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Intra- and interspecific polymorphisms of *Leishmania donovani* and *L. tropica* minicircle DNA

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Abstract A pair of degenerate polymerase chain reaction (PCR) primers (LEI-1, TCG GAT CC[C,T] [G,C]TG GGT AGG GGC GT; LEI-2, ACG GAT CC[G,C] [G,C][A,C]C TAT [A,T]TT ACA CC) defining a 0.15-kb segment of *Leishmania* minicircle DNA was constructed. These primers amplified not only inter- but also intraspecifically polymorphic sequences. Individual sequences revealed a higher intraspecific than interspecific divergence. It is concluded that individual sequences are of limited relevance for species determination. In contrast, when a data base of 19 different sequences was analyzed in a dendrographic plot, an accurate species differentiation was feasible.

Introduction

Traditionally, *Leishmania tropica* is considered to cause cutaneous leishmaniasis, whereas the visceral type is believed to result from an infection with *L. donovani*. Recently, however, increasing evidence suggests that *L. tropica*, too, can lead to visceral leishmaniasis (Kreutzer et al. 1993; Magill et al. 1993). Thus, it appears desirable to discriminate between the two species not only by their clinical manifestations but also on a genomic level.

In an effort to achieve such discrimination, kinetoplast minicircle DNA was selected as a target sequence for polymerase chain reaction (PCR) amplification. Minicircles are present in each cell in multiple copies (Lee et al. 1993), thus leading to a higher sensitivity, which is often required for detection of *Leishmania* in clinical specimens. Kinetoplast minicircle DNA of *Trypanosoma* is known to be polymorphic (Rogers and Wirth 1988; Stuart 1983); however, especially in *Leishmania*, the extent on the nucleotide levels is poorly described. Except for *L. mexicana amazonensis* (Rogers and Wirth 1988; Lee et al. 1993) and *L. tarentolae* (Kidane et al. 1984), published sequences of *Leishmania* minicircle DNA report only single examples for each species (Barker et al. 1986; Smith et al. 1989; Laskay et al. 1991; Blackwell 1992; de Bruijn and Barker 1992).

The finding that intraspecific heterogeneity, even within a single isolate, can exceed interspecific heterogeneity necessitated that an approach be found that is not dependent on single sequences. In this report a data-base approach to species differentiation is presented.

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Materials and methods

DNA extraction

Isolates from patients were available either as promastigote cell cultures cryoconserved in liquid nitrogen or in the amastigote form in hamster spleen cells stored at –70° C in dimethylsulfoxide (DMSO). Diagnoses of five *Leishmania donovani* (D1-D5) and six *L. tropica* (T1-T6) infections were based on clinical symptoms and travel history. The *L. mexicana* strain LV4 was kindly provided by W. Solbach, Erlangen, and is referred to as M1. All isolates were processed by pelleting 400 µl of the cell suspension, resuspending the pellet in 400 µl TET buffer [50 m*M* TRIS-HCl (pH=8.0), 25 m*M* ethylenediaminetetraacetic acid (EDTA), 100 m*M* NaCl, 0.4% Triton X-100], and adding 4 µl (20 mg/ml) proteinase K (Appligene, Illkirch, France). After incubation at 50° C for 90 min followed by a centrifugation step, the DNA was purified from the supernatant by silica-gel adsorption (Geneclean; BIO 101, La Jolla) and eluted in 100 µl water.

PCR amplification

Primers LEI-1 and LEI-2 were obtained from Genzentrum (Martinsried, Germany). PCR amplifications were performed employing a "hot-start" technique in which 38.2 µl DNA solution and 1 µl each of both primers (50 µ*M* each) were overlaid with two drops of mineral oil (Sigma) and denatured at 96° C for 2 min. After cooling of the solution to 80° C, a mixture of 5 µl 10x buffer [100 m*M* TRIS-HCl (pH=8.3), 500 m*M* KCl], 1.5 µl 50 m*M* $MgCl_2$, 0.5 µl deoxynucleotide triphosphate (dNTP) mix (25 m*M* each, United States Biochemical Corporation, Cleveland), and 0.3 µl (8 u/µl) *Taq* polymerase (United States Biochemical Corporation, Cleveland) was added. A total of 35 cycles of denaturing at 92° C for 60 s, annealing at 53° C for 60 s, and extension at 72° C for 90 s were performed. Reaction products were visualized in a 1.3% agarose gel containing 0.2 µg ethidium bromide/ml.

