Geographical distribution and epidemiological features of Old World *Leishmania infantum* and *Leishmania donovani* foci, based on the isoenzyme analysis of 2277 strains

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SUMMARY

A series of 2277 *Leishmania* strains from Old World visceral leishmaniasis foci, isolated between 1973 and 2008, were studied by isoenzyme analysis. The strains were obtained from humans, domestic and wild carnivores, rodents and phlebotomine sandflies, and came from 36 countries. In all, 60 different zymodemes were identified and clustered by a phenetic analysis into 3 different groups corresponding to the typically visceralizing species *L. donovani* (20 zymodemes, 169 strains), *L. archibaldi* (3 zymodemes, 46 strains) and *L. infantum* (37 zymodemes, 2,062 strains). The taxonomic position of these isoenzymatic groups is discussed in view of contradictory results obtained from recent molecular studies.

Key words: visceral leishmaniasis, *Leishmania infantum*, *Leishmania donovani*, *Leishmania archibaldi*, isoenzymatic identification, Old World.

INTRODUCTION

In a previous paper (Pratlong *et al*. 2009) the geographical distribution and epidemiological features of Old World dermotropic *Leishmania* species, *L. major, L. tropica* and *L. aethiopica,* usually responsible for cutaneous leishmaniasis were analysed. The study was based on isoenzyme analysis of 1048 strains, collected during 24 years in 33 countries of Africa, and the Near and Middle East. The analysis of 2277 commonly viscerotropic strains collected during 35 years from 36 countries of Europe, Asia and Africa is presented here.

Visceral leishmaniasis was originally described as being due to a protozoon named *L. donovani* by Laveran and Mesnil (1903). In 1908, Nicolle distinguished a second taxon, *L. infantum*, on clinical and epidemiological arguments. These taxa autonomy remained controversial during years until the development of multilocus enzyme electrophoresis (MLEE). Lanotte *et al*. (1981) separated *L. infantum* from *L. donovani* with only 2 zymodemes for each one. Later, Moreno *et al*. (1986) individualized 2 phenetic groups within the *L. donovani* complex *s.l.*, with 8 zymodemes for *L. donovani* and 9 for *L. infantum*, on isoenzymatic and epidemiological arguments (zoonotic versus anthroponotic cycles and geographical distribution). As the number of strains described increased, the number of zymodemes increased correspondingly and, more recently, Pratlong *et al*. (2001) described 31 zymodemes for *L. infantum*, 16 zymodemes for *L. donovani* and introduced a new group, *L. archibaldi,* with 3 zymodemes.

The present study was based on the analysis of 2277 strains, isolated between 1973 and 2008, cryopreserved in the International Cryobank of *Leishmania* of Montpellier, France. In addition to providing a substantial amount of new isoenzymatic data, this study aims to evaluate the relevance of the previously described groups. The controversial taxonomic position of *L. archibaldi*, illustrated by the recent molecular studies, is discussed.

MATERIALS AND METHODS

Leishmania *strains*

The studied sample included 2277 *Leishmania* strains isolated between 1973 and 2008 either in our laboratory or by other teams (see Acknowledgements section). They were all cryopreserved in liquid nitrogen, in the International Cryobank of *Leishmania* of Montpellier, France (number 879 in the data base

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Table 1. Numbers of *Leishmania* strains according to country

Country	Strains
Albania	3
Algeria	138
Cerntral African Republic	1
China	7
Croatia	1
Cyprus	68
Djibouti	4
Egypt	4
Ethiopia	25
France	918
Georgia	1
Germany	1
Greece	129
India	32
Iran	5
Iraq	4
Israel	19
Italy	51
Kenya	12
Lebanon	5
Malta	1
Monaco	8
Morocco	26
Portugal	241
Saudi Arabia	3
Senegal	2
Spain	217
Sri Lanka	7
Sudan	144
Syria	2
Tunisia	165
Turkey	10
Ukraine	1
United-Kingdom	1
Uzbekistan	8
Yemen	13

of the World Data Centre for Microorganisms of the World Federation for Culture Collection). The strains were mainly isolated from humans ($n=1364$, 59·9%), but also from mammalian reservoir hosts: dogs ($n=808$, 35·5%), foxes ($n=4$), racoon dog ($n=1$), cats ($n=6$) and rats ($n=5$), and phlebotomine sandflies ($n=88$). They originated in 36 countries, over a wide geographical area, between West-Africa and China (Table 1). The western part of the Mediterranean Basin was well represented, due to field investigations carried out either by our laboratory in France ($n=918$ strains), or by collaborating teams: in Portugal ($n=241$ strains), Spain ($n=217$), Algeria ($n=138$ strains), Greece ($n=129$) and Tunisia ($n=165$). The strains from countries with fewer samples were obtained from occasional clinical human cases. The studied strains were analysed along with 60 zymodeme reference strains and the MHOM/FR/78/LEM75 *L. infantum* MON-1, as basic reference zymodeme. The World Health Organization (WHO) codes and enzyme profiles of all these strains are shown in Table 2.

Isoenzymatic identification

Starch gel electrophoresis was performed according to the method described by Rioux *et al*. (1990), using the following 15 enzyme systems: malate dehydrogenase, MDH, EC 1.1.1.37 ; malic enzyme, ME, EC 1.1.1.40; isocitrate dehydrogenase, ICD, EC 1.1.1.42; 6-phosphogluconate dehydrogenase, PGD, EC 1.1.1.44; glucose-6-phosphate dehydrogenase, G6PD, EC 1.1.1.49; glutamate dehydrogenase, GLUD, EC 1.4.1.3; NADH diaphorase, DIA, EC 1.6.2.2; purine nucleoside phosphorylase, NP_1, EC 2.4.2.1; purine nucleoside phosphorylase, NP_2, EC 2.4.2.*; glutamate-oxaloacetate transaminases, GOT_1 and GOT_2, EC 2.6.1.1; phosphoglucomutase, PGM, EC 5.4.2.2; fumarate hydratase, FH, EC 4.2.1.2; mannose phosphate isomerase, MPI, EC 5.3.1.8; glucose phosphate isomerase, GPI, EC 5.3.1.9. Isoelectrofocusing was used as a complementary technique with greater resolving power than electrophoresis (Piarroux *et al*. 1994).

