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LEISHMANIA MAJOR–LIKE PARASITE, A PATHOGENIC AGENT OF CUTANEOUS LEISHMANIASIS IN PARAGUAY

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Abstract. *Leishmania* parasites isolated from two patients with cutaneous leishmaniasis from geographically different localities in Paraguay have been characterized by enzyme electrophoresis (zymodeme) and digestion profiles of kinetoplast DNA with restriction enzymes (schizodeme). Both Paraguayan isolates showed identical zymodeme profiles to each other using 14 enzymes (glutamic pyruvate transaminase, glutamic oxaloacetic transaminase, enolase, fumarate hydratase, glucose phosphate isomerase, glucose-6-phosphate dehydrogenase, malate dehydrogenase, malic enzyme, mannose phosphate isomerase, nucleoside phosphorylase, peptidase-D, 6-phosphogluconate dehydrogenase, phosphoglucomutase, and pyruvate kinase). Although two Paraguayan isolates showed different zymodeme profiles from those of six *Leishmania* reference strains of Old and New World *Leishmania* species, they showed identical zymodeme profiles to those of an *L. major*–like parasite from Ecuador. These observations were confirmed by schizodeme analysis using three restriction endonucleases (*Msp* I, *Hae* III, and *Taq* I). These results indicate that *Leishmania* parasites isolated in Paraguay are identified as an *L. major*–like parasite, and it is necessary to consider the existence of *L. major*–like parasites when classifying *Leishmania* isolates from the New World.

American cutaneous and mucocutaneous leishmaniasis are endemic in Paraguay, especially in the southeastern part of the country.[1–3] According to case reports from the country, the clinical manifestations of the disease differ markedly from a single, localized cutaneous lesion to disfiguring and mucocutaneous lesions. These diverse clinical forms may be due to different agents, and the degree of susceptibility of the patient may be an important factor.[4] To date, two different species of the parasite (*Leishmania mexicana amazonensis* and *L. braziliensis*) have been identified from Paraguayan patients with this disease.[5,6] We previously reported two strains of *Leishmania* isolated from patients with cutaneous leishmaniasis in Paraguay.[7] The present paper describes the further identification of these isolates by the analyses of zymodemes and schizodemes using more reference strains.

MATERIALS AND METHODS

Parasites. The two *Leishmania* isolates from Paraguay and the *Leishmania* reference strains used in the present study are listed in Table 1; the geographic locations of the *Leishmania* isolates from Paraguay are shown in Figure 1. Isolated *Leishmania* parasites were initially cultured in N,N,N biphasic medium containing rabbit blood; *Leishmania* promastigotes were then cultured in serum free GIT medium (Wako Pure Chemicals, Osaka, Japan) supplemented with 12.4 μM hemin (Sigma, St. Louis, MO) at 24°C. The parasites at the initial stationary phase were harvested by centrifugation at 3,000 rpm for 10 min at 4°C and washed several times with cold, sterilized phosphate-buffered saline, pH 7.2. The cell pellet was frozen at −80°C until use. *Leishmania donovani chagasi* (ATCC No. 30881), *L. braziliensis panamensis* (ATCC No. 30879), and *L. mexicana* (ATCC No. 30883), used in the schizodeme analysis as reference strains, were obtained from the American Type Culture Collection (Rockville, Maryland).

Zymodeme analysis. Suspensions were made by homogenizing 5×10^8 promastigotes in phosphate buffer, pH 7.0, using an ultrasonicator, then subjected to 12% starch gel electrophoresis (Connought Starch, Toronto, Ontario, Canada). The electrophoretic conditions and buffer solutions used for the enzyme tested are shown in Table 2. After electrophoresis, the gel was

TABLE I

Isolates and reference strains of Leishmania *parasites used in the present study*

