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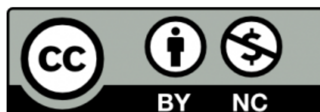

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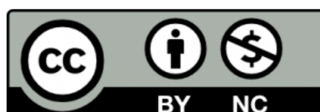

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DESCRIPTION OF *LEISHMANIA (LEISHMANIA) FORATTINII* SP. N., A NEW PARASITE INFECTING OPOSSUMS AND RODENTS IN BRAZIL

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A new parasite species of Leishmania *is described,* L. (Leishmania) forattinii *sp. n., which was isolated from a pooled triturate of liver and spleen of a opossum (*Didelphis marsupialis aurita*) and from skin samples from a rodent (*Proechimys iheringi denigratus*), captured in primary forest on the Atlantic Cost of Brazil. Our results on the basis of biological and molecular criteria indicate that this taxonomically distinct parasite is a new species of the* L. mexicana *complex, but closely related to* L. (L.) aristidesi *Lainson & Shaw, 1979, as revealed by phenetic and phylogenetic numerical analyses of the enzyme data.* L. forattinii *was clearly distinguishable from other* Leishmania *species of the genus using enzyme electrophoresis, monoclonal antibodies, molecular karyotypes, analysis of restriction enzyme digestion patterns of kinetoplast DNA (kDNA), as well as the use of kDNA hybridization procedures.*

Key words: *Leishmania (L.) forattinii* sp. n. – Protozoa – Kinetoplastida – Trypanosomatidae – mammalian reservoirs – molecular characterization – monoclonal antibodies – isoenzyme electrophoresis – kinetoplast DNA analysis – molecular karyotype analysis

Leishmaniasis is endemic in many areas of tropical and subtropical America, where it constitutes an important public health problem (Grimaldi et al., 1989; WHO, 1990). The disease in this region is basically a zoonosis; humans are only an incidental host in the life cycle of the various human pathogenic parasite species (Lainson & Shaw, 1987; Shaw & Lainson, 1987). During surveys of potential reservoir hosts of leishmanial parasites done in forested areas in Brazil, four leishmanial parasites were isolated from wild mammals (Yoshida et al., 1979; Barretto et al., 1985). Preliminary biological and molecular characterization of the leishmanial isolates indicated that these parasites are of the *L. mexicana* complex (Barretto et al., 1985; Yoshida et al., 1985; Grimaldi et al., 1987). Here we have further studied two of these isolates using molecular techniques for the characterization of *Leishmania*, as well as determined their phenetic relationships to reference strains of *Leishmania* by numerical taxonomic analyses.

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MATERIALS AND METHODS

Parasites examined – Identification of the *Leishmania* strains used in this study is given in Table. The strains (MDID/BR/77/Conchas; MPRO/BR/83/MTB-584/585) of the proposed new species were originally isolated from a pooled triturate of liver and spleen of a opossum (*Didelphis marsupialis aurita*) and from skin samples taken from a rodent (*Proechimys iheringi denigratus*), both captured in humid tropical forest on regions endemic for American cutaneous leishmaniasis in Brazil, respectively in the municipality of Coonchas, state of São Paulo (Yoshida et al., 1979) and the municipality of Três Braços, state of Bahia (Barretto et al., 1985). Promastigotes of each strain were grown in roller bottles at 25 °C in Schneider's *Drossophila* medium (Hendricks et al., 1978), supplemented with 20% heat-inactivated fetal bovine serum. Parasites in the log phase of growth were harvested by centrifugation (1,500 x g for 10 min at 4 °C) and

TABLE

Origin and identification of reference strains and selected *Leishmania* species or strain variants employed in this study

