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Polymorphism analysis of the internal transcribed spacer and small subunit of ribosomal RNA genes of *Leishmania mexicana*

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Abstract *Leishmania mexicana* causes a wide spectrum of clinical diseases. In spite of the variety of clinical forms, no data exist regarding genetic polymorphism of *L. mexicana*. We analyzed the polymorphism of the internal transcribed spacer (ITS) and the small subunit rRNA genes of 3 reference strains and 24 Mexican isolates of *L. mexicana*, by means of polymerase chain reaction and subsequent digestion by restriction enzymes.

All strains of *L. mexicana* had invariant patterns for both the ITS and the small subunit of rRNA genes. *Leishmania amazonensis* and *Leishmania venezuelensis* displayed polymorphism only in the ITS. The high degree of identity of this region was confirmed by sequencing DNA from three *L. mexicana* isolates. There was almost complete identity of the sequence for the ITS region of *L. venezuelensis* and that of strains of *Leishmania major*, suggesting that these species may be more closely related than previously thought.

The sequences reported in this article are available in GenBank with the accession numbers AF339752, AF339753, AF466380, AF466381, AF466382 and AF466383.

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Introduction

Leishmania mexicana is highly endemic in Mexico and can cause various clinical forms of cutaneous leishmaniasis: localized cutaneous leishmaniasis (LCL), diffuse cutaneous leishmaniasis (DCL), and atypical pseudodiffuse cutaneous leishmaniasis (PDCL). We wanted to determine whether this wide clinical spectrum is associated with genetic variation amongst *L. mexicana* strains isolated from Mexico.

Molecular diversity within *Leishmania* species has been analyzed by several methods (Macedo et al. 1992; Fernandes et al. 1994, 1999; Luis et al. 1998; Mauricio et al. 1999) and comparison of the sequences of ribosomal RNA (rRNA) genes has been valuable in phylogenetic studies (Sogin et al. 1986; Ramirez and Guevara 1987; Hernández et al. 1990; Briones et al. 1992; Fernandes et al. 1993; Landweber and Gilbert 1994; Maslov et al. 1994; Uliana et al. 1994; Cupolillo et al. 1995; Schönian et al. 2000).

There are between 150 and 200 copies of these rRNA genes in the ribosomal genome of *Leishmania* (Villalba and Ramírez 1982). Some segments are never transcribed and other segments have transcripts that are rapidly degraded. These segments have no apparent coding function and can accumulate mutations to evolve rapidly (Long and Dawid 1980).

The structure of a ribosomal gene locus has two regions of DNA that code for the 18S (small subunit; SSU) and 28S (large subunit; LSU) rRNAs. The 5′-end of the region coding for the 18S has a segment called the external transcribed spacer (ETS), which extends from the 5′-end of the 18S sequence to the promoter. The internal transcribed spacer (ITS) is located between the 3′-end of the region coding for the 18S sequence and the 5′-end of the region coding for the 28S sequence (Long and Dawid 1980). This ITS region is again subdivided into the ITS 1 and ITS 2 sequences separated by the intervening, but included, sequence coding for the 5.8S RNA (El Tai et al. 2000). The ITS regions are less

conserved between species than the rRNA genes and are therefore suitable for the characterization of phylogenetically closely related organisms such as different species of the same genus (Hillis and Dixon 1991). In a recent analysis of ITS sequences to explore phylogenetic relationships within the genus *Leishmania*, Dávila and Momen (2000) postulated sufficient variation to permit discrimination between species at the interspecific level. Both the ETS and the ITS, together with the two regions coding for the 18S and the 28S RNAs, are transcribed to make the precursor molecule of rRNA. However, transcripts of ETS and ITS are rapidly degraded, and they seem to have no function in the cell (Sollner-Webb and Tower 1986). The last part of the ribosomal gene is a segment of DNA which includes the region of the promoter up to the 3′-end of the region encoding the 28S RNA, called the non-transcribed spacer (Long and Dawid 1980).

The SSU gene has been sequenced for more than 100 species (Schnare et al. 1986; Sogin et al. 1986; Dams et al. 1988; Looker et al. 1988; Hernández et al. 1990; Briones et al. 1992). Several papers indicate that the SSU rDNA is conserved from a phylogenetic point-of-view in the animal kingdom (Sogin 1994) and also in the Kinetoplastidae (Schnare et al. 1986; Looker et al. 1988; Hernandez et al. 1990; Uliana et al. 1991; Briones et al. 1992; Van Eys et al. 1992). Van Eys et al. (1992) sequenced part of the rDNA of the SSU from nine different species of *Leishmania*, comparing them with those of *Trypanosoma brucei*, *T. cruzi* and *Crithidia fasciculata*. Among the *Leishmania* species, only a few point mutations were found in the central region of the SSU.

