Genetic heterogeneity and phylogenetic status of *Leishmania* (*Leishmania*) *infantum* zymodeme MON-1. Epidemiological implications.

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SUMMARY

Leishmania (*Leishmania*) *infantum* zymodeme MON-1 is responsible for the majority of visceral leishmaniasis cases around the Mediterranean basin, albeit that it causes also cutaneous forms. The MON classification is based on starch gel multilocus enzyme electrophoresis (MLEE) typing. The aim of this work was to explore further the genetic diversity and phylogenetic status of this zymodeme by alternative typing techniques. Fourteen *L.(L.) infantum/L.(L.) chagasi* stocks identified as MON-1 by MLEE in reference laboratories, three *L. infantum* stocks attributed to other zymodemes (MON-24, MON-29, MON-33) and reference standard stocks belonging to other species (*L.* (*L.*) *major*, *L.* (*L.*) *tropica* and *L.* (*L.*) *donovani*) were characterised by two different markers: MLEE on cellulose acetate plates and Random Amplified Polymorphic DNA (RAPD). We have obtained 10 different genotypes with RAPD and 6 different genotypes with MLEE on cellulose acetate plates for the fourteen *L. infantum/L. chagasi* MON-1 stocks studied. MLEE and RAPD data gave quite congruent phylogenetic results: *L. infantum* zymodeme MON-1 was shown to be polyphyletic and genetically heterogeneous. This work confirms the necessity of using different markers to build up a robust phylogeny. Finally the epidemiological and clinical implications of these results are discussed.

Key words: *Leishmania* (*Leishmania*) *infantum*, zymodeme MON-1, Random Amplified Polymorphic DNA (RAPD), Multilocus Enzyme Electrophoresis (MLEE) on cellulose acetate plates, Discrete Typing Unit (DTU), phylogeny.

INTRODUCTION

The parasites belonging to the *Leishmania* genus are the causative agents of leishmaniasis. These diseases constitute a priority for WHO in terms of public health. With regard to *L.* (*L.*) *infantum* (Nicolle, 1908), many new cases are now appearing in Southern Europe, due to the frequent association between this species and HIV infection (Alvar *et al.*, 1992). This species belongs, together with *L.* (*L.*) *chagasi* (Cunha and Chagas, 1937), *L.* (*L.*) *donovani* (Laveran and Mesnil, 1903) and *L. (L.) archibaldi* (Castellani and Chalmers, 1919), to the *L. donovani* complex. These four species are described as responsible for visceral leishmaniasis, although they are often isolated from cutaneous lesions too. *L. infantum* has been subdivided into zymodemes MON with starch gel Multilocus Enzyme Electrophoresis technique (MLEE) by the French WHO reference laboratory of Montpellier (Laboratoire d'Ecologie Médicale et Pathologie Parasitaire, France) (Rioux *et al.*, 1990), (Lanotte *et al.*, 1981). The MON classification is based on 15 enzymatic systems (DIA (NADH diaphorase), FH (fumarate hydratase), G6PD (glucose-6-phosphate deshydrogenase), GLUD (glutamate deshydrogenase), GOT1 & GOT2 (glutamate oxaloacetate transaminase), GPI (glucose phosphate isomerase, IDH (isocitrate deshydrogenase), MDH (malate deshydrogenase), ME (malic enzyme), MPI (mannose-phosphate isomerase), NHI & NHD (nucleoside hydrolase), 6PGD (6-phosphogluconate deshydrogenase) and PGM (phosphoglucomutase). A zymodeme is defined as a collection of stocks that have the same isozyme profile (Godfrey and Kilgour, 1976).

The present study focused on *L. infantum* species and especially on the zymodeme MON-1. The zymodeme MON-1 is the more common and the more widespread zymodeme in the Mediterranean basin (Moreno *et al.*, 1986). Being responsible for the majority of visceral leishmaniasis cases, together with the zymodeme MON-80 (Aoun *et al.*, 1999; Marty *et al.*, 1994) and the zymodeme MON-34 (Harrat *et*

al., 1996). The zymodeme MON-1 however, can also produce cutaneous leishmaniasis cases (Marty *et al.*, 1998) as do other zymodemes of the *L. donovani* complex.