Stock	Designation[a]	Species	Geographic origin
L67	MDID/BR/77/Conchas	*L. forattinii*	São Paulo, Brazil
L93	MHOM/BR/81/PUL	*Leishmania (L)* sp. n.	Pará, Brazil
L107	MHOM/BR/71/MT	*Leishmania (L)* sp. n.	Mato Grosso, Brazil
L113	MHOM/VE/00/L54	*L. amazonensis*	Guarico, Venezuela
L114	MHOM/VE/00/L64	*L. amazonensis*	Merida, Venezuela
L117	MHOM/BZ/58/LM5	*L. mexicana*	Belize, Belize
L168	MHOM/BR/73/MT(D)	*L. amazonensis*	Amazonas, Brazil
L181	MPRO/BR/82/RV203	*Leishmania (L)* sp. n.	Espírito Santo, Brazil
L182	MPRO/BR/82/RV208	*Leishmania (L)* sp. n.	Espírito Santo, Brazil
L185	MHOM/BR/77/LTB0016	*L. amazonensis*	Bahia, Brazil
L278	MHOM/BR/84/JJV	*L. amazonensis*	Bahia, Brazil
L289	MPRO/BR/83/P584/85	*L. forattinii*	Bahia, Brazil
L561	MHOM/BZ/82/BEL21[b]	*L. mexicana*	Belize, Belize
L564	MORY/PA/68/GML3[b]	*L. aristidesi*	Darien, Panama
L565	MHOM/BR/75/M4147[b]	*L. guyanensis*	Pará, Brazil
L566	MHOM/BR/75/M2903[b]	*L. braziliensis*	Pará, Brazil
L567	MCAV/BR/45/L88[b]	*L. enriettii*	Paraná, Brazil
L568	MHOM/VE/74/PMH-17[b]	*L. venezuelensis*	Lara, Venezuela
L569	MHOM/BR/73/M2269[b]	*L. amazonensis*	Pará, Brazil
L571	MHOM/SU/58/str.OD[b]	*L. tropica*	Azerbaijan, USSR
L575	IFLA/BR/67/PH8[b]	*L. amazonensis*	Pará, Brazil
L577	MNYC/BZ/62/M379[b]	*L. mexicana*	Cayo, Belize
L579	MHOM/BR/80/PP75[b]	*L. chagasi*	Bahia, Brazil
L581	MHOM/SU/73/5 ASKH[b]	*L. major*	Turkmen, USSR
L583	MHOM/VE/57/LL1[b]	*L. pifanoi*	Venezuela
L584	MHOM/VE/76/JAP78[b]	*L. garnhami*	Merida, Venezuela
L612	MHOM/BR/85/ASS	*L. amazonensis*	Bahia, Brazil
L613	MHOM/BR/85/EAS	*L. amazonensis*	Bahia, Brazil
L614	MHOM/BR/85/JSS	*L. amazonensis*	Bahia, Brazil
L617	MHOM/BR/85/ABC	*L. amazonensis*	Bahia, Brazil
L632	MCAV/BR/45/L88unc	*L. enriettii*	Paraná, Brazil
L717	MHOM/CO/84/CL-018	*L. mexicana*	Pueblo Rico, Colombia
L725	MHOM/BR/86/MPS	*L. amazonensis*	Bahia, Brazil
L728	MHOM/BR/86/JGR	*L. amazonensis*	Bahia, Brazil
L747	MHOM/BR/85/HI-2	*L. braziliensis*	Espírito Santo, Brazil
L880	MHOM/BR/87/IM3147	*L. amazonensis*	Amazonas, Brazil
L881	IHAR/CO/85/CL500[b]	*L. colombiensis*	Santander, Colombia
L882	IHAR/CO/85/CL501	*L. colombiensis*	Santander, Colombia
L886	MHOM/IL/79/LRCL251	*L. major*	Jericho, Israel
L888	MCHO/EC/82/Lsp1[b]	*L. equatorensis*	Guayas, Ecuador
L890	MHOM/EC/86/Paute	*L. major*-like	Azuay, Ecuador
L894	MHOM/EC/87/G-07	*L. panamensis*	Pichincha, Ecuador
L1226	MHOM/EC/88/Pt24	*L. major*-like	Azuay, Ecuador
L1227	MHOM/EC/88/Pt25	*L. mexicana*	Azuay, Ecuador
L1228	MHOM/EC/88/Pt27	*L. mexicana*	Azuay, Ecuador
L1231	MHOM/EC/88/PT115	*L. major*-like	Azuay, Ecuador
L1233	MCAN/EC/88/PtInu2	*L. mexicana*	Azuay, Ecuador
L1240	MHOM/CO/87/CL-380	*L. chagasi*	Tolima, Colombia
L1363	IAYA/EC/89/PAI1	*L. mexicana*	Azuay, Ecuador

a: designation code. Host (M = Mammalia: CAN = *Canis familiaris;* CAV = *Cavia porcellus;* CHO = *Choloepus hoffmani;* DID = *Didelphis marsupialis;* HOM = *Homo sapiens;* NYC = *Nyctomys sumichrasti;* ORY = *Oryzomys capito;* PRO = *Proechimys iheringi;* I = Insecta: AYA = *Lutzomyia ayacuchensis;* FLA = *Lu. flaviscutellata;* HAR = *Lu. hartmani*)/ country of origin/year of isolation/original code.

b: *Leishmania* reference strain (WHO, 1990; Kreutzer et al., 1991; Grimaldi et al., 1992).

washed twice in phosphate-buffered saline (PBS), pH 7.3. The final pellet was used for preparation of samples for parasite characterization, using the molecular techniques described below.

Morphological examination – Giemsa-stained preparations of log phase promastigotes (grown in Schneider's medium) and amastigotes (from experimenttally infected hamsters) of the parasite from São Paulo were examined by light microscopy.

Experimental infection of animals – Stationary-phase promastigotes of the new parasite (strain L67) were inoculated intradermally (with a dose of 2 x 10^6 organisms in 0.1 ml) into the noses of young adult inbred BALB/c and C57BL/6 mice. The size and appearance of leishmanial lesions and metastases were evaluated at weekly intervals. Nose lesion size was expressed as the diameter of the raised nodular lesion. At death, fragments of primary lesions were removed and prepared for electron microscopy following the procedures as described elsewhere (Grimaldi et al., 1984). The remaining tissue from the cutaneous lesion was fixed in neutral 10% formalin. Paraffin sections were prepared and stained with haematoxylin and eosin.

Molecular characterization – The molecular procedures used for characterizing the parasites (isoenzyme electrophoresis, serodeme analysis using species-specific monoclonal antibodies, restriction endonuclease fragment patterns of k-DNA, Southern blot hybridization with species-specific k-DNA probes, and molecular karyotype analysis) have been described in detail in previous publications (Pacheco et al., 1990; Grimaldi et al., 1991; 1992; Cupolillo et al., 1993; Tavares et al., 1992). Numerical analyses of enzyme data followed the epistemology of Rioux et al. (1990). The phenetic analysis was performed to establish the relationships between this and other *Leishmania* species (Cupolillo et al., 1993).

