Mem. Inst. Oswaldo Cruz, Rio de Janeiro, Vol. *87* (2): 221-228, apr./jun. 1992

DESCRIPTION OF *LEISHMANIA EQUATORENSIS SP. N.* (KINETOPLASTIDA: TRYPANOSOMATIDAE), A NEW PARASITE INFECTING ARBOREAL MAMMALS IN ECUADOR

GABRIEL GRIMALDI, Jr.; RICHARD D. KREUTZER*; YOSHIHISA HASHIGUCHI**; EDUARDO A. GOMEZ***; TATSUYUKI MIMORY**** & ROBERT B. TESH*****

Departamento de Imunologia, Instituto Oswaldo Cruz, Av. Brasil 4365, 21045-900, Rio de Janeiro, RJ, Brasil
*Department of Biology, Youngstown State University, Youngstown, Ohio 44555, U.S.A.
**Department of Parasitology, Kochi Medical School, Nankoku, Kochi 781-51, Japan
***Departamento de Parasitologia, Instituto Nacional de Higiene y Medicina Tropical, Apartado 3961, Guayaquil, Ecuador ****Department of Parasitic Diseases, Kumamoto University School of Medicine, Kumamoto 860, Japan *****Department of Epidemiology and Public Health, Yale University School of Medicine, New Haven, Conecticut 06510, U.S.A.

Characterization is given of a new parasite, Leishmania equatorensis *sp. n., which was isolated from the viscera of a sloth (*Choloepus hoffmanni*) and a squirrel (*Sciurus granatensis*), captured in humid tropical forest on the Pacific Coast of Ecuador. Data based on biological and molecular criteria, as well as numerical zymotaxonomical analysis, indicate that this parasite is a new species of the* L. braziliensis *complex.* L. equatorensis *is clearly distinguishable from all other known species within this complex, using the following molecular criteria: reactivity patterns with specific monoclonal antibodies, isoenzyme electrophoresis, and restriction-endonuclease fragment patterns of kinetoplast DNA (k-DNA).*

Key words: *Leishmania equatorensis* sp. n. – Protozoa – Kinetoplastida – Trypanosomatidae – mammalian reservoirs – molecular characterization – monoclonal antibodies – isoenzyme electrophoresis – kinetoplast DNA analysis

Cutaneous and mucocutaneous leishmaniases are endemic in Ecuador; the disease occurs in both lowland and Andean regions of the country (Hashiguchi, 1987; Grimaldi et al., 1989; Mimori et al., 1989; Armijos et al., 1990; Hashiguchi et al., 1991). To date, six different species of the parasite (*Leishmania braziliensis, L. panamensis, L. guyanensis, L. mexicana, L. pifanoi* and *L. amazonensis*) have been isolated from Ecuadorian patients with the disease (Mimori et al., 1989; Armijos et al., 1990; Hashiguchi et al., 1991); isolations of *L. mexicana* and *L. amazonensis* have also been made from domestic and wild animals (Hashiguchi et al., 1985; 1991; Mimori et al., 1989).

During a survey of potential reservoir hosts of leishmanial parasites done in Ecuador in 1982, two unidentified parasites were isolated from a sloth (*Choloepus hoffmanni*) and from a squirrel (*Sciurus granatensis*) captured in Naranjal, Department of Guayas (Hashiguchi et al., 1985; Hashiguchi, 1987). In preliminary studies, these two isolates appeared to be identical to each other, but they were distinct from the currently recognized New Worid *Leishmania.* On the basis of their growth characteristics, the parasites were provisionally assigned to the *L. braziliensis* complex (Hashiguchi et al., 1985). In order to better understand their taxonomic position in the genus, we have further studied these isolates and have compared them with well characterized World Health Organization (WHO) reference strains (WHO, 1984), using a large panel of species-specific monoclonal antibodies in a radioimmune binding assay, isoenzyme electrophoresis, and restriction-endonuclease fragment patterns of

Grant support: Overseas Scientific Research Program of the Ministry of Education, Science and Culture, Japan (grants 61041059, 62043055 and 63041096); CNPq (Brazil); National Institutes of Health of the United States (grants AI-20108, AI-21049 and AI-23004); UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases; and the MacArthur Foundation.

Received 9 September 1991.
Accepted 1 November 1991.

k-DNA. The present paper gives the results of these studies and describes the two parasites as members of a new species, belonging to the *L. braziliensis* complex and for which we propose the name *Leishmania equatorensis* sp. n.

MATERIALS AND METHODS

Parasites examined – Identification of the *Leishmania* parasites used in this study is given in Table I. The two strains of the proposed new species (MCHO/EC/82/L sp1 and MSCI/EC/82/L sp2) were originally isolated from pooled liver and spleen samples taken from a sloth (*Choloepus hoffmanni*) and a squirrel (*Sciurus granatensis*), both captured in a humid tropical forest located about 1,100 m above sea level near Naranjal, Department of Guayas, Ecuador on 23 February 1982 (Hashiguchi et al., 1985; Hashiguchi, 1987).