On the other hand, Cupolillo et al. (1995) studied the ITS region of the subgenus *Viannia* of New World *Leishmanias* in several isolates of eight different species. Using intergenic region typing in the ITS region, they derived molecular evolutionary trees for the isolates analyzed. This analysis showed that within the genus *Viannia*, the isolates of two species *L. (V.) naiffi* and *L. (V.) braziliensis*, were highly polymorphic, whereas the other species were tightly clustered.

The group of Schönian used the ITS region to determine the polymorphism of numerous clinical samples from *L. donovani* and the genetic variability of strains of the *L. tropica* complex with different PCR-based methods. Although heterogeneity within the ITS sequences was apparent by some methods, no clear correlation could be discerned between the genetic variants and either the geographical origin of the strains or the clinical manifestations of human disease (El Tai et al. 2000; Schönian et al. 2001). In contrast with Cupolillo's findings, amplified ITS regions of different strains of *L. donovani*, *L. infantum* and *L. chagasi* showed no intraspecific variation when digested with ten different restriction enzymes.

We studied both the ITS and the SSU regions of three World Health Organization (WHO) reference strains: *L. mexicana*, *L. venezuelensis* and *L. amazonensis*, as well as 24 Mexican isolates of *L. mexicana* obtained from patients with LCL, DCL and PDCL. We used the polymerase chain reaction (PCR), restriction length polymorphism analysis (RFLP) with different endonucleases, and also DNA sequencing of PCR fragments to measure the extent of variation in different parts of the rDNA loci. Our results show that the ITS region of *L. mexicana* is more similar to that of *L. donovani* than that of *L. (V.) braziliensis*. Surprisingly, the entire sequence for the ITS region determined for *L. venezuelensis* was almost identical to that of several *L. major* strains, which are usually classified among the Old-World species (Lainson and Shaw 1987). The possible significance of this finding is discussed.

Materials and methods

Parasites

The parasites (Mexican isolates) used in this study are listed in Table 1. They were obtained in various states of Mexico, where *Leishmania* is endemic, from patients with LCL, DCL and PDCL. All isolates were typed either by monoclonal antibodies and isoenzymes at the Centro Internacional de Entrenamiento e Investigaciones Médicas (CIDEIM), Cali, Colombia, or in our laboratory using monoclonal antibodies generously donated by Dr. Farrokh Modabber from the Leishmania Vaccine Steering Committee (WHO, Geneva, Switzerland).

Dr. Nancy G. Saravia (CIDEIM) donated reference strains of the New World species *L. mexicana*, *L. amazonensis* and *L. venezuelensis* selected by the WHO (Table 1). All *Leishmania* were cultured in RPMI 1640 supplemented with 5% fetal calf serum at 26°C.

DNA purification

Promastigotes were harvested from mass culture by centrifugation at 6,000 *g* for 10 min, and washed twice in cold phosphate-buffered saline, pH 7.2. The pellets, containing approximately 3–5×10^9 organisms, were lysed in 150 mM Tris, pH 7.5, 50 mM EDTA, pH 8.0, and 1% Sarkosyl at room temperature (1 ml of lysis buffer/10^9 parasites) with gentle shaking. Proteinase K was added to a final concentration of 100 μg ml^{-1} and the lysate incubated at 55°C for 2 h. The DNA solutions were subjected twice to a phenol:chloroform:isoamyl alcohol (25:24:1) extraction followed by ethanol precipitation. The pellet was solubilized in 10 mM Tris, pH 7.5, 1 mM EDTA, pH 8.0. After sequential incubation with RNAse and proteinase K, DNA was extracted with phenol:chloroform:isoamyl alcohol and precipitated with ethanol. The pellet was washed with 75% ethanol, dried and solubilized in ultra pure water. DNA treated in this way was used for PCR. Kinetoplast DNA of several *Leishmania* cultures was purified as described previously (Berzunza-Cruz et al. 2000).

