# *Leishmania donovani* complex: genotyping with the ribosomal internal transcribed spacer and the mini-exon

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SUMMARY

Intergenic region typing by restriction analysis of the ribosomal internal transcribed spacer (ITS) and mini-exon provide diagnostic markers for some *Leishmania*. Here, we evaluate restriction analysis of these targets for genotyping and phylogenetic analysis within the *Leishmania donovani* complex (agents of visceral leishmaniasis). Each method was useful for genotyping of both *L. donovani* complex strains and Old World *Leishmania* species. The targets produced less robust groups than gp63 intergenic regions, but support the need for re-evaluation of the taxonomy of the *L. donovani* complex.

Key words: restriction fragment length polymorphism, genotyping, Kinetoplastida, *Leishmania donovani*, ribosomal internal transcribed spacer, mini-exon.

## INTRODUCTION

We have previously identified 6 strain groups in the *Leishmania donovani* complex, based on intergenic regions of gp63, named MIRT1-MIRT6 (*mspC* intergenic region type). The 6 groups comprised, respectively; mainly strains from Kenya; from India; from Sudan; *L. infantum*; Saudi Arabia/Ethiopia, and a Chinese strain (Mauricio *et al*. 2001). Restriction analysis of the ribosomal internal transcribed spacer (ITS) for several American *Leishmania* species has correlated well with isoenzyme and mini-exon data (Cupolillo *et al*. 1995) and has been used to genotype the *L. donovani* complex in Sudan (El Tai *et al*. 2001). The mini-exon gene has been exploited for both PCR diagnosis (Hassan *et al*. 1993; Katakura *et al*. 1998) and identification of *Leishmania* complexes (Ramos *et al*. 1996; Harris *et al*. 1998). In the *L. donovani* complex the mini-exon non-transcribed spacer, varies from 422 bp to 467 bp (Fernandes *et al*. 1994), is highly variable and thus a potential target for intra-specific genotyping. ITS and mini-exon restriction analyses were used here to test the existence of the groups described by gp63 intergenic regions.

## MATERIALS AND METHODS

DNA was extracted (Kelly, 1993) from *L. donovani* complex strains (Table 1), selected to represent geographical and zymodeme diversity, and from outgroup strains *L. aethiopica* MHOM/ET/70/L96, *L. major* MHOM/SU/73/5-ASKH and *L. tropica* MHOM/SU/74/K27. The ribosomal internal transcribed spacer (ITS) was amplified with the primers described by Cupolillo *et al*. (1995) and PCR conditions as used by Stothard *et al*. (1996). The primers described by Fernandes *et al*. (1994) were used to amplify the mini-exon repeat unit as in Ramos *et al*. (1996). Desired PCR products were purified after electrophoresis using the QIAEX II™ kit (Qiagen), cut with *Alu*I, *Mse*I, *Taq*I, *Eco*RI and *Sph*I (ITS), *Msp*I (mini-exon), and *Bst*UI, *Cfo*I and *Hae*III (both) and separated by polyacrylamide gel electrophoresis as described (Mauricio *et al*. 2001). Intense fragments $<900$ bp were scored as 1 if present (fragment restricted) and as 0 if absent (fragment was not restricted). Trees were built from data matrices of individual and pooled data by PHYLIP 3.6 alpha2 (Felsenstein, 1993), using Nei & Li distance for restriction fragment data, as implemented by RESDIST for an average restriction site size of 4 nucleotides, with both neighbour-joining (NEIGHBOR) and maximum parsimony (PARS). Bootstrap analysis was by SEQBOOT.

## RESULTS AND DISCUSSION

For the *L. donovani* complex ITS PCR products were approximately 1 kb, for *L. major* 1·1 kb, for *L. aethiopica* 1·05 kb and for *L. tropica* 1·05 kb and 0·9 kb. Mini-exon PCR products varied in size between 0·4 and 0·5 kb and were not species specific. Double mini-exon bands were obtained for all 5 Indian *L. donovani* strains as described for strain DD8 (Fernandes *et al*. 1994). Thus DD8 is a good reference strain for group 2 (Indian) of the *L. donovani* complex, readily identifiable by mini-exon PCR, but not good for *L. donovani* as a whole.