For comparing enzymatic polymorphism by country, a polymorphism index (PI) was calculated: the number of zymodemes/the number of strains studied in the corresponding country, according to Gramiccia (2003). This PI was calculated only for countries with a sufficient sample size (more than 100 strains). Strains from Italy were excluded due to a sampling bias (selection of the strains sent to the Montpellier Cryobank).

Taxonomic methods

Phenetic analysis was based on 15 isoenzyme loci, 60 zymodemes and 51 characters (electromorphs); 3 *L. tropica* zymodemes were used as outgroup (Table 2). On the hypothesis that *Leishmania* is 'mainly' diploid (Martínez-Calvillo *et al*. 2005), multiband patterns obtained in starch gels were considered to be heterozygous and electromorph values were duplicated. Genetic clustering was done using the Neighbor-Joining (NJ) method implemented in the PHYLIP3.6 package (Felsenstein, 2004). The robustness of the nodes was statistically tested by bootstrap analysis with 1000 replicates (Felsenstein, 1985).

RESULTS

Isoenzyme analysis differentiated the 2277 strains into 60 zymodemes which were split in 3 groups through phenetic clustering : *L. infantum*, *L. donovani* and *L. archibaldi*, corresponding with the classically-known Old World viscerotropic *Leishmania* species. (Table 2, Fig. 1). In the Neighbour-Joining tree the *L. archibaldi* group was highly supported (Bootstrap value=81%), the *L. donovani* and *L. infantum* groups were moderately supported (Bootstrap values of 56 and 47%

respectively). The *L. donovani* cluster aggregated 20 zymodemes and appeared well structured. The *L. infantum* cluster comprised 37 zymodemes and was only weakly structured, leading to a multifurcated topology. There was only a partial correlation between the isoenzymatic sub-groups and the geographical origin of the strains.

Among the 3 groups defined, *L. infantum* has the widest geographical distribution (between China and West Africa) and the largest number of strains ($n=2062$). *L. donovani* has also a wide distribution (between China and East Africa), while *L. archibaldi* has a restricted geographical range, essentially limited to East Africa. These 2 last species have limited numbers of strains in our sample ($n=169$ and 46 respectively).

Leishmania infantum

The majority of *L. infantum* strains were obtained from human cases (1168 strains, 56·6%). These human strains were mostly from cases of visceral leishmaniasis (77·3%), but also from tegumentary cases (20·8% cutaneous and 0·4% mucosal lesions); the clinical features of 1·5% of the strains were unknown. The other hosts were dogs ($n=796$, 38,6%), foxes ($n=4$) and a raccoon dog ($n=1$), cats ($n=6$), and rats ($n=5$), and phlebotomine sandflies ($n=81$, 3·9%), principally 47 *Phlebotomus perniciosus* and 32 *P. ariasi*.

Thirty-seven different zymodemes were described (Table 2) and all of them were found in humans, with the exception of MON-278, the 2 strains of which occurred only in dogs. Only 12 zymodemes out of 37 reported were found in dogs, namely MON-1, MON-11, MON-24, MON-27, MON-30, MON-34, MON-77, MON-98, MON-108, MON-267, MON-278 and MON-281. Out of these zymodemes, MON-1 was largely predominant, with the highest number of strains ($n=1611$, 78·2%) and the widest geographical distribution, including Southern Europe, Africa and the Near and Middle-East (Fig. 2). This zymodeme was present in humans, dogs, foxes, cats, rats and sandflies.

The second zymodeme with a wide geographical range, corresponding to the Mediterranean Basin (Fig. 2), was MON-24 ($n=132$, 6·4%), which was found in humans, dogs, and *P. ariasi* and *P. perfiliewi* sandflies. The 761 other strains belonged to 35 zymodemes with more restricted geographical distributions.

The strains studied were from 28 countries, ranging between Senegal and Portugal in the west and China in the east. Of these, 6, belonging to the Mediterranean Basin, had a sample size greater than 100 strains: Algeria, France, Greece, Portugal, Spain and Tunisia (Table 3). The polymorphism index of Spain was the highest and the France one the lowest (Table 4).

Leishmania donovani

The *L. donovani* sample included 169 strains, the majority of which were from human cases ($n=158$ strains, 93·5%). The remaining strains were from dogs ($n=6$, 3·5%) and sandflies ($n=5$, 3%). The human strains mostly came from visceral leishmaniasis cases ($n=134$, 84·8%), but also from cutaneous ($n=15$, 9·5%) and Post Kala-azar Dermal Leishmaniasis (PKDL) cases ($n=9$, 5·7%).

The strains studied were from 13 countries, ranging from East-Africa to China (Fig. 3). The number of strains by country was relatively small, Sudan being the only country with a sample of more than 50 strains due to dedicated epidemiological surveys.

The isoenzyme analysis showed 20 zymodemes, without predominance of any one (Table 2). The number of zymodemes by country ranged from 5 for the most polymorphic (Ethiopia, India and Sudan) to 1 (Cyprus, Sri Lanka and Ukraine) (Table 5). *Leishmania donovani* MON-37 was the zymodeme showing the widest geographical range, from Sri Lanka and India in the east to Cyprus and East Africa in the west (Fig. 3).