Table 1 Amplified sequences obtained with primers LEI-1 and LEI-2

D1X1	TCTGCAAAATCGG-AAAAATGGGTGCAGAAATCCCGTTCAAAAATCGGCC AAAAAT--GCCAAAAATCAGCTCCGGGGCGGGAAACTGGGGGTT	91
D1X4	TCTGCGAAAACCG-AAAAATGGGTGCAGAAATCCCGTTCAAAAATCGGCC AAAAAT--GCCAAAAATGGGCTCCGGGGCGGGAAACTGGGGGTT	91
D1X10	TCTGCGAAAACCG-AAAAATGGGTGCAGAAATCCCGTTCAAAAATCGGCC AAAAAT--GCCAAAAATGGCCTCCGGGGCGGGAAACTGGGGGTT	91
D1X8	TCTGCGAAAATCG-AAAAATGGGTGCAGAAATCCCGTTCAAAAATCGGCC AAAAAT--GCCAAAAATGGCCTCCGGGGCGGGAAACTGGGGGTT	91
D1X7	TCTGCGAAAATCG-AAAAATGGGTGCAGAAATCCCGTTCAAAAAAAGGCC AAAAAT--GCCAAAAATCGGCTCCGGGGCGGGAAACTGGGGGTT	91
D5X1	TCTGCGAAAAGCG-AAAAATGGGTGCAGAAATCCCGTTCAAAAATCGGCC AAAAAT--GCCAAAAATCGGGTCCGGGGCGGGAAACTGGGGGTT	91
D1X5	TCTGCGAAAACCG-AAAAATGGGTGCAGAAATCCCGTTCAAAAAATGCCC AAAAAT--GCCAAAAATCGGGTCCGAGGCGGGAAACTGGGGGTT	91
D2X1	TCTGCGAAAATCG-AAAAATGGGTGCAGAAATCCCGTTCAAAAAATGCCC AAAAAT--GCCAAAAATCGGGTCCGAGGCGGGAAACTGGGGGTT	91
D1X9	TCTGCGAAAACCG-AAAAATGGGTGCAGAAATCCCGTTCAAAAATCGGCC AAAAAT--GCCAAAAATCGGGTCGGAGGCGGGAAACTGGGGGTT	91
D3X1	TCTGCGAAAACCG-AAAAATGGGTGCAGAAATCCCGTTCAAAAAATGCCC AAAAAT--GCCAAAAATCGGGTTCGGGGCGGGAAACTGGGGGTT	91
D4X1	TCTGCGAAAACCG-AAAAATGGGTGCAGAAATCCCGTTCAAAAAATGCCC AAAAAT--GCCAAAAATCGGCTCCGGGGCGGGAAACTGGGGGTT	91
D2X2	TCTGCGAAAATCG-AAAAATGGGTGCAGAAATCCCGTTCAAAAAACGGTC AAAAAT--GCCAAAAATCGGCTCGGAGGCGGGAAACTGGGGGTT	91
D1X2	TCTGCGAAATCGG-AAAAATGGGTGCAGAAATCCCGTTCATTTTTTGGCC AAAATT--GCCATTTTTGGGCTCCGGGGCGGGAAACTGGGGGTT	91
D1X3	TCTGCGAAATCGG-AAAAATGGGTGCAGAAATCCCGTTCATTTTTTGGCC AAAATT--GCCATTTTTGGGCTCCGGGGCGGGAAACTGGGGGTT	91
D1X6	TCTGCGAAATCGG-AAAAATGGGTGCAGAAATCCCGTTCATTTTTTGGCC AAAATT--GCCATTTTTGGGCTCCGGGGCGGGAAACTGGGGGTT	91
T1X1	TCTGCGAAAATCG-AAAAATGGGTGCAGAAATCCCGTTCATTTTTGGCCG AAAATT--GGCATTTTTGGGCTCGGAGGCGGGAAACTAGGGGTT	91
T1X4	TCTGCGAAAAGCG-AAAAATGGGTGCAGAAATCCCGTTCATTTTTGGCCG AAAATT--GGCATTTTTGGGCTCGGAGGCGGGAAACTAGGGGTT	91
T2X1	TCTGCGAAATTCG-AAAAATGGGTGCAGAAACCCCGTTCATTTTTGGCCG AAAATT--GCCATTTTTGGGCTCCGGGGCGGGAAACTAGGGGTT	91
T3X1	TCTGCGAAAATCG-AAAAATGGGTGCAGAAACCCCGTTCATTTTTGGTCG AAAAAC--GCCATTTTTGGGCTCGAGGGCGGGAAACTAGGGGTT	91
T1X2	TCTGCGAAAATCG-AAAAATGGGTGCAGAAATCCCGTTCATATTTTCCCG GAAAAT--GCCATTTTTGGGCTCCGGGGCGGGAAACTAGGGGTT	91
T1X3	TCTGCGAAAAGCG-AAAAATGGGTGCAGAAATCCCGTTCATTTTTGGCCG GAAAAT--GCCATTTTTGGGCTCGGAGGCGGGAAACTAGGGGTT	91
T3X2	TCTGCGAAAACCG-AAAAATGGGTGCAGAAATCCCGTTCATTTTTGGCCG GAAAAT--GCCATTTTTGGGCTCGGAGGCGGGAAACTAGGGGTT	91
T4X1	TCTGCGAAAACCG-AAAAATGGGTGCAGAAATCCCGTTCATTTTTGGCCG GAAAAT--GCCATTTTTGGGCTCGGAGGCGGGAAACTAGGGGTT	91
T2X2	TCTGCGAAAACCG-AAAAATGGGTGCAGAAATCCCGTTCATTTTTGGCCG GAAAAT--GGCATTTTTGGGCTCGAGGGCGGGAAACTAGGGGTT	91
T5X1	TCTGCGAAAACCG-AAAAATGGGTGCAGAAATCCCGTTCATTTTTGGCCG GAAAAT--GCCATTTTTGGGCTCCGGGGCGGGAAACTAGGGGTT	91
T4X2	TCTGCGAAAACCG-AAAAATGGGTGCAGAAATCCCGTTCATAATTTGGCG AAAAAT--GCCATTTTTGGGGTCGGAGGCGGGAAACTAGGGGTT	91
T6X1	TCTGCGAAATTCG-AAAAATGGGTGCAGAAATCCCGTTCATAATTTGGCG AAAAAT--GCCATTTTTGGCCTCGAGGGTGGGAAACTAGGGGTT	91
M1X1	TCTGCGGAAACCGGAAAAATGAGCGCAGAAACCCCGTTCATAATTTGGGG GAAATTCGGCCGAAAACAG-CTCGGGGCGGGGAAACTGGGGGTT	93
M1X2	TCTGCGGAAACCGGAAAAATGAGTGCAGAAACCCCGTTCATAATTTGGGG GAAATTCGGCCGAAAACAG-CTCGGGGCGGGGAAACTGGGGGTT	93
M1X3	TCTGCGGAAACCGGAAAAATGAGTGCAGAAACCCCGTTCATAATTTGGGG GAAATTCGGCCGAAAACAG-CTCGGGGCGGGGAAACTGGGGGTT	93
M1X4	TCTGCGGAAACCGGAAAAATGAGTGCAGAAACCCCGTTCATAATTTGGGG GAAATTCGGCCGAAAACAG-CTCGGGGCGGGGAAACTGGGGGTT	93
M1X5	TCTGCGGGGAGGGCAAAAATGAGTGCAGAAACCCCGTTCATAATTTGGGG GAAATTCGGCCGAAAACAG-CTCGGGGCGGGGAAACTGGGGGTT	93