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Table 1. *Leishmania donovani* complex strains, codes and typing results for mini-exon (ME) and ITS

| Code | WHO code | Zymodeme MON/LON | Profiles ME | Groups and profiles (a–i) ITS¶ | ITS + ME | All† |
|---|---|---|---|---|---|---|
| 1 | MHOM/TU/1980/IPT-1* | 1/49 | *12* | 4a | 4 | 4 |
| 2 | MHOM/BR/1974/PP75* | 1/49 | *13* | 4a | 4 | 4 |
| 3 | MCER/BR/1981/M6445 | —/— | 15 | 4a | 4 | 4 |
| 4 | MHOM/CN/1980/Strain A | 34/— | *16* | 4a | 4 | 4 |
| 5 | MHOM/CY/1963/L53 | 24§/— | *14* | 4a | 4 | 4 |
| 6 | MHOM/ES/1987/Lombardi | 24§/— | *20* | 4a | 4 | N.D. |
| 7 | MCAN/FR/1982/Pharoah | —/49 | 15 | 4a | 4 | 4 |
| 8 | MHOM/HN/1988/HN122 | —/— | 15 | 4a | 4 | 4 |
| 9 | MHOM/HN/1993/HN336 | —/— | 15 | 4a | 4 | 4 |
| 10 | MHOM/PA/1978/WR285 | —/— | 15 | 4a | 4 | 4 |
| 11 | IARI/PT/1989/IMT171 | 24/— | *19* | 4a | 4 | 4 |
| 12 | MHOM/IN/1980/DD8* | 2/41 | 7 | 1/2/3d | 1/2 | 2 |
| 13 | MHOM/CN/0000/Wangjie-1 | 35/— | 1 | 5/6i | 6 | 6 |
| 14 | MHOM/ET/1967/HU3 (LV9) | 18/46 | *23* | 1/2/3e | 1/2 | 3 |
| 15 | MHOM/ET/0000/Ayele 5 | 31§/52 | *27* | 5/6b | 5 | 5 |
| 16 | MHOM/ET/0000/Hussen | 31§/42 | *22* | 5/6c | 5 | 5 |
| 17 | MHOM/ET/0000/Ayele 8 | —/56 | *2* | 1/2/3a | 1/2 | 1 |
| 18 | MHOM/ET/1972/Gebre 1 | 82/50 | *10* | 1/2/3g | 3 | 3 |
| 19 | MHOM/ET/1982/Bekele | —/42 | *29* | 5/6a | 5 | 5 |
| 20 | MHOM/ET/1984/Addis 142 | —/(56) | *5* | 1/2/3/a | 1/2 | 1 |
| 21 | IMAR/KE/1962/LRC-L57 | 37/44 | *3* | 1/2/3b | 1/2 | 1 |
| 22 | MCAN/KE/0000/D2 | —/45 | 1 | 1/2/3/a | 1/2 | 1 |
| 23 | MHOM/ET/1984/Addis 164 | 83/— | *11* | 5/6f | 5 | 5 |
| 24 | MHOM/KE/0000/Neal-R1 | —/56 | 1 | 1/2/3/a | 1/2 | 1 |
| 25 | MHOM/KE/1967/MRC(L)3 | 37§/44 | 4 | 1/2/3e | 1/2 | 1 |
| 26 | MHOM/KE/1980/Ndandu 4A | —/44 | 1 | 1/2/3/a | 1/2 | 1 |
| 27 | MHOM/KE/1973/MRC74 | 2§/51 | 4 | 1/2/3e | 1/2 | 1 |
| 28 | MHOM/IN/1977/Chowd-X | —/— | 6 | 1/2/3c | 1/2 | N.D. |
| 29 | MHOM/IN/1979/STL1-79 | —/41 | 6 | 1/2/3c | 1/2 | 2 |
| 30 | MHOM/IN/1982/Nandi 1 | —/41 | 6 | 1/2/3c | 1/2 | 2 |
| 31 | MHOM/IN/1982/Patna 1 | —/(41) | 7 | 1/2/3d | 1/2 | 2 |
| 32 | MCAN/IT/1976/DORA | —/50 | *17* | 1/2/3f | 3 | 3 |
| 33 | MHOM/LB/1984/Salti 4 | —/(46) | *21* | 1/2/3g | 3 | 3 |
| 34 | MHOM/SA/1981/Jeddah KA | 31/42 | *9* | 5/6e | 5 | 5 |
| 35 | MHOM/SA/1987/VL6 | —/(42) | *28* | 5/6g | 5 | 5 |
| 36 | MHOM/SA/1987/VL23 | —/(42) | 26 | 5/6h | 5 | 5 |
| 37 | MHOM/SA/1987/VL29 | —/(42) | 26 | 5/6c | 5 | 5 |
| 38 | MHOM/SD/0000/Khartoum | 18/46 | 24 | 1/2/3f | 3 | 3 |
| 39 | MHOM/SD/1985/A22 | —/(48) | *25* | 1/2/3f | 3 | 3 |
| 40 | MHOM/SD/1982/GILANI | 30/48 | 8 | 1/2/3i | 3 | 3 |
| 41 | MHOM/SD/1987/UGX-marrow | 31§/— | *18* | 5/6d | 5 | 5 |
| 42 | MHOM/PT/1992/IMT180 | 18/— | 8 | 1/2/3h | 3 | 3 |