PCR amplification

For PCR amplification of the ITS region we used oligonucleotides IR1 and IR2 designed by Cupolillo et al. (1995). The two ITS sequences located between the SSU and the LSU rRNA genes were amplified with a primer complementary to the conserved sequences from the 3′-end of the SSU rRNA and the 5′-end of the LSU rRNA sequences of *T. brucei*, *T. cruzi* and *C. fasciculata*.

For amplification by PCR of a part of the SSU rRNA genes, we used oligonucleotides S1 and S5 designed by Uliana et al. (1991). S1 consisted of the first 20 nucleotides on the 5′-end and S5 corresponded to a 17-base oligonucleotide complementary to a sequence

Table 1. Mexican isolates of *Leishmania mexicana* used in this study

L. mexicana isolate	Geographic area	International code
GS	Tabasco-Mexico	MHOM/MX/84/SET GS
FD	Tabasco-Mexico	MHOM/MX/93/INDRE FD
MC	Tabasco-Mexico	MHOM/MX/88/HRC MC
RHA	Tabasco-Mexico	MHOM/MX/93/INDRE RHA
JS	Tabasco-Mexico	MHOM/MX/88/HRC JS
RC	Tabasco-Mexico	MHOM/MX/94/INDRE RC
AMC	Tabasco-Mexico	MHOM/MX/94/INDRE AMC
JV	Tabasco-Mexico	MHOM/MX/94/INDRE JV
NBO	Tabasco-Mexico	MHOM/MX/94/INDRE NBO
MOD	Tabasco-Mexico	MHOM/MX/94/INDRE MOD
CGM	Tabasco-Mexico	MHOM/MX/94/INDRE CGM
GBB	Campeche-Mexico	MHOM/MX/98/UAC GBB
527	Campeche-Mexico	MHOM/MX/92/UADY 527
68	Campeche-Mexico	MHOM/MX/92/UADY 68
250	Campeche-Mexico	MHOM/MX/93/UADY 250
815	Campeche-Mexico	MHOM/MX/96/UADY 815
RR	Campeche-Mexico	MHOM/MX/98/UNAM RR
CV	Quint. Roo-Mexico	MHOM/MX/83/UADY CV
CTC	Quint. Roo-Mexico	MHOM/MX/94/INDRE CTC
AM	Veracruz-Mexico	MHOM/MX/92/INDRE AM
AG	Veracruz-Mexico	MHOM/MX/92/INDRE AG
HF	Veracruz-Mexico	MHOM/MX/85/ISET HF
RMH	Chiapas-Mexico	MHOM/MX/93/INDRE RMH
NAY8	Nayarit-Mexico	MHOM/MX/96/INDRE NAY8
Reference strains	Source	
L. mexicana	CIDEIM-Colombia	MNYC/BZ/62/M379
L. venezuelensis	CIDEIM-Colombia	MHOM/VE/80/PMH3
L. amazonensis	CIDEIM-Colombia	IFLA/BR/67/PH8

located 780 base pairs (bp) downstream from the 5′-end of the 18S rRNA sequence of *L. donovani* (see Looker et al. 1988).

The PCR amplification of both regions was performed in 100 μl of the following buffer: 20 mM Tris-HCl, pH 8.4, 50 mM KCl; 1.5 mM $MgCl_2$; 125 μM of each dATP, dCTP, dTTP and dGTP; 200 ng of each primer; 10 ng of genomic DNA and 2.5 U of *Taq* DNA polymerase (Gibco BRL).

Amplification was performed using a Perkin-Elmer thermocycler 2400 with different conditions according to the oligonucleotides used: for IR1 and IR2, 30 cycles of 1 min at 94°C, 1 min at 58°C and 2 min at 72°C; and for S1 and S5, 30 cycles of 1 min at 94°C, 1 min at 38°C and 2 min at 72°C.

Restriction analysis

PCR products were extracted with the CONCERT rapid PCR purification system (Gibco BRL), following the manufacturer's instructions. DNA recovery and PCR efficiency were analyzed by electrophoresis in 1.5% agarose gels. Approximately 100 ng of DNA amplified by PCR of the ITS or the SSU were digested with 1 of 21 restriction enzymes. Digestion products of the ITS were observed with the following enzymes: *Alu*I, *Cfo*I, *Eco*RI, *Hae*III, *Hpa*II, *Rsa*I, *Mbo*I, and *Tha*I (Fig. 1A, B), and the SSU was fragmented by *Alu*I, *Cfo*I, *Dra*I, *Hae*III, *Hinc*II, *Hpa*II, and *Tha*I (Table 2 and Fig. 2). Digestion products were analyzed by electrophoresis in 2% agarose.

