanese/Ethiopian lineages should be proven by analyzing additional strains from this area and other genetic markers. Including additional strains, especially from the Middle East, should help to develop a substantiated hypothesis of the mode of spread of the particular *L. donovani* lineages.

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