Intergenic and External Transcribed Spacers of Ribosomal RNA Genes in Lizard-infecting *Leishmania*: Molecular Structure and Phylogenetic Relationship to Mammal-infecting *Leishmania* in the Subgenus *Leishmania* (*Leishmania*)

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To establish the relationships of the lizard- and mammal-infecting Leishmania, *we characterized the intergenic spacer region of ribosomal RNA genes from* L. tarentolae *and* L. hoogstraali. *The organization of these regions is similar to those of other eukaryotes. The intergenic spacer region was approximately 4 kb in* L. tarentolae *and 5.5 kb in* L. hoogstraali. *The size difference was due to a greater number of 63-bp repetitive elements in the latter species. This region also contained another element, repeated twice, that had an inverted octanucleotide with the potential to form a stem-loop structure that could be involved in transcription termination or processing events. The ribosomal RNA gene localization showed a distinct pattern with one chromosomal band (2.2 Mb) for* L. tarentolae *and two (1.5 and 1.3 Mb) for* L. hoogstraali. *The study also showed sequence differences in the external transcribed region that could be used to distinguish lizard* Leishmania *from the mammalian* Leishmania. *The intergenic spacer region structure features found among* Leishmania *species indicated that lizard and mammalian Leishmania are closely related and support the inclusion of lizard-infecting species into the subgenus* Sauroleishmania *proposed by Saf'janova in 1982.*

Key words: lizard leishmania - RNA polymerase I - rRNA processing - repetitive DNA - rRNA spacer evolution

Leishmania tarentolae and *L. hoogstraali* are bloodstream kinetoplastids of lizards. Their geographical distribution is restricted to the Old World. Lizard-infecting *Leishmania* are closely related to mammalian-infecting *Leishmania*, but their exact taxonomic position and phylogenetic relationship remains uncertain. Recently, biochemical and biological data have been used in comparative studies of lizard and mammalian *Leishmania* species (Simpson & Holz 1988, Briones et al. 1992, Fu & Kolesnikov 1994, Croan & Ellis 1996, Previato et al. 1997, Noyes et al. 1997, Croan et al. 1997, Shaw 1997, Noyes et al. 1998) indicating a close relationship.

Lizard-infecting *Leishmania* play an important role in epidemiological studies of leishmaniasis. Amastigote forms of *L. adleri* (another lizard *Leishmania* species) were detected in volunteers at the site of inoculation five days after infection with *L. adleri* promastigotes. Serological studies also showed common antigens among *L. adleri* and *L. hoogstraali* and some mammalian *Leishmania* species. This observation has an implication in the kala-azar epidemiology since false positives could be generated due to naturally-transmitted *L. adleri* (see revision in Wilson & Southgate 1979, Telford 1995). In addition, amastigote forms have been detected in phagocytic cells of naturally and experimentally infected lizards (Dollahon & Janovy 1974, Elwasila 1988).

Earlier studies using the small subunit ribosomal RNA (rRNA) molecule helped to establish the lizard *Leishmania* proximity to mammalian *Leishmania* (Briones et al. 1992) relative to other trypanosomatid genera. However, the small subunit sequences are not suitable for resolution of species within the same genera. On the other hand, the rRNA gene intergenic spacer region (IGS) and the external transcribed spacer (ETS) are under less selective pressure and thus provide a means to distinguish evolutionary relationships among more closely-related species. The IGS/ETS region of trypanosomatids have been widely studied (White et al. 1986, Grondal et al. 1990, Zomerdijk et al. 1991, Rudenko et al. 1991, Dietrich et al. 1993, Martinez-Calvillo & Hernandez 1994, Tyler-Cross et al. 1995, Uliana et al. 1996, Gay et al. 1996, Yan et al. 1999, Downey & Donelson 1999, Schnare et al. 2000) and shows a general organization similar to the majority of eukaryotes.

In the present work, the molecular structure of the IGS region from two lizard-infecting *Leishmania* species is described and compared to the corresponding sequences

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Note: Nucleotide sequence data reported here are available in the GenBank database under the accession numbers AY026525 and AF345631-33.