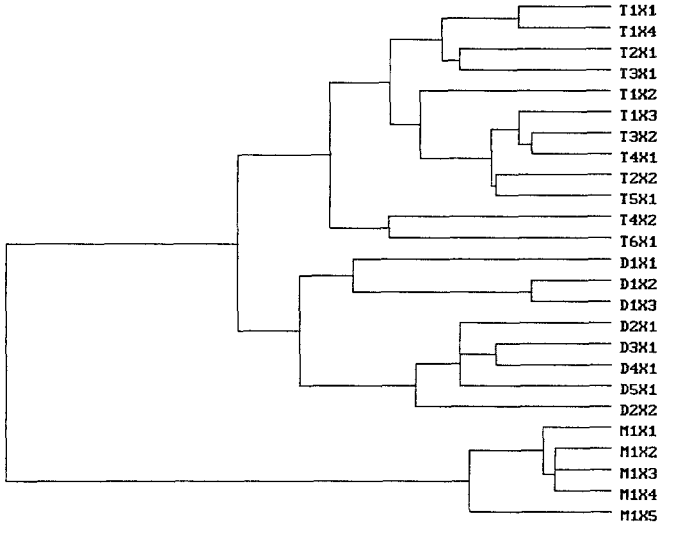

Fig. 1 Dendrogram of the amplified sequences. Sequences starting with *D, T,* and *M* correspond to *Leishmania donovani, L. tropica,* and *L. mexicana,* respectively

Cloning and sequencing

PCR products (0.15 kb) were excised from the agarose gel and the DNA purified by silica-gel adsorption (QIAEX; Qiagen Corp., Chatsworth). Using the flanking *BamHI* sites of LEI-1 and LEI-2, the products were ligated into *BamHI*-cut pBluescript II HSK-vectors (Stratagene, La Jolla) and used to transform XL1-Blue cells (Stratagene, La Jolla) by electroporation. Sequencing was performed according to Sanger et al. (1977) using a Sequenase 2.0 kit (United States Biochemical Corporation, Cleveland).