Morphological examination – Fixed preparations of the new parasites (cultured log-phase promastigotes and amastigotes from experimentally infected hamsters) were examined to determine their morphology and size, using the methods described by Shaw & Lainson (1976).

Preparation of samples for biochemical studies – Promastigotes of each parasite strain listed in Table I were grown in roller bottles at 23-24 °C in Schneider's *Drosophila* 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 washed twice in phosphate-buffered saline (PBS), pH 7.3. The final pellet was used for preparation of samples for parasite characterization, using the three molecular techniques described below.

Isoenzyme electrophoresis – Cellulose electrophoresis procedures and preparation of enzyme activities for the identification of *Leishmania* parasites are done routinely in our laboratory and have been reported previously (Kreutzer et al., 1987). The leishmanial strains were characterized twice, examining the following enzymes: LDH; MDH; ME; ICD; 6PGDH; G6PDH; GSR_1/GSR_2; GOT/ASAT; ALAT; HK; PFK; PK; PGM_1/PGM_2; EST; ACP_1/ACP_2; PEPD/LP; FUM; MPI; and GPI. For enzyme abbreviations, see Kreutzer et al. (1991).

Genetic analysis – Taxonomic classification of the Ecuadorian parasites was based on comparisons of the enzyme data from these isolates and from selected species complexes of *Leishmania,* including the reference strains (Table I). Statistical analysis of the enzyme data, as described by Ney (1972), was used for the calculations of genetic identity (I) and distance (D) between the new parasite and other established species within the genus *Leishmania.*

Monoclonal antibodies and indirect radioimmune assay – The monoclonal antibodies used in this study have been described previously (Grimaldi et al., 1987; 1991; Hashiguchi et al., 1991; Kreutzer et al., 1991). Characterization of the *Leishmania* was performed with an indirect radioimmune binding assay using whole parasite lysates as antigen. The technique has been described in detail before (Grimaldi et al., 1987).

Schizodeme analysis of k-DNA – Kinetoplast DNA was isolated from 5 x 109 promastigotes by the rapid method of Morel and others (1980). Samples of excreted k-DNA (approximately, 2 μg) for schizodeme analysis were digested with an excess of the appropriate restriction enzymes (*Hinf* I, *Alu* I, *Msp* I, or *Tag* I) and prepared for electrophoresis, using the method described previously (Hashiguchi et al., 1991). The products of digestion were separated by electrophoresis in a vertical direction, at a constant current of 8 mA, on a linear 5 to 12% acrylamide gel, until the bromophenol blue marker dye reached the bottom of the gel (usually overnight). The gels were stained in ethidium bromide (0.5 μg/ml) and photographed with UV transillumination.

RESULTS

Initial isolation and growth characteristics – The two strains of *L. equatorensis* (MCHO/EC/82/L sp1 and MSCI/EC/82/L sp2) were isolated from pooled liver-spleen homogenates from a sloth and a squirrel, respectively. Primary isolation of the parasites was made in tubes of blood agar (Walton et al., 1972); the method was described previously (Hashiguchi et al., 1985). Once established, both parasite strains grew well in cultures of blood agar and in Schneider's *Drosophila* medium with fetal bovine serum. Inoculation of cultured promastigotes into the noses of hamsters (*Mesocricetus auratus*) produced local swelling without metastasis; appearance of the lesions

TABLE I

Origin and identification of reference strains and *Leishmania* isolates from Ecuador which were characterized by monoclonal antibodies, isoenzyme and schizodeme (kDNA) analyses in this study

Stock	Designation[a]	Species	Geographic origin
L562	MHOM/PA/71/LS94[b]	*L. panamensis*	Canal Zone, Panama
L565	MHOM/BR/75/M4147[b]	*L. guyanensis*	Pará, Brazil
L566	MHOM/BR/75/M2903[b]	*L. braziliensis*	Pará, Brazil
L571	MHOM/SU/58/str.OD[b]	*L. tropica*	Azerbaijan, USSR
L577	MNYC/BZ/62/M379[b]	*L. mexicana*	Cayo, Belize
L581	MHOM/SU/73/5 ASKH[b]	*L. major*	Turkmen, USSR
L747	MHOM/BR/85/HI-2	*L. braziliensis*	E. Santo, Brazil
L880	MHOM/BR/87/IM3147	*L. amazonensis*	Amazonas, Brazil
L881	IHAR/CO/85/CL500[c]	*L. colombiensis*	Santander, Colombia
L867	MHOM/VE/60/LtRod[d]	*"L. pifanoi"*	Venezuela
L886	MHOM/IL/79/LRC-L251	*L. major*	Jericho, Israel
L888	MCHO/EC/82/Lsp1	*L. equatorensis*	Guayas, Ecuador
L889	MSCI/EC/82/Lsp2	*L. equatorensis*	Guayas, Ecuador
L892	MPOT/EC/87/G-03	*L. amazonensis*	Bolivar, Ecuador
L894	MHOM/EC/87/G-07	*L. panamensis*	Pichincha, Ecuador
L1023	MHOM/BR/81/M6426[b]	*L. lainsoni*	Pará, Brazil
L1365	MHOM/BR/79/M5533[b]	*L. naiffi*	Pará, Brazil