Species	Designation	Where isolated	Clinical forms*
Paraguayan isolates			
Leishmania sp.	MHOM/PR/87/JE15	Lima, San Pedro	CL
Leishmania sp.	MHOM/PR/87/BN40	Residenta, Canindeyu	CL
Reference strains			
L. donovani chagasi†		Tegucigalpa, Honduras	VL
L. braziliensis panamensis†		Canal Zone, Panama	CL
L. mexicana†		San Antonio, Texas	MCL
L. mexicana amazonensis‡		Unknown	
L. major–like§	MHOM/EC/89/G-09	Quininde, Ecuador	CL
World Health Organization reference strains			
L. braziliensis braziliensis¶	MHOM/BR/75/M2904	Para, Brazil	CL
L. braziliensis panamensis¶	MHOM/PA/71/LS94	Canal Zone, Panama	CL
L. braziliensis guyanensis¶	MHOM/BR/75/M4147	Para, Brazil	CL
L. mexicana mexicana¶	MNYC/BZ/62/M379	Cayo, Belize	CL
L. mexicana amazonensis¶	MHOM/BR/73/M2269	Para, Brazil	CL
L. mexicana pifanoi¶	MHOM/VE/57/LL1	Venezuela	DCL
L. mexicana garnhami¶	MHOM/VE/76/JAP78	Venezuela	CL
L. donovani chagasi¶	MHOM/BR/74/M2682	Brazil	VL
L. major	MHOM/SU/73/5-ASKH	Turkmen, USSR	CL
L. tropica	MHOM/SU/58/strain OD	Azerbaijan, USSR	CL

* CL = cutaneous leishmaniasis; VL = visceral leishmaniasis; MCL = mucocutaneous leishmaniasis; DCL = diffuse cutaneous leishmaniasis.
† Obtained from the American Type Culture Collection (Rockville, MD).
‡ A gift from Department of Parasitology, Tokai University (Isehara, Kanagawa, Japan).
§ Classified by schizodeme and zymodeme analyses.
¶ Used only for zymodeme analysis.

sliced and stained according to the method of Agatsuma and others[8] modified as shown in Table 2. The following 14 enzymes were examined: glutamic oxaloacetic transaminase (GOT) E. C. 2.6.1.1., glucose-6-phosphate dehydrogenase (G6PD) E.C. 1.1.1.49., enolase (ENO) E.C. 4.2.1.11., phosphoglucomutase (PGM) E. C. 2.7.5.1., fumarate hydratase (FH) E. C. 4.2.1.2., glucose phosphate isomerase (GPI) E. C. 5.3.1.9., glutamic pyruvate transaminase (GPT) E.C. 2.6.1.2., mannose phosphate isomerase (MPI) E.C. 5.3.1.8., nucleoside phosphorylase (NP) E.C. 2.4.2.1., peptidase-D (PEP-D) E.C. 3.4.11.5., malate dehydrogenase (MDH) E.C. 1.1.1.37., malic enzyme (ME) E.C. 1.1.1.40., 6-phosphogluconate dehydrogenase (6PGD) E.C. 1.1.1.44., and pyruvate kinase (PK) E.C. 2.7.1.40.

Schizodeme analysis. A total of 2–3 × 10^9 promastigotes were used for the extraction of kinetoplast DNA (kDNA), and the preparation of kDNA was carried out according to the method of Goncalves and others.[9] Two or three hundred nanograms of kDNA were digested with an excess of restriction enzymes, *Msp* I, *Hae* III (Nippon Gene, Toyama, Japan), and *Taq* I (TaKaRa, Kyoto, Japan). The digests were separated by electrophoresis in 4–15% linear gradient polyacrylamide gels at 100 V in a Tris/borate/EDTA buffer system. The gels were stained in ethidium bromide (0.5 μg/ml) and photographed using a red filter.

RESULTS

Electrophoretic studies of two enzymes, GPT and ENO, in the Paraguayan isolates and World Health Organization (WHO) reference strains were carried out. Figure 2 shows that the both Paraguayan isolates (lanes 11 and 12) are apparently different from the *L. braziliensis* complexes (lanes 1–3), the *L. mexicana* complexes (lanes 5–9), and *L. donovani chagasi* (lane 4). Interestingly, the Paraguayan isolates showed profiles identical to those of the Ecuadorian strain (G-O9, lane 10). Therefore, to identify the two Paraguayan strains, zymodeme patterns were compared using another 12 enzymes and the other WHO strains, including the wild strain from Ecuador (G-O9). Figure 3 shows the electrophoretic profiles of the representative five enzymes, and it is evident that all enzyme profiles

FIGURE 1. Geographic location of two *Leishmania* isolates from Paraguay.