RESULTS

In vitro cultivation and morphometric studies – Primary isolations of *L. forattinii* were made in tubes of blood agar (Jaffe et al., 1984) and both parasite strains (designated with stock codes L67 and L289) grew well in cultures of blood agar and Schneider's medium with fetal bovine serum. The growth characteristics *in vitro* and the general morphology (Figs 1a, 1b) of the new species were similar to those of other *L. mexicana* complex parasites. All measurements in micrometers of log-phase promastigotes and amastigotes of strain L67 were made using the methods described by Shaw & Lainson (1976) and the data are shown below.

Pathogenicity for laboratory animals – The behavior of the new parasite was indistinguishable from other species of this group, based on its virulence and development in laboratory animals. Thus, inoculation of genetically resistant C57BL/6 mice with *L. forattinii* resulted in control and resolution of infection. In contrast, in susceptible BALB/c mice the lesions developed progressively as nonulcerating nodules of variable size which metastasize in the extremities. During the acute phase of the disease there was an accumulation of neutrophil and eosinophil granulocytes, and some immature cells of the mononuclear phagocyte system (MPS). The granulocytes apparently destroyed many parasites. Following this stage a chronic inflammation developed consisting mainly of a progressive infiltration of immature cells of the MPS which coalesced and evolved into well-developed macrophages forming loosely organized granuloma. In susceptible BALB/c mice, the parasites not destroyed by granulocytes during the acute phase penetrated the mononuclear phagocytes and multiplied intracellularly, thus leading to accumulation of parasitized, vacuolated macrophages (Fig. 1b). In contrast, in the resistant C57BL/6 mice there was formation of tuberculoid-type granulomas containing few amastigotes which resulted in control and resolution of cutaneous lesions.

Zymodeme analysis – The two *L. forattinii* strains showed similar electromorphs (bands of enzyme activity as revealed by electrophoresis) with all 18 enzymes examined (Fig. 2a), and they were grouped into a new zymodeme (IOC-11). The enzyme profiles of the new species shared more electromorphs with parasites of the *L. mexicana* complex and fewer with other species complexes of *Leishmania*. The enzymes useful in distinguishing *L. forattinii* are listed below. Affinities between zymodemes were calculated using the Jaccard's similarity coefficient and were transformed into a phenogram, by the unweighted paired-group method using arithmetical avarage (UPGMA). In the phenogram (Fig. 2b) the new zymodeme (IOC-11) could be clustered within the *L. mexicana* species complex. This information