a: designation code: Host (M for Mammalia: CHO = *Choloepus hoffmani;* DAS = *Dasypus novemcinctus;* HOM = *Homo sapiens;* NYC = *Nyctomys sumichrasti;* POT = *Potus flavus;* and SCI = *Sciurus granatensis;* I for Insecta: HAR = *Lutzomyia hartmani)*/country of origin/year of isolation/original code.
b: WHO reference strain.
c: reference strain of a parasite isolated both in Colombia and Panama, which was recently classified as a new species, *L. colombiensis*, of the *L. braziliensis* complex (Kreutzer et al., 1991).
d: this strain has also previously been characterized as *"L. major*-like", based on studies comparing isoenzymes, reactivity patterns with specific monoclonal antibodies, and kDNA fragment patterns (Hashiguchi et al., 1991).

took 1-3 months, depending on the size of the inoculum.

The behavior of *L. equatorensis* is indistinguishable from other members of the *L. braziliensis* complex, based on its virulence and development in laboratory animals. In contrast to *L. mexicana* complex parasites which usually produce a tumorous inflammation at the site of inoculation in hamsters, infections with species of the *L. braziliensis* complex, including *L. equatorensis*, grow relatively slowly and show few parasites in the lesions. The general morphology of *L. equatorensis* amastigotes and promastigotes, as determined by light microscopy, is also indistinguishable from other species of the *L. braziliensis* complex.

Characterization by enzyme electrophoresis – As shown in Fig. 1, the two Ecuadorian isolates (identified as L888 and L889 in the Fig.) had identical allomorphs (bands of enzyme activity as observed by electrophoresis) to each other. For most of the 22 enzymes examined, the profiles were different from those of all other *Leishmania* species tested. The exceptions were two recently described species of the *L. braziliensis* complex: *L. lainsoni* from northern Brazil (Silveira et al., 1987) and *L. colombiensis* from Colombia and Panama (Kreutzer et al., 1991). Similarity was noted between *L. equatorensis* and the latter two parasites with the enzymes GPI and MPI, plus 6PGDH with *L. colombiensis;* these enzymes can be used to distinguish most other *Leishmania* species. However, data from these three plus any one of the enzymes ACP, GOT, LP, MDH, ME, PGM, EST, and PK could distinguish the new parasite from either *L. lainsoni* or *L. colombiensis.*

To define the taxonomical position of the new Ecuadorian parasite in relation to other *Leishmania* complex species, genetic identities (I) and distances (D) were calculated from their enzyme data. In general, pairwise comparisons of parasites defined as separate species show higher values in their genetic distance index (usually with D values > 0.500) than when different strains of the same parasite species are compared. For example, a D