The restriction patterns of kinetoplast DNA with the enzymes *Hae*III and *Alu*I were analyzed as described (Berzunza-Cruz et al. 2000).

DNA sequencing

The ITS regions of all reference strains and the three isolates GS, NBO, and RR (Table 1) were sequenced. The amplified PCR products were sequenced using an Applied Biosystems automatic sequencer at the Molecular Biology Unit of the Instituto de Fisiología Celular, U.N.A.M. The corresponding GenBank accession numbers are AF339752, AF339753, AF466380, AF466381, AF466382, and AF466383.

Data analysis

Identity of the sequences was compared using the FASTA software (Pearson and Lipman 1988) and multiple alignment was performed with the ALIGN Program (Pearson et al. 1997). Phylograms were constructed using neighbor-joining analysis of 51 *Leishmania* ITS rDNA sequences using version 2.1 of the MEGA 2 (molecular evolutionary genetic analysis) software (Kumar et al. 2001 and see Fig. 3).

Results

PCR amplification of the ITS in the three reference *Leishmania* strains and all the Mexican isolates of *L. mexicana* were analyzed with 21 restriction enzymes. Digestion products were detected with eight of these enzymes (data not shown). Each of the reference strains of *L. amazonensis*, *L. venezuelensis* and *L. mexicana* showed a different digestion pattern whereas all the Mexican isolates of *L. mexicana* showed an identical fragment pattern profile to the reference strain of *L. mexicana* (Fig. 1A, B). PCR amplification of the SSU in the three reference strains and all the isolates of *L. mexicana* were analyzed with the same 21 restriction enzymes used for the ITS region. Only seven restriction enzymes gave a digestion pattern in the SSU region (Table 2). All the SSU products gave the same identical restriction pattern with each of the seven enzymes tested (Fig. 2).

