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GENETIC POLYMORPHISM WITHIN THE *LEISHMANIA DONOVANI* COMPLEX: CORRELATION WITH GEOGRAPHIC ORIGIN

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Abstract. Random amplified polymorphic DNA (RAPD) was used to detect intraspecific diversity for the *Leishmania donovani* complex. Fifty-two decameric to 21-meric primers of arbitrary sequence were applied to 15 strains that belong to nine zymodemes. Strains belonging to the species *L. major* and *L. tropica* were used as outgroups. A total of 902 amplicons generated by RAPD were scored. Most primers produced species-specific profiles, only 0.6% amplicons were shared by all species, while 4.3% amplicons were common for all 15 strains of the *L. donovani* complex. Well-supported trees have been constructed, which show a rather strong correlation between the genetic polymorphism of studied strains and their geographic origin. In all obtained trees, *L. infantum* was paraphyletic. The RAPD profiles suggest that MON-30 belongs to *L. donovani.* Moreover, the genetic distance between the *L. archibaldi* strain and other leishmanias does not warrant existence of a separate species.

INTRODUCTION

The *Leishmania donovani* complex, the etiologic agent of visceral leishmaniasis, is reported to include three species (*L. donovani*, *L. infantum*, and *L. archibaldi*), the concept of which is based on multilocus enzyme electrophoresis.[1,2] However, this method has several shortcomings,[3] and is being questioned by various DNA-based methods: sequencing of ribosomal intergenic regions,[4] mini-exons,[5] and gp63 genes,[6] microsatellite analysis,[7] kinetoplast DNA minicircles,[8] and polymerase chain reaction–restriction fragment length polymorphism of gp63.[9,10] Data from these analyses have been used to build phylogenetic trees, which in general did not correlate with taxonomy based on multilocus enzyme electrophoresis. However, DNA-based phylogenies were all derived from a small number of characteristics and a high level of genetic homogeneity within the complex makes it rather difficult to reconstruct (robust) phylogenetic relationships. For comparison of closely related *Leishmania* strains, the limited reproducibility of random amplified polymorphic DNA (RAPD) seems to be outweighed by the amount of information obtained. The RAPDs have provided the most polymorphic characteristics, but have been applied with only a small number of primers. Nevertheless, RAPD has enabled the detection of intraspecific diversity for *L. donovani* and *L. infantum*.[6,11–14]

Here we present a RAPD analysis of the *L. donovani* complex using 52 primers that provided numerous distinct amplicon profiles. Well-supported RAPD based trees of this complex were obtained for the first time and revealed a significant correlation between genetic polymorphism and geographic origin in *L. infantum/donovani*.

MATERIALS AND METHODS

Total DNA was obtained from 15 *L. donovani* complex strains (10 *L. infantum* and 5 *L. donovani*; full strain information at http://www.paru.cas.cz/structure/LMP/leish.htm), one *L. major* strain, and one *L. tropica* strain (Table 1) isolated from autochthonous patients in different geographic regions, using a DNA isolation kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Amplicons were obtained with 62 random decameric to 21-meric primers (Table 2). Each 25-μL RAPD reaction contained 5 ng of genomic DNA, 3 mM $MgCl_2$, 25 pmol of primer, and 0.5 units of *Taq* polymerase in the appropriate buffer. The amplification cycle was 94°C for four minutes; then 45 cycles at 94°C for one minute, 35°C for two minutes, and 72°C for three minutes; and at 72°C for 10 minutes. The RAPD products were separated by electrophoresis on ethidium bromide–stained 1.5% agarose gels in 0.5× Tris-acetate-EDTA buffer (20 mM Tris-acetate, 0.5 mM EDTA) at 80 V for four hours. The size of the scored amplicons ranged from 100 base-pairs to 4 kilobases.