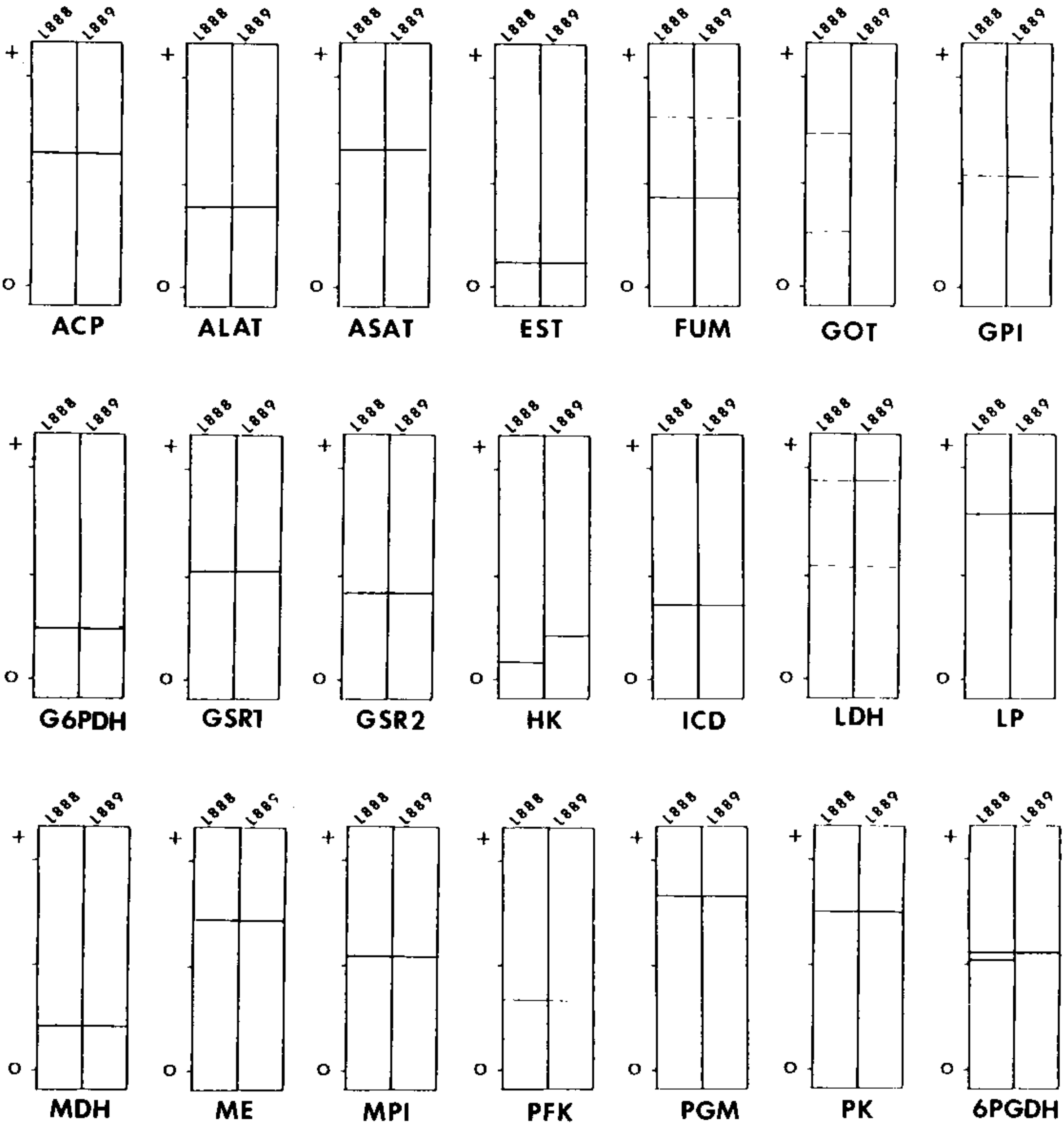

Fig. 1: diagrammatic representation of enzyme profiles of the two *Leishmania equatorensis* isolates, L888 (MCHO/EC/82/L sp1) and L889 (MSCI/EC/82/L sp2).

value of 1.030 was obtained when *L. panamensis* and *L. braziliensis* strains were compared, contrasting with the result obtained when different populations of *L. panamensis* from Panama and Colombia were compared (D = 0.023). Similar comparisons of the enzyme profiles of strains MCHO/EC/82/L sp1 and MSCI/EC/82/L sp2 with other *Leishmania* species produced D values $\geqslant$ 1.200, which indicate that the aforementioned parasites represent, as a group, a separate species. Although distinct, the new parasite species from Ecuador is genetically closest to *L. colombiensis* (I = 0.561) and to *L. lainsoni* (I = 0.410).

Reactivity with monoclonal antibodies – The two Ecuadorian isolates (designated with stock codes L888 and L889) were also characterized by serodeme analysis, using a large panel of specific monoclonal antibodies (Table II). The specificity of these monoclonal antibodies for species of the *L. braziliensis, L. mexicana, L. tropica,* and *L. donovani,* complexes have been described before (Grimaldi et al., 1987; 1991; Kreutzer et al., 1991; Hashiguchi et al., 1991). As shown in Table II, only two monoclonal antibodies (B4 and B8), which are specific for members of the *L. braziliensis* group, bound to the L888 and L889

TABLE II

Radioimmune binding assay results[a], employing *Leishmania* species-specific monoclonal antibodies, with reference strains and *Leishmania* sp n isolates from Ecuador

Stock code	Species[b]	Monoclonal antibodies[c]										
		B4	B8	B13	B19	B7	B12	B5	B3	B11	B16	B18
Reference strains												
L1023	*L. lainsoni*	1 7	1.8	6.5	1 3	2.4	1.4	1.3	1.0	0.8	0.7	0 8
L566	*L braziliensis*	3.9	27.8	17.6	1 6	14.2	21 0	10 8	21 0	2 0	23.2	26 7
L562	*L panamensis*	17.0	7.4	15.6	2.3	16.0	8.9	12.6	16.0	12.6	2.6	2 4
L565	*L guyanensis*	2.5	2.3	10.6	13.2	19 7	3.2	1.6	1 8	1 4	2 3	1 9
L1365	*L naiffi*	1.3	2.2	3 4	0.9	6 8	6.2	0.5	1 2	0 5	1 0	0 7
L881	*L colombiensis*	27.1	4.8	5 4	1.5	1 1	1 2	1.0	2.2	1 1	1.1	1.1
Ecuador isolates[d]												
L888	*Leishmania* sp n.	18.4	12.1	1.3	1.7	1.6	1 2	1 0	2 1	1 2	1 4	1 2
L889	*Leishmania* sp n.	19.6	13.6	1.0	1.4	1.2	1.2	1 1	1 8	1 6	1 3	1 4

a: results shown express the ratio of cpm bound antibody/cpm bound control; values $>$ 3 were considered positive.