The aim of this study was to re-examine the phylogenetic individualisation and genetic homogeneity of the *L. infantum* zymodeme MON-1. Two genetic markers were used for phylogenetic analyses: MLEE on cellulose acetate plates (Godfrey and Kilgour, 1976) and random amplified polymorphic DNA (RAPD) technique (Williams *et al.*, 1990).

MATERIALS AND METHODS

Leishmania *stocks*

Table 1 The Origin of the stocks is detailed in Table 1. The stocks were cultivated at 26°C in RPMI 1640 medium supplemented with 10 % heat-inactivated foetal calf serum, 1 % glutamine and 0.5% gentamycin in disposable flasks. Parasites were harvested in logarithmic phase by centrifugation (5,000g for 30 min at 4°C) and washed twice in PBS (Phosphate-Buffered Saline: 0.01M Na2HPO3; 0.01M Na2H2PO4; 0.15M NaCl, pH 7.3). Parasite pellets were kept at -80°C.

MLEE analysis

MLEE experiments were performed on cellulose acetate plates (Helena Laboratories) according to Godfrey & Kilgour (1976), Ben Abderrazak *et al*. (1993) with slight modifications (Bañuls, 1998). Cellular pellets were lysed in an equal volume of enzyme stabiliser (EDTA, dithiotreitol, ε-aminocaproic, aa 2mM) on an ice bed for 15 min. The lysates were centrifuged at 13,000g for 10 min at 4°C and the water soluble fraction was removed and stored at -80°C. Fifteen enzyme systems were studied, they were as follows:

Aconitase (ACON, E.C.4.2.1.3.), alanine aminotransferase (ALAT, E.C.2.6.1.2.), glucose-phosphate isomerase(GPI, E.C.5.3.1.9.), glucose-6-phosphate dehydrogenase

(G6PD, E.C.1.1.1.49.), glutamate oxaloacetate transaminase (GOT, E.C.2.6.1.1.), isocitrate dehydrogenase (IDH, E.C.1.1.1.42.), malate dehydrogenase NAD^+ (MDH, E.C1.1.1.37.), malate dehydrogenase ($NADP^+$) or malic enzyme (ME, E.C.1.1.1.40.), mannose-phosphate isomerase (MPI, E.C.5.3.1.8.), nucleoside hydrolase NHI (inosine substrate) and NHD (deoxyinosine substrate) (E.C.2.4.2.*), 6-phosphogluconate dehydrogenase (6PGD, E.C.1.1.1.44.), phosphoglucomutase (PGM, E.C.2.7.5.1.), peptidase 1, substrate 1-leucyl-leucine-leucine (PEP1, E.C.3.4.11) and peptidase 2, substrate 1-leucyl-1-alanine (PEP2, E.C.3.4.11), superoxyde dismutase (SOD, E.C.1.15.1.1.). This gives a total of sixteen loci since NHI system shows an activity at two distinct loci (*Nhi* 1 and *Nhi* 2, *Nhi* 1 being the fastest migrating locus).

The difference between the panels of enzymatic systems for MLEE on starch gel (MON classification) and for MLEE on acetate plates is based on: ACON, ALAT, PEP (1 & 2) and SOD for acetate plates and GLUD, DIA and FH for starch gel.

Random Amplified Polymorphic DNA

The technique was performed according to Williams *et al.* (1990). DNA was extracted by phenol/chloroform (Sambrook, Fritsch & Maniatis, 1989) and DNA concentration was estimated by spectrophotometry at 260 nm. Primers giving reproducible patterns have been selected according to a previous screening performed in our laboratory on 120 decameric primers (Bañuls *et al.*, 1999a). The following sixteen primers from Operon Technologies (Alameda, California) have been used to study the sample (5'-3': A1-CAGGCCCTTC, A7-GAAACGGGTG, A10-GTGATCGCAG, B10-CTGCTGGGAC, B11-GTAGACCCGT, F10-GGAAGCTTGG, F13-GGCTGCAGAA, N20-GGTGCTCCGT, R2-CACAGCTGCC, R7-ACTGGCCTGA, R8-CCCGTTGCCT, R15-GGACAACGAG, U2-CTGAGGTCTC, U3-CTATGCCGAC, B4-GGACTGGAGT, B15-GGAGGGTGTT). Each RAPD reaction (60µl) contained: 20ng template DNA,