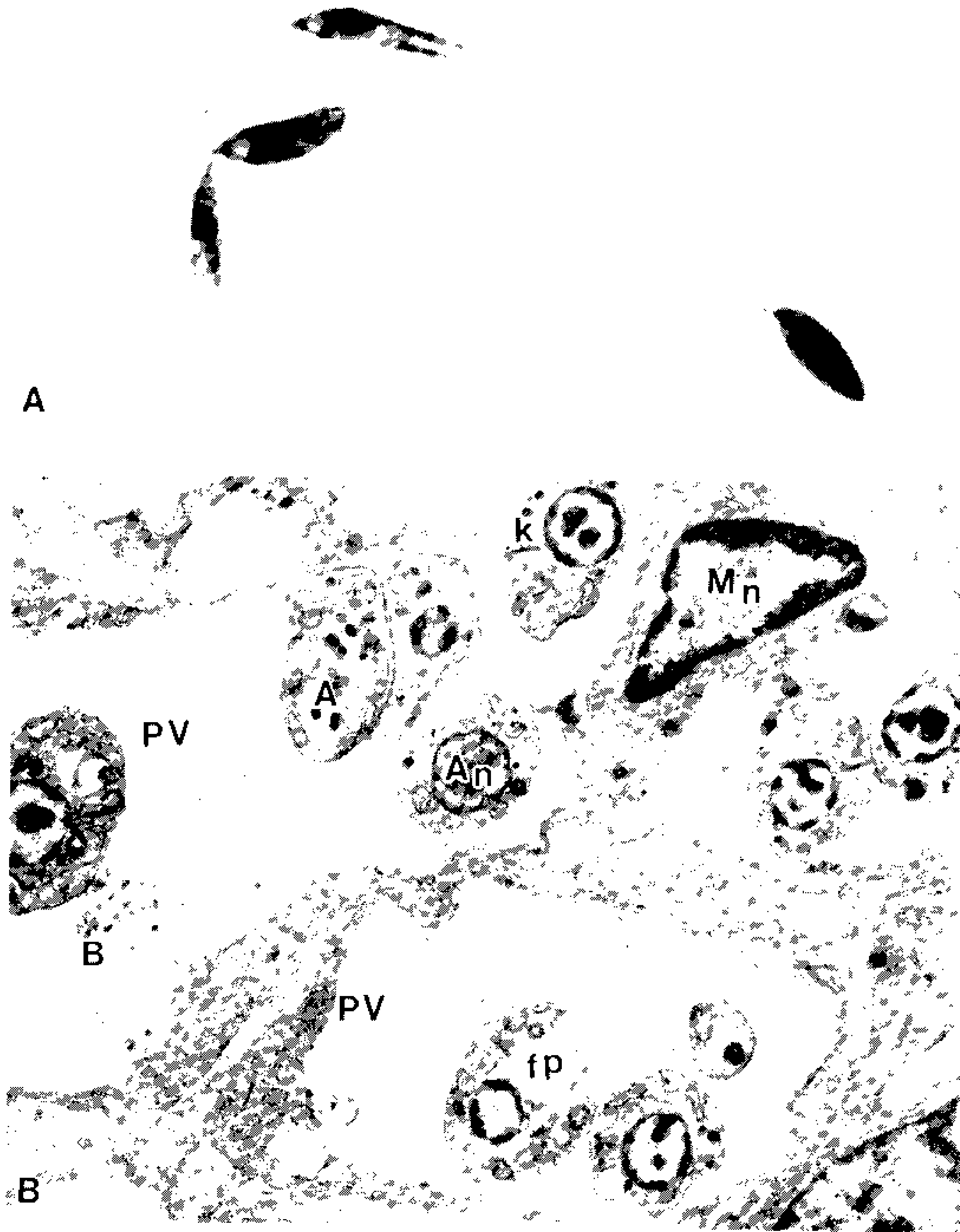

Fig. 1-A: promastigotes of *Leishmania forattinii* from a 7 day-old culture in Diffco NNN blood-agar medium (smear stained by Giemsa's method; x 600). B: electron micrograph of chronic cutaneous lesion (5 months) in BALB/c mouse, showing parasitized vacuolated macrophages containing amastigotes (x 6,000). The following structures may be observed: macrophage nucleus (Mn), parasitophorous vacuole (PV), amastigote (A), parasite nucleus (An), kinetoplast (k), and flagelar pocket (fp).

is confirmed by data from Cupolillo et al. (1993), who also showed in a cladistic analysis that *L. forattinii* is related to *L. aristidesi*.

Reactivity with monoclonal antibodies – Both *L. forattinii* strains were characterized by serodeme analysis, using a large panel of monoclonal antibodies. Characterization of the parasites were performed with an indirect radioimmune binding assay using whole parasite lysates as antigen (Grimaldi et al., 1987). The specificity of these monoclonal antibodies for species, the *L. mexicana*, *L. braziliensis*, *L. tropica*, and *L. chagasi*, have been described

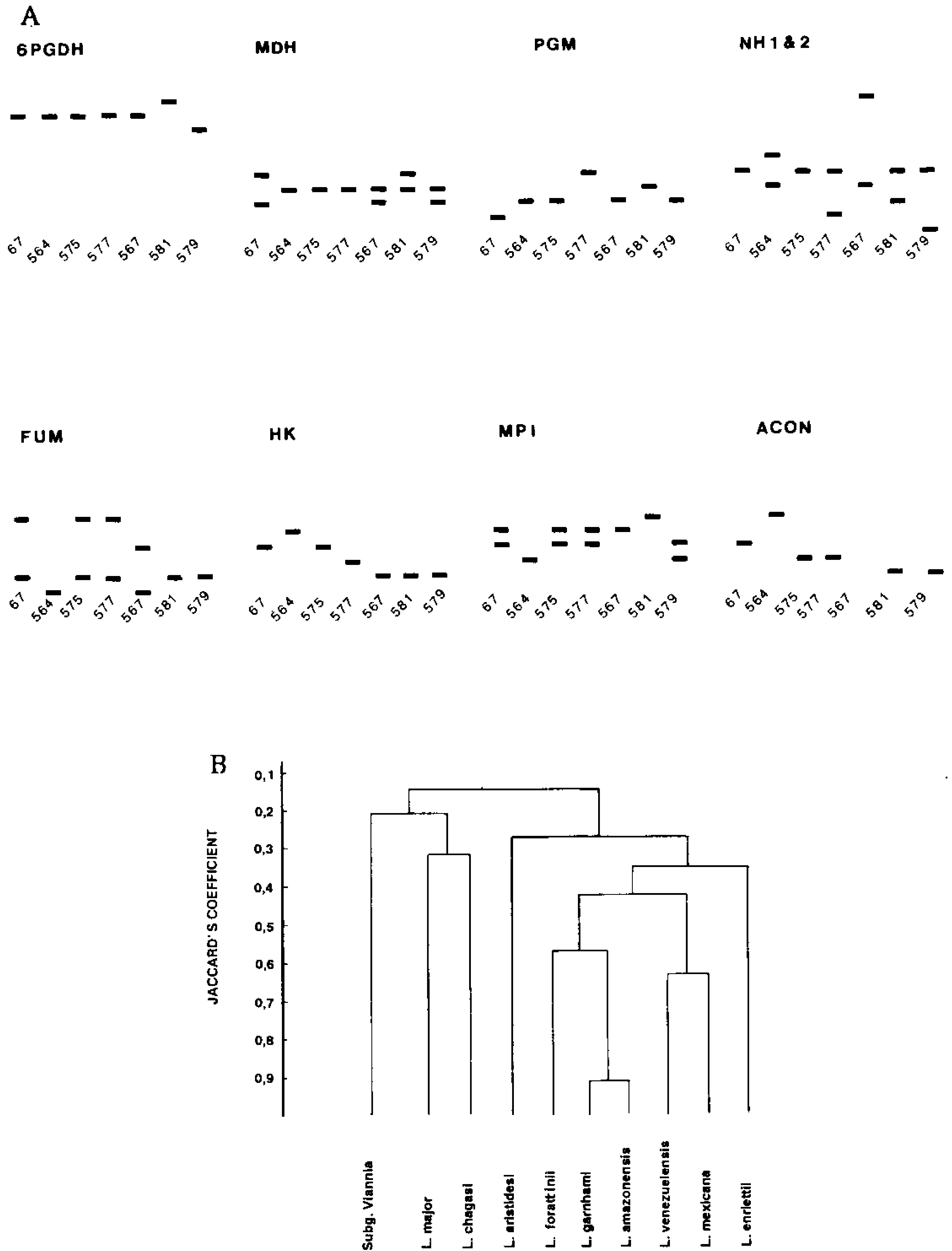

Fig. 2-A: diagrammatic representation of the electrophoretic patterns of the eight enzyme loci analyzed in this study, distinguishing the showing the similarities and differences in the enzyme profiles of *Leishmania forattinii* with other *Leishmania* reference strains. The origin and identification of the Leishmania strains studied are given in the Table. B: phenogram showing the Jaccard's coefficient of similarity between groups and/or species of *Leishmania* and the new parasite, *L. forattinii*.