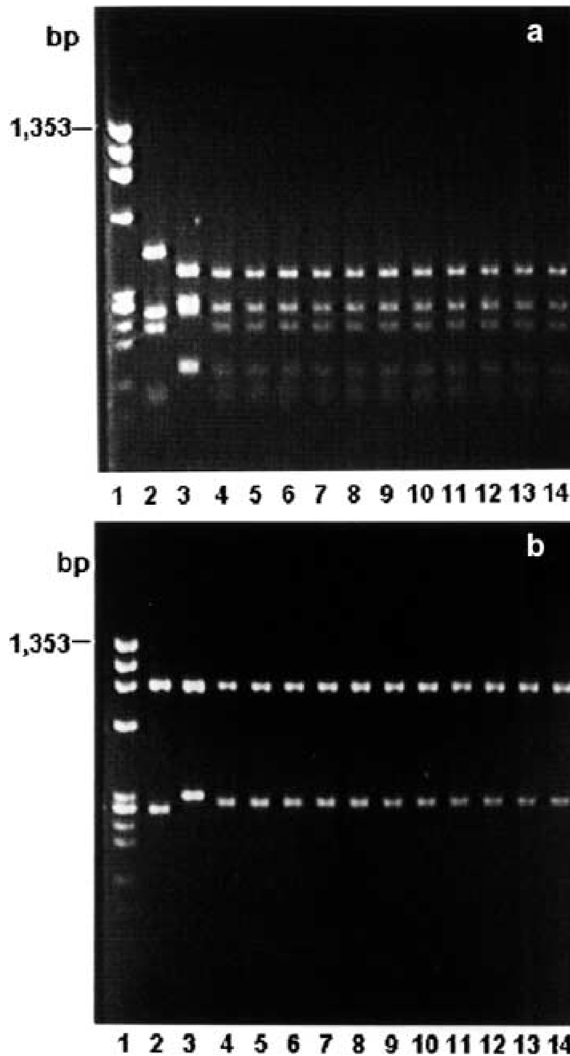

Fig. 1a, b. Restriction patterns of the amplified internal transcribed spacer (ITS) region of different *Leishmania* reference strains and Mexican isolates of *L. mexicana*. **a** *Cfo*I. Lanes: *1* Markers (ΦX174 RF DNA/*Hae*III); *2 L. amazonensis* (PH8); *3 L. venezuelensis* (PMH3); *4 L. mexicana* (M379); *5* GS, *L. mexicana* (DCL); *6* AG, *L. mexicana* (DCL); *7* AM, *L. mexicana* (DCL); *8* MC, *L. mexicana* (LCL); *9* AMC, *L. mexicana* (PDCL); *10* 815, *L. mexicana* (LCL); *11* 250, *L. mexicana* (LCL); *12* CTC, *L. mexicana* (LCL); *13* NAY8, *L. mexicana* (LCL); *14* RMH, *L. mexicana* (LCL). **b** *Eco*RI. Lanes: *1* Markers (ΦX174 RF DNA/*Hae* III); *2 L. amazonensis* (PH8); *3 L. venezuelensis* (PMH3); *4 L. mexicana* (M379); *5* GS, *L. mexicana* (DCL), *6* AG, *L. mexicana* (DCL); *7* AM, *L. mexicana* (DCL); *8* MC, *L. mexicana* (LCL), *9* AMC, *L. mexicana* (PDCL); *10* 815, *L. mexicana* (LCL), *11* 250, *L. mexicana* (LCL); *12* CTC, *L. mexicana* (LCL); *13* NAY8, *L. mexicana* (LCL); *14* RMH, *L. mexicana* (LCL)

The sequence comparison of the complete ITS region of the GS, NBO, and RR isolates gave identities between 99.8 and 100% (Table 3). These isolates were 99.7% identical to the reference *L. mexicana* strain and 94.3% and 81.5% to the reference *L. amazonenzis* and *L. venezuelensis* strains, respectively. Sequence analysis of the ITS regions accurately predicted all of the observed endonuclease restriction fragments (data not shown).

Table 2. Digestion of the small subunit (SSU) region with restriction endonucleases. The first column indicates the restriction enzymes used in this study. The second, third and fourth columns show the number of fragments and the sum of their size in bp for *L. mexicana* and its isolates, *L. amazonensis* and *L. venezuelensis*, respectively

Enzyme	*L. mexicana* (M379) and Mexican isolates of *L. mexicana*	*L. amazonensis* (PH8)	*L. venezuelensis* (PMH3)
	Fragments/bp	Fragments/bp	Fragments/bp
*Hpa*II	4/850	4/850	4/850
*Alu*I	3/515	3/515	3/515
*Cfo*I	3/860	3/860	3/860
*Tha*I	3/810	3/810	3/810
*Dra*I	2/810	2/810	2/810
*Hinc*II	2/810	2/810	2/810
*Hae*III	1/400	1/400	1/400

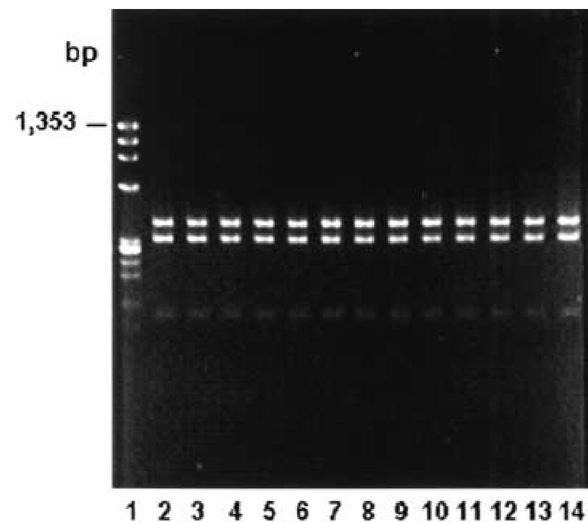

Fig. 2. *Cfo*I restriction patterns of the amplified small subunit (SSU) region of different *Leishmania* strains. Lanes: *1* Markers (ΦX174 RF DNA/*Hae*III); *2 L. amazonensis* (PH8); *3 L. venezuelensis* (PMH3); *4 L. mexicana* (M379); *5* GS, *L. mexicana* (DCL); *6* AG, *L. mexicana* (DCL); *7* AM, *L. mexicana* (DCL); *8* MC, *L. mexicana* (LCL); *9* AMC, *L. mexicana* (PDCL); *10* 815, *L. mexicana* (LCL); *11* 250, *L. mexicana* (LCL); *12* CTC, *L. mexicana* (LCL); *13* NAY8, *L. mexicana* (LCL); *14* RMH, *L. mexicana* (LCL)