100µM each dNTP, 6µl 10X Taq polymerase Buffer, 12pmol decameric primer, 0.9U Taq polymerase (Roche, Germany), 49.4µl nuclease free water. Forty-five cycles (denaturation: 1 min at 94°C; annealing: 1 min at 36°C; elongation: 1 min at 72 °C) were followed by a final step of 7 min at 72°C in a PTC-100 Thermocycler (MJ research, Inc., Massachusetts, USA). RAPD products were visualised on 1.6 % agarose gels and revealed with ethidium bromide under UV.

Data analysis:

All bands obtained on RAPD and MLEE gels were numbered and scored as presence or absence data. For MLEE data, band number 1 is the fastest one and for RAPD, band number 1 is the slowest. MLEE and RAPD data were computed with the *Genetics Toolbox* software designed in our laboratory (S. Noël and F. Chevenet). Genotypic diversity (number of multilocus genotypes on the total number of stocks) and mean genetic diversity *H* were calculated according to Selander & Levin (1980). Mean genetic diversity *H* is computed with the formula:

$$H=\Sigma h/r$$

where $h= 1-\Sigma q_i^2$, q is the frequency of each allele for a locus i and r the total number of loci.

Estimation of genetic differences among the stocks was performed using the Jaccard's distance (Jaccard, 1908), computed with the following formula:

$$D_{ij} =1-[a/(a+b+c)]$$

Where a is the number of common bands between the stocks i and j, b is the number of bands present in i and absent in j and c is the number of bands present in j and absent in i.

All the distances were regrouped in 2 distance matrices (one for RAPD and one for MLEE data). Dendrograms have been constructed from these matrices with the neighbour joining method (Saitou and Nei, 1987). Parsimony analysis of Wagner

(Felsenstein, 1985) (with 100 replicates) was also used to build phylogenetic trees based on MLEE data compiled with RAPD data and the robustness of the nodes was tested by a bootstrap analysis. Dendrograms were analysed with the Treedyn software (Chevenet, 2000). Correlation between the results obtained with MLEE and RAPD was evaluated by a non parametric test (Mantel, 1967) with 10^4 repetitions.

RESULTS

MLEE analysis

We have obtained 47 different electrophoretic bands with the sixteen enzyme loci. The genotypic diversity was 0.43 within *L. infantum/L. chagasi* MON-1 and 0.5 within the whole *L. donovani* complex. The mean genetic diversity (*H*) was 0.083, 0.095 and 0.21 for *L. infantum/L. chagasi* MON-1, the whole *L. donovani* complex and the whole sample, respectively. Only five enzyme loci were polymorphic within *L. infantum/L. chagasi*

Fig. 1 MON-1: 6*pgdh*, *Mdh*, *Nhi*2, *Got*, *Pep*1 (see Fig. 1 as example*)*, which corresponds to a polymorphism rate 0.312. For the *L. donovani* complex, we found four loci giving

Fig. 2 specific electromorphs: *G*6*pdh*, *Gpi*, *Pgm*, *Sod* (see Fig. 2 as example), though there was no loci giving specific electromorphs either for *L. infantum* or for MON-1. Considering all these data, we have observed that (i) in some cases MLEE loci on acetate plate gave more resolutive patterns: some MON-1 stocks showed different patterns for 6PGDH, GOT, MDH, NHI2, PEP1 on acetate plates; and (ii) in some other cases MLEE on starch gel was more resolutive than MLEE on acetate plates: the three no MON-1 stocks had the same pattern as several MON-1 stocks with MDH system on acetate plates but were different from MON-1 stocks on starch gel.