Leishmania archibaldi

The *L. archibaldi* sample was limited to 46 strains, the majority of which were from human cases ($n=38$, 82·6%). The remaining strains were distributed between dogs ($n=6$, 13·0%) and sandflies ($n=2$, 4·4%). Most of the human strains were from visceral leishmaniasis cases ($n=32$, 84·2%), but some were also from cutaneous ($n=2$) and PKDL cases ($n=1$).

The strains studied were from 4 countries in East-Africa and Lebanon (Fig. 2). With the exception of Sudan ($n=38$ strains), the numbers of strains by country were small: Ethiopia ($n=2$), Kenya ($n=1$) and Lebanon ($n=5$). The isoenzyme analysis showed 3 zymodemes: MON-82, MON-257 and MON-258.

DISCUSSION

Since their introduction in 1980, MLEE has largely contributed to the construction of a comprehensive taxomony of the *Leishmania* parasites, which highly improved the knowledge of the geographical distribution and epidemiological features of leishmaniasis.

The present work is a retrospective study based on a 35 years of collection of the strain samples. Such a large sample collection provides a valuable addition to the knowledge of *Leishmania* geographical distribution and epidemiology. However, this type of collection evidently introduces some sampling bias, related to the geographical localization of the collection and of its main providers. The high number of *L. infantum* strains is due to our own field work and to collaboration with western Mediterranean

Table 2. Zymodemes of *Leishmania* reference strains used

	Zymodeme	MDH	ME	ICD	PGD	G6PD	GLUD	DIA	NP1	NP2	GOT1	GOT2	PGM	FH	MPI	GPI
L. donovani																
MHOM/IN/00/DEVI	**2**	104	100	100	93	100	100	100	140	100	113	113	100	100	100	100
MHOM/IQ/73/IF3	**3**	104	100	100	100	100	100	100	140	100	113	113	100	100	100	86
MHOM/ET/67/HU3	**18**	112	100	100	100	100	100	100	140	100	113	113	100	100	100	100
MHOM/SA/81/JEDDAH-KA	**31**	104	100	100	100	100	100	100	140	100	113	113	100	100	110	86
MHOM/KE/75/H9	**32**	104	100	100	100	100	100	100	150	100	113	113	100	100	100	100
MHOM/CN/00/WANGJIE-1	**35**	104	100	100	100	100	150	100	150	100	113	113	100	100	100	86
MHOM/KE/55/LRC-L53	**36**	104	100	100	100	100	100	100	100	100	113	113	100	100	100	100
IMAR/KE/62/LRC-L57	**37**	104	100	100	100	100	100	100	140	100	113	113	100	100	100	100
MHOM/IN/54/SC 23	**38**	100	100	100	100	100	100	100	100	100	113	113	100	100	100	100
MHOM/SU/84/MARZ-KRIM	**73**	104	100	100	100	100	100	100	140	100	113	113	100	110	100	100
MHOM/ET/84/ADDIS 164	**83**	104	100	100	100	100	100	100	140	100	113	113	100	98	110	86
MHOM/MT/33/MALTA 33	**84**	112	100	100	100	100	100	100	150	100	113	113	100	100	100	100
IALE/CN/88/Turfan 10	**138**	104	100	100	100	100	120	100	140	100	113	113	100	100	100	86
MHOM/YE/93/LEM2677	**191**	104	100	100	100	100	100	100	140	100	113	113	100	105	100	86
MHOM/DJ/97/AVR1	**268**	104	100	100	100	100	100	100	140	100	113	113	88	100	100	100
MHOM/SD/98/LEM3581	**274**	112	100	100	100	100	100	100	140	100	113	113	100	100	110	95/80
MHOM/SD/99/LEM3793	**276**	112	100	100	100	100	100	100	140	100	113	113	100	100	110	100
MCAN/SD/2000/LEM3942	**277**	112	100	100	100	100	100	100	140	100	113	113	100	100	105/95	100
MHOM/IL/79/LANSBERG-LRC-L264	**280**	104	100	100	100	100	100	100	140	100	113	113	100	100	97	100
MHOM/DJ/2000/DJG002	**287**	104	80	100	100	100	100	100	140	100	113	113	88	100	100	100
L. archibaldi																
MHOM/ET/72/GEBRE1	**82**	112	100	100	100	100	100	100	140	100	110	110	100	100	100	100
MHOM/SD/97/LEM3129	**257**	112	100	100	100	100	100	100	140	100	110	110	100	100	110	100
MHOM/SD/97/LEM3463	**258**	112	100	100	100	100	100	100	140	100	110	110	100	93	110	100
L. infantum																
MHOM/FR/78/LEM75	**1**	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
MHOM/FR/80/LEM189	**11**	104	100	100	100	105	100	100	130	100	100	100	100	100	100	100
MHOM/DZ/82/LIPA 59	**24**	104	100	100	100	100	100	100	140	100	100	100	100	100	100	100
MHOM/IT/79/ISS 7	**27**	100	100	100	100	100	100	100	130	100	100	100	100	100	100	100
MHOM/ES/83/BCN 2	**28**	104	100	100	100	102	100	100	140	100	100	100	100	100	100	100
MHOM/ES/81/BCN 1	**29**	104	100	100	100	105	100	100	140	100	100	100	100	100	100	100
MHOM/SD/82/GILANI	**30**	112	100	100	100	100	100	100	140	100	100	100	100	100	100	100
MHOM/FR/82/LEM356	**33**	104	100	100	100	105	100	100	100	100	100	100	100	100	100	100
MHOM/FR/84/LEM538	**34**	104	100	100	100	100	100	100	100	100	100	100	100	100	100	100
MHOM/IT/86/ISS 218	**72**	100	100	100	100	100	100	100	100	100	100	100	109	100	100	100
MCAN/ES/86/LEM935	**77**	100	100	100	100	102	100	100	100	100	100	100	100	100	100	100
MHOM/MT/85/BUCK	**78**	104	100	100	100	100	100	100	140	100	100	100	100	110	100	100
MHOM/DZ/83/LEM425	**80**	104	100	100	100	100	100	100	130	100	100	100	100	100	100	100
MHOM/SD/00/3S	**81**	112	100	100	100	100	100	100	150	100	100	100	100	100	100	100
MHOM/EG/87/RTC 2	**98**	100	90	100	100	100	100	100	100	100	100	100	100	100	100	100
MCAN/FR/87/RM1	**108**	100	100	100	100	100	100	120	100	100	100	100	100	100	100	100
MHOM/IT/85/ISS 175	**111**	104	100	100	100	100	100	100	140	100	100	100	100	100	127/100	100
MHOM/IT/90/ISS 510	**136**	104	100	100	100	100	100	100	140	100	100	100	100	110	127/100	100
MHOM/FR/91/LEM2298	**183**	104	100	100	100	100	100	100	100	100	100	100	100	100	100	115