b: stock identification (classification) established by isoenzyme and/or schizodeme analyses.

c: from the hybridoma clones: B4, VI-2A5-A4; B8, VII-3E12-D3; B13, XLIV-5H2-A10; B19, XLIV-5A2-B9; B7, VI-2A4-E3; B12, XIII-3H6-A12; B5, VII-2C5-C5; B3, VI-4D10-D12; B11, VII-5G3-F3; B16, XIII-3E6-B11; and B18, XIV-2A5-A10.

d: no significant cross-reactivity was obtained to any of these strains with a large panel of monoclonal antibodies species-specific for *L. mexicana* and *L donovani* complex parasites. However, these strains did cross-react with some monoclonals (T1, XLVI-5B8-A8; T2, XLVI-4H12-C2; T3, XLVI-5A5-D4; T4, LXVIII-1A4-G1; and T8, LXVII-3E12-F8), which were produced against strains of *L major*.

stocks. No significant cross-reactivity was obtained to any of these strains with a large panel of monoclonal antibodies which are specific for *L. mexicana* and *L. donovani* complex parasites. On the basis of these reactivity patterns, we confirmed the identification of this parasite, as a new species of the *L. braziliensis* complex. However, as noted with *L. colombiensis* (Kreutzer et al., 1991), another new species in the *L. braziliensis* complex, the two *L. equatorensis* strains cross-reacted with monoclonal antibodies T1, T2, T3, T4, and T8 (data not shown), which were produced against members of the *L. tropica* complex.

Schizodeme analysis of k-DNA – A comparison of k-DNA fragment profiles from stocks, representing selected *Leishmania* complex parasites and the two isolates from Ecuador, was done by schizodeme analysis of restriction enzyme digests of k-DNAs, fractioned by gradient acrylamide gel electrophoresis. As shown in Fig. 2, the stocks L888 and L889 possessed identical k-DNA fragment patterns with all four restriction endonucleases tested (*Hinf* I, *Alu* I, *Msp* I, and *Tag* I). The k-DNA fragment profiles of these parasites were distinct from those seen with all strains of the *L. braziliensis* and *L. mexicana* complexes examined. However, based on the degree of heterogeneity within minicircles obtained with each of the restriction enzymes, *L. equatorensis* seems to be much closer to species of the *L. braziliensis* complex, than to those of any other group. In addition, on the basis of their k-DNA fingerprints, the above Ecuadorian isolates are distinct from the WHO *L. major* and *L. tropica* reference strains, despite their similarities by serodeme analysis (reactivity to monoclonal antibodies) with the latter two Old World species.

DISCUSSION

Results of recent epidemiological surveys and characterization of leishmanial isolates obtained from a variety of sources (humans, animals and sand flies) have demonstrated the diversity within this genus, particularly in sylvan regions of the Neotropics (Lainson, 1981; Momen et al., 1985; Grimaldi et al., 1987; 1989; 1991; Lainson & Shaw, 1987; 1989; Shaw & Lainson, 1987; Silveira et al., 1987; Lainson et al., 1989; Hashiguchi et al., 1991; Kreutzer et al., 1991). Some of the New World *Leishmania* species are associated with disease