RAPD analysis

We have obtained 117 different electrophoretic bands with the 16 primers used. The genotypic diversity was 0.71 within *L. infantum/L. chagasi* MON-1. RAPD analysis allowed to reveal a more important genetic diversity within the MON-1 (0.205) compared to the MLEE data. The mean genetic diversity was 0.37 in the whole sample and 0.24 within the *L. donovani* complex. Six primers gave polymorphic patterns within

Fig. 3 *L. infantum/L. chagasi* MON-1 (U2, R15, F10, B4, B10, A1) (see Fig. 3 as example*)*. The corresponding polymorphism rate was 0.5. There was five primers giving specific

Fig. 4 amplicons of the *L. donovani* complex (A7, A10, B15, F13, U3) (see Fig. 4 as example) but we did not found specific amplicons for MON-1.

Phylogenetic analysis

Fig. 5 The two neighbour joining trees (Fig. 5) designed from MLEE and RAPD distance matrices showed similar patterns. All stocks belonging to the *L. donovani* complex fell into one cluster. In both trees, the MON-1 stocks were not grouped into a specific cluster and were mixed among stocks from other zymodemes (Fig. 5). For example, the *L. donovani* stock was associated with the two *L. chagasi* MON-1 stocks. On the Wagner

Fig. 6 parsimony tree (Fig. 6), a strong bootstrap value (100%) supported the node of the whole *L. donovani* complex. This complex was characterised by several specific markers (five RAPD amplicons and four MLEE electromorphs). Within this complex, no relevant additional subdivision was revealed by the bootstrap analysis.

Correlation test

Overall strong agreement between the MLEE and RAPD trees was ascertained by the results of the nonparametric correlation test (Mantel, 1967). All p values were $< 10^{-4}$, either for the whole sample (r: 0.95), for the *L. donovani* complex (r: 0.73) or for the MON-1 stocks (r: 0.80) considered separately.

DISCUSSION

The parasites of the *L. donovani* complex are responsible for very different clinical expressions and present different epidemiological features. In this framework, the aim of this study was to explore in depth the genetic structure of the zymodeme MON-1 at the microevolutionary level. It is considered that MON-1 has a considerable epidemiological relevance. The main goal was therefore to verify the phylogenetic individualisation of MON-1 within the *L. donovani* complex and its genetic homogeneity. The epidemiological and clinical implications of the genetic and phylogenetic results are discussed.

Individualisation of the L. donovani *complex*

Our results confirmed the genetic individualisation of the *L. donovani* complex against the other species (*L. tropica* and *L. major*). The phylogenetic construction (Fig. 5) and the

four specific MLEE and five specific RAPD markers (tags) show that the *L. donovani* complex can be considered as a DTU (Discrete Typing Unit) according to the definition of Tibayrenc (Tibayrenc, 1998). These data confirmed those published in Mauricio & Howard (1999); indeed they postulated that the *L. donovani* complex has a monophyletic origin on the basis of RAPD data and *msp*C sequence.

Phylogenetic relationship between L. donovani *complex species*

The controversy still exists around the species and zymodeme classification belonging to the *L. donovani* complex. Indeed, it was widely accepted that the *L. donovani* complex is divided in four different species (*L. infantum, L. chagasi*, *L. donovani*, *L. archibaldi*). Moreover, recent data raise the question of the genetic individualisation of *L. donovani*

and *L. infantum* (Rioux. J.A. Personal communication; Bañuls *et al.*, 1999b) and of *L. archibaldi*. It is difficult to know clearly the real phylogenetic status of each species. Mauricio *et al.* (1999) suggest that *L. infantum/L. chagasi* is a monophyletic group (with the RAPD primer H1 5'-3':CGCGCCCGCT as specific tag). The same author showed by PCR-RFLP (ITS and mini-exon) and RAPD that *L. chagasi* could not be distinguished from *L infantum* (Mauricio *et al.*, 2000). In 1986, other authors considered that *L. infantum* and *L. chagasi* pertained to the complex *L. infantum* and *L. donovani* was a distinct complex (Moreno *et al.*, 1986). Our results cannot distinguish the three species: *L. donovani*, *L. chagasi* and *L. infantum*. By the use of more resolutive molecular tools such as microsatellites and larger samples of *L. donovani*, *L. chagasi* and *L. archibaldi*, this fundamental point should be resolved.

