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Characterization of *Leishmania (Leishmania) waltoni* n.sp. (Kinetoplastida: Trypanosomatidae), the Parasite Responsible for Diffuse Cutaneous Leishmaniasis in the Dominican Republic

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Abstract. *Leishmania* parasites isolated, between 1979 and 1988 by the late Bryce Walton, from Dominican Republic (DR) patients with diffuse cutaneous leishmaniasis, were characterized using a panel of 12 isoenzymes, 23 monoclonal antibodies, small subunit ribosomal DNA (SSu rDNA), and multilocus sequence analysis (MLSA). The isoenzyme and monoclonal antibody profiles and the MLSA results showed that the Dominican Republic parasites were distinct from other described *Leishmania* species. This new species belongs to the *mexicana* complex, which is distributed in central and parts of northern South America. It is suggested that the parasites uniqueness from other members of the *mexicana* complex is related to it being isolated on an island for millions of years. If *Leishmania (Leishmania) waltoni* fails to adapt to some imported mammal, such as the house rat, it will be the only *Leishmania* to be classified as an endangered species. The excessive destruction of habitats on Hispaniola threatens the survival of its vectors and presumed natural reservoirs, such as the rodent hutias and the small insectivorous mammal solenodon. The concept of *Leishmania* species is discussed in the light of recent evaluations on criteria for defining bacterial species.

INTRODUCTION

Diffuse cutaneous leishmaniasis in Latin America has been associated with three *Leishmania* species that all belong to the subgenus *Leishmania (Leishmania)*—*L. (L.) amazonensis*, *L. (L.) mexicana*, and *L. (L.) pifanoi*.¹ In the Dominican Republic (DR), three cases of diffuse cutaneous leishmaniasis (DCL) were reported in 1975² and, during the following 10 years, 22 additional cases were diagnosed.³

An epidemiological investigation³ carried out between May 1981 and April 1984 led to the incrimination of *Lutzomyia christophei* as the probable vector. This sandfly species was collected in the vicinity of several human cases and shown to be experimentally susceptible to infection with the parasite. The incrimination of an animal reservoir remained unsolved after this study, while four *Rattus rattus* out of 44 were found to be seropositive for antibodies against the DR parasite.

Five isolates were obtained from patients by the late Bryce Walton between 1979 and 1988, and given in parallel to both Jeffrey Shaw (Instituto Evandro Chagas, Belem, Brazil) and David Evans (London School of Tropical Medical and Hygiene, London, United Kingdom). Initial studies^{4,5} showed that the parasite belonged to the genus *Leishmania*. Profiles⁵ of excreted factor and the electrophoretic mobility of malate dehydrogenase distinguished them from *L. (L.) amazonensis*. However, nuclear and kinetoplastid DNA buoyant densities⁴ indicated that two DR isolates were closer to *L. (L.) amazonensis* but distinct from *L. (L.) mexicana*.

In this study, we report the characterization of five isolates of the DR *Leishmania* by isoenzymatic electrophoresis, multilocus sequence analysis (MLSA), and a panel of 23 monoclonal antibodies and examination of the small subunit ribosomal

DNA (SSu rDNA) for two of them. Numerical taxonomic analysis, including cladistic study enabled us to determine the precise taxonomic position of this parasite, which we consider as a new taxon within the *L. (L.) mexicana* complex.

MATERIALS AND METHODS

Studied strains. Five strains isolated from DCL human cases from the DR were cryopreserved in both the Cryobank of the Department of Medical Protozoology, London School of Tropical Medicine and Hygiene (LSTMH), and the Instituto Evandro Chagas's cryobank, where monoclonal and rDNA examinations were performed.

Those obtained from the LSTMH collection are stored at the International Cryobank and Identification Center for *Leishmania* in Montpellier, under Biobank No. *BB-0033-00052 (Montpellier, France). These strains were studied using isoenzymatic electrophoresis, MLSA, and numerical taxonomic analysis.

The strain code numbers are as follows: MHOM/DO/79/CECILIO, MHOM/DO/79/CONSTANCIA, MHOM/DO/88/025, MHOM/DO/0000/452-A, and MHOM/DO/0000/450-B.