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from the mammal-infecting *Leishmania*. These data refine the phylogenetic positioning of the lizard *Leishmania* and support their status as a subgenus of *Leishmania*.

MATERIALS AND METHODS

Organisms - Promastigotes of *L. tarentolae* (ATCC 30267) and *L. hoogstraali* (RHEM/SD/1963/NG26) from the Trypanosomatid Bank at Universidade de São Paulo were cultivated at 25°C in LIT medium (Camargo 1964).

DNA and RNA extractions, isolation of the IGS/ETS region and sequencing - Genomic DNA from *Leishmania* species was purified as previously described (Uliana et al. 1991). Total RNA was extracted as described (Chomczynski & Sacchi 1987).

A genomic *Apa*I-*Bgl*II fragment encompassing ~3 kb of the IGS/ETS region of *L. tarentolae* was isolated from a cosmid library (Fig. 1C, clone pAB 2.8). Clone pLt1.3 (Fig. 1C) was obtained by the isolation of an *Alu*I fragment from pAB 2.8. For *L. hoogstraali*, a 9-kb *Bgl*II fragment was isolated from a partial genomic library, constructed using inserts of 7 to 10 kB produced by a complete digestion of genomic DNA with *Bgl*I. This fragment contains ~2 kb from the large subunit rRNAs subunits and the whole IGS/ETS region. Fig. 1B shows clones pLh 6500, pLh 3400 and pLh 8A, containing small fragments, used in this work. These clones were mapped with several restriction enzymes, subcloned into pUC 18/19 series (Fig. 1) and sequenced by the dideoxynucleotide method (Sanger et al. 1977). The sequences were determined by a combination of manual and automated sequencing using an ABI 377 or 310 sequencer (Perkin Elmer).

Chromosomal analysis - Chromosome bands were separated by pulsed-field gel electrophoresis (Schwartz & Cantor 1984) in 1.2% agarose gels and 0.5x TBE buffer. The running conditions were: three phases pulses of 180, 240 and 300 s at 6 V/cm for 18 h each pulse at 10°C. Blots were probed with the *Acc*I fragment (Fig. 1C) and with the small subunit rRNA from *L. tarentolae.*

Sequence and phylogenetic analysis - The sequences were aligned with CLUSTALW 1.6 (Thompson et al. 1994) and the matrix obtained contained 1119 characters and 6 taxa. *Crithidia fasciculata* was used as the outgroup.

Phylogenetic trees were constructed using PAUP version 4.03b (Swofford 1999) for distance and parsimony methods. Maximum likelihood trees were obtained with BASEML program from the PAML 2.0 package (Yang 1999) using the K80 model of DNA substitution with gamma distribution ($\alpha = 0.85$). Bootstrap values were calculated for 100 replicates.

RESULTS

IGS/ETS sequence comparison - Based in a Northern blot experiment the approximate size of the ETS region of *L. hoogstraali* and *L. tarentolae* was determined (data not shown) and used to positioned the data to align and compare this region with the corresponding sequences of *L. (L.) amazonensis*, *L. (L.) chagasi*, *L. (L.) donovani* and *C. fasciculata* (Fig. 2, Table I). The comparison showed blocks of sequence similarity among the *Leishmania* species and *Crithidia.* Motifs were identified corresponding to those described previously in *L. (L.) amazonensis* (Uliana et al. 1996) and two regions (Fig. 2) of absolute identity to the proposed base-pairing sites of