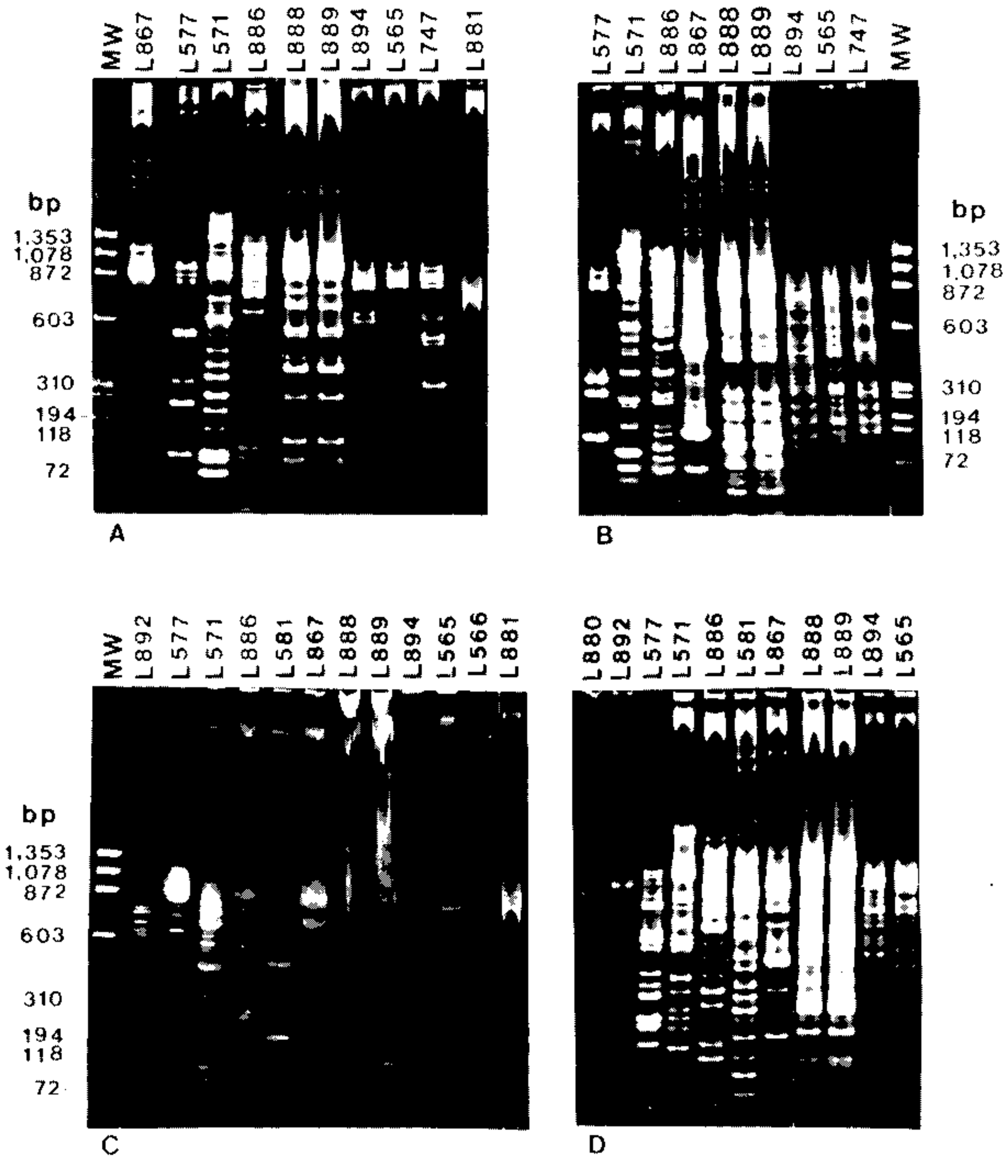

Fig. 2: acrylamide gradient (5-12%) gel electrophoresis comparison of k-DNA fragment patterns, generated with the restriction enzymes *Hinf* I (a), *Alu* I (b), *Msp* I (c), and *Tag* I (d), among selected species of *Leishmania* and the two new strains (L888 and L889) from Ecuador. The stock codes of the *Leishmania* strains studied by k-DNA analysis are indicated above the lanes; their identification is given in Table I. The apparent sizes of k-DNA fragments are estimated relative to fragments from a *Hae* III digest of 0 X 174 RF DNA and their molecular weights are indicated in base pairs (bp) beside the figures. Undigested k-DNA network is visible in the slots at the top of the gels, whereas presumptive maxicircle DNA fragments and minicircle oligomers migrate at > 6 kb and are visible just below. The linearized minicircles migrate at about 0.8 kb and, in some lanes, the minicircle digests appear as bands (ladders) between 0.05 and 0.7 kb.

in humans, while others appear to be restricted to lower orders of mammals, such as rodents and edentates (Lainson & Shaw, 1987; Shaw & Lainson, 1987; 1989; Grimaldi et al., 1989; 1991; Lainson et al., 1989; Grimaldi & McMahon-Pratt, 1991; Kreutzer et al., 1991). As the newer molecular techniques for parasite characterization become more widely used, undoubtedly additional *Leishmania* species will be recognized.

In the current paper, we have characterized two leishmanial isolates from sloth and squirrel captured in Ecuador, in comparison to reference strains of other *Leishmania* species. On the basis of their distinct molecular make-up we considered these isolates as a new species of *Leishmania* within the *L. braziliensis* complex. However, some similarity was noted between the aforementioned Ecuadorian parasites and two other recently described species

in the *L. braziliensis* complex: *L. lainsoni* from northern Brazil (Silveira et al., 1987) and *L. colombiensis* from Colombia and Panama (Kreutzer et al., 1991).

Because of clear differences between these two Ecuadorian isolates and the approximately 3,000 other New World *Leishmania* strains that we have examined by the aforementioned molecular techniques, it is proposed that these parasites be considered as a new species, designated *Leishmania equatorensis* sp. n. The public health importance of *L. equatorensis* sp. n. remains to be determined. To date, it has only been isolated from arboreal mammals; no human infections with the parasite have been identified. Likewise, the sand fly vector(s) are unknown. A formal description of the new species follows:

Leishmania equatorensis sp. n.