Reference strains for isoenzyme characterization and MLSA. The above strains were compared with the following 18 MON zymodeme reference strains: MON-40 (MNYC/BZ/62/M379), MON-121(MHOM/MX/89/RIOS), MON-152(MHOM/MX/85/SOLIS), MON-153(MHOM/BZ/85/BEL65), MON-154(IYLE/GT/81/23L), MON-155(MHOM/PA/00/GML637), MON-156(MHOM/BZ/82/BEL21), MON-110(MHOM/EC/87/EC-103), MON-194 (MHOM/00/92/LPN88), and MON-195(MHOM/MX/93/CRE47) for *L. (L.) mexicana*; MON-41(IFLA/BR/67/PH8), MON-132(MHOM/BR/73/M2269), MON-157(IFLA/TT/71/71-110), MON-41(MHOM/PA/87/GML416), and MON-41(MHOM/CO/82/CELIS) for *L. (L.) amazonensis*; MON-133(MORY/PA/68/GML-3) for *L. (L.) aristidesi*; and MON-97(MCAV/BR/45/L88) and MON-227(MCAV/BR/95/CUR3) for *L. enriettii*. The enzymatic profiles of all these reference strains are given in Table 1.

Biochemical characterization. Isoenzymatic characterization based on starch gel electrophoresis was used according to

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TABLE 1
Enzymatic profiles of the zymodeme reference strains used in the isoenzyme analysis

<i>Leishmania taxa</i>	WHO code	NRCL*code	ME	PGD	G6PD	DIA	NPI	NP2	GOT1	GOT2	PGM	FH	MPI	GPI	ZYMODEMEMON
<i>L. amazonensis</i>	IFLA/BR/67/PH8	LEM 395	105	110	65	98	0	90	85	105	111	95	115	45	41
	MHOM/BR/73/M2269	LEM 690	105	116-110	75-65	98	0	90	70	112	111	95	115	45	132
	IFLA/TT/71/1-110	LEM 2246	105	110	65	98	0	90	85	105	111	95	106	45	157
	MHOM/PA/87/GML416	LEM 2237	105	110	65	98	0	90	85	105	111	95	115	45	41
	MHOM/CO/82/CELS	LEM 2247	105	110	65	98	0	90	85	105	111	95	115	45	41
	MORY/PA/68/GML-3	LEM 693	95-85	110	65	98	0	87	85	100	111	95	80	45	133
<i>L. arisidresi</i>	MHOM/MX/89/RIOS	LEM 1871	100	110	65	75	295	90	80	105	133	95	135	35	121
	MHOM/MX/85/SOLIS	LEM 2223	106-100	110	70-65	75	295	90	80	105	133	95	135	35	152
	MHOM/BZ/85/BEL65	LEM 2224	100	110	65	75	295	90	80	105	133-123	95	135	35	153
	IYLE/GT/81/23L	LEM 2225	100	110	65	75	295	90	80	105	133	95	145-120	35	154
	MHOM/PA/00/GML637	LEM 2263	100	115	70	75	295	87	80	105	109.4	95	105	35	155
	MHOM/BZ/82/BEL21	LEM 695	100	110	65	75	295	90	80	105	133	95	150	35	156
<i>L. mexicana</i>	MHOM/EC/87/EC-103	LEM 1221	109	115	70	75	290	87	75	114	109.4	95	115-103	38	110
	MHOM/00/92/LPN88	LEM 2573	100	110-95	65	75	295	90	80	105	133-117	95	135	35	194
	MHOM/MX/93/CRE47	LEM 2695	100	110-95	65	75	295	90	80	105	133	95	135	35	195
	MNYC/BZ/62/M379	LEM 280	100	110	65	75	295	90	80	105	133	95	118-110	35	40
	MCAV/BR/45/L88	LEM 1120	65	110	83	20	0	135	120	120	111	75	137	79	97
	MCAV/BR/95/CUR3	LEM 3045	65	110	92	20	0	135	120	120	111	75	137	79	227

Rioux and others (1990), using the following 12 enzymes: malic enzyme (ME): EC1.1.1.40, phosphogluconate dehydrogenase (PGD): EC 1.1.1.44; glucose-6-phosphate dehydrogenase (G6PD): EC 1.1.1.49; diaphorase NADH, DIA: EC 1.6.2.2; nucleoside purine phosphorylase 1 (NP₁): EC 2.4.2.1 and 2 (NP₂): EC 2.4.2.*; glutamate-oxaloacetate transaminase 1 and 2 (GOT₁ and GOT₂): EC 2.6.1.1; phosphoglucomutase (PGM): EC 5.4.2.2; fumarate hydratase (FH): EC 4.2.1.2; mannose-phosphate isomerase (MPI): EC 5.3.1.8; and glucose-phosphate isomerase (GPI): EC 5.3.1.9.

Cladistic analysis. A cladistic analysis of the New World subgenus *Leishmania* was carried out to define the position of the DR isolates. The zymodemes were considered as operational taxonomic units and each enzymatic system as a multi-valent character, each electromorph being a character state. The construction was based on Hennig's principles⁶ and parsimony using Felsenstein's MIX software (Difco B45 – Becton Dickinson, Franklin Lakes, NJ).