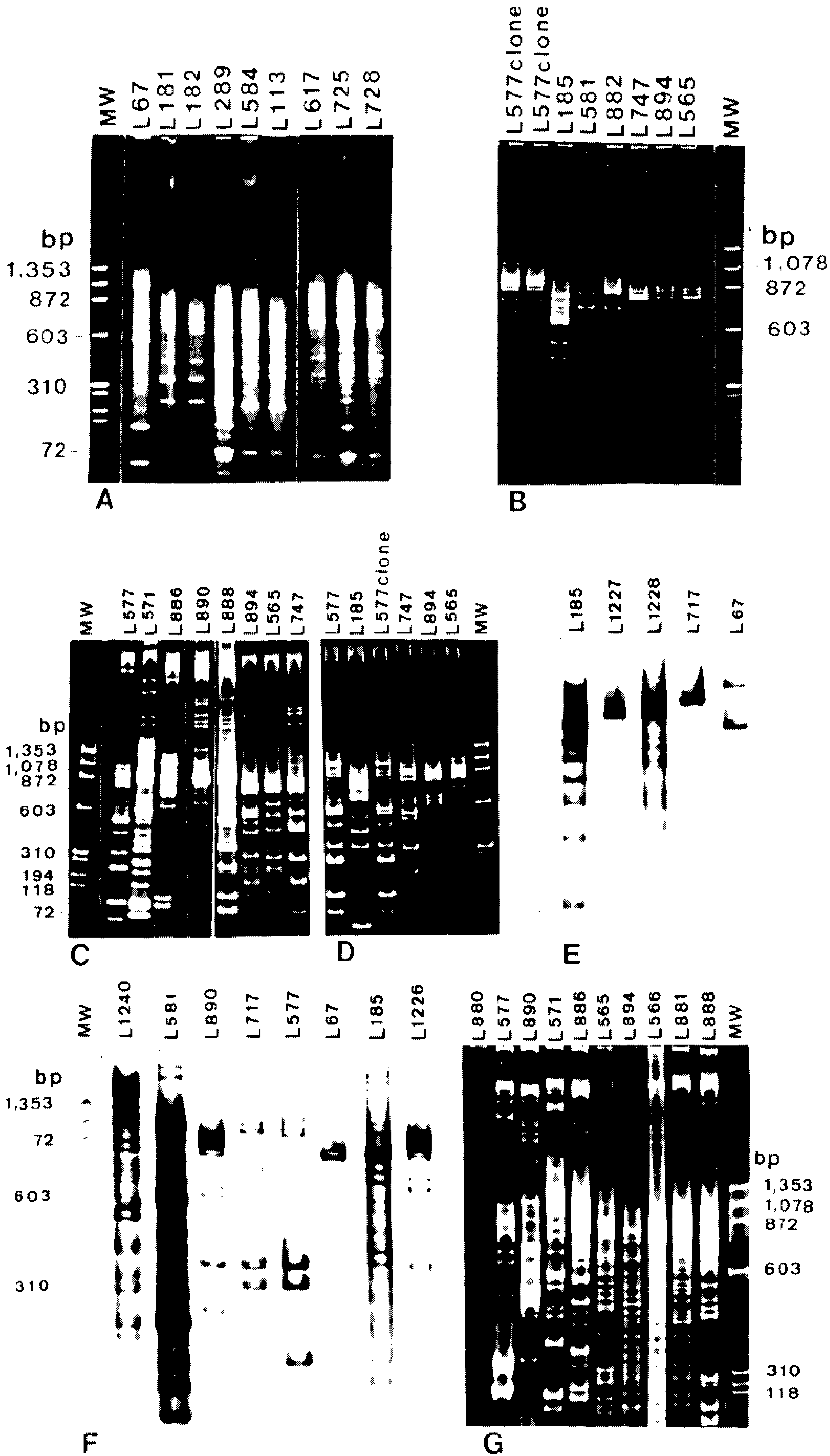

Fig. 3: acrylamide gradient (5-12%) gel electrophoresis comparison of kDNA fragment patterns, generated with the restriction enzyme *Msp* I (a, b), *Hinf* I (c, d, e) and Alu I (f, g), among selected species complexes of *Leishmania* and the two strains (L67 and L289) of *L. forattinii* from Brazil. The identification of the *Leishmania* strains studied by k-DNA analysis is given in the Table. Molecular weights of k-DNA fragments are indicated in basepairs (bp) besides the gels.