TABLE 2

Buffer systems and electrophoretic conditions for the 14 enzymes examined

Enzymes*	Electrode buffer	Gel buffer dilution	Conditions
GOT, G6PD	0.04 M sodium citrate, 6.6 mM *N*-(3-aminopropyl)-morpholine, pH 6.0	1:20	20 mA, 4 hr
ENO, PGM	0.1 M Tris, 0.1 M maleic acid, 10 mM EDTA, 10 mM $MgCl_2$, 0.13 M NaOH, pH 7.4	1:10	20 mA, 14 hr
FH, GPI	Same as for ENO and PGM	1:10	15 mA, 14 hr
GPT, MPI, NP, PEP-D	Same as for ENO and PGM	1:10	10 mA, 14 hr
MDH, ME, PK, 6PGD	0.135 M Tris, 43 mM sodium citrate, pH 7.5	1:10	30 mA, 5 hr

* For definition of enzymes, see Materials and Methods.

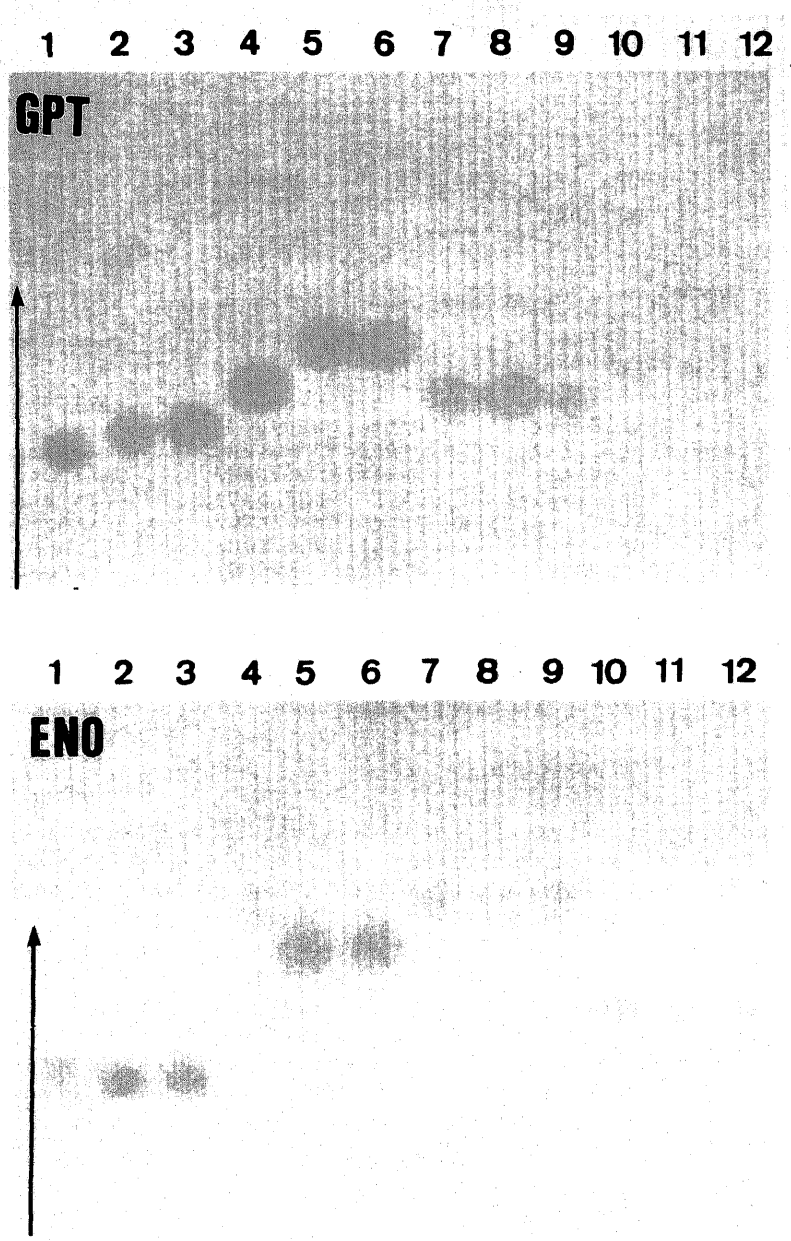

FIGURE 2. Zymodeme profiles obtained for two enzymes from *Leishmania* isolates and reference strains. Lane 1, *L. braziliensis braziliensis* (M2904); lane 2, *L. braziliensis panamensis* (LS94); lane 3, *L. braziliensis guyanensis* (M4147); lane 4, *L. donovani chagasi* (M2682); lane 5, *L. mexicana mexicana* (M379); lane 6, *L. mexicana pifanoi* (LL1); lane 7, *L. mexicana amazonensis* (M2269); lane 8, *L. mexicana amazonensis* (from Tokai University, Isehara, Kanagawa, Japan); lane 9, *L. mexicana garnhami* (JAP78); lane 10, *L. major*–like (G-O9); lanes 11 and 12, Paraguayan isolates (BN 40 and JE 15). GPT = glutamic pyruvate transaminase; ENO = enolase. **Arrows** indicate the direction of electrophoresis (cathode to anode).