Reference strains for monoclonal and rDNA studies. The DR strains were compared with the following species: *L. (L.) mexicana* (MNYC/BZ/62/M379 and MHOM/BZ/82/BEL21), *L. (L.) pifanoi* (MHOM/VE/76/ESTHER), *L. (L.) garnhami* (MHOM/VE/76/JAP78), *L. (L.) amazonensis* (IFLA/BR/67/PH8), *L. forattinii* (MDID/BR/82/RV288), *L. (L.) aristidresi* (MORY/PA/68/GML3), *L. (L.) venezuelensis* (MHOM/VE/81/PMH17), *L. (L.) infantum chagasi* (MHOM/BR/74/PP75), and *Endotrypanum* (MCHO/BR/79/M5725). These strains were chosen as they represent taxa that the DR strains need to be differentiated from.

Indirect antibody fluorescent protocol for leishmanial monoclonal antibodies. Promastigotes of all strains were grown in blood agar base medium (Difco B45).⁷ Log phase parasite were washed in phosphate buffered saline (PBS) pH7.2 (2.5 mM NaH₂PO₄, 7.4 mM Na₂HPO₄, and 14 mM NaCl) three times by centrifugation at 5,000 G for 10 minutes at 4°C. The pellet was suspended in PBS (4°C) to give a final concentration of 10⁴ parasites/mL. Ten microliter of this suspension was placed in each orifice of teflon-coated slides. They were air dried, fixed for 15 minutes in analytical grade acetone and stored at 20°C in plastic bags containing silica gel.

A total of 23 monoclonal antibodies were used (B2, B5, B12, B13, B18, B19, M2, M11, M12, CO1, CO2, CO3, L1^{8,9}; T3, D13^{10,11}; WIC.79.3¹²; N2, N3, LA2, WH1, WA2, V1^{13,14}). The B and N series react selectively with species of the subgenus *L. (Viannia)*; M2, T3, D13, M11, M12, WIC.79.3, WA2, and V1 react selectively with parasites of the subgenus *L. (Leishmania)* and *Endotrypanum*; CO1, CO2, CO3, and L1 are group specific and react selectively with members of both *Leishmania* subgenera, *Endotrypanum*, and some species of *Trypanosoma*. The protocol followed a three-step biotin/avidin procedure¹⁵ that allows antibodies to be used at higher dilutions. After the application of the leishmanial monoclonal antibody biotinylated anti-mouse (H + L) is used and then fluorescence is obtained using fluorescent-labeled avidin.

Examination of SSU rDNA. DNA was extracted from washed cultures and the SSU rDNA was amplified¹⁶ using the S1/S4 or S12/S4 primers and then with the S8, S9, and S10 rDNA probes.¹⁷ S8 reacts with *L. (L.) amazonensis* and *L. (L.) major* complex parasites, S9 with parasites of the *L. (L.) mexicana* and *L. (L.) donovani* complexes, and S10 with *L. (Viannia)* species and the *Endotrypanum* group that included *L. colombiensis*.

TABLE 2
Enzymatic profiles of the five Dominican Republic *Leishmania* strains

WHO code	NRCL* code	ME	PGD	G6PD	DIA	NP ₁	NP ₂	GOT1	GOT2	PGM	FH	MPI	GPI	ZYMODEME	MON-
MHOM/DO/00/452A	LEM2579	109-87	110	70	260-70	180	90	80	0	118	84	135	32	192	
MHOM/DO/79/Cecilio	LEM2580	109-87	110	70	260-70	180	90	80	0	118	84	135	32	192	
MHOM/DO/79/Constancia	LEM2581	109-87	110	70	260-70	180	90	80	0	118	84	135	32	192	
MHOM/DO/00/450B	LEM2582	109-87	110	70	260-70	180	90	80	0	118	84	135	32	192	
MHOM/DO/88/025	LEM3365	90	110	70	260-70	180	90	80	105	118	84	135	32	259	

WHO = World Health Organization; ME = malic enzyme; PGD = phosphogluconate dehydrogenase; G6PD = glucose-6-phosphate dehydrogenase; DIA = diaphorase; NP₁ = nucleoside purine phosphorylase 1; NP₂ = nucleoside purine phosphorylase; GOT₁ and GOT₂ = glutamate-oxaloacetate transaminase 1 and 2; PGM = phosphoglucomutase; FH = fumarate hydratase; MPI = mannose-phosphate isomerase; GPI = glucose-phosphate isomerase.

*National Reference center for *Leishmania*.