* WHO reference strain.
† Pooled analysis of ITS, mini-exon and gp63 intergenic regions.
§ Strains typed for the first time.
¶ Different profiles for each group are shown as letters.
WHO codes: CSSS/cc/YYYY/N: host(**C**- class; **SSS** – species)/**c**ountry/**Y**ear/strain **N**ame. Host: I, insect; M, mammal; ARI, *Phlebotomus ariasi*; CAN, *Canis familiaris*; CER, *Cerdocyon thous*; HOM, *Homo sapiens*; MAR, *Phlebotomus martini*. Country: BR, Brazil; CN, China; CY, Cyprus; ES, Spain; ET, Ethiopia; FR, France; HN, Honduras; IN, India; IT, Italy; KE, Kenya; LB, Lebanon; PA, Panama; PT, Portugal; SA, Saudi Arabia; SD, Sudan; TU, Tunisia.
N.D., Not done.

Restriction profiles of ITS and mini-exon PCR products distinguished between the *L. donovani* complex, *L. major*, *L. aethiopica* and *L. tropica* as described (Schonian *et al.* 2001). Within the *L. donovani* complex differences in fragment size were more evident with mini-exon than with ITS restriction analysis (Fig. 1). Mini-exon was also more polymorphic than ITS (29 *vs.* 20 different profiles – Table 1). With the exception of *Hae*III the 2 bands of Indian *L. donovani* had indistinguishable mini-exon restriction profiles. In some cases both ITS and mini-exon generated a total fragment size greater than the amplification products, characteristic of multiple alleles. Although it is generally believed