Fifty-two primers had a sufficient number of scorable amplicons and good reproducibility, tested by repeating most reactions. Although profiles were reproducible, the intensity of individual bands was subject to significant differences, which may be due to small differences in DNA and primer concentrations. High-quality DNA was always used because degradation of DNA altered amplification profiles.

The RAPD profiles were manually scored as presence or absence data. Phylogenetic trees were constructed using minimum evolution as an optimality criterion and NeiLi or UpHolt distance matrices (as implemented in PAUP 4b10, Swofford, 1998; Sinauer Associates, Sunderland, MA).

RESULTS

Forty-three primers were used with all strains shown in Table 1. Figure 1 shows a representative RAPD gel (other gels are available at http://www.paru.cas.cz/structure/LMP/Figures.htm) and each generated between 1 and 34 amplicons scored by size, in a total of 770. Nine additional primers were used with a smaller set of strains and produced 132 scored amplicons. Thus, a total of 902 amplicons has been evaluated. Scores obtained with individual primers are available at http://www.paru.cas.cz/structure/LMP/RAPD_primers.htm. Most primers produced profiles specific for *L. major*, *L. tropica*, or *L. aethiopica*, and the *L. infantum*/*L. donovani* complex. The length of the primers did not correlate with the number of genetic markers. Only 0.6% of the amplicons were shared by all four species, while 4.3% were common for the 15 strains of the *L. donovani* complex. When this complex was subdivided into *L. infantum* and *L. donovani* (based on multilocus enzyme electrophoresis),[1,2] 16.6% and 6.9% were species specific, respectively.

TABLE 1
Characterization of analyzed *Leishmania* spp. strains*

Name	Code	Strain	Zymodeme	Country	Type of infection
L. infantum	LG1	MHOM/FR/1978/LEM75	MON1	France	VL
L. infantum	LG2	MHOM/FR/1995/LPN114	MON1	France	VL
L. infantum	LG3	MHOM/1993/PM1	MON1	Spain	VL
L. infantum	LG4	MHOM/FR/1997/LSL29	MON1	France	CL
L. infantum	LG5	MHOM/ES/1986/BCN16	MON1	Spain	CL
L. infantum	LG6	MHOM/PT/2000/IMT260	MON1	Portugal	CL
L. infantum	LG7	MHOM/FR/1996/LEM3249	MON29	France	VL
L. infantum	LG8	MHOM/ES/1991/LEM2298	MON183	Spain	VL
L. donovani	LG9	MHOM/IN/0000/DEVI	MON2	India	VL
L. donovani	LG10	MHOM/IN/1996/THAK35	MON2	India	VL
L. archibaldi	LG11	MHOM/ET/1972/GEBRE 1	MON82	Ethiopia	VL
L. donovani	LG12	MHOM/SD/1982/GILANI	MON30	Sudan	VL
L. donovani	LG13	MHOM/ET/0000/HUSSEN	LON42	Ethiopia	VL
L. infantum	LG14	MHOM/FR/1980/LEM189	MON11	France	VL
L. infantum	LG15	MHOM/MT/1985/BUCK	MON78	Malta	VL
L. major	M	LV561	?	Israel	CL
L. tropica	T	VEDHA	?	Turkey	CL

* VL = visceral leishmaniasis; CL = cutaneous leishmaniasis.

The power to discriminate between the *L. donovani* complex and the outgroups (*L. tropica* or *L. aethiopica* and *L. major*) was primarily observed for primers A12, B13, ILO875, and TA-150. Seven primers (A5, B5, B6, B7, B8, 198, and TA-150) distinguished *L. infantum* from *L. donovani* strains. Selected amplicons of varying specificity, obtained with the above-mentioned primers, are being cloned and sequenced with the aim of obtaining species-specific regions. The profiles most polymorphic for the strains LG1 to LG8 (*L. infantum sensu stricto*) were generated by primers B1, B3, B9, B10, B20, and 193. Moreover, primers A15, B12, and TA-150 distinguished between Indian *L. donovani* and Mediterranean *L. infantum*. Importantly, none of the primers used distinguished between *L. infantum*, *L. donovani*, and *L. archibaldi*. Moreover, primer H1 produced a *L. donovani*-specific band of approximately 600 basepairs.