Multilocus sequence analysis. In all, 15 *Leishmania* strains from South and central America (including the five *L. waltoni* strains) were analyzed using loci 03.0980, 12.0010, 14.0130, and 31.2610 of four housekeeping genes.¹⁸ Sequences were deposited into the GenBank database under the following accession numbers: KC158811, KC158589, KC159255, KC159699, KC849477-KC849479, KC849511-KC849513, KC849613-KC849615, KC849647-KC849649, KC960499, KC960504, KC960509, KC960513, and KM555296-KM555339. The four loci were concatenated and duplicated to avoid information loss due to ambiguous states. The Maximum likelihood tree was constructed using PhyML, version 3.0^{19,20} with the generalized time reversible (GTR) model for nucleotide substitutions including a proportion of invariables sites and gamma-distributed rate variation across sites. One *L. infantum* strain (LEM 75) was used as an out-group and 1,000 bootstrap replicates were performed to estimate nodes support.

RESULTS

Enzymatic characters. The enzymatic profiles of the five DR *Leishmania* strains showed the existence of two different new zymodemes: MON-192 (four strains) and MON-259 (one strain) (Table 2).

These strains were compared with the 10 zymodeme reference strains of *L. (L.) mexicana* complex: six *L. (L.) mexicana* electromorphs are found in the DR strains (PGD¹¹⁰, G6PD⁷⁰, NP2⁹⁰, GOT1⁸⁰, GOT2¹⁰⁵, and MPI¹³⁵), three of them (PGD, NP2, and GOT1) regularly found in the majority of the zymodemes (Table 2).

The comparison with the three zymodemes of the *L. (L.) amazonensis* complex showed three common electromorphs: PGD¹¹⁰ and NP2⁹⁰, already found in *L. (L.) mexicana*, and GOT1¹⁰⁵ found in two zymodemes of *L. (L.) amazonensis*. Concerning *L. (L.) aristidesi* and *L. enriettii* they both have a single electromorph in common with the DR strains, which is also common to *L. (L.) mexicana* and *L. (L.) amazonensis* (PGD¹¹⁰).

Three electromorphs are specific of the DR *Leishmania* zymodemes: DIA²⁶⁰⁻⁷⁰, NP1¹⁸⁰, and GPI³² in both zymodemes MON-192 and MON-259. Moreover, an autapomorph is present for the enzyme ME¹⁰⁹⁻⁸⁷ in MON-192.

Phylogenetic characters. The cladistic analysis leads to the definition of a plesiomorphic state (PGD¹¹⁰) for the New World *Leishmania* subgenus: i.e., *L. (L.) mexicana*, *L. (L.) amazonensis*, *L. (L.) aristidesi* and *L. enriettii* complexes. Two states (NP2⁹⁰ and GOT2¹⁰⁵) are common to the two complexes *L. (L.) mexicana* and *L. (L.) amazonensis*, and another state (GOT1⁸⁰) is found only in *L. (L.) mexicana* complex.^{21,22}

The phylogenetic tree shows the inclusion of the two DR zymodemes within the *L. (L.) mexicana* complex. However, they are located on a branch distinct from the *L. (L.) mexicana sensu stricto* species at 12 evolutionary steps (Figure 1).

Multilocus sequence analysis. The four single copy-coding DNA sequences analyzed gave a 2,520 bp-long concatenated sequence. Excluding the *L. infantum* LEM75 reference strain used as an out-group, 36 polymorphic sites, including 23 informative parsimonious sites and 13 singletons, were identified among the 15 *Leishmania (Viannia)* strains analyzed. The five *L. waltoni* strains analyzed were almost genetically identical and only one polymorphism was detected in the LEM2581 strain among the 2,520 bp analyzed. The Maximum likelihood tree (Figure 2) has shown a well-supported *L. waltoni* strain's genetic cluster (BP = 99%).

Monoclonal immunofluorescent antibody tests. The results of the monoclonal immunofluorescent antibody (IFAT) tests are summarized in Table 3. The profile of the new parasite is distinct from those of the named South American species of the subgenus *L. (L.) Leishmania*. There were no reactions with the monoclonals M12 and WA2 that recognise *L. (L.) mexicana* or with M2 that has so far been shown to be