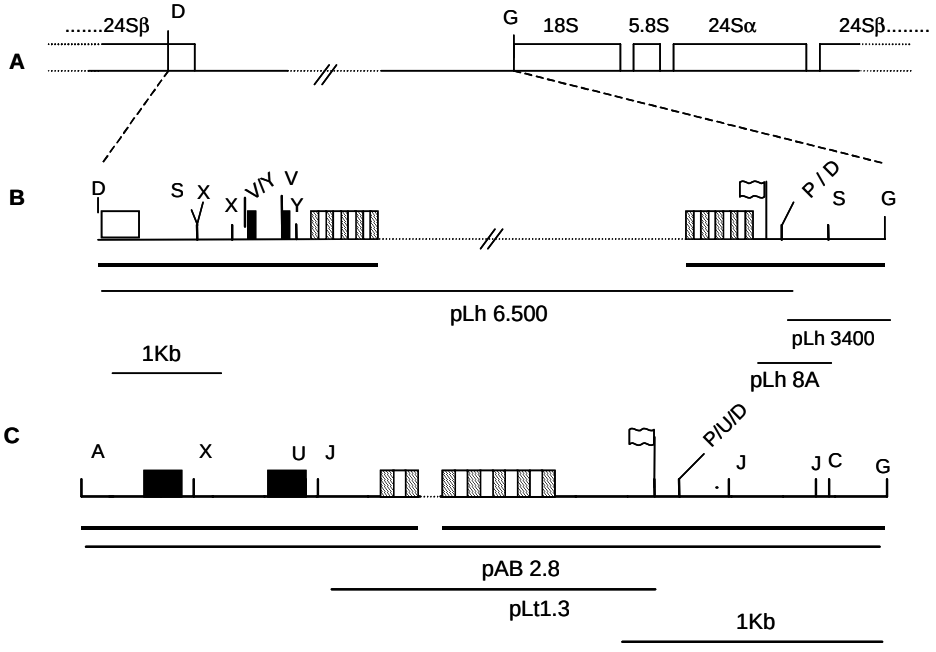

Fig. 1: scheme of the general ribosomal RNA gene arrangement (A) with the respective intergenic spacer/external transcribed spacer region of *Leishmania hoogstraali* (B) and *L. tarentolae* (C). The blank box in B represents the 3' end of the rRNA gene 24Sβ. A, *Apa*I; B, *Bam*HI; C, *Hin*cII; D, *Hin*dIII; G, *Bgl*II; J, *Acc*I; P, *Pst*I; S, *Sma*I; U, *Alu*I; V, *Pvu*II; X, *Xho*I; Y, *Dde*I. The 63 bp repetitive elements are represented in stippled and white rectangles, the flag maps the estimated transcription start point, the black rectangles indicate the element containing the inverted octanucleotides. The thick line corresponds to the sequenced region.

the U3 small nucleolar RNA (snoRNA) of *C. fasciculata* (Schnare et al. 2000) and *L. tarentolae* (Shi et al. 1994). A *Hin*dIII site in the estimated ETS region of *L. tarentolae* and *L. hoogstraali* (Fig. 1) could be used to discriminate lizard isolates from the mammalian isolates.

L. tarentolae and L. hoogstraali IGS/ETS structure - The IGS/ETS structure was determined by sequence analysis of the corresponding genomic segments from each species (Fig. 1): ~4 kb for *L. tarentolae* and 5.5 kb for *L. hoogstraali*.

The relevant IGS/ETS fragments were digested with restriction enzymes that had 4-bp recognition sites. The patterns obtained for both species with *Dde*I revealed the presence of repetitive element in both regions. A non-stoichiometric quantity of a ~70-bp fragment accumulated with partial-restriction kinetics, and the sum of all fragments generated by complete digestion was less than the size of the original fragment (data not shown).

Determining the nucleotide sequence of the IGS fragments allowed us to fully characterize the repetitive elements. For both species, the first 63-bp repeat is located ~200 bp upstream of the estimated transcription start point (Fig. 1B, C, hatched rectangles). By sequence analysis we detected approximately 12 repeats for *L. tarentolae* and 40 for *L. hoogstraali*.

These repetitive elements showed approximately 90% sequence identity between lizard *Leishmania* species (Table I, upper section). In contrast, comparison with the same repetitive elements in the IGS of mammalian *Leishmania* showed a very low degree of similarity (Table I, upper section). Fig. 3 shows a sequence comparison of the repetitive elements found in each species.