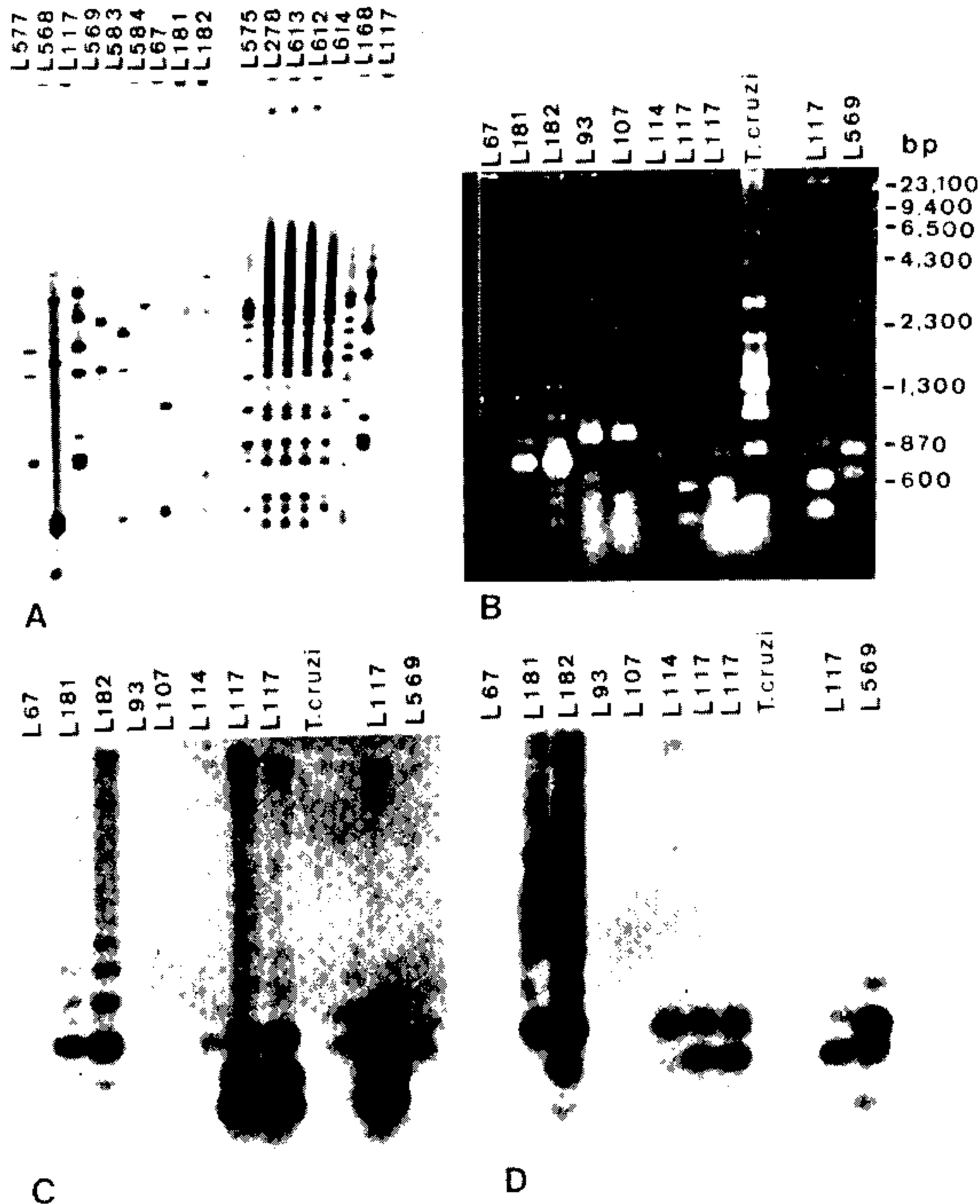

Fig. 4: acrylamide (A) and agarose (B) gradient gel electrophoresis comparisons of kDNA fragment patterns generated with the restriction enzyme *Bsp* I among selected species complexes of *Leishmania* and the L67 strain of *L. forattinii*; also shown are Shouthern blot analyses of k-DNAs fragments (B) hybridized with *L. mexicana* (C) and *L. amazonensis* (D) total k-DNA probes. The origin and identification of the *Leishmania* strains studied are given in the Table.

(Grimaldi et al., 1987; 1991; Hashiguchi et al., 1991). Only some monoclonal antibodies (M2, M4, M8 and M9), which are *L. mexicana*-group specific, bound to *L. forattinii*. The reactivity patterns of both isolates were quantitatively similar, except for that obtained with the monoclonal antibody M4, which showed lower values for the leishmanial strain isolated in Bahia. No significant cross-reactivity was obtained to any of these strains with a large panel of monoclonal antibodies specific for other species complexes of *Leishmania*.

Schizodeme and hybridization analyses of k-DNAs – Comparisons of kDNA restriction enzyme fragment profiles from stocks representing selected species complexes of *Leishmania* and the parasite isolates from São Paulo (stock L67) and Bahia (stock L289) were carried out by polyacrylamide gradient gel electrophoresis. The *L. forattinii* strains possessed identical k-DNA fragment patterns with the endonucleases *Hinf* I, *Alu* I, and *Bsp* I (data not shown), but these profiles were distinct from those of all strains examined (Figs 3, 4). However, variation between the two strains of the new parasite was demonstrated by their different schizodeme profiles with the enzymes *Msp* I (Fig. 3A) and *Mbo* I (data not shown). In addition, *L. forattinii* did not cross-hybrid-