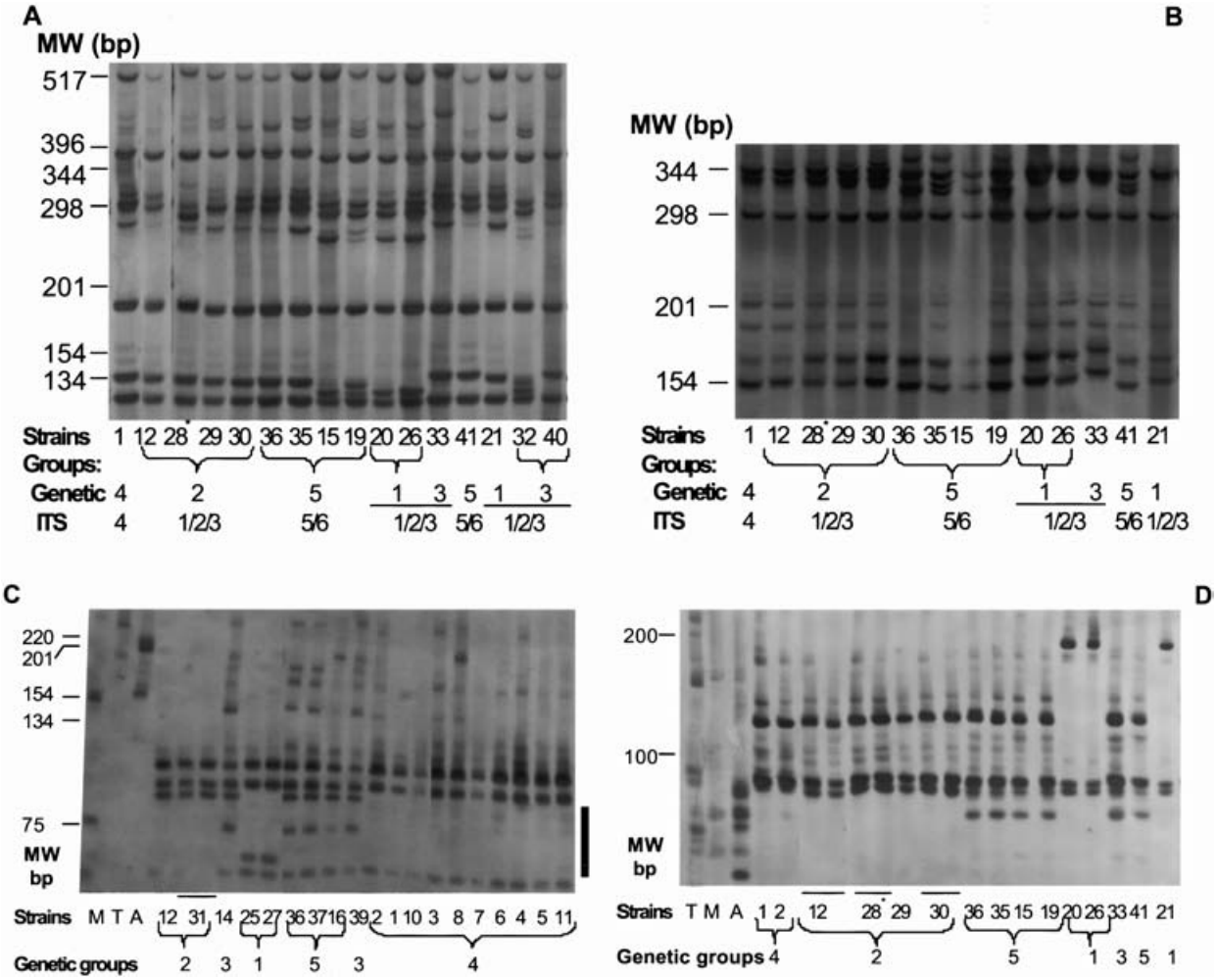


Fig. 1. Gel photos of restriction profiles of ITS (A: *Bst*UI; B: *Taq*I) and mini-exon (C: *Hae*III; D: *Msp*I) for *Leishmania donovani* complex and outgroup strains. Genetic groups (from pooled ITS, mini-exon and gp63 intergenic regions) are shown for each profile. ITS genetic groups are shown for ITS profiles. Lanes are either from the same gel or from simultaneous electrophoreses, with reference strains on each gel. Strains are as in Table 1. Absence of bands in the marked region of C is characteristic of group 4. Strain 28 (marked with *) was included here in genetic group 2 because of its characteristic mini-exon profile (see Table 1).

that multicopy genes become identical in each genome, *L. tropica* ITS and Indian *L. donovani* mini-exon clearly had at least 2 sequence types.

Individual ITS and pooled ITS and mini-exon data produced 2 robust groups of strains (Fig. 2): *L. infantum* and strains of group MIRT5+6 (here ITS 5+6). In the pooled ITS and mini-exon analysis strains of group MIRT3 also formed a robust group (here ITS 3). Group numbering was kept consistent across the different data sets. Strains included in *L. infantum* (MON-30, strain 40) by isoenzyme based phylogenies (Rioux *et al*. 1990) did not cluster with *L. infantum*, nor was MON-82 (strains 18 and 32, *L. archibaldi*) distinct: these 2 zymodemes clustered preferentially with *L. donovani* MON-18 (strains 14, 33, 38, 42). Upon combined analysis of ITS, mini-exon and both gp63 intergenic regions the 6 groups described previously became strongly supported (Fig. 3). Strains with higher than expected total fragment size clustered with the expected group.

Group-specific markers, including for *L. infantum* if defined as group 4, were identified from ITS and mini-exon, particularly using *Bst*UI and *Taq*I for ITS (Fig. 1A, B) and *Hae*III and *Msp*I for mini-exon (Fig. 1C, D). Further details are available upon request.

Neither ITS nor mini-exon produced as many robust groups as gp63 based restriction analyses. ITS has microsatellite regions (El Tai *et al*. 2001), which are prone to reverse mutations and therefore may confuse the phylogenetic signal; mini-exon is very GC rich and has short single-base repeats. The increased number of robust groups when data were pooled suggests that the lower bootstrap support for individual ITS and mini-exon trees is due to the relatively small number of characters. ITS and mini-exon are thus less reliable for *L. donovani* typing than our gp63 based restriction analysis but, because they are multicopy, they may be useful when a suspected VL case is negative by amplification of gp63