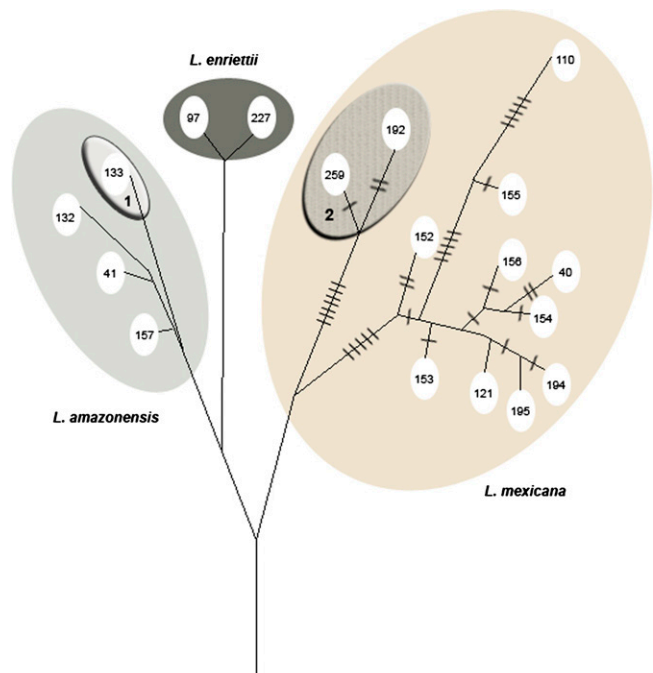


FIGURE 1. Phylogenetic tree of the New World *Leishmania* subgenus, with mention of the evolutionary steps. (1: *L. (L.) aristidesi*, 2: *L. (L.) waltoni*).

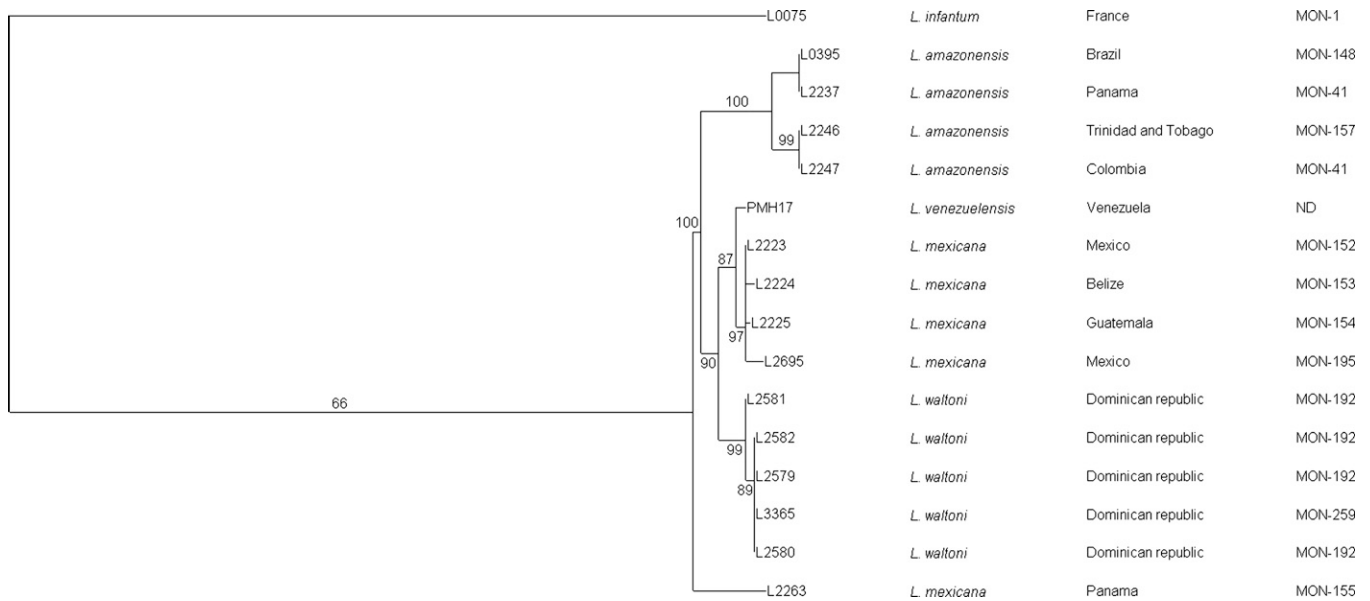


FIGURE 2. Maximum likelihood tree of the concatenated nucleotide sequences of 16 *Leishmania* strains. Significant bootstrap values are shown in percentage on each node. The species/taxonomic names, the geographical origins and the zymodemes are shown for each strain. The five *L. (L.) waltoni* strains fall into a highly supported cluster (BP 99%). The L numbers correspond to the National Reference Code *Leishmania* (NRCL) codes: for correspondence to the World Health Organization (WHO) codes, see Table 1. ND = not determined.

specific for parasites of the *L. (L.) amazonensis* complex. This clearly differentiated the DR strains from parasites belonging to the *L. (L.) amazonensis* complex. The reaction with T3 puts it closer to *L. (L.) mexicana* and *L. (L.) major*. There are two distinct serodemes of the DR strains: one composing of strains 1137 and 1139 that do not react with the WIC-79.3 and CO2 and the other that includes the type strain that reacts with both these monoclonals.