Diagnosis

Type hosts: *Choloepus hoffmanni* and *Sciurus granatensis.*

Locality in host: spleen and liver; not found in the skin.

Type locality: Naranjal, Department of Guayas, Ecuador.

Strain designation: MCHO/EC/82/L spl.

Sand fly vector: unknown.

Enzyme profiles: the enzymes GPI, MPI, and 6PGDH separate this parasite from all currently recognized *Leishmania* species, except *L. colombiensis.* However, by adding data from any one of the enzymes ACP, GOT, LP, MDH, ME, PGM, EST, and PK, *L. equatorensis* can be distinguished from the latter parasite.

Reactivity with monoclonal antibodies: as with some other *L. braziliensis* complex species, this parasite cross-reacts with monoclonal antibodies B4 (VI-2A5-A4) and B8 (VII-3E12-D3), which were produced against *L. panamensis* (strain MHOM/PA/74/WR120). The new parasite also cross-reacted with monoclonal antibodies T1, T2, T3, T4, and T8, which were produced against members of the *L. tropica* complex.

Schizodeme analysis of k-DNA: when digested with *Hinf* I, *Alu* I, *Msp* I, and *Tag* I, the k-DNA fragment profiles of this parasite were readily distinguished from those seen with other *Leishmania* species examined, including *L. colombiensis* and *L. lainsoni* as well as the WHO *L. major* and *L. tropica* reference strains.

Behaviour in hamster: produced local swelling without ulceration or metastasis in hamsters within 1-3 months after intradermal inoculation.

In vitro culture: grows well in blood agar and Schneider's *Drosophila* medium.

Host specificity: to date, only *Choloepus hoffmanni* and *Sciurus granatensis* have been found infected.

Type material: cryopreserved promastigotes were deposited in the following cryobanks: Departamento de Parasitologia, Instituto Nacional de Higiene y Medicina Tropical, Guayaquil, Ecuador; Department of Parasitology, Kochi Medical School, Kochi, Japan; Department of Epidemiology and Public Health, Yale University School of Medicine, New Haven, CT, U.S.A.; and Department of Immunology, Instituto Oswaldo Cruz, Rio de Janeiro, Brazil.

ACKNOWLEDGEMENTS

To Dr Diane McMahon-Pratt, Yale University School of Medicine, for providing the monoclonal antibodies used in this study. To our collaborators at the Instituto Nacional de Hygiene y Medicina Tropical, Guayaquil, Ecuador.

REFERENCES

ARMIJOS, R. X.; CHICO, M. E.; CRUZ, M. E.; GUDERIAN, R. H.; KREUTZER, R. D.; BERMAN, J. D.; ROGERS, M. D. & GROGL, M., 1990. Human cutaneous leishmaniasis in Ecuador: identification of parasites by enzyme electrophoresis. *J. Trop. Med. Hyg., 42:* 424-428.

GRIMALDI, Jr. G.; DAVID, J. R. & McMAHON-PRATT, D., 1987. Identification and distribution of New World *Leishmania* species characterized by serodeme analysis using monoclonal antibodies. *Am. J. Trop. Med. Hyg., 36:* 270-187.

GRIMALDI, Jr. G. & McMAHON-PRATT, D., 1991. Leishmaniasis and its etiologic agents in the New World: An overview, p. 73-118. In T. Sun (ed.), *Progress in Clinical Parasitology,* Vol. II. Field & Wood, Philadelphia.

GRIMALDI, Jr. G.; MOMEN, H.; NAIFF, R. D.; McMAHON-PRATT, D. & BARRETT, T. V., 1991. Characterization and classification of leishmanial

parasites from humans, wild mammals, and sand flies in the Amazon Region of Brazil. *Am. J. Trop. Med. Hyg., 44:* 645-661.

GRIMALDI, Jr. G.; TESH, R. B. & McMAHON-PRATT, D., 1989. A review of the geographic distribution and epidemiology of leishmaniasis in the New World. *Am. J. Trop. Med. Hyg., 41:* 687-725.

HASHIGUCHI, Y., 1987. Studies on New World leishmaniasis and its transmission, with particular reference to Ecuador, p. 44-69. *Res. Rep. Ser. No 1.* Kochi, Japan: Kyowa Printing.

HASHIGUCHI, Y.; GOMEZ, E. A.; de CORONEL, V. V.; MIMORI, T. & KABATA, M., 1985. *Leishmania* isolated from wild mammals caught in endemic areas of leishmaniasis in Ecuador. *Trans. R. Soc. Trop. Med. Hyg., 79:* 120-121.