Phylogenetic trees were constructed using the following doublets of strains (Table 1) as outgroups: 1) *L. major* and *L. tropica*; 2) strains DEVI and THAK 35 (*L. donovani* from India); and 3) strains LEM 189 and BUCK (*L. infantum*). Overall, the analysis showed well-resolved trees with topology slightly influenced by the choice of outgroup. When the tree was rooted with *L. major* and *L. tropica*, *L. infantum* strains LEM 189 and BUCK formed a sister group to the remaining strains (Figure 2) with the longest interior branches. The three African strains were a strongly supported group (86% bootstrap), the relationship of which with the clade consisting of Indian *L. donovani* was not fully resolved (Figure 2). The crown group, composed solely of strains isolated from humans originating from the Iberian peninsula, was supported by 98% bootstrap with this outgroup.

Exclusion of *L. major* and *L. tropica* from the dataset reduced the number of non-shared characteristics and thus increased bootstrap support. Almost no differences were observed between the UpHolt and NeiLi matrices. Upon rooting with LEM 189 and BUCK, the first branch was composed of African strains, followed by one with both Indian *L. donovani* strains (Figure 3A). *Leishmania donovani* strains isolated from India and Sudan were clearly paraphyletic. The crown group (bootstrap support 98%) contained Mediterranean strains (Figure 3A).

Trees rooted with DEVI and THAK 35 (Indian *L. donovani*) were split into two strongly supported branches, with internal topology being apparently insensitive to the outgroup selected (Figure 3B). *Leishmania donovani* strains were not monophyletic in any of the analyzed trees, nor were the *L. infantum* MON-1 strains. Interestingly, all three MON-1 strains isolated from cutaneous forms of the disease formed a clade, albeit also containing one strain from a visceral case (Figures 2 and 3).

In a separate RAPD analysis with a higher number of strains but with less primers (C2, C4, C5, C6, D8, D10, H1, H4, and L2) using conditions previously described,[6] all primers distinguished the *L. donovani* complex from outgroup strains (*L. major* and *L. aethiopica*), while primers C4 and H1 produced *L. infantum*-specific profiles. In the resultant trees, two groups, *L. infantum* strains and *L. donovani* strains originating from India, were robust and a group with strains of Kenyan origin was detected but not strongly supported. Strains from Ethiopia/Saudi Arabia or Sudan did not form monophyletic assemblies.

DISCUSSION

The RAPD analysis of strains of the *L. donovani* complex presented here detected a correlation with geographic origin, regardless of whether a high number of studied strains or primers was used in a given dataset. With a single exception, *L. infantum* strains originating from France, Spain, and Portugal were strongly monophyletic. Moreover, the African and Indian strains constituted two monophyletic groups.

Strain Gilani (MON-30) did not cluster with *L. infantum*, as it would be expected from the report of Rioux and others,[1] but with Gebre 1 (MON-82). This suggests that strains MON-30 are not *L. infantum* and casts doubts on the existence of the species *L. archibaldi*. Along with gp63 restriction fragment length polymorphism data,[9] recently obtained sequences of microsatellites and the chitinase gene of *L. infantum*, *L. donovani*, and *L. archibaldi* strains provided strong support for monophyly of most strains of Sudanese origin, regardless of their species classification (Noyes HA, unpublished data).[3]