Genetic heterogeneity and polyphyletism of MON-1 within the L. donovani *Complex*

Analysis of the *L. infantum* species by other molecular methods than MLEE on starch gel allowed study of the genetic diversity and the phylogenetic status of the zymodeme MON-1. The different calculations showed a comparable polymorphism in MON-1 and in the whole sample of *L. infantum* as well as a significant genotypic diversity. The use of two different markers (better resolution than MLEE on starch gel) allowed to be displayed an additional and non-negligible genetic variability within the zymodeme MON-1. From a phylogenetic point of view, MLEE and RAPD data gave congruent results confirmed by the Mantel test (r = 0.80 within MON-1; $p<10^{-4}$). Analysis by phenetic and phylogenetic methods underscored that zymodeme MON-1 did not correspond to a DTU. The present study did not allow the separation of the zymodeme MON-1 from the other zymodeme (MON-24, MON-33, MON-29) responsible for cutaneous forms in competent host (Aoun *et al.*, 2000; Harrat *et al.*, 1996; Rioux *et al.*, 1985). MON-1 stocks are not grouped into a specific cluster, indeed other MON

zymodemes are inserted among them (Fig. 5). Moreover, the *L. chagasi* stock characterised as MON-1 is more distant from *L. infantum* than *L. infantum* stocks belonging to other zymodemes. These data suggest that, in spite of the MLEE on starch gel classification, zymodeme MON-1 has not a monophyletic status for the studied markers which is probably due to an insufficient resolution of the 15 enzyme loci used for MON identification.

Clinical and epidemiological implications

The *L. donovani* species is described as producing in the majority of cases, a visceral clinical form and evolving only in an anthroponotic cycle. The two other species, *L. chagasi* and *L. infantum* (among which MON-1), evolve in an anthropozoonotic cycle and have the potentiality to produce asymptomatic infections, benign cutaneous forms as well as lethal visceral forms of the disease. The additional genetic polymorphism within MON-1 obtained in this study, suggests the possibility of testing for statistical association between the clinical variability observed in patients and the parasite genotypes. This kind of work must be performed on a large *Leishmania* sample representative of the clinical variability, and informations about patient (immunological status, disease symptoms) must be taken into account. Moreover, the polyphyletic nature of MON-1 and the additional genetic variability revealed in this work also raise questions about the reservoir implicated in the parasite's transmission, the origin of transmission of the disease and the vector species incriminated. The general conclusion from our data is that MON-1 is not the result of a recent, common clonal descent, which has to be taken into account in all epidemiological surveys. The common ancestor of all MON-1 genotypes may be ancient, and it is common to other strains pertaining to different zymodemes. For such epidemiological surveys, markers with a higher discriminative power are preferable (Tibayrenc, 1999). This is the case for microsatellites or multi-gene sequencing

(Minodier *et al.*, 1997; Rossi *et al.*, 1994; Vickerman, 1994), which showed also their usefulness for the intraspecific analysis because of their fast molecular clock.

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Table 1. *Leishmania* spp. stocks