Examination of SSU rDNA. The results of the SSU rDNA hybridizations are shown in Table 4. The *L. (L.) amazonensis* reference strain (M2269) hybridized only with S8, the *L. (L.) mexicana* reference strain (M7369) was positive only with S9. Two strains of *L. (L.) major* included in the study showed hybridization with S8, the same pattern observed for *L. (L.) amazonensis*. The *L. (L.) infantum* hybridization profile was the same as *L. (L.) chagasi* and *L. (L.) donovani*, being positive for S9 and S16. The reference strains not previously tested, *L. (Viannia) lainsoni* and

L. (V.) shawi, of the *L. (Viannia)* subgenus, hybridized with S10. A strain of *Endotrypanum schaudini* hybridized with the S10 probe. The common reactivity between *Viannia* species and *Endotrypanum* means that it is impossible to use the S10 probe for identifying sylvatic reservoirs hosts and vectors. It is interesting that both have a peripylarian development yet antigenically *Endotrypanum* is closer to *L. (L.) amazonensis*.²³ The two strains of the new parasite hybridized with the S9 probe but were negative with the S16 probe that is the same as parasites belonging to the *mexicana* complex.

DISCUSSION

As a result of the phylogenetic analysis, the DR strains are characterized by three states (DIA²⁶⁰⁻⁷⁰ NPI¹⁸⁰ GPI³²), which belong exclusively to all of them. These synapomorphic characters confirm the individualization of the group and its cohesion. Similarly, the monoclonal antibody profile clearly

TABLE 3

Results of the indirect immunofluorescent test with a selected number of *Leishmania*-specific monoclonal antibodies that react with American parasites belonging to the subgenus *L. (Leishmania)* and the genus *Endotrypanum*

Species	M2	M11	M12	WA2	T3	WIC	CO2	V1	L1	N2
<i>L. waltoni</i> (1137)	-	-	-	-	+	-	-	-	+	-
<i>L. waltoni</i> (1139)	-	-	-	-	+	-	-	-	+	-
<i>L. waltoni</i> (452-A)	-	-	-	-	+	+	+	-	+	-
<i>L. waltoni</i> (450-B)	-	-	-	-	+	+	+	-	+	-
<i>L. waltoni</i> (Celio)	-	-	-	-	+	+	+	-	+	-
<i>L. venezuelensis</i> (PMH17)	-	-	-	-	-	-	-	-	+	-
<i>L. mexicana</i> (M379)	-	-	+	+	+	+	+	-	+	-
<i>L. mexicana</i> (BEL21)	-	+	+	+	+	+	+	-	+	-
<i>L. pifanoi</i> (ESTHER)	-	+	+	+	+	-	+	-	+	-
<i>L. garnhami</i> (JAP78)	+	+	-	-	+	-	+	-	+	-
<i>L. amazonensis</i> (PH8)	+	+	-	+	-	-	+	-	+	-
<i>L. aristedesi</i> (GML3)	+	+	-	+	-	-	-	-	+	-
<i>L. forattinii</i> (RV288)	+	+	-	-	-	-	-	-	+	-
<i>L. major</i> (P)	-	-	-	-	+	+	-	-	+	-
<i>Endotrypanum</i> (M5725)	-	-	-	-	+	+	-	-	+	+

TABLE 4

Hybridisation of probes S8, S9, S10 and with DNA of strains considered as belonging to the *mexicana* and *amazonensis* complexes

Species (original code)	S8	S9	S10	S16
<i>Leishmania (Leishmania) waltoni</i> (1137)	-	+	-	-
<i>L. (L.) waltoni</i> (1139)	-	+	-	-
<i>L. (L.) mexicana</i> (M379)	-	+	-	-
<i>L. (L.) mexicana</i> (BEL21)	-	+	-	-
<i>L. (L.) pifanoi</i> (ESTHER)	-	+	-	-
<i>L. (L.) garnhami</i> (JAP78)	+	-	-	-
<i>L. (L.) amazonensis</i> (PH8)	+	-	-	-
<i>L. (L.) major</i> (P)	+	-	-	-
<i>L. (L.) chagasi</i> (PP75)	-	+	-	+
<i>L. (V.) braziliensis</i> (M2904)	-	-	+	-
<i>Endotrypanum</i> (M5725)	-	-	+	-

distinguishes the DR strains from other named species of the subgenus *L. (Leishmania)*. These results lead us to consider the Dominican parasite as a new taxon, naming it *Leishmania (Leishmania) waltoni*, in honour to the late Bryce Walton, who isolated the DR strains that are the basis of this study. As a member of the American Armed Forces, he had unique access to leishmaniasis patients and his studies of these cases were important in developing clinical tests, such as the use of the immunofluorescent test for monitoring the treatment of cutaneous leishmaniasis.²⁴ He was also one of the first researchers to draw attention to a link between the severity of leishmanial infections and genetic differences of human populations.²⁵ Throughout his professional life he was interested in all aspects of leishmaniasis, especially in the New World, and was responsible for stimulating research on the disease within both military units and public health organizations, such as the World Health Organization and the Pan-American Health Organization, serving as a consultant to both.