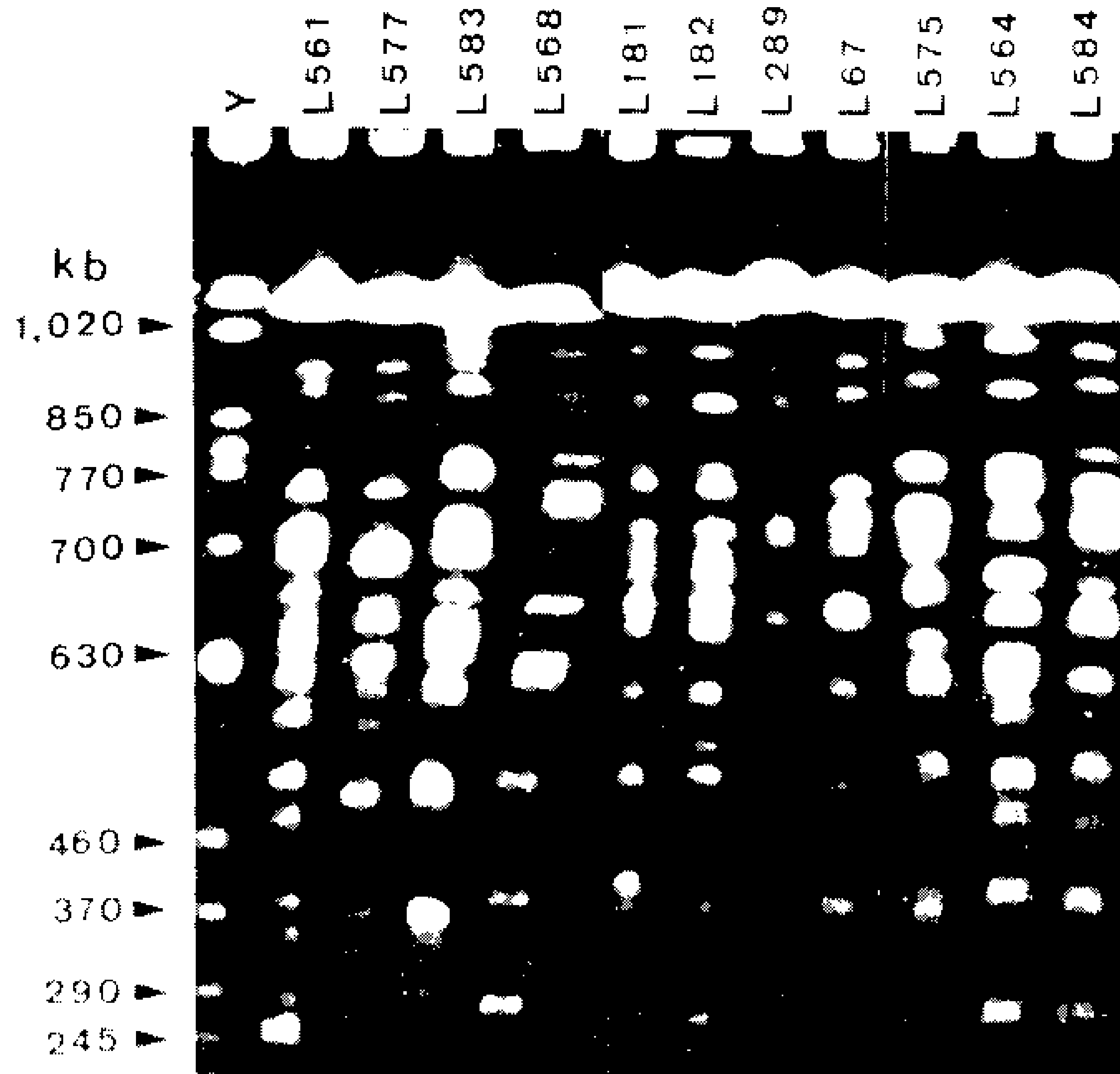

Fig. 5: CHEF electrophoresis separation of chromosomes-sized DNA molecules under 1,000 kb from *L. forattinii* and other *L. mexicana* complex species. The stocks (see Table for their origins) were run in 1.5% agarose gel for 48 hr (with pulse times of 120 s for 3 hr, 90 s for 30 hr, and 60 s for 15 hr), at 175 V and 12 °C, using a Pulsaphor electrophoresis unit from Pharmacia-LKB. The apparent sizes of DNA molecules are estimated relative to yeast chromosomes used as markers.

ize with either *L. mexicana* (Fig. 4C) or *L. amazonensis* (Fig. 4D), when using radiolabelled total k-DNA probes.

Molecular karyotype analysis – Although *L. forattinii* and other species of the *L. mexicana* complex showed a specific number of size-concordant DNA molecules, clear karyotypic differences exist among these parasites (Fig. 5). However, chromosomes were polymorphic in both the number and size of bands between the two strains of the new parasite.

DISCUSSION

Parasitic protozoa of the genus *Leishmania* (Kinetoplastida: Trypanosomatidae) are a biologically diverse group of microorganisms (Lainson & Shaw, 1987). Taxonomic studies of *Leishmania* isolates from the New World indicate diversity within this genus (Grimaldi et al., 1989). A number of new *Leishmania* species have been described recently from sylvan areas of Neotropics (Silveira et al., 1987; Lainson et al., 1989; Lainson & Shaw, 1989; Kreutzer et al., 1991; Grimaldi et al., 1992). Some of those parasites cause disease in humans (Silveira et al., 1987; Lainson et al., 1990; Kreutzer et al., 1991; Naiff et al., 1991; Shaw et al., 1991); others are seemingly restricted to lowers orders of mammal (Lainson & Shaw, 1987; Grimaldi et al., 1989; 1992). Some other taxonomically distinct taxa, isolated from wild mammals and vectors in the Brazilian Amazon Region, have also been described as unnamed new species (Lainson & Shaw, 1987; Grimaldi et al., 1989; 1991).