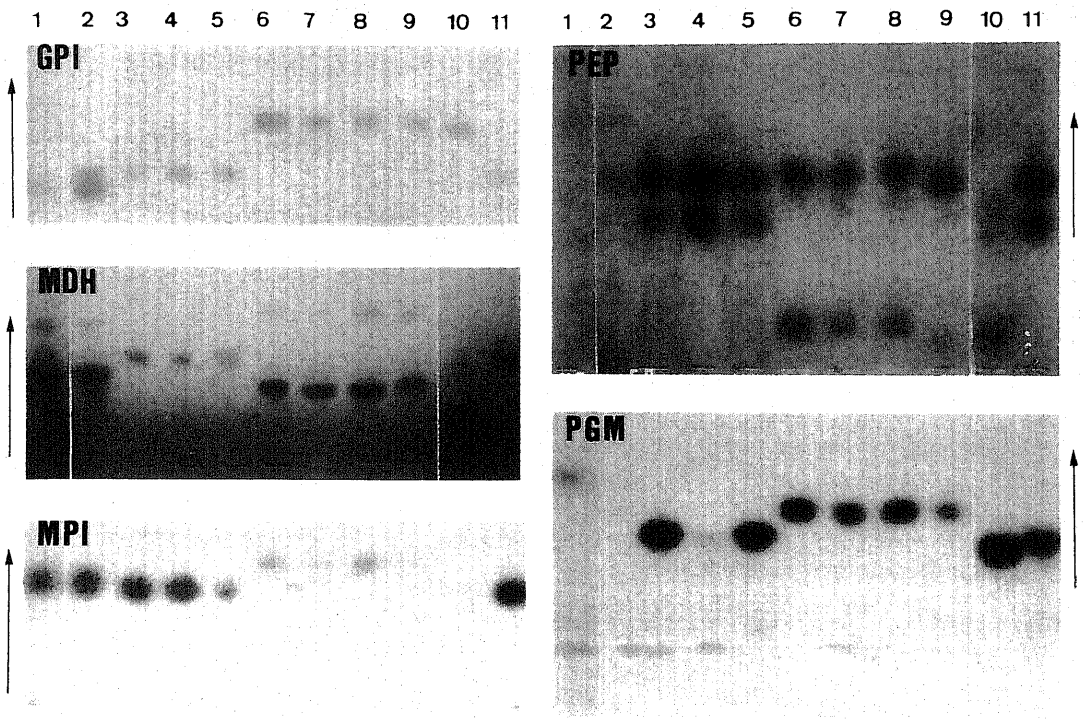

FIGURE 3. Electrophoretic profiles for five enzymes from *Leishmania* isolates and reference strains. Lane 1, *L. mexicana mexicana* (M379); lane 2, *L. mexicana pifanoi* (LL1); lane 3, *L. mexicana amazonensis* (M2269); lane 4, *L. mexicana amazonensis* (from Tokai University, Isehara, Kanagawa, Japan); lane 5, *L. mexicana garnhami* (JAP78); lane 6, *L. major*–like (G-O9); lanes 7 and 8, Paraguayan isolates (BN 40 and JE 15); lane 9, *L. major* (5-ASKH); lane 10, *L. tropica* (strain OD); lane 11, *L. donovani chagasi* (M2682). GPI = glucose phosphate isomerase; MDH = malate dehydrogenase; MPI = mannose phosphate isomerase; PEP = peptidase-D; PGM = phosphoglucomutase. **Arrows** denote the direction of electrophoresis (cathode to anode).

examined showed that the two Paraguayan isolates are identical to each other and to the Ecuadorian strain (lane 6), which is called *L. major*–like. These two Paraguayan isolates were also found to have enzyme profiles similar to those of *L. major*. However, the PEP patterns were distinct from *L. major* (lane 9 in Figure 3), and the other enzymes examined in this study showed that the Paraguayan isolates are indistinguishable from *L. major*–like parasite (G-O9) and *L. major* from the Old World. On the basis of these zymodeme patterns, it is strongly suggested that the Paraguayan isolates are both *L. major*–like parasites.

To confirm these results, schizodeme analyses were done. The fragment patterns obtained by restriction enzyme digestion of kDNA (schizodeme) are unique for each parasite as the degree of heterogeneity with kDNA minicircles varies among species or strains of *Leishmania*.[10] As shown in Figure 4, the two Paraguayan isolates shared identical schizodeme profiles obtained using three restriction enzymes (*Msp* I, *Hae* III, and *Taq* I). The *Msp* I digestion of the kDNA resulted in two major fragments, 1 kilobase and 800 basepairs (bp) (panel a), *Hae* III digestion yielded two prominent bands at approximately 210–220 bp (panel b), and three major bands of 770, 360 and 350 bp were obtained by *Taq* I digestion (panel c). When the schizodeme patterns of Paraguayan isolates were compared with those of the Ecuadorian *L. major*–like parasite, the profiles of prominent bands were identical (Figure 4, lanes 5–7). However, several minor bands (e.g., 520 bp with *Msp* I, 550 and 900 bp with *Hae* III, and 580 bp with *Taq* I digests) seen in the Ecuadorian *L. major*–like parasite were not seen in the Paraguayan isolates. Furthermore, the relative abundance of the > 612-bp band observed in *Taq* I digests when com-