MHOM/IT/91/ISS 666	**185**	104	100	100	100	100	100	130	100	100	100	100	110	127/100	100		
MHOM/IT/92/ISS 683	**187**	104	100	100	100	100	100	140	100	100	100	100	105	127/100	100		
MHOM/IT/93/ISS 800	**188**	104	100	100	100	100	100	140	100	100	100	100	105	127	100		
MHOM/IT/93/ISS 826	**189**	104	100	100	100	100	100	130	100	100	100	100	105	100	100		
MHOM/IT/93/ISS 833	**190**	104	93	100	105	100	100	100	100	100	100	100	100	100	100		
MHOM/ES/88/LLM 175	**198**	104	100	100	105	100	100	140	100	100	100	100	100	100	115		
MHOM/ES/92/LLM 373	**199**	104	100	100	102	100	100	140	100	100	100	100	100	100	105		
MHOM/IT/93/ISS 822	**201**	104	100	100	100	100	100	130	100	100	100	100	100	127/100	100		
MHOM/IT/94/ISS 1036	**228**	104	100	100	100	100	100	140	100	100	100	100	100	100	105		
MHOM/00/97/L 2767	**253**	100	100	100	95	100	100	100	100	100	100	100	100	100	100		
MHOM/SD/97/LEM 3472	**267**	112	100	100	100	100	100	140	100	100	100	100	100	110	100		
MCAN/SD/2000/LEM3941	**278**	112	100	100	100	100	100	140	100	100	100	100	100	105/95	100		
MHOM/PS/1999/LRC-L773	**281**	140	100	100	100	100	100	100	100	100	100	100	100	100	100		
MHOM/ES/2000/LLM-938	**282**	100	100	100	100	100	100	100	100	100	100	100	105	100	100		
MHOM/ES/99/LLM-887	**283**	104	100	100	100	100	100	130	100	100	100	100	100	100	105		
MHOM/ES/99/LLM-855	**284**	104	100	100	100	100	100	100	100	100	100	100	100	100	105		
MHOM/ES/97/LLM-615	**285**	104	100	100	105	100	100	100	100	100	100	100	100	100	115		
MHOM/ES/96/LLM-558	**302**	104	100	100	100	100	100	140	100	100	100	100	100	100	115		
L. tropica																	
MRAT/IQ/72/ADHANIS 1	**5**	112	95	100	93	82	80	100	450	100	135	90	100	110		76	
MHOM/PK/00/LV 691	**7**	112	95	100	93	82	95	110	450	100	135	90	100	110/100		76	
MHOM/TN/80/LEM 163	**8**	100	100	100	93	82	110	100	300	100	127	90	100	110		76	

country teams. Despite the fact that the sample of *L. donovani*, including *L. archibaldi*, is less represented, the number of collected strains, however, gives an indication of the global polymorphism.

In spite of an increase of the number of strains and of the corresponding zymodemes, the global structure of the taxonomic groups previously defined was remarkably confirmed. The present study confirmed the existence of 3 taxonomic groups, in contradiction to several molecular-based approaches. We will discuss separately the isoenzyme data obtained for these groups and will compare them with data in the literature.

In the Old World, *L. infantum* is a zoonotic species, which has domestic canids as main reservoir hosts, and is transmitted by various sandfly species, mainly belonging to the subgenera *Larroussius* and *Adlerius* in the Old World. It is typically responsible for visceral leishmaniasis but, in endemic regions, purely cutaneous cases occur occasionally (Dedet and Pratlong, 2009). This species has a wide range, which reflects the large sample of 2062 strains analysed in this work, originating from 28 countries extending from Senegal and Portugal in the west to China in the east. Our sample included 796 strains isolated from dogs (38·6% of the strains), which confirms the zoonotic character of this taxon. The higher number of human strains (56·6% of the total strains) is based on isolations mainly obtained during human diagnosis. A majority of the human strains (77·3%) were isolated from VL cases.