In this study, we have characterized two leishmanial parasites from opossum and rodent captured in the Atlantic Forest of Brazil with other *Leishmania* species. On the basis of biological and molecular features, the stocks were characterized as a distinct species within the *L. mexicana* complex. Because of clear differences between the two isolates and the 3,000 other *Leishmania* strains that we have examined so far, it is proposed that these parasites be considered as a new species, designated *L. forattinii* sp. n. The numerical analysis demonstrated that *L. forattinii* was clustered within the aforementioned group of parasites. This information was confirmed by data from Cupollilo et al. (1993), who also showed in a cladistic analysis that *L. forattinii* is related to *L. aristidesi*.

Species of *Proechimys* and *Didelphis* have been previously found infected with leishma-

nial parasites, including *L. amazonensis, L. braziliensis, L. guyanensis* and *L. chagasi* (Lainson & Shaw, 1987; Grimaldi et al., 1989). Other leishmanial strains with similar characteristics to *L. forattinii* were recovered from *Proechimys* captured in the same region (Barretto et al., 1985). In addition, several species of wild caught sandflies were collected in the same site; experimental infections suggested that one or more of these sandfly species encountered in high numbers (*Lu. ayrosai* and *Lu. yuilli*) may be the vector(s) of the new parasite (Barretto et al., 1986). However, to date, no human infection with *L. forattinii* has been identified.

Description and Diagnosis

Type Hosts: Didelphis marsupialis aurita and *Proechimys iheringi denigratus.*

Locality in host: spleen and liver; also found in the skin.

Type locality: humid tropical forest within municipality of Conchas, São Paulo State, Brazil.

Strain Designation: MDID/BR/77/Conchas.

Amastigotes: Length 4.52 ± 0.42 (3.44-5.18); width 4.24 ± 0.41 (2.98-5.16; nucleus length 2.22 ± 0.27 (1.61-2.90); nucleus width 1.67 ± 0.26 (1.13-2.10); kinetoplast length 1.03 ± 0.14 (0.64-1.44); kinetoplast width 0.49 ± 0.07 (0.35-0.66).

Promastigotes: total body length 12.50 ± 1.52 (7.78-14.90); width 2.71 ± 0.33 (1.65-3.26); nucleus length 2.26 ± 0.30 (1.76-292); nucleus width 2.08 ± 0.31 (1.22-2.61); nucleus centre to anterior tip 4.48 ± 0.67 (2.91-6.24); nucleus centre to posterior tip 7.76 ± 1.52 (4.14-10.08); free flagellum 15.11 ± 3.30 (10.43-24.83).

Development in Sandfly: typical suprapylarial pattern in experimentally infected *Lu. ayrosai* or *Lu. yuilli* samples from Três Braços, Bahia; with abundant active forms of the parasite in the stomach, pharynx and reaching the prosboscis of the insect (Barretto et al., 1986).

Enzyme profiles: the enzyme 6PGDH separates this parasite from all *Leishmania* species, except those of the *L. mexicana* complex. However, using the enzymes MDH, ME, PGM, NH1 & 2, FUM, HK, MPI, ACON, PEP 3, and IDHNAD the latter parasite species can be distinguished one from another (Fig. 2a).

Reactivity with monoclonal antibodies: as some other *L. mexicana* complex species, *L. forattinii* cross-reacts with monoclonal antibodies M2 (IX-2H7-E10), M4 (IX-1F9-D8), M8 (LXVIII-4D8-B7), AND M9 (XLV-2B5-H7), which were produced against *L. (L.) mexicana* (M8) and *L. (L.) amazonensis* (M2, M4, and M9).

Schizodeme analysis of k-DNA: the k-DNA fragment profiles of this parasite are readily distinguished from those of all other *Leishmania* complex parasites examined (including *L. aristidesi* reference strain) when digested with *Msp* I, *Hinf* I, *Alu* I, *Mbo* I, and *Bsp* RI.

Molecular karyotype analysis: each stock of the new parasite had a distinct karyotype; the differences found between these strains and other parasites of the *L. mexicana* complex are not clear enough for parasite identification.

Behaviour in animals: lesions produced in hamsters or BALB/c mice are similar to those which occur in susceptible animals inoculated with parasites of the *L. mexicana* complex; large, non-ulcerated histiocytoma-like nodules rich in large amastigote forms.

Behaviour in in vitro culture: grows luxuriantly in either blood-agar slants or Schneider's Drosophila liquid medium.

Host specificity: known, so far, only from *Didelphis marsupialis* aurita and *Proechimys iheringi denigratus.*

Type material: hapantotype slides (amastigotes and promastigotes) held in the Departamento de Parasitologia, Universidade Estadual Paulista, Botucatu, SP and Departamento de Patologia, Universidade de Brasília, DF, Brazil. Cultures held in cryobanks at the Departamento de Imunologia, Instituto Oswaldo Cruz, Rio de Janeiro and the Departamento de Parasitologia, Instituto Evando Chagas, Belém, PA, Brazil.

Etymology: Leishmania (L.) forattinii sp. n. is named in honour of Professor O. P. Forattini, in tribute to his extensive work on the eco-epidemiology of cutaneous leishmaniasis in São Paulo State, Brazil.

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