TABLE 2
List of primers used

A-01 5′-CAGGCCCTTC-3′	B-01 5′-GTTTCGCTCC-3′	192 5′-GCAAGTCACT-3′
A-02 5′-TGCCGAGCTG-3′	B-02 5′-TGATCCCTGG-3′	193 5′-TGCTGGCTTT-3′
A-03 5′-AGTCAGCCAC-3′	B-03 5′-CATCCCCCTG-3′	181 5′-ATGACGACGG-3′
A-04 5′-AATCGGGCTG-3′	B-04 5′-GGACTGGAGT-3′	160 5′-CGATTCAGAG-3′
A-05 5′-AGGGGTCTTG-3′	B-05 5′-TGCGCCCTTC-3′	198 5′-GCAGGACTGC-3′
A-06 5′-GGTCCCTGAC-3′	B-06 5′-TGCTCTGCCC-3′	159 5′-GAGCCCGTAG-3′
A-07 5′-GAAACGGGTG-3′	B-07 5′-GGTGACGCAG-3′	164 5′-CCAAGATGCT-3′
A-08 5′-GTGACGTAGG-3′	B-08 5′-GTCCACACGG-3′	ILO875 5′-GTCCGTGAGC-3′
A-09 5′-GGGTAACGCC-3′	B-09 5′-TGGGGGACTC-3′	ILO872 5′-CCCGCCATCT-3′
A-10 5′-GTGATCGCAG-3′	B-10 5′-CTGCTGGGAC-3′	TAF300 5′-CACCTCAAAACATACCCC-3′
A-11 5′-CAATCGCCGT-3′	B-11 5′-GTAGACCCGT-3′	TA610 5′-TCAACCGATTACAAACCA-3′
A-12 5′-TCGGCGATAG-3′	B-12 5′-CCTTGACGCA-3′	TA150 5′-ATGCGATGAGTGGTTGAG-3′
A-13 5′-CAGCACCCAC-3′	B-13 5′-TTCCCCCGCT-3′	PLID2-9 5′-CAAAAGTCCCCACCAATCCC-3′
A-14 5′-TCTGTGCTGG-3′	B-14 5′-TCCGCTCTGG-3′	C2 5′-GAAACGGGTG-3′
A-15 5′-TTCCGAACCC-3′	B-15 5′-GGAGGGTGTT-3′	C4 5′-AATCGGGCTG-3′
A-16 5′-AGCCAGCGAA-3′	B-16 5′-TTTGCCCGGA-3′	C5 5′-CTCACGTAGG-3′
A-17 5′-GACCGCTTGT-3′	B-17 5′-AGGGAACGAG-3′	C6 5′-CTGATCGCAG-3′
A-18 5′-AGGTGACCGT-3′	B-18 5′-CCACAGCAGT-3′	D8 5′-AGCCAGCGAA-3′
A-19 5′-CAAACGTCGG-3′	B-19 5′-ACCCCCGAAG-3′	D10 5′-GTTGCGATCC-3′
A-20 5′-GTTGCGATCC-3′	B-20 5′-GGACCCTTAC-3′	H1 5′-CGCGCCCGCT-3′
H4 5′-TGCCGAGCTG-3′		L2 5′-CGGACGTCGC-3′

Leishmania infantum (thus defined as excluding MON-30) was not monophyletic due to the position of strains LEM 189 and BUCK. These two strains were genetically distant from the other *L. infantum* and the trees constrained for monophyly of *L. infantum* had significantly lower bootstrap support than unconstrained trees. This is an unexpected result, since every method used so far to study the *L. donovani* complex has shown that *L. infantum* without MON-30 strains