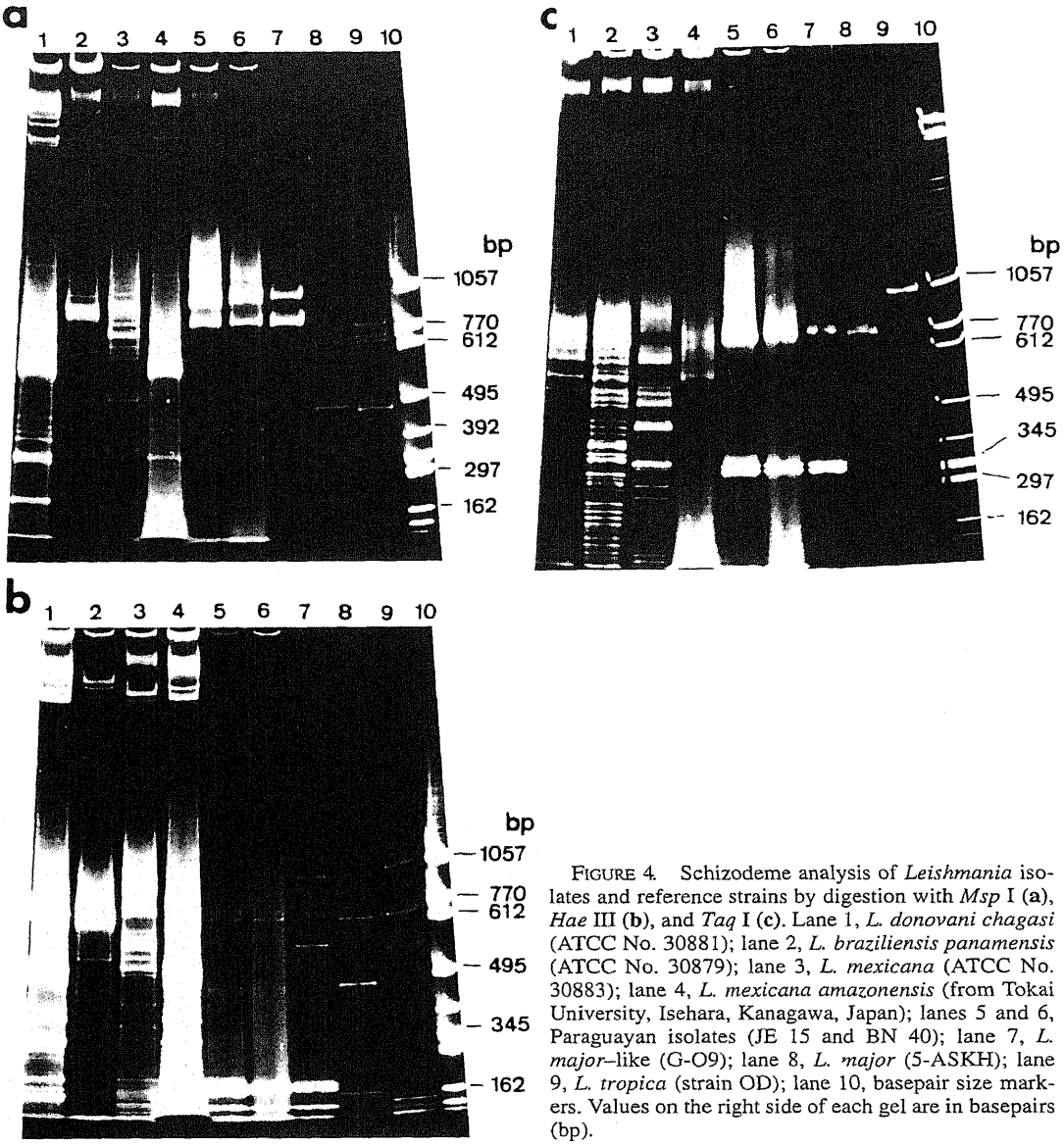

FIGURE 4. Schizodeme analysis of *Leishmania* isolates and reference strains by digestion with *Msp* I (**a**), *Hae* III (**b**), and *Taq* I (**c**). Lane 1, *L. donovani chagasi* (ATCC No. 30881); lane 2, *L. braziliensis panamensis* (ATCC No. 30879); lane 3, *L. mexicana* (ATCC No. 30883); lane 4, *L. mexicana amazonensis* (from Tokai University, Isehara, Kanagawa, Japan); lanes 5 and 6, Paraguayan isolates (JE 15 and BN 40); lane 7, *L. major*–like (G-O9); lane 8, *L. major* (5-ASKH); lane 9, *L. tropica* (strain OD); lane 10, basepair size markers. Values on the right side of each gel are in basepairs (bp).

pared with the two bands at 345–360 bp also appeared somewhat different between Paraguayan and Ecuadorian parasites. These differences may be due to the heterogeneity or divergence of the kDNA minicircle sequences.[10–14] The Paraguayan isolates were easily distinguishable from *L. major* and *L. tropica* from the Old World and *L. braziliensis, L. mexicana,* and *L. donovani* complexes from the New World. Therefore, the Paraguayan isolates are identical to an *L. major*–like parasite on the basis of the both results on zymodeme and schizodeme analyses, although there are minor variations in the kDNA digests.