The ITS sequences obtained for the GS, NBO, RR isolates, and the reference *L. amazonensis*, *L. mexicana* and *L. venezuelensis* strains were aligned with all other available sequences for *Leishmania* of the ITS1, 5.8S rRNA gene and ITS2 regions using the ALIGN software with default parameters (Pearson et al. 1997). Interestingly, when analyzed with the FASTA program (Pearson and Lipman 1988), the sequence of the ITS region of *L. venezuelensis* has 99.214, 99.269, 94.789 and 94.636% identity with the sequence of the same region reported for *L. major* strains Friedlin, NLB089A, Vin and 5ASKH, respectively. In a phylogram generated with MEGA 2 software (Kumar et al. 2001) the ITS

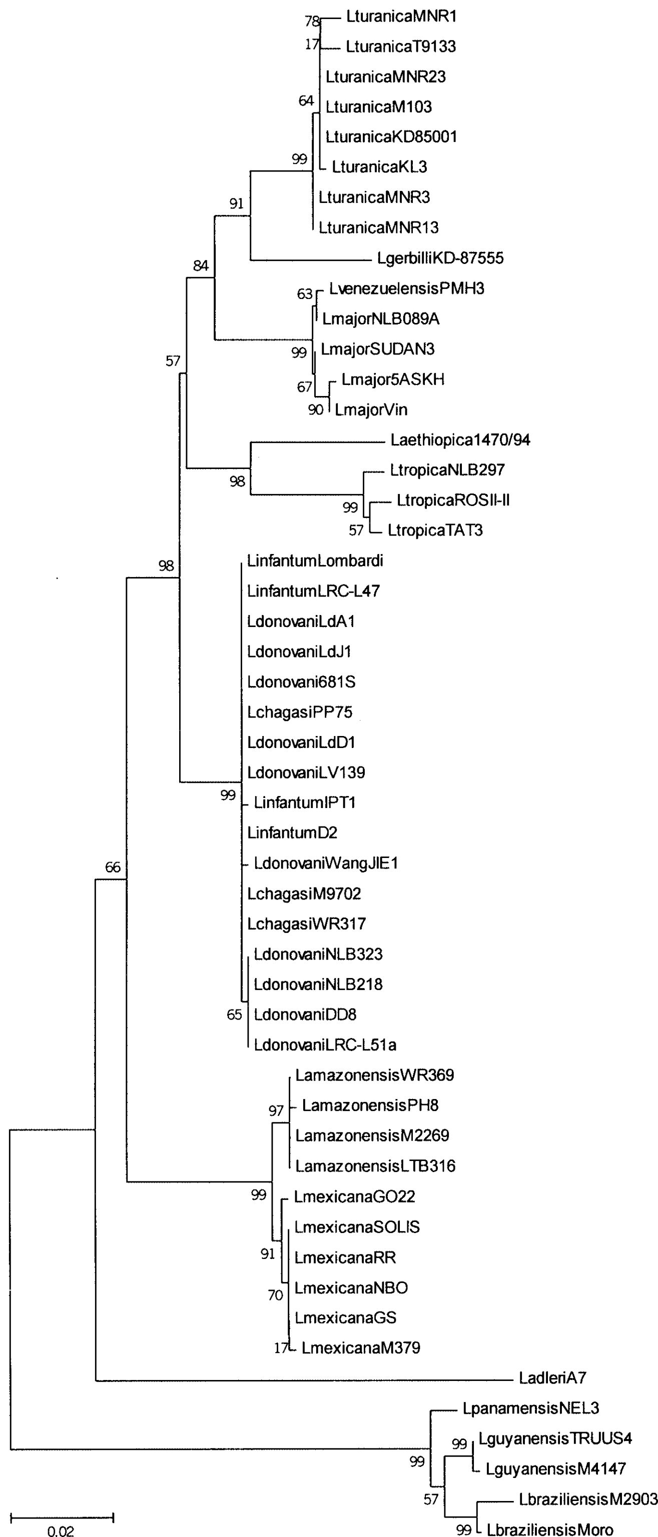

Fig. 3. Phylogram of 51 *Leishmania* ITS rRNA sequences. The unrooted tree was constructed using Version 2.1 of the MEGA 2 software (Kumar et al. 2001) with the "neighbor joining" option selected and with the Kimura-2-parameter model of substitution. For gaps and missing data, both complete- and pairwise-deletion were tested. The number of bootstraps replicates was 1,000

sequence of *L. venezuelensis* clustered with the sequences of *L. major* rather than with those of *L. mexicana* (see Fig. 3 and Discussion).