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            +584
AMA           AGCTACACA--TGTGTAGAGAGCNCTGCGCCGAACTAGTCGTGCNTNCTGAACATGTTTT
CHA           AGC....TATATG...AG..A..GCTGC..C.AA..AGT.A.G.G.G.A..........
DON           AGC....TATATG...AG..A..GCTGC..C.AA..AGT.A.G.G.G.A..........
TAR           AGC....TGTATG...AG..A..GTGNN..C.AA..AGT.G.G.G.G.T..........
HOO           AGC....TGTATG...AG..A..GCTGC..C.AA..AGT.G.G.G.G.T..........
CRIT          ---....TAT-CC...C-..T..GCTGC..T.GG..TAC.G.A.G.G.C..........

                                                    I
AMA           GTGTGTGCTGCCGTGTCTACACGT-TTGCAGCGGAAACTCGGTTGAACCAGAAACAGAAC
CHA           ..............GTC.AC..G.-.......G............A.CCA..AA.A.A..
DON           ..............GTC.AC..G.-.......G............A.CCA..AA.A.A..
TAR           ..............GTC.AC..-.-.......G............A.CCA..AA.A.A..
HOO           ..............GTC.AC..G.-.......G............A.CCA..AA.A.A..
CRIT          ..............TCT.TT..G.G.......A............G.ATG..GT.G.C..

                                                          II
AMA           TTGATTGTTGCTGCATTTCGAGGAATTTCAGCCAAACAAGAGGTCAACAACCAAGCCGCG
CHA           .......T.......T...................A..................C..G.G
DON           .......T.......T...................A..................G..G.G
TAR           .......T.......T...................-..................-..G.-
HOO           .......T.......T...................A..................G..G.G
CRIT          .......A.......C...................A..................G..A.G
                                                                        +759
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Fig. 2: sequence comparison of a segment of the external transcribed spacer. AMA corresponds to *Leishmania* (*L.*) *amazonensis*, CHA corresponds to *L.* (*L.*) *chagasi*, DON correspondes to *L.* (*L.*) *donovani*, TAR corresponds to *L. tarentolae*, HOO corresponds to *L. hoogstraali* and CRIT corresponds to *Crithidia fasciculata*. GeneBank accession numbers for *L.* (*L.*) *amazonensis*, *L.* (*L.*) *chagasi*, *L.* (*L.*) *donovani* and *C. fasciculata* are U21687, U42465, L38572 and Y00055, respectively. Regions I and II correspond to the proposed site of base pairing with the U3 snoRNA in *C. fasciculata* (Schnare et al. 2000) and *L. tarentolae* (Shi et al. 1994). Numbering is in relation to the transcription start point of *L. amazonensis* (Uliana et al. 1996). Dots (.) represent nucleotide identity, dashes (-) represent gaps introduced to improve the alignment obtained with Clustalw (1.60) program.

TABLE

Percentage of similarity among the six external transcribed spacer sequences (lower part) and in the 63bp repetitive elements (upper part)

	L. tarentolae	*L. hoogstraali*	*L. (L.) amazonensis*	*L. (L.) chagasi*	*L. (L.) donovani*
Leishmania tarentolae	-	90	36	34	36
L . hoogstraali	93	-	35	34	36
L. (L.) amazonensis	85	87	-	68	68
L. (L.) chagasi	85	86	88	-	96
L. (L.) donovani	86	88	90	97	-
Crithidia fasciculata	60	62	59	58	59

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HOO  TGTGCAGCTACGCGGGCACG**CTCAG**CGAGGCATTGTCTGGCGTCGCAGCAGTGCAGTATTGGC-
TAR  T..G.AGCCATGCAGG.ACG**C..AG**TGA.GCATT.T.TGG..CNGCAGT.G.G.GGTATTGGC-
AMA  A..A.TTGGACATTCC.--TT..GCCAC.CCAGA.-.CGT..-GAGGCC.C.C.TTCGCGTCCC
CHA  A..A.TGGAGCATTCC.GTTT..GCTGC.AGGAA.A.CCT..CGGGAGC.T.G.TTCGCGCCCC
DON  A..A.TGGAGCATTCG.GTTT..GCTGC.AGGGA.A.CCT..CGGGAGC.T.G.TTCGCGCCCC
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Fig. 3: repetitive elements found in the intergenic spacer region of the rRNA genes of *Leishmania* species. Species identification as in Fig. 2. Bold type is the *Dde*I site. Dots (.) represent nucleotide identity, dashes (-) represent gaps introduced to improve the alignment obtained with Clustalw (1.60) program.