A high enzymatic polymorphism was detected, with 37 zymodemes identified, of which MON-1 was the predominant one, particularly around the Mediterranean basin. This has already been reported by different studies in various Mediterranean countries: France (Pratlong *et al*. 2004; Marty *et al*. 2007), Portugal (Campino *et al*. 2006), Spain (Jimenez *et al*. 1995; Martin-Sanchez *et al*. 2004), Algeria (Harrat *et al*. 1996), Tunisia (Belhadj *et al*. 2002, Kallel *et al*. 2008) and Italy (Gramiccia *et al*. 1992; 1995; Gramiccia, 2003). Out of these 37 zymodemes, 25 were not isolated from dogs, and were considered as small variants of zymodeme MON-1, scarce in humans and the reservoir of which remains unknown. The dog is incontestably the reservoir host of *L. infantum* MON-1, in the Mediterranean basin, where it represents between 75 and 98·4% of the canine strains depending on the countries (reviewed by Aït Oudhia *et al*. 2011) (87·2% in the present study). MON-1 has also been found in foxes in Portugal (Campino *et al*. 2006), and previous studies described it as *L. infantum* wild reservoir host in different countries, including France (Rioux *et al*. 1968), Italy (Bettini *et al*. 1980), Spain (Portus *et al*. 2002) and Portugal (Abranches *et al*. 1983).

The enzymatic polymorphism of *L. infantum* is in reality somewhat greater than detected in our sample. Nine additional *L. infantum* zymodemes have been

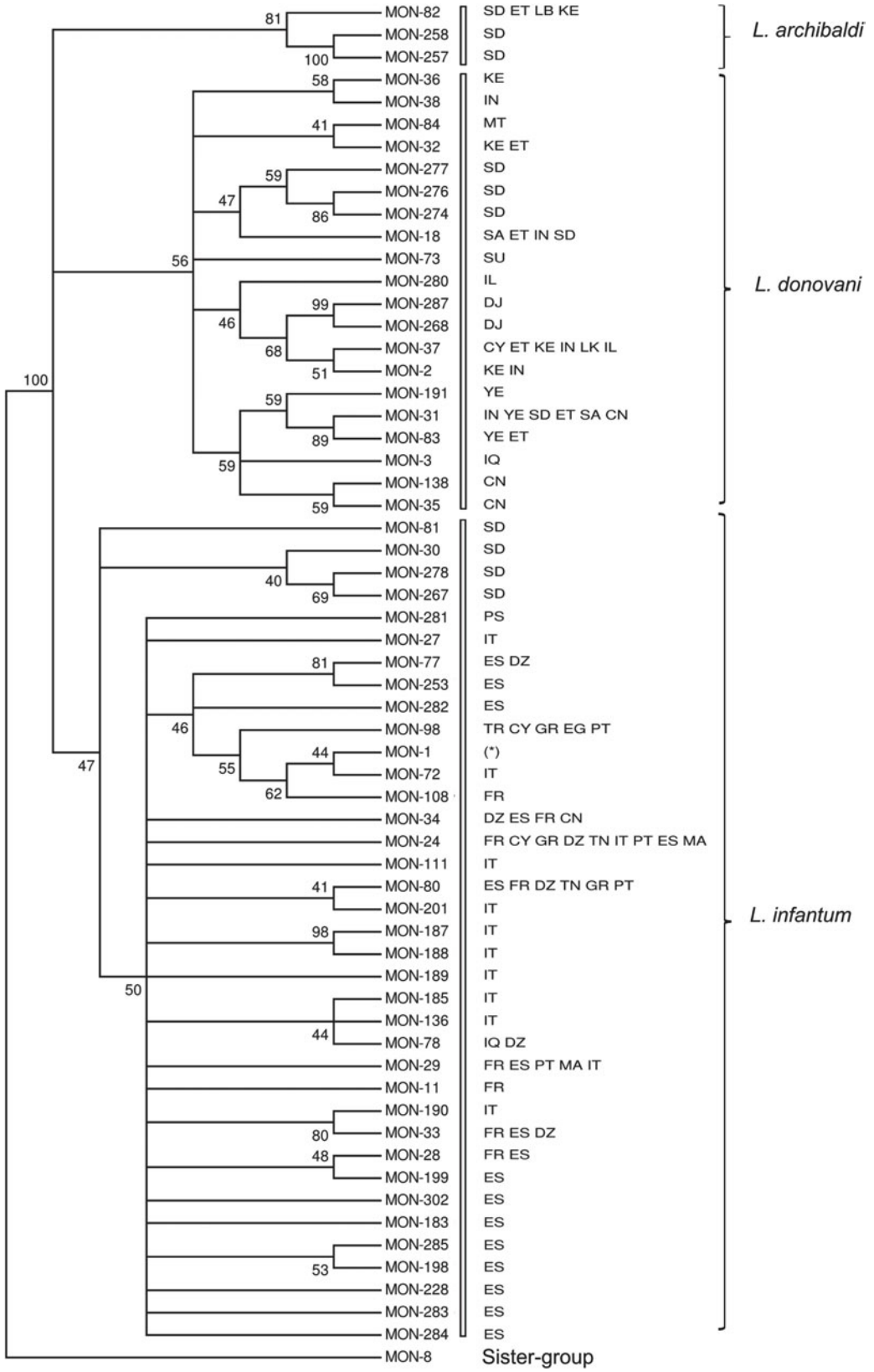

Fig. 1. Neighbour-Joining (NJ) dendrogram obtained from the matrix of the presence/absence of enzyme character-states using the Neighbour software implemented in the Phylip package on15 iso-enzymatic systems. The robustness of the nodes (bootstrap values) was evaluated with 1000 replicates. Sixty zymodemes representative of the genetic diversity of *Leishmania archibaldi, L. donovani* and *L. infantum* were analyzed. *Leishmania tropica* zymodemes Mon-5, MON-7 and MON-8 were used as an out-group. Branches supported by bootstrap values below 40 were collapsed and a bootstrap value is indicated on the figure for each node. For each zymodeme, the geographical origin of the strains was indicated using the WHO code. (*) zymodeme MON-1 was isolated from DZ, CY, HR, SU, IT, GR, PT, FR, IL, ES, SY, IR, MA, SN, TN, TR, YE, CF, US, UZ, UK, DE and AL.