HASHIGUCHI, Y.; GOMEZ, E. A.; CORONEL, V. V.; MIMORI, T.; KAWABATA, M.; FURUYA, M.; NONAKA, S.; TAKAOKA, H.; ALEXANDER, J. B.; QUIZHPE, A. M.; GRIMALDI, Jr. G.; KREUTZER, R. D. & TESH, R. B., 1991. Andean leishmaniasis in Ecuador caused by infection with *Leishmania mexicana* and *L. major*-like parasites. *Am. J. Trop. Med. Hyg., 44:* 205-217.

HENDRICKS, L. D.; WOOD, D. E. & HADJUK, M. E., 1978. Haemoflagellates: commercially available liquid media for rapid cultivation. *Parasitology, 74:* 309-316.

KREUTZER, R. D.; CORREDOR, A.; GRIMALDI, Jr. G.; GROGL, M.; ROWTON, E. D.; YOUNG, D. G.; McMAHON-PRATT, D.; GUZMAN, H. & TESH, R. B., 1991. Characterization of *Leishmania colombiensis* sp. n. (Kinetoplastida: Trypanosomatidae), a new parasite infecting humans, animals and phlebotomine sand flies in Colombia and Panama. *Am. J. Trop. Med. Hyg., 44:* 662-675.

KREUTZER, R. D.; SOURATY, N. & SEMKO, M. E., 1987. Biochemical identities and differences among *Leishmania* species and subspecies. *Am. J. Trop. Med. Hyg., 36:* 22-32.

LAINSON, R., 1981. Epidemiologia e ecologia de leishmaniose tegumentar na Amazônia. *Hiléia Méd., 3:* 35-40.

LAINSON, R.; BRAGA, R. R.; de SOUZA, A. A. A.; POVOA, M. M.; ISHIKAWA, E. A. Y. & SILVEIRA, F. T., 1989. *Leishmania (Viannia) shawi* sp. n., a parasite of monkeys, sloths and procyonids in Amazonian Brazil. *Ann. Parasitol. Hum. Comp., 64:* 200-207.

LAINSON, R. & SHAW, J. J., 1987. Evolution, classification and geographical distribution, p. 1-120. In W. Peters & R. Killick- Kendrick (eds), *The Leishmaniases in Biology and Medicine,* Vol. 1. Academic Press, London.

LAINSON, R. & SHAW, J. J., 1989. *Leishmania (Viannia) naiffi* sp. n., a parasite of the armadillo, *Dasypus novemcinctus* (L.) in Amazonian Brazil. *Ann. Parasitol. Hum. Comp., 64:* 3-9.

MIMORI, T.; GRIMALDI, Jr. G.; KREUTZER, R. D.; GOMEZ, E. A.; McMAHON-PRATT, D.; TESH, R. B. & HASHIGUCHI, Y., 1989. Identification, using isoenzyme electrophoresis and monoclonal antibodies, of *Leishmania* isolated from humans and wild animals of Ecuador. *Am. J. Trop. Med. Hyg., 40:* 154-158.

MOMEN, H.; GRIMALDI, Jr. G.; PACHECO, R. S.; JAFFE, C. L.; McMAHON-PRATT, D. & MARZOCHI, M. C. A., 1985. Brazilian *Leishmania* stocks phenotypically similar to *Leishmania major. Am. J. Trop. Med. Hyg., 34:* 1076-1084.

MOREL, C.; CHIARI, E.; PLESSMAN CAMARGO, E.; MATTEI, D. M.; ROMANHA, A. J. & SIMPSON, L., 1980. Strains and clones of *Trypanosoma cruzi* can be characterized by pattern of restriction endonuclease products of kinetoplast DNA minicircles. *Proc. Nat. Acad. Sci. USA, 77:* 6810-6814.

NEY, M., 1972. Genetic distance between populations. *Am. Nat., 106:* 283-291.

SHAW, J. J. & LAINSON, R., 1976. Leishmaniasis in Brazil: XI. Observations on the morphology of *Leishmania* of the *braziliensis* and *mexicana* complexes. *J. Trop. Med. Hyg., 76:* 9-13.

SHAW, J. J. & LAINSON, R., 1987. Recent advances in studies on the ecology and epidemiology of New World leishmaniasis, p. 291-363. In W. Peters & R. Killick-Kendrick (eds), *The Leishmaniases in Biology and Medicine,* Vol. 1. Academic Press, London.

SILVEIRA, F. T.; SHAW, J. J.; BRAGA, R. R. & ISHIKAWA, E., 1987. Dermal leishmaniasis in the Amazon region of Brazil: *Leishmania (Viannia) lainsoni* sp. n. a new parasite from the State of Pará *Mem. Inst. Oswaldo Cruz, 82:* 289-292.

WALTON, B. C.; BROOKS, W. H. & ARJONA, I., 1972. Serodiagnosis of American leishmaniasis by indirect fluorescent antibody test. *Am. J. Trop. Med. Hyg., 21:* 296-299.

WORLD HEALTH ORGANIZATION, 1984. The Leishmaniases. *WHO Techn. Rep., Ser No 701,* Geneva, p. 140.