The identity of the strain of *L. venezuelensis* was ascertained by restriction endonuclease analysis of kinetoplast DNA using different enzymes. Digestion with *Hae*III and particularly with *Alu*I produced patterns that clearly distinguished *L. venezuelensis* from both *L. major* and *L. mexicana* (Fig. 4A, B).

Discussion

We analyzed two regions of the rRNA genes of Mexican isolates of *L. mexicana* as well as of WHO reference strains *L. mexicana, L. amazonensis* and *L. venezuelensis*. These three species are supposed to be closely related, and together with *L. pifanoi*, *L. aristidesi*, *L. garnhami* and *L. forattinii* have been classified as a distinct group sometimes known as the *L. mexicana* complex. We were interested in analyzing heterogeneity in a relatively conserved region of the DNA in order to establish whether the different clinical manifestations observed in patients infected in Mexico with *L. mexicana* could be correlated with polymorphism within the *L. mexicana* species. Additionally, we were interested in analyzing whether *L. mexicana* showed polymorphism in a very conserved region of the rDNA, as Cupolillo et al. (1995) described for two species of the subgenus *Viannia*. These investigators found that *L. (V.) naiffi* and *L. (V.) braziliensis* were highly polymorphic in the ITS region, in contrast to *L. (V.) panamensis* isolates, which showed no polymorphism in this region. In accordance with this latter observation, Schönian and her group also showed that strains of both the *L. donovani* and the *L. tropica* complexes had some variability within the ITS region but when PCR-amplified ITS products were subjected to restriction analysis no polymorphism could be detected (El Tai et al. 2000; Schönian et al. 2001).

No data exist on the degree of polymorphism within the *L. mexicana* complex. Our PCR product digestion of the rDNA ITS region with 21 endonucleases showed that all the strains of *L. mexicana*, including the WHO reference strain, had an identical pattern (like the *L. donovani* and the *L. tropica* complexes mentioned above). This pattern differed completely from the patterns of the other two closely related species examined: *L. amazonensis* and *L. venezuelensis*. Thus, we observed three digestion patterns with eight different enzymes: one corresponding exclusively to the *L. mexicana* strains, a second to *L. amazonensis* and a third to *L. venezuelensis* (Fig. 1).

The analysis of the rDNA SSU region of the 3 WHO reference strains as well as of the 24 Mexican isolates showed no polymorphism with the same endonucleases tested. Different enzymes produced fragments of various sizes but these were always identical in each of the strains tested (Fig. 2; Table 2).

Table 3. Percent identity between sequences of the internal transcribed spacer (ITS) region between *L. mexicana* reference strains and three Mexican isolates

	L. amazonen1zis (PH8)	*L. venezuelensis* (PMH3)	*L. mexicana* (M379)	*L. mexicana* isolate GS	*L. mexicana* isolate NBO	*L. mexicana* isolate RR
PH8	100	80.6	94.3	94.3	94.2	94.3
PMH3		100	81.3	81.5	81.4	81.5
M379			100	99.8	99.6	99.8
GS				100	99.8	100
NBO					100	99.8
RR						100