Description of new taxon. *Leishmania (Leishmania) waltoni* sp.n., Shaw JJ, Pratlong F and Dedet JP

Type host: man.

Locality in host: skin.

Type locality: Lagunita Cuchillo, El Seybo Province, Dominican Republic.

Strain designation: MHOM/DO/88/025

Promastigote measurements: body length: $7.8 \pm 1.7 \mu$; body width: $1.9 \pm 0.4 \mu$; flagellum length: $5.3 \pm 1.6 \mu$

Suspected sandfly vector: *Lutzomyia christophei*

Reservoir(s): unknown

Enzyme profiles: ME⁹⁰, PGD¹¹⁰, G6PD⁷⁰, DIA²⁶⁰⁻⁷⁰, NP1¹⁸⁰, NP2⁹⁰, GOT1⁸⁰, GOT2¹⁰⁵, PGM¹¹⁸, FH⁸⁴, MPI¹³⁵, GPI³² corresponding to zymodeme MON-259.

Monoclonal antibodies: T3 + ve, WIC-79.3 + ve, CO1 + ve, L1 + ve, M2-ve, M11-ve, M12-ve, WA2-ve, CO2-ve, V1-ve, N2-ve.

rDNA Probes: Reactions with SSU rDNA probes: S9 + ve, S8-ve, S10-ve, S16-ve.

Multilocus Sequence Analysis (MLSA): GenBank Acc.no for loci 03.0980, 12.0010, 14.0130 and 31.2610 are KM555303, KM555314, KM555325, and KM555336 respectively.

Growth in vitro: easy to grow, doubling time 6 hours on SDM79 medium (PAA Laboratories GmbH, Austria) supplemented with 15% fetal calf serum and 7 μ g/mL hemin.

Growth in vivo: produces a small lesion in the hamster (*Mesocricetus auratus*) similar in size to those of *L. (L.) venezulensis* and *L. (Viannia)* spp., unlike the large,

parasite rich *L. (L.) mexicana* and *L. (L.) amazonensis* histiocytomata. There is no evidence of metastasis.

Pathology in man: producing multiple nodular skin lesions, with long-term evolution without spontaneous cure. Resistant to treatment by antimonials and amphotericin B.

Type material: cryopreserved promastigotes deposited in the International Cryobank and Identification Center for *Leishmania*, Laboratoire de Parasitologie, Montpellier (France).

The divergence between *L. (L.) waltoni* and *L. (L.) mexicana* is relatively recent, as the number of evolutive steps is reduced (12), but sufficient to individualize the taxon. It presumably results from island isolation, and poses several questions concerning the date of the appearance of leishmaniasis in Hispaniola Island. The most likely scenario would seem to be the introduction of a *mexicana*-like parasite from central America or the northern coastal region of Venezuela. This probably occurred well before man appeared in the region, which was only some 40,000 years ago.²⁶ Sandflies have been recorded from Miocene (24Ma) in amber²⁷ from the Dominican Republic and the island arch, of which present day Hispaniola is part, has been in place since the middle Eocene (49Ma).²⁸ Fossil records indicate that the island had a rich mammalian fauna consisting of rodents, insectivores, monkeys, and sloths,²⁹ suggesting periodic links with the mainland. *Leishmania (L.) waltoni*'s original reservoir may have been native rodents such as hutias (*Plagiodontia* spp.) that were abundant in caves and forests. Four species are recorded from Hispaniola but only one or two have survived the post-Colombian colonization. Looking for evolutionary differences between *L. (L.) waltoni* and *L. (L.) mexicana* using molecular markers could serve as a basis for calibrating leishmanial evolutionary events. It is perhaps ironical that *L. (L.) waltoni* could become extinct if it remains limited to its endemic silvatic hosts and fails to adapt to non-endemic rodents such as domestic rats.

A 1380-bp fragment of the coding region of the heat shock protein 70 (Hsp70) gene that is commonly used in restriction fragment length polymorphism (RFLP) analysis was recently sequenced³⁰ and its authors concluded "As such no subspecies can be defined based on Hsp70, and *L. mexicana* would be the single recognized species." There are a number of valid reasons for not accepting this conclusion. The tree in this article showed good bootstrap values supporting the separation of the reference strain of *L. (L.) mexicana* from the *L. (L.) amazonensis* strains with the exception of one strain designated as *L. (L.) mexicana* from Peru. No other identification methods were used to validate its identity. In an article published in 1994¹⁶ the authors could not distinguish between the standard strains of *L. (L.) mexicana* and *L. (L.) amazonensis* using probes based upon SSU rDNA sequences. However, in a later study,¹⁷ these same probes distinguished *L. (L.) mexicana* and *L. (L.) amazonensis*. In the second study, a new line of the same *L. (L.) mexicana* strain was obtained from a source where its identity had been validated. The problem was that somewhere the *L. (L.) mexicana* strain had become contaminated with a parasite that reacted with the S8 *amazonensis* probe. There is a constant danger of strains being contaminated by others. For instance, a *L. (S.) adleri* isolate turned out to be *L. (L.) major*³¹ and a strain of *L. (L.) infantum* was in fact *Endotrypanum*.³² Differences of this magnitude are easily