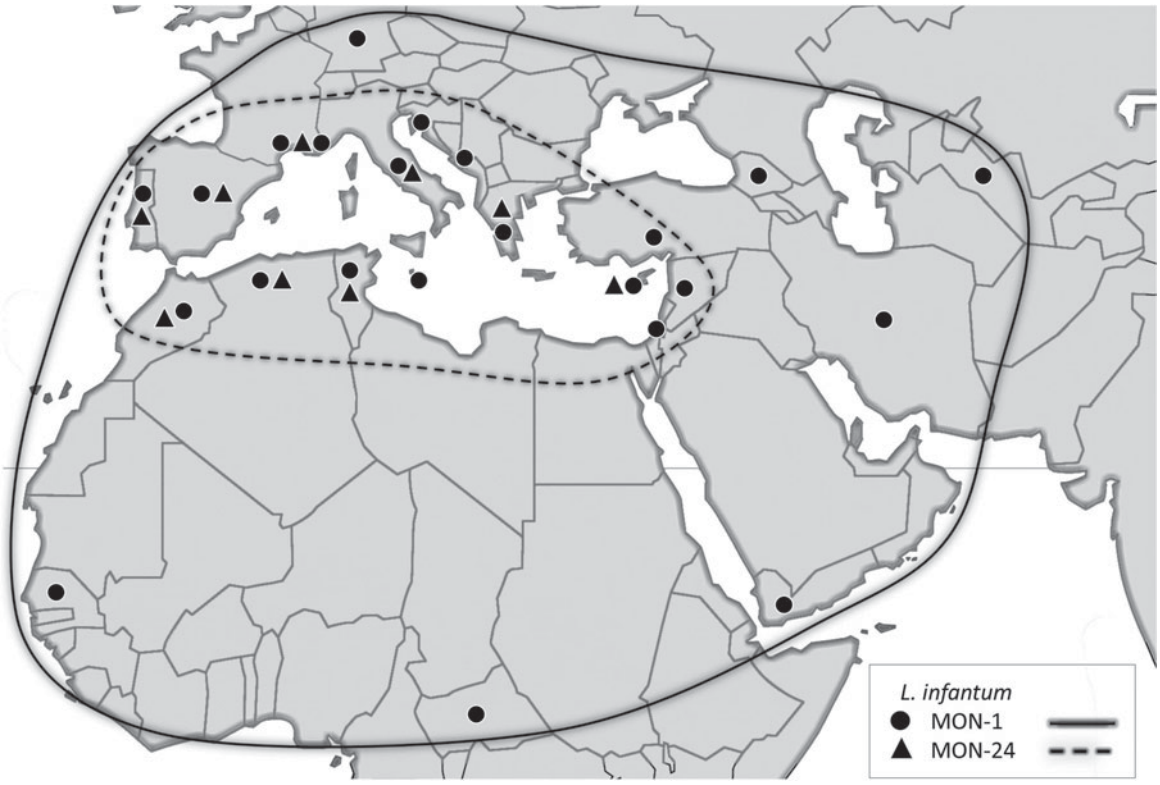

Fig. 2. Old World geographical distribution of *Leishmania infantum* zymodemes MON-1 and MON-24.

identified in other labs on the basis of their differences with the known MON-zymodemes and qualified as 'variants' by the authors : GR-2 (MON-77 var MDH[104]), GR-8 (MON-77 var MDH[104], GPI[105]), GR-11 (MON-199 var NP1[130]), GR-13 (MON-183 var NP1[140]), GR-14 (MON-198 var G6PD[102]), GR-17 (MON-80 var ME[93]), GR-19 (MON-24 var NP1[150]) (Martin-Sanchez *et al*. 2004), and MON-1 var DIA[110], MON-189 var NP1[140] (Gramiccia, 2003).

Concerning the enzymatic polymorphism, a high level has already been reported in southern Spain, where 20 zymodemes were detected for 161 strains (Martin-Sanchez *et al*. 2004) and 22 zymodemes in a sample covering all Spain during 1984–2001 (Chicharro, *et al*. 2003). Similarly, a high polymorphism was described in strains isolated from sandflies in Southern Spain (Martin-Sanchez *et al*. 1995, 2004). In the last paper, 16 zymodemes were found for 45 strains isolated from sandflies (Martin-Sanchez *et al*. 2004). In Italy, a comparative study of PI carried out by Gramiccia (2003) showed regional variations, with a particularly high PI in Sicily (14 zymodemes for 108 patients, PI = 0·13,) when compared to the rest of Italy (7 zymodemes for 253 patients, PI = 0·03). Whatever the country, the polymorphism was partially attributed to particular zymodemes found in HIV co-infected patients (Pratlong *et al*. 2003; Chicharro *et al*. 2003 and Gramiccia, 2003). This was particularly evident in Sicily, where the PI was 0·3 versus 0·13, respectively in HIV-positive and HIV-negative patients (Gramiccia, 2003).

In Sudan, 39 *L. infantum* strains belonging to 4 different zymodemes were detected in our work. The existence of this taxon in East Africa has been largely debated. Jamjoom *et al*. (2004) proposed to explain the presence of *L. infantum* zymodemes in Sudan as a consequence of a recent mutation of the GOT gene from local *L. donovani* stocks, a possible example of convergence according to these authors, *L. donovani* is classically an anthroponotic species, restricted to the Old World, where it is found from East Africa to Central Asia and China. It is transmitted by various sandfly species belonging to the subgenera *Euphlebotomus, Synphlebotomus* and *Larroussius*. It is mainly responsible for visceral leishmaniasis and characterized by the subsequent occurrence of PKDL. It can also exceptionally be responsible for localized cutaneous leishmaniasis (Pratlong *et al*. 1995). Epidemic outbreaks have been described in India, as well as in Sudan.