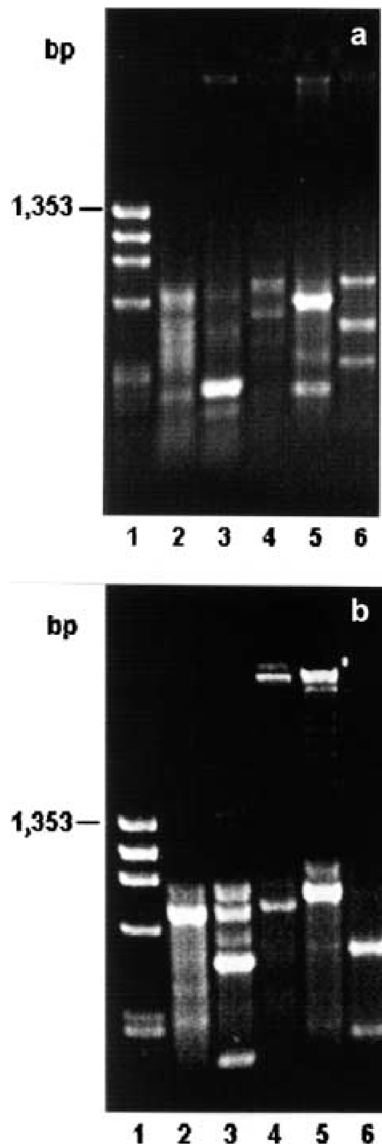

Fig. 4. Restriction patterns of kinetoplast DNA of *Leishmanias* from different species. **a** *Hae* III. Lanes: *1* Markers (ΦX174 RF DNA/*Hae*III); *2 L. major* (5ASKH); *3 L. venezuelensis* (PMH3); *4 L. amazonensis* (PH8); *5 L. mexicana* (M379); *6* GS, *L. mexicana* (SOLIS). **b** *Alu* I. Lanes: *1* Markers (ΦX174 RF DNA/*Hae*III); *2 L. major* (5ASKH); *3 L. venezuelensis* (PMH3); *4 L. amazonensis* (PH8); *5 L. mexicana* (M379); *6* GS, *L. mexicana* (SOLIS)

To our knowledge, no previous data on the degree of polymorphism within the *L. mexicana* complex have been published. Our results show that there are important differences between the three species examined, similar to those between species of the subgenus *Viannia*. The polymorphism in the ITS region and the absence of polymorphism within the SSU region between the three species of the subgenus *Leishmania*, are strikingly opposed to the greater polymorphism found within *L. (V.) naiffi* and *L. (V.) braziliensis* of the subgenus *Viannia*, but in agreement with the findings for *L. (V.) panamensis*, *L. donovani* and *L. tropica*.

When the identity of the ITS sequences was initially analyzed, the isolates had the highest percentages between themselves and had lower values when compared with the reference strains, just as expected (Table 3). A surprising result was obtained in initial alignments of ten sequences of the ITS region for strains of the *L. mexicana* complex (GS, RR, SOLIS, NBO, M379, GO22, M2269, WR369, LTB316 and PMH3). In phylograms generated from these alignments, *L. venezuelensis* strain PMH3 always clustered independently of the other nine strains (data not shown). As already mentioned, FASTA analysis of this last sequence revealed virtually 100% identity with the sequence for the complete ITS region of several strains of *L. major*.

Dávila and Momen (2000) used 36 sequences of the ITS region to explore phylogenetic relationships within *Leishmania*. In view of the complete identity between the sequences of *L. venezuelensis* and *L. major*, it was interesting to reanalyze the phylogeny proposed by these authors with these new data and 15 new sequences available in GenBank. Our analysis confirms previous observations made by these authors including the clustering of *L. infantum*, *L. chagasi* and *L. donovani*, the separate clusters of *L. tropica* and *L. aethiopica* and *L. major*, and the clade containing *L. mexicana* and *L. amazonensis* (Dávila and Momen 2000). All sequences reported in this work clustered exactly where expected, with the exception of *L. venezuelensis*, which appears in the *L. major* branch (Fig. 3). The possibility that a strain of *L. major* had mistakenly been confused with *L. venezuelensis* or that one species was cross-contaminated with the other is unlikely, in view of the distinct patterns obtained by re-

striction analysis of kinetoplast DNA of both species (Fig. 4 and Momen et al. 1985).

L. venezuelensis has always been classified with the *L. mexicana* complex. This classification has been based on the clinical manifestations of the disease (it seems to produce only LCL), characteristics of cultivation in blood-agar medium, monoclonal antibody reactivity and zymodemes (Lainson and Shaw 1987). Almost no data on DNA analysis of this strain exist, and the sequence for the ITS region is the first determined for this species of *Leishmania*. Thus, the very strong relationship with *L. major* is remarkable.

It is widely recognized that *L. major* and *L. mexicana* share many features and can be easily mistaken for one another (Lainson 1983). There are also several reports of *L. major*-like parasites in Brazil (Momen et al. 1985), Ecuador, Venezuela (Hashiguchi et al. 1991) and Paraguay (Yamasaki et al. 1994).

The very high degree of conservation of one of the more variable parts of the rRNA between *L. venezuelensis* and *L. major*, together with their widely different distribution, leads to the speculation that perhaps this parasite was recently introduced into the New World in an analogous manner to *L. infantum* (Gardener 1977; Killick-Kendrick et al. 1980). It is clear that more information at all levels, but particularly at the level of DNA sequences, is needed to confirm or modify the classification of *L. venezuelensis* within the *L. mexicana* complex.

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