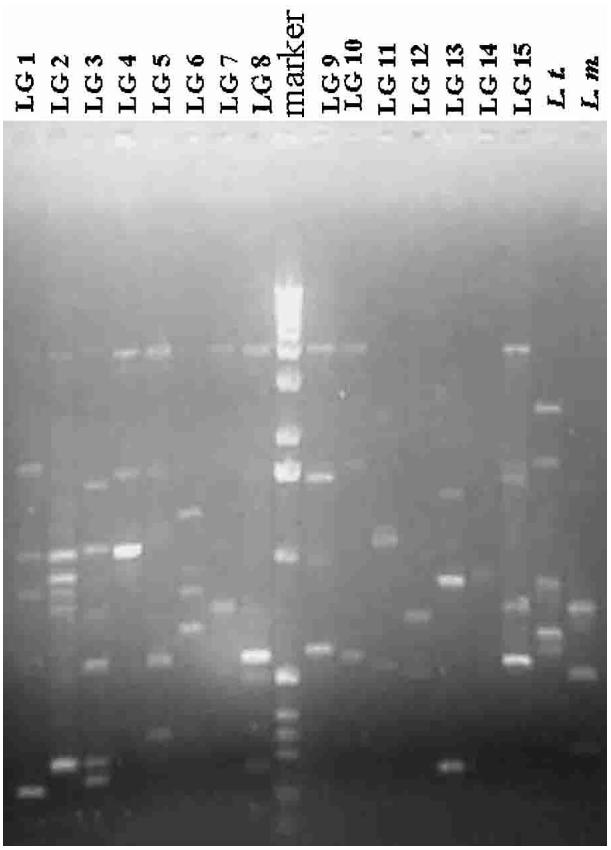

FIGURE 1. Random amplified polymorphic DNA amplification patterns of *Leishmania* spp. strains (Table 1) with primer B20. Marker = 100-basepair ladder (Gibco-BRL, Gaithersburg, MD); *L. t.* = *L. tropica*; = *L. m.* = *L. major*.

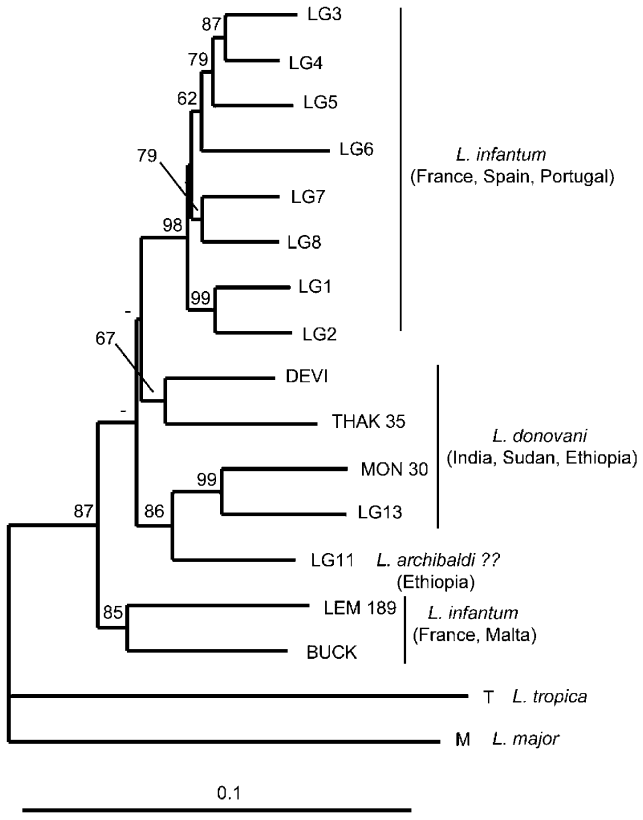

FIGURE 2. Phylogenetic tree constructed using minimum evolution as an optimality criterion and UpHolt distance matrices, with *Leishmania major* and *L. tropica* used as outgroups. The bootstrap test was carried out using 1,000 replicates. The scale for substitution/site is given under the tree. The geographic origin of the strains is indicated.