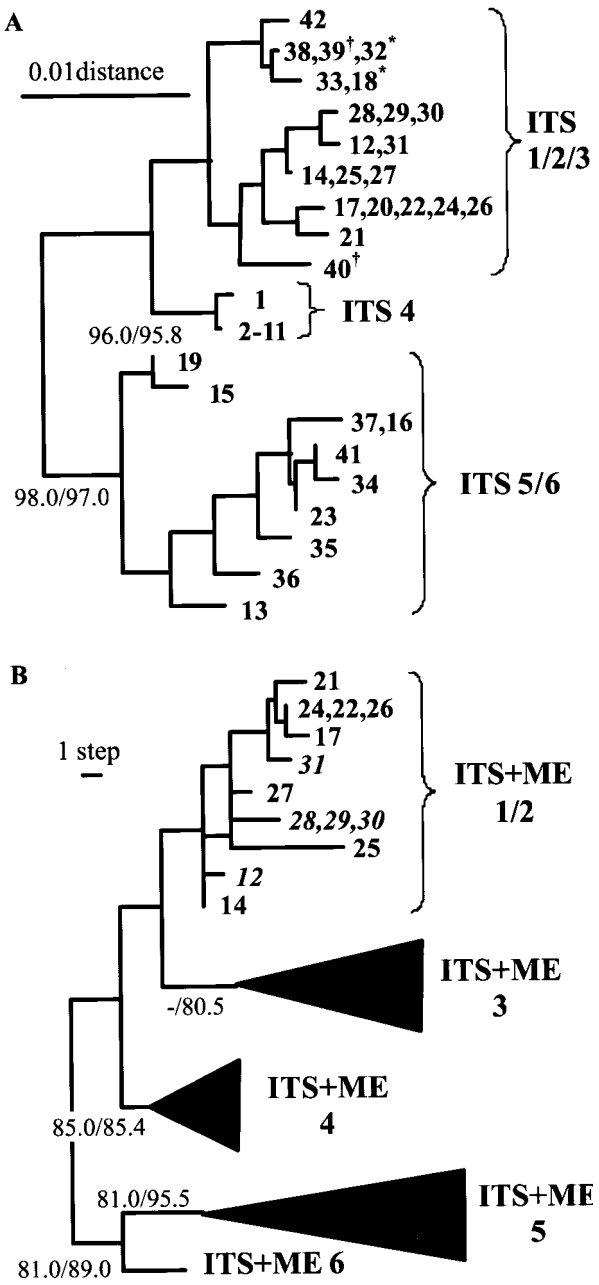


Fig. 2. Analysis of genotypes. (A) Neighbour-joining tree of ITS, showing ITS groups. (B) Maximum parsimony tree of ITS and mini-exon pooled data, showing pooled ITS and mini-exon (ITS+ME) groups. Trees are unrooted and bootstrap values >80% are shown for neighbour-joining and parsimony at each tree.

intergenic regions, and, because they are smaller, when DNA is somewhat degraded.

ITS restriction analysis confirmed that MON-30 strains do not cluster with *L. infantum* (group 4) and the ITS+mini-exon analysis did not support MON-82 as a separate species (*L. archibaldi*). Our results also confirmed that *L. donovani* is not a homogeneous clade. Discrimination of genetic groups within *L. donovani* provides a basis for improved understanding of the epidemiology of visceral leishmaniasis and to extrapolate correctly biological,

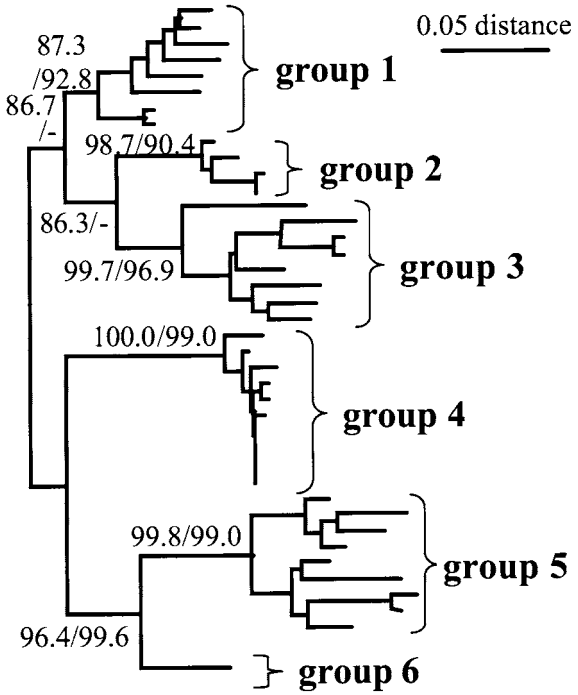


Fig. 3. Neighbour-joining unrooted tree built using pooled ITS, mini-exon and gp63 intergenic region data. Bootstrap values above 75% (1000 replicates) are shown for assigned genetic groups and main nodes. Individual strains are not shown on the tree, but group assignment is shown in Table 1 (under 'All').

clinical or immunological studies done with single strains.

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