Although our sample of 169 strains is much smaller than that of *L. infantum*, it is representative of the distribution of the species, which involves 13 countries extending from East Africa to China. The only country for which we have a large sample is Sudan, where we carried out an epidemiological survey (Dereure *et al*. 2003; Pratlong *et al*. 2001). The strains of India and Sri Lanka came from collaborations with local teams (Thakur *et al*. 2001; Karunaweera *et al*. 2003). The majority of the strains

Table 3. Numbers of *Leishmania infantum* strains according to country and to zymodeme number

Zymodeme number / Country	1	11	24	27	28	29	30	33	34	72	77	78	80	81	98	108	111	136	183	185	187	188	189	190	198	199	201	228	253	267	278	281	282	283	284	285	302	Total
Albania	3																																					3
Algeria	82		42					1	3		2	1	7																									138
CAR	1																																					1
China									5																													5
Croatia	1																																					1
Cyprus	61		1												1																							63
Egypt															4																							4
France	793	15	10		3	47		42	1				1			6																							918
Georgia	1																																					1
Germany	1																																					1
Greece	98		1										1		29																							129
Iran	5																																					5
Iraq												1																										1
Israel	16																																					16
Italy	38		3	2						1							1	1		1	1	1	1			1												51
Malta												1																										1
Monaco	8																																					8
Morocco	22		4																																			26
Portugal	232		3			4							1		1																							241
Senegal	2																																					2
Spain	124	1	6		6	16		5	1		39		2						6						1	1		1	3				1	1	1	1	1	217
Sudan							30							1																6	2							39
Syria	2																																					2
Tunisia	99		62										4																									165
Turkey	7														3																							10
United-Kingdom	1																																					1
Uzbekistan	8																																					8
Yemen	5																																					5

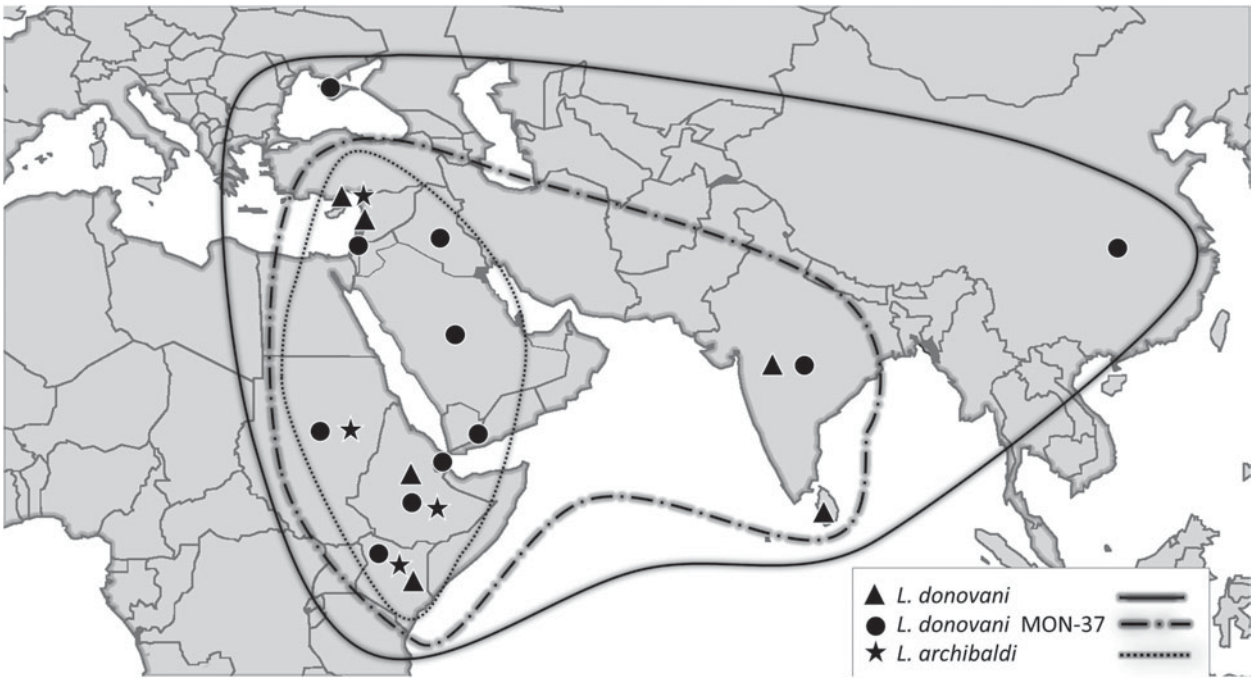

Fig. 3. Geographical distribution of *Leishmania donovani* and *L. archibaldi*, and particularly *L. donovani* MON-37.

Table 4. Polymorphism index of the *Leishmania infantum* zymodemes according to the Mediterranean countries in which the sample was superior to 100

Mediterranean Countries	Number of strains	Number of Zymodemes	Polymorphism index
Algeria	138	7	0·05
France	918	9	0·01
Greece	129	4	0·03
Portugal	241	5	0·02
Spain	217	19	0·09
Tunisia	165	3	0·02

are of human origin (93·5%) in accordance with the role of the reservoir played by humans. For India, 88% of the strains isolated after 1970 were MON-2 zymodeme. This low polymorphism is in accordance with the data reported by Alam *et al*. (2009) and Downing *et al*. (2012), and might be explained as a side-effect of the 1960s anti-malaric campaigns.