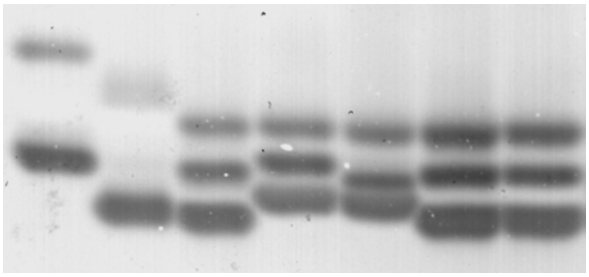
International Code	Zymodeme	Locality of isolation	Locality of infection
L. infantum			
MHOM/FR/78/LEM75	1	Beziers	--
MHOM/FR/92/LEM2572	1	Toulouse	Milan (IT)
MHOM/FR/92/LEM2301	1	Toulouse	Marseille (FR)
MHOM/TN/80/IPT1	1	Monastir	--
MHOM/MA/67/ITMAP263	1	--	--
MHOM/FR/92/LPN83	1	Nice	--
MHOM/FR/93/LPN92	1	Nice	--
MHOM/FR/94/LPN94	1	Nice	--
MHOM/FR/94/LPN105	1	Nice	Bastia (FR)
MHOM/FR/95/LPN119	1	Nice	Cannes (FR)
MHOM/FR/96/LPN129	1	Nice	Vallauris (FR)
MHOM/FR/96/LPN132	1	Nice	Mouns Sartoux (FR)
MHOM/DZ/82/LIPA59*	24	El Asnam	--
MHOM/ES/81/BCN1*	29	Monserrat	--
MHOM/FR/82/LEM356*	33	Amélie-les bains	--
L. chagasi			
MHOM/BR/79/L101	1	--	--
MHOM/BR/74/PP75	1	Bahia	--
L. donovani			
MHOM/ET/67/HU3	18	Humera	--
L. tropica			
MHOM/SU/74/K27	60	--	--
L. major			
MHOM/SU/73/5ASKH	4	Askhabad	--

Key for countries:

FR: France; TN : Tunisia; MA: Morocco; DZ: Algeria;

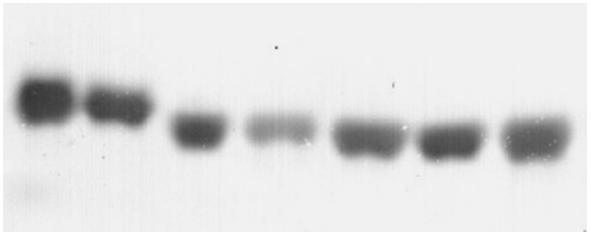
ES: Spain; BR: Brazil; ET: Ethiopia; IN: India; SU: former Soviet Union.

* *L. infantum* LIPA59, BCN1, LEM356 were isolated from cutaneous lesions, the other stocks pertaining to the *L. donovani* complex were isolated from visceral lesions.



1 2 3 4 5 6 7

Fig. 1. MLEE profile revealed with the MDH enzymatic system. From left to right: Lane 1: *L. major*, 5ASKH; lane 2: *L. tropica*, K27; lane 3: *L. infantum*, LEM75 (MON-1); lane 4: *L. chagasi*, L101 (MON-1); lane 5: *L. donovani*, HU3; lane 6 and 7: *L. infantum*, BCN1 (MON-29), LIPA59 (MON-24). The lanes 3 and 4 reveal polymorphic patterns within zymodeme MON-1.



1 2 3 4 5 6 7

Fig. 2. MLEE profiles obtained for the PGM enzymatic system From left to right: Lane 1: *L. tropica,* K27; lane 2: *L. major*, 5ASKH;lane 3: *L. donovani*, HU3; lane 4 and 5: *L. infantum*, LEM75, BCN1; lane 6 and 7: *L. chagasi*, PP75, L101. This system is a specific tag of the *L. donovani* complex. The five stocks pertaining of the *L. donovani* complex have the same band and this band is absent for *L. tropica* and *L. major.*

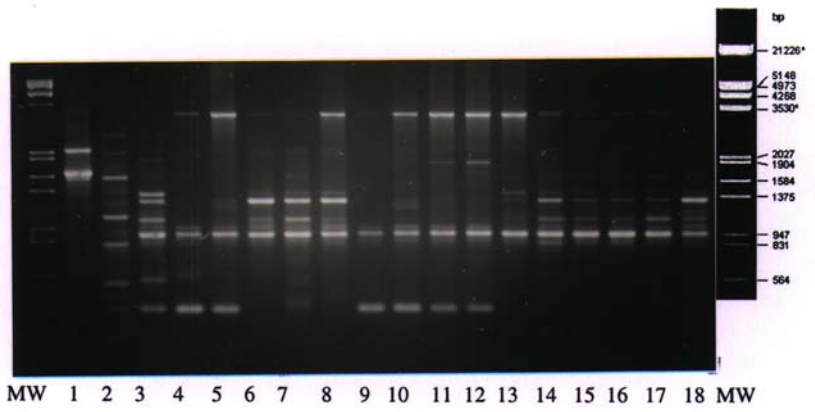


Fig. 3. RAPD patterns obtained with the primer F10. From left to right: Lane 1: *L. tropica*, K-27; lane 2: *L. major*, 5ASKH; lane3: *L. donovani*, HU3; lane 4-8: *L. infantum*, LEM2301 (MON-1), LEM2572 (MON-1), LPN119 (MON-1), IPT1 (MON-1), ITMAP263 (MON-1); lane 9: *L. chagasi*, PP75 (MON-1); lane 10-18: *L. infantum*, LIPA59 (MON-24), BCN1 (MON-29), LEM356 (MON-33), LPN132 (MON-1), LPN92 (MON-1), LPN94 (MON-1), LPN105 (MON-1), LPN129 (MON-1), LPN83 (MON-1); MW: Molecular Weight. Note the polymorphic patterns within *L. infantum/L. chagasi* MON-1.

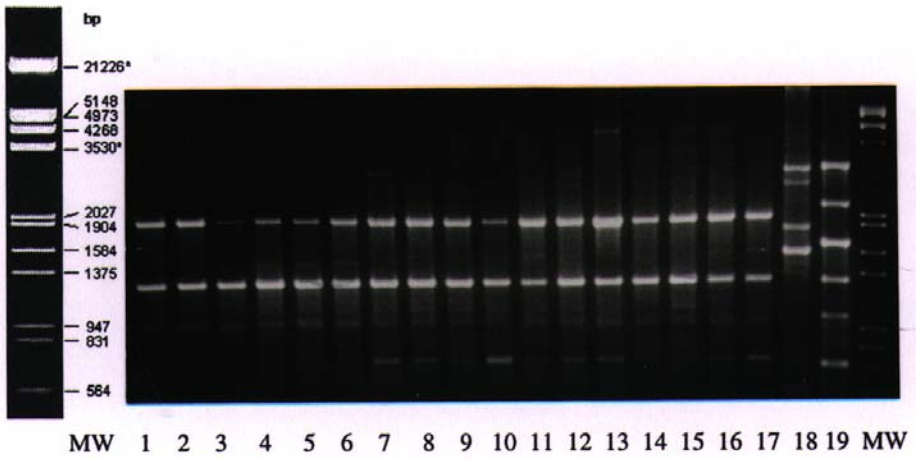


Fig. 4. RAPD patterns obtained with the primer A10. All the patterns are identical for the stocks pertaining to the *L. donovani* complex, A10 primer is specific of the complex (tag). From left to right: Lanes 1-3: *L. infantum*, LIPA59 (MON-24), BCN1 (MON-29), LEM356 (MON-33); Lane 4: *L. chagasi,* PP75; Lane 5-16: *L. infantum,* LEM2301 (MON-1), LEM2572 (MON-1), LPN119 (MON-1), IPT1 (MON-1), ITMAP263 (MON-1), LPN132 (MON-1), LPN92 (MON-1), LPN94 (MON-1), LPN105 (MON-1), LPN129 (MON-1), LPN83 (MON-1), LEM75 (MON-1); Lane 17: *L. donovan*i, HU3; Lane 18: *L. major*, 5ASKH; Lane 19: *L. tropica*, K-27; MW: Molecular Weight.

Fig. 5.Two neighbour joining dendrograms obtained from RAPD (left) and MLEE (right) data. The mentioned loci correspond to specific loci (tag) of the *L. donovani* complex. In order to align the two dendrograms, the branch corresponding to the LPN83 stock in MLEE tree has been rotated.

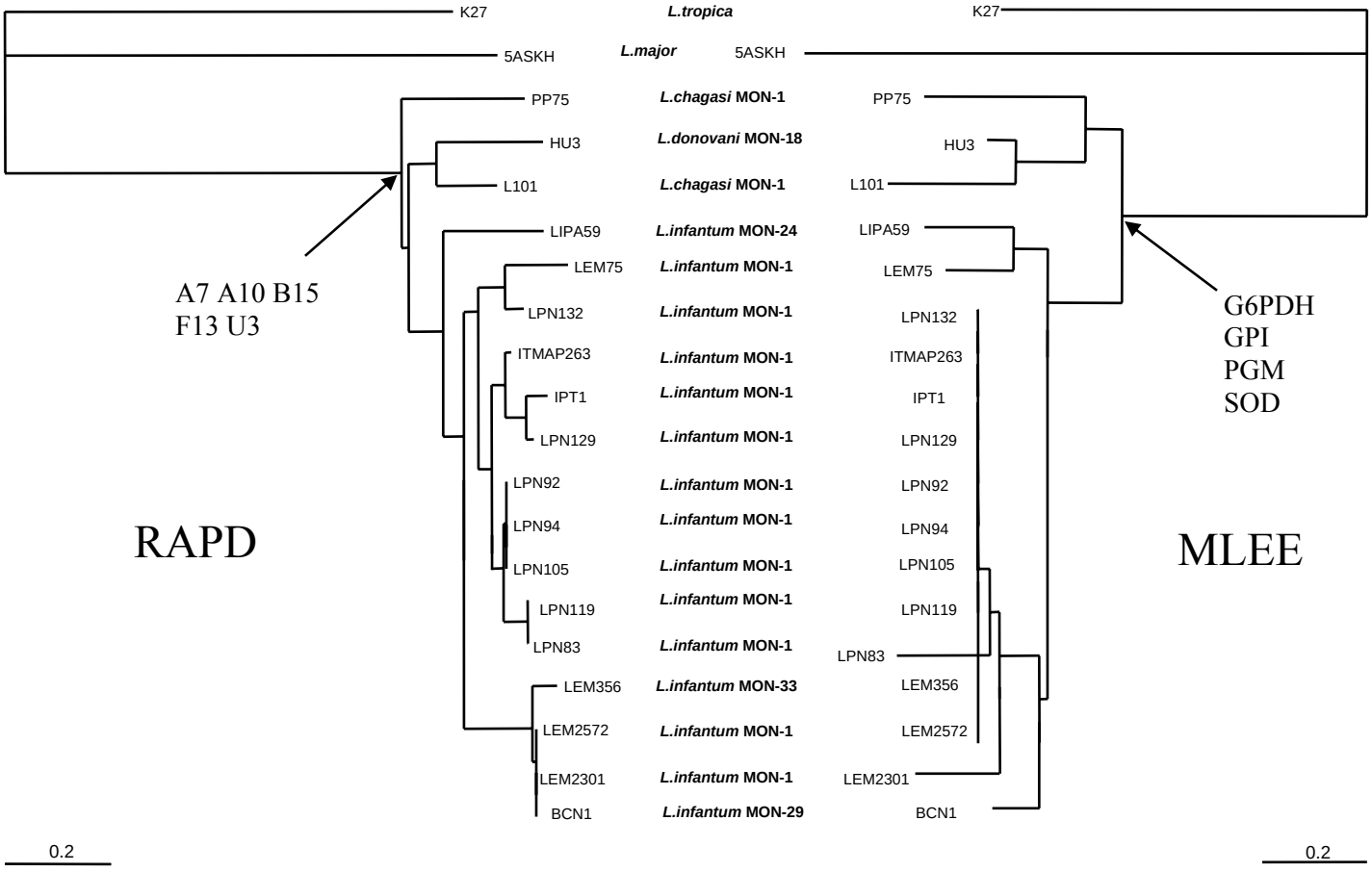


Fig. 6. Phylogenetic tree constructed by bootstrapped (100 replicates) parsimony analysis of Wagner. Boostrap values for all nodes are given. The stocks pertaining to the zymodeme MON-1 fall into different groups and these groups are not supported by strong nodes. The node of the DTU "*L. donovani* complex" is supported by a strong bootstrap value (100%).

* LNP 92, LPN94 and LPN105 have the same genotype with MLEE and RAPD techniques.