The results of the probed pulsed-field gel electrophoresis blots (Fig. 4) indicated that the repetitive elements co-localized to the same chromosomal bands as the rRNA of both species and were not dispersed throughout the genome. The pulse-field pattern also revealed that the rRNA gene maps to chromosomal bands of different size (Fig. 4) in *L. tarentolae* (2.2 Mb) and *L. hoogstraali* (1.5 and 1.3 Mb). Hybridization experiments using high stringency conditions indicated the presence of these elements in *L. adleri* and *L. gymnodactyli* (other lizard *Leishmania* species). The 63-bp repetitive elements from lizard *Leishmania* did not hybridize with *L. (L.) amazonensis* nor *L. (L.) mexicana* chromosomal bands (data not shown).

Upstream from the 63-bp repetitive elements, an inverted octanucleotide motif repeated twice was found in both species (Fig. 1B, C, black rectangles). A similar sequence was described in the same IGS position in *L. (L.) infantum* (Requeña et al. 1997) and recently, it was also found in the IGS region of *C. fasciculata* (Schnare et al. 2000). A comparison among these sequences showed that the repeats in *C. fasciculata* and *Leishmania* species are not as conserved as among the *Leishmania* species repeats (Fig. 5A). The potential to form a stem-loop structure as identified in the *L. (L.) infantum* sequence was also found for the two blocks of *L. tarentolae*, *L. hoogstraali* and *C. fasciculata* (Fig. 5B).

Phylogeny - Trees derived from distance, parsimony and maximum likelihood (Fig. 6) methods exhibited identical topologies and showed that the *Leishmania* of lizards studied are very close to each other. The lizard *Leishmania* form a sister group to the mammalian *Leishmania* of the subgenus *L.* (*Leishmania*) analyzed with *C. fasciculata* used as the outgroup. The bootstrap values obtained for each method (Fig. 6, nodes) support the hypothesis that lizard *Leishmania* and mammalian *Leishmania* species of the subgenus *L.* (*Leishmania*) are sister groups.

DISCUSSION

In this study we have determined the sequence of the IGS/ETS upstream from the small subunit rRNA of two lizard-infecting *Leishmania* species for comparison with their mammal-infecting relatives. The size of the IGS region determined for both species is approximately the same as the size found for *L. (L.) infantum* (5 kb, Requeña et al. 1997) and *L. (L.) donovani* (4 kb, Lodes et al. 1995), indicating that the size of this region is conserved between