By contrast with *L. infantum*, only 20 zymodemes were found for *L. donovani*. The enzymatic polymorphism appeared lower, which might be due to the smaller number of strains (about 10 times smaller). With the exception of Sudan, the absence of field epidemiological surveys prevents the evaluation of the enzymatic polymorphism in each country. In this group, the only new findings are the extension of zymodeme MON-37 from East Africa, to India and Sri Lanka (Karunaweera *et al*. 2003), and more recently to south-eastern Europe, in Cyprus (Antoniou *et al*. 2008). The molecular studies of different strains belonging to this zymodeme are in favour of an alternate hypothesis of a paraphyletic origin (Alam *et al*. 2009).

L. donovani var. *archibaldi* was named by Castellani and Chalmers (1919) for the parasite responsible for Sudanese VL, on clinical, epidemiological and geographical grounds. The VL in Sudan was subsequently attributed to either *L. donovani* or *L. archibaldi*. The name *L. archibaldi* was reintroduced by Rioux *et al*. (1990) for a single zymodeme MON-82. Later, Pratlong *et al*. (2001) discussing the taxonomic position of *L. archibaldi,* stressed 2 options: to consider *L. archibaldi* as a new complex or as a subunit of *L. donovani* s.l. Subsequently, the taxonomic status of *L. archibaldi* has been controversial, and considered by molecular tools as *L. donovani* (Kuhls *et al*. 2007). However, a recent paper still individualized the *L. archibaldi* taxon by a microsatellite loci approach (Rougeron *et al*. 2011).

Among the 46 strains studied in the present paper, the same 3 zymodemes occurred as found in the 2001 sample of 6 strains, showing that the increase in sample size did not change the polymorphism. *Leishmania archibaldi* appears to be mainly located in East Africa.

More recently, independently of the isoenzymes, several molecular methods have been used for the taxonomic description and identification of *Leishmania*. Molecular analyses were performed both on coding genes (HSP70, PolA, RPOII, CytoB, GP63, genes coding for 10 isoenzymes) and non-coding DNA sequences (repeated sequences, ribosomal ITS, microsatellites). Except in a few studies (Kuhls *et al*. 2007; Lukes *et al*. 2007; Mauricio *et al*. 2006), the number of analysed strains

Table 5. Numbers of *Leishmania donovani* and *L. archibaldi* (*) strains according to country and to zymodeme number

Zymodeme number / Country	2	3	18	31	32	35	36	37	38	73	82*	83	94	138	191	257*	258*	268	274	276	277	280	287	Total
China						1								1										2
Cyprus								5																5
Djibouti																		3					1	4
Ethiopia			2	6	5			9			2*	1												25
Georgia										1														1
India	26		1	1				3	1															32
Iraq		3																						3
Israel								2														1		3
Kenya	1				4		2	4			1*													12
Lebanon											5*													5
Saudi Arabia			1	2																				3
Sri Lanka								7																7
Sudan			60	2							27*					10*	1*		2		1	2		105
Yemen				6								1			1									8

and discriminatory genetic markers was generally low, resulting in poor information content and some contradictory outcomes. However, the general messages were quite similar in the different studies and were confirmed in large-scale studies involving multiple genetic markers.

First of all, a good congruence was observed between the genetic data and the geographical distribution of the strains. As stressed by Lukes *et al.* (2007), *L. donovani, L. infantum* and *L. archibaldi* from Sudan were globally intermingled in the molecular trees and networks, while the European *L. infantum* strains (mainly from Italy, France and Spain) were identified as a cluster. In different studies, *L. archibaldi* was not supported (Gelanew *et al.* 2010) and the authors proposed to include it in *L. donovani*. The differences between the isoenzymatic and the molecular outcomes could partially be explained by the existence of discrepancies between the isoenzyme-coding gene sequences and the phenotype (electromorphs) observed. Looking at the genetic basis of the enzymatic polymorphism by studying the isoenzyme gene sequences, Mauricio *et al.* (2006) and Zemanova *et al.* (2007) showed that there was a good correlation between the amino-acid sequence polymorphism and related changes in enzyme mobility. Nevertheless, in some cases, distinct genotypes produced identical isoenzymatic phenotypes, and different isoenzymatic phenotypes were coded by identical genotypes.

The contribution of each enzyme to the structure of the global tree was unequal and could be divided into 3 groups: (i) not contributing (ICD, NP2) or poorly contributing (PGD, ME, GLUD, DIA and PGM) to the structure, (ii) shared between the 3 species (G6PD, NP1, FH, MPI, MDH and GPI) and (iii) differentiating the 3 taxa *L. infantum, L. donovani* and *L. archibaldi* (GOT_1 and GOT_2). The exclusion of the 2 GOTs produces a different tree in which the zymodemes belonging to the 3 taxa are intermingled, giving *in fine* a single large group without evident structure (data not shown). This loss of structure is related to the poor support of the tree.

In conclusion, the classification based on isoenzymes does not show the *L. donovani*/*L. infantum* merging resulting from molecular approach, and maintains the existence of 3 taxonomic groups. Except for a case of convergent evolution (Jamjoom *et al.* 2004), there is no convincing explanation for such a discrepancy. As proposed by Schönian *et al.* (2010) and Van der Auwera *et al.* (2011), a revision of the taxonomy of *Leishmania* is needed for taking into account the outcomes of new genetic studies. Such a development might be based on multiple gene sequencing; it must be highly congruent and uncontroversial, and must be applied to the largest possible sample of strains, as has been the case for isoenzyme analysis.

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