Sequence analysis

Sequence comparisons and the dendrographic plot were obtained using the program CLUSTAL in the PC/GENE software package (IntelliGenetics, Mountain View) employing a method developed by Higgins and Sharp (1988). Computation parameters were set to a K-tuple value of 5, a gap penalty of 5, a window size of 10, and a filtering level of 2.5. All sequences described originated from separate PCR amplifications.

Results

Leishmania kinetoplast minicircle DNA sequences amplified with the primers LEI-1 and LEI-2 are listed in Ta-

Table 2 Number of identical amplification products produced by multiple PCR on single isolates of *Leishmania donovani, L. tropica,* and *L. mexicana*

	Isolate		
	D1	T1	M1
Individual PCR reactions	10	4	5
Identical sequences	3	0	3

ble 1. The primer sequences themselves are excluded. Only 5 of 32 sequences were redundant (D1X3 and D1X6, with D1X2; T4X1, with T3X2; and M1X3 and M1X4, with M1X2). A dendrogram derived from a maximum of 25 of these sequences showed isolates belonging to the same *Leishmania* species grouped in separate branches (Fig. 1). The number of identical bases between all *L. donovani* sequences was 67 (74%), with the lowest individual intraspecific identity being 81% (between sequences D1X2 and D2X1). The *L. tropica* sequences were 80% identical, with the lowest individual intraspecific identity being 87% (between sequences T1X4 and T6X1). The highest interspecific identity was 92% (between isolates T2X1 and D1X2).

Cloning of the products from individual PCR reactions done with isolates D1 *(L. donovani),* T1 *(L. tropica),* and M1 *(L. mexicana)* resulted in multiple sequences for the same isolate (Table 2).

Discussion

Primer construction

Both primers are located on interspecifically conserved sequences described previously (de Bruijn and Barker 1992) with emphasis given to the 3′-end being the less degenerate one. This observation is in agreement with the findings by Sommer and Tautz (1989) that the 3′-terminal nucleotides have to match perfectly for successful priming. To achieve the greatest possible sensitivity, the primers were synthesized degenerately, that is, as mixtures of 4 (LEI-1) and 16 (LEI-2) individual sequences. This not only facilitated the acquisition of amplification products from all five *Leishmania donovani* and all six *L. tropica* isolates as well as the *L. mexicana* strain, but these primers were also capable of detecting the described intraspecific as well as intrastrain variability.

Intrastrain polymorphism

A marked polymorphism within individual isolates was observed with identities being as low as 82% (between sequences D1X2 and D1X5). In an attempt to evaluate the intrastrain polymorphism detectable by the primers LEI-1 and LEI-2, we performed ten individual PCR amplifications of the *L. donovani* isolate D1. Because only three of the ten independently amplified sequences were identical, it can be assumed that the total number of different sequences that can be amplified with LEI-1 and LEI-2 must be considerably greater than the eight described herein. This might be true to a lesser degree for *L. mexicana* strains, where three of only five sequences were identical (Table 2). The identity between the individual sequences amplified from isolate D1 (76%) is similar to the intraspecific value (74%). Possible explanations could be an exchange of genetic information from a gene pool shared by the members of the respective species or species-specific mechanisms for the generation of sequences diversity.

Intraspecific polymorphism

The primers LEI-1 and LEI-2 detected a marked intraspecific polymorphism with identities of only 74% and 80% within *L. donovani* and *L. tropica,* respectively. With the possible exception of D1X2, which showed a high homology to *L. tropica* sequences, the homogeneous distribution of the computer-generated, pairwise similarity scores obtained by matching each sequence to every other within the same species (data not shown) does not readily allow for the distinction of different classes of sequences within a given species.

Interspecific polymorphism

The number of bases conserved between all *L. donovani* and *L. tropica* sequences was 58 (64%). However, only 2 of the 33 nonconserved bases would allow for a species differentiation between the *L. donovani* and *L. tropica* sequences presented herein, whereas the remaining 31 nonconserved bases would not. It can be extrapolated that the number of discriminating nucleotides will decrease even further as the number of available sequences increases. No "specific" mutation at all could be considered if *L. mexicana* were to be differentiated as a third species. Therefore, individually found sequences are of little value in distinguishing between *Leishmania* species. This observation suggests that the usual way of establishing individual consensus sequences in an effort to distinguish between the different species will be of little value for minicircle DNA.

As a possible alternative, we suggest the use of the presented sequences (possibly without identical and highly similar sequences, leaving the following 19 sequences: T1X1, T1X2, T2X1, T2X2, T3X1, T4X1, T4X2, T5X1, T6X1, D1X1, D1X2, D2X1, D2X2, D3X1, D4X1, D5X1, M1X1, M1X2, and M1X5) as a data base to construct a dendrogram such as that shown in Fig. 1, since all of the given sequences fall within separate branches representing the individual species. It can be speculated that further sequences will follow suit.

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