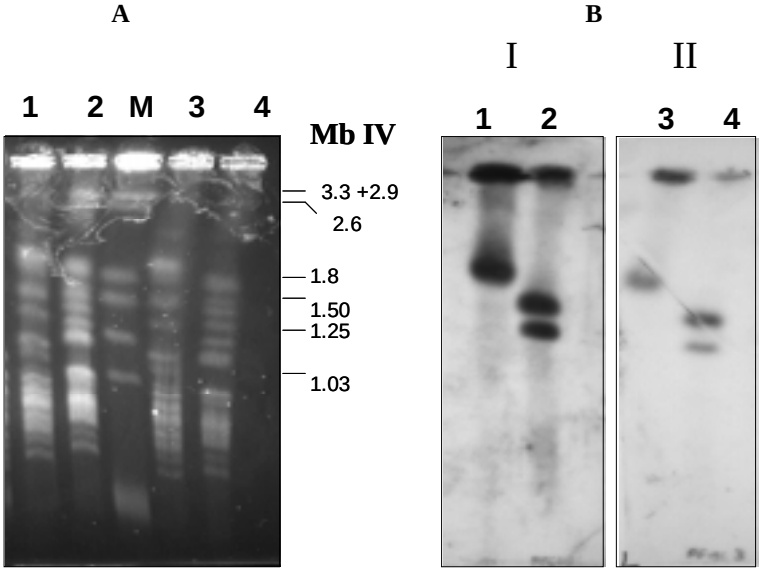

Fig. 4: chromosomal pattern of *Leishmania tarentolae* (1 and 3) and *L. hoogstraali* (2 and 4) rRNA gene localization. A: chromosomal bands after ethidium bromide staining. M corresponds to Megabase IV (Invitrogen); B: autoradiogram of the chromosome blot in panel A. In panel I the fragment *Acc*I/*Acc*I from *L. tarentolae* (Fig. 1A) was used as the probe and in panel II the 18S subunit DNA was used as the probe. Washing conditions: 2x SSC/0.1% SDS at 45°C. Exposure time: 5 days at -20°C for panel I and 4 days at -20 °C for panel II.

A

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INF1   CAACGGACCCGA**CAACCAGC**TGTGTGCGCGTATATATTTACTATA------CGTCTGCATACACG--**GCTCGTTG**TCGGGTCCTTGCCCTTTT
INF2   CACCCGACCCGG**CAACCAGC**TG----------------------------CGCCCAAACGCGCG--**GCTCGTTG**TCGGGTCCTTGCCTTTTT
INF3   CACCCGACCCGG**CAACCAGC**TG----------------------------CGCCCAAACGCGCG--**GCTCGTTG**TCGGGTCCTTGCCTTTTT
TAR1   CCCCCGACCCGG**CAACCAGC**TG----------------------------CGACCACAACGGCGCA**GCTCGTTG**TCGGGTCCTTGCCCTTTT
TAR2   CCCCCGACCCGG**CAACCAGC**TG----------------------------CGACCACAACGGCGCA**GCTCGTTG**TCGGGTCTTTGCCCTTTC
HOO1   CAAAGGACCCGA**CAACCAGC**TGTGTGTTGTGCGTGCGTATAGGTTTATACGTGCACACATGTANGCA**GCTCGTTG**TCGGGTCCTTGCCCTTTN
HOO2   CACCCGACCCGG**CAACCAGC**TG----------------------------CGACCACAACGGCGCA**GCTCGTTG**TCGGGTCCTTGCCCTTTC
CRIT   --CATCCCCCGA**CAACCAGC**CA----------------------------------GCTCACATG**GCTCGTTG**TCGGGG--TTTGCCTTTC
          **** ********                                                ************   **  * ***
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B