DISCUSSION

The pathogenic parasites that cause cutaneous and mucocutaneous leishmaniases in Central and South America are generally classified into *L. braziliensis* and *L. mexicana* complexes.[15] Both *L. braziliensis* and *L. mexicana amazonen-*

FIGURE 5. Distribution of *Leishmania major*–like parasites in the New World. The map was made on the basis of the following references and our study. Localities 1, 2–5, and 6 and 7 are from Grimaldi and others,[5] Deane and Grimaldi,[23] and Hashiguchi and others,[16] respectively. Localities 8 and 9 are from the present study.

sis identified have already been reported as causative agents of this disease in Paraguay.[5, 6] However, two Paraguayan *Leishmania* parasites isolated from patients with cutaneous leishmaniasis were neither *L. braziliensis* nor *L. mexicana* by zymodeme and schizodeme analyses. They were identical to the Ecuadorian *L. major*–like parasite by zymodeme analysis, with minor differences seen in schizodeme analyses. Restriction enzyme patterns and sequence analysis have shown that kDNA minicircles within a single parasite are heterogeneous and can be divided into different classes in trypanosomatid protozoa.[11–13] Furthermore, a single minicircle from *L. mexicana amazonensis* contains sequences conserved across most *Leishmania* species and sequences conserved only within the *L. mexicana* complex.[14] Hashiguchi and others reported that schizodeme profiles of Ecuadorian and Venezuelan *L. major*–like parasites were not quite identical, but that both *Leishmania* species were identified as *L. major*–like parasites by zymodeme and schizodeme analyses.[16] These variant schizodeme profiles may result in the heterogeneity or divergence of kDNA minicircles.

Leishmania major–like parasites were origi-

nally designated as Brazilian *Leishmania* (Type III) because they were indistinguishable from *L. major* reference strains by zymodeme analysis.[17] Recently, *L. major*–like parasites were also discovered in the Pacific lowland and Andes regions of Ecuador.[16, 18] In Venezuela, *L. pifanoi* was originally reported to be a causative agent associated with diffuse cutaneous leishmaniasis (DCL).[5, 19] However, recent molecular studies indicate that the strain of *L. mexicana pifanoi* (MHOM/VE/00/L20) from a DCL case is phenotypically similar to the reference strain of *L. major,* but can be differentiated by kDNA restriction enzyme profiles.[17, 20] We have also isolated *L. major*–like parasites from patients from geographically different regions in Paraguay: one from Lima, Departamento de San Pedro, and the other from Residenta, Departamento de Canindeyu. Taken together, these results indicate that *L. major*–like parasites might be widely distributed in the New World (Figure 5).

As noted above, the Paraguayan isolates are indistinguishable from *L. major* by zymodeme analysis with all enzymes examined except for PEP. However, the kDNA restriction enzymes profiles of the Paraguayan isolates are quite different from those of *L. major,* indicating that the Paraguayan isolates are *L. major*–like parasites that should be described as a new species. Recently, Katakura and others have reported that the DNA karyotypes of Ecuadorian *L. major*–like parasites are quite different from those of *L. major* from the Old World,[21] and from those reported for five other *L. major* stocks (MRHO/SU/59/P, MHOM/IL/67/Jericho II, strain D-1, strain WR300, and strain Bokkara).[22] This suggests that the *L. major*–like parasite in the New World differs from *L. major* in the Old World in terms of both kinetoplast and chromosomal DNA organization.[21] Although the origin of the *L. major*–like parasite in the New World is unclear, it might occur indigenously in the New World as suggested by Hashiguchi and others.[16] The present study demonstrates that an *L. major*–like parasite is widely distributed in the New World (Venezuela, Ecuador, Brazil, and Paraguay). Thus, the existence of an *L. major*–like parasite as a causative agent of cutaneous leishmaniasis should be considered when identifying *Leishmania* isolates from the New World.

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