detectable but may go unnoticed in dealing with closely related taxa such as ecologically isolated populations.

On the basis of SSu rDNA analysis, the *mexicana* clade as described in 1987³³ is clearly composed of two distinct genetic complexes.¹⁷ The first corresponds to *L. (L.) mexicana* and occurs throughout central America and the northern rim of South America and the second corresponds to *L. (L.) amazonensis* and is found throughout South America. Within these two groups some parasites have been given specific status. The validity of the *mexicana* and *amazonensis* complexes is supported by a number of characters such as functional differences in the domains of the rDNA promoter regions,³⁴ isoenzyme analysis,²² and cytochrome b.³⁵ A trend that should be avoided is embarking on taxonomic revisions using a single molecular character that may or may not reflect the true taxonomic status of the species.

The MLSA showed clearly that the *L. (L.) waltoni* forms a distinct group within the *mexicana* complex, which is supported by a high bootstrap value. Interestingly *L. (L.) venezuelensis* that shares biological similarities with *L. (L.) waltoni* is closer to *L. (L.) mexicana*. This suggests that this species may have diverged at a later date from the ancestral *mexicana* stock.

Statistically monoclonal antibodies have a greater discriminatory power than isoenzymes for distinguishing *Leishmania* species.³⁶ This is confirmed in this study since with the exception of *L. (L.) amazonensis* and *L. (L.) aristedesi* all the named indigenous species of the subgenus *L. (Leishmania)* could be differentiated. They are perhaps some of the most useful tools for identifying *Leishmania* but since their epitopes are generally not completely known, they are of limited use in taxonomy. When WIC-79.3 was originally described, it was simply known as a promastigote surface antigen but it was later shown that it recognizes part of *L. (L.) major* lipophosphoglycan. There are monoclonals against other surface antigens such as GP63, and genetic studies have shown that polymorphism of its gene loci allow specific markers for subgenera, species, and geographical populations.³⁷ There is therefore a solid link explaining the efficiency of monoclonals with genetic variation but it has yet to be exploited for identification.

It is questionable whether all the species described within the two clades merit specific status but there are notable biological differences between some of them and little is known of their individual zoonotic cycles. Before any decisions are reached both the biology and genetics of more strains from the different type areas from both animal and vectors need to be studied.

Recently it was suggested³⁸ that going back to using subspecies could resolve present-day problems of leishmanial taxonomy. This is an interesting idea but one of the reasons that Lainson and Shaw³³ abandoned the subspecies was that at that time it was becoming obvious that the genetic diversity was much greater than what had previously been expected. However, going back to using subspecies may be justified for parasites such as *L. (V.) braziliensis* that is composed of many distinct geographical populations. The basic building blocks of taxonomies are species but the definition of a *Leishmania* species causes great difficulties since the major form of their propagation is clonal.³⁹ Hybrid strains have been found^{40–42} suggesting that occasionally genetic exchange does occur under certain ecological conditions. However, this does not negate the use of species names since they represent a stable

method of communication and with modern computer technology they can be easily followed in name-based databases and infrastructures that collate biological information.⁴³ Bacterial species are equally difficult to define⁴⁴ since their reproduction, like the *Leishmania*, is clonal with occasional genetic exchange. In the present genomic era it has been suggested⁴⁵ that a bacterial species should be a biologically meaningful cluster. For obligatory pathogens the ecological niches are very restricted and result in detectable clusters. This is perhaps a good starting point for us to begin examining the species concept for *Leishmania* and it is within this framework that we have given a specific name to the Dominican parasite.

The present observations confirm once again the importance that geographical isolation has on the evolution and diversity of living organisms. Its importance in the evolution of the genus *Leishmania* has received little attention due to the difficulty of knowing where such barriers begin and end. However, the importance of ecological niche barriers within an ecotope has been shown by the difference in sympatric arboreal and terrestrial cycles, such as those of *L. (V.) guyanensis* and *L. (L.) amazonensis* in Amazonia, that involve vectors and reservoirs whom have different ecological preferences.

Like many *Leishmania*, the reservoirs of *L. (L.) waltoni* are unknown, but it is amazing that