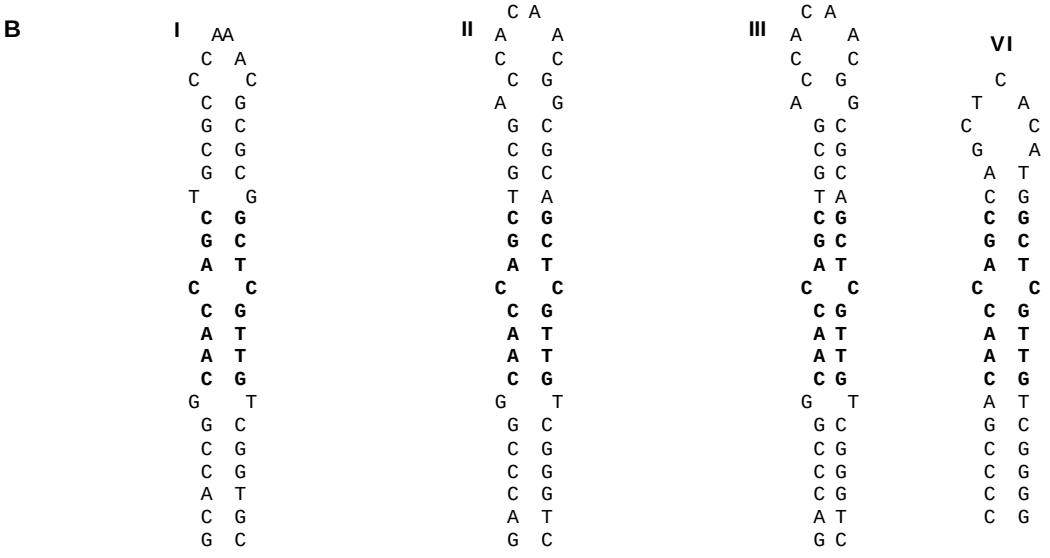

Fig. 5: analysis of the three repetitive elements found downstream from the *Leishmania* (*L.*) *infantum* large subunit rRNA (INF 1, 2 and 3), *L. tarentolae* (TAR 4 and 5), *L. hoogstraali* (HOO 6 and 7) and *Crithidia fasciculata* (CRIT). A: alignment of the elements. Bold letters correspond to the inverted octanucleotides. Asterisks (*) below the alignment indicate base identity in each position among the eight sequences; dashes (-) represent gaps introduced to maximize the alignment; B: sequences shown in A can form similar stem loop structures. I. *L.* (*L.*) *infantum* (Requeña et al. 1997); II. *L. tarentolae.* III. *L. hoogstraali.* IV. *C. fasciculata* (from R3 sequence described in Schnare et al. 2000).

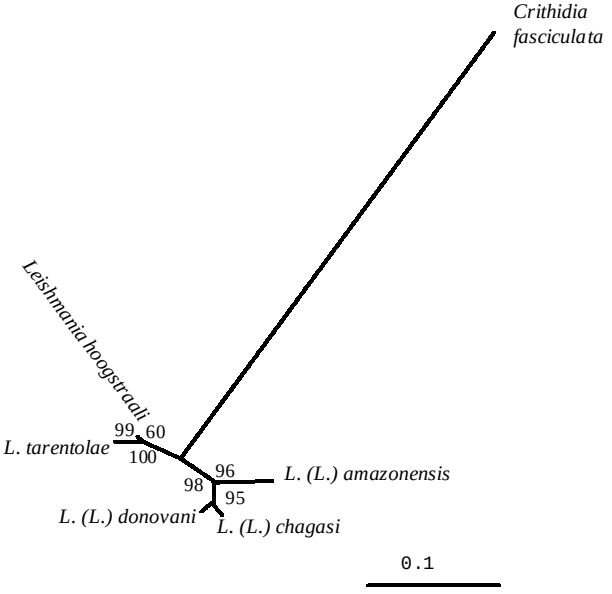

Fig. 6: topology obtained with the maximum likelihood method for the external transcribed spacer sequence alignment. Distance method (performed with the neighbor-joining algorithm) and parsimony method gave similar topologies, although the bootstrap values obtained differed: numbers at the left of each node represent maximum likelihood values, numbers at the right represent neighbor-joining values and numbers under each node represent parsimony values. The bar indicates distance for maximum likelihood.

lizard and mammalian *Leishmania* species; the size observed in other eukaryotes is variable. The differences in length can be attributed to a variable number of 63-bp repetitive-element found in each lizard species.

The estimated ETS region of each species (~1000 bp) was shown to be very similar in size to *L. (L.) amazonensis* (1050 bp, Uliana et al. 1996), *L. (L.) chagasi* (1060 bp, Gay et al. 1996), *L. (L.) donovani* (1020 bp, Yan et al. 1999), *C. fasciculata* (1050 bp, Grondal et al. 1990) and *T. congolense* (961 bp, Downey & Donelson 1999). For *T. cruzi* and *T. brucei* the ETS size is significantly larger [1550 bp, Dietrich et al. (1993) and 1200 bp, White et al. (1986), respectively].

The comparison of ETS regions showed conserved motifs between lizard and mammalian *Leishmania* species and *C. fasciculata* (data not shown), including the proposed sites of base-pairing with the U3 snoRNA of *C. fasciculata* (Schnare et al. 2000) and *L. tarentolae* (Shi et al. 1994) (Fig. 2). The presence of similar motifs suggests a common processing mechanism of the pre-rRNA molecule.

As in many other eukaryotes, conserved, repeated elements upstream of the predicted transcription start point were detected in both species. However, when compared to mammalian *Leishmania* species, the degree of similarity dropped three fold (Table). The repeat number varied among the *Leishmania* species: two populations of 35 and 70 copies in *L.* (*L.) amazonensis* (Uliana et al. 1996)

and 39 copies in *L.* (*L.*) *donovani* (Yan et al. 1999), while for lizard *Leishmania* approximately 12 copies of the 63-bp repeat in the *L. tarentolae* IGS and approximately 40 copies in *L. hoogstraali* were found. The size of the repeated elements was conserved in *Leishmania* (~ 60 bp), while 28 copies of a 19-bp element were found located upstream of the transcription start point in *C. fasciculata* (Schnare et al. 2000), indicating that the repeat size is not necessarily conserved in the kinetoplastids. In *L. (L.) chagasi* these elements showed a modest enhancer activity in a transient transfection assay (Gay et al. 1996).

The chromosomal analysis (Fig. 4) showed that the rRNA genes and the 63-bp repetitive elements mapped to the same chromosomal bands for both species. However, the detection of two chromosomal bands of different size in *L. hoogstraali* (1.3 and 1.5 Mb) does not discriminate whether they reside on homologous or different chromosomes. A similar pattern of two chromosomal bands for the rRNA genes was also identified for some *Viannia* subgenus species (Inga et al. 1998), as well as in *L. (L.) donovani* (Yan et al. 1999).

The analysis of an element located downstream of the large subunit 2 (24Sβ) of *L. tarentolae*, *L. hoogstraali*, *L. (L.) infantum* (Requeña et al. 1997) and *C. fasciculata* (Schnare et al. 2000) showed high degrees of similarity, mainly in the inverted octanucleotides (Fig. 5A, bold) and in the nucleotides that can form potential stem-loop structures (Fig. 5B).

This conservation indicates a possible biological role to this element, probably in the transcription termination of pre-rRNA or in subsequent processing events. The sequence of the element is conserved among *Leishmania* species, demonstrating the close relationship between lizard and mammalian *Leishmania*. An intriguing aspect of these motifs is that they have inverted octanucleotides identical to blocks found in l phage and *Escherichia coli* (Thaler & Stahl 1988), where they define hot spots for recombination. Thus, the repeated sequences could be involved in recombination events, as evidenced by the correlation of the repetitive sequences to the heterogeneity in the copy number of the rRNA genes.

The alignment of ETS sequences showed a high degree of sequence similarity between lizard *Leishmania* and mammal-infecting *Leishmania* (Table, lower section). The analysis of the data using diverse phylogenetic approaches resulted in the generation of identical trees showing that the lizard *Leishmania* are very closely related to *L. (L.) donovani*. These results are in agreement with biogeographic data, since both lizard *Leishmania* and *L. (L.) donovani* are found only in the Old World (Shaw 1994). Similar phylogenetic relationships for these organisms were found using the DNA polymerase I genes and RNA polymerase II genes of different *Leishmania* species (Croan et al. 1997). Furthermore, functional results involving the exact transcription starting point, the ETS and the promoter region of the rRNA gene of lizard *Leishmania* showed some degree of heterologous recognition of these regions between lizards and mammalian-infecting species (Orlando et al., manuscript in preparation), corroborating their close relationship also in a functional level, as this recognition is thought to be highly species-specific (Grummt et al. 1982, Sommerville 1984).

Both the molecular data presented and the inferred phylogenetic relationships are in agreement with the inclusion of the lizard *Leishmania* in the subgenus *Sauroleishmania*, as proposed by Saf'janova, using biological criteria (Saf'janova 1982).

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