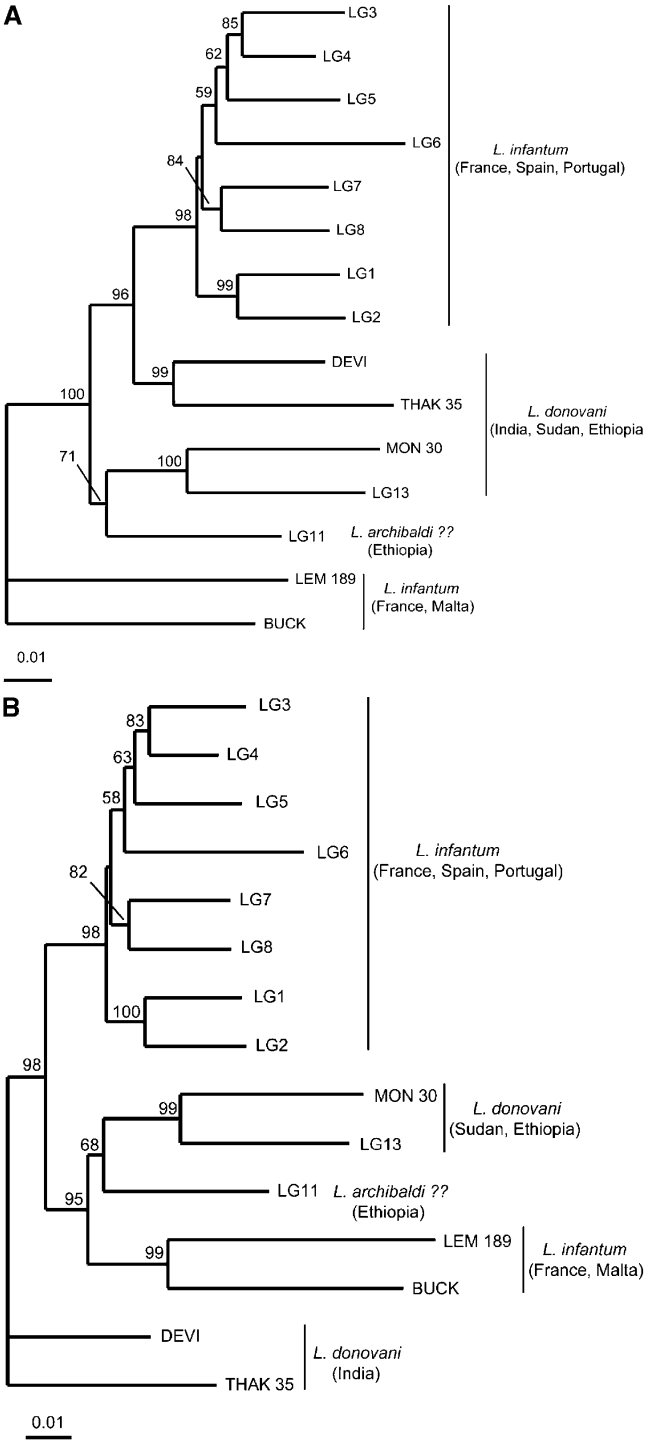

FIGURE 3. Phylogenetic trees constructed using minimum evolution as an optimality criterion and **A**, UpHolt distance matrices or **B**, NeiLi distance matrices, with the dataset narrowed to the *Leishmania donovani* group only. The bootstrap test was carried out using 1,000 replicates. The scale for substitution/site is given under the tree. The geographic origin of the strains is indicated. *Leishmania infantum* strains LEM 189 and BUCK served as outgroups in **A** and *L. donovani* strains DEVI and THAK 35 served as outgroups in **B**.

is indeed monophyletic.[6,9,15] It should be stressed, however, that strain BUCK seems to be one of the most divergent strains in this species.[9]

It should be emphasized that current taxonomy of the *L. donovani* complex relies on differences in the mobility of selected enzymes in starch gels and that this mobility can be altered by a single point mutation. As such, multilocus enzyme electrophoresis–based taxonomies rely on a small number of characteristics, in contrast with the relationships among parasites proposed here, which are based on hundreds of different characteristics. Unfortunately, only a relatively small number of strains have so far been extensively studied by molecular means and much remains to be done. However, we present here more data that support a call for a taxonomic re-evaluation of the *L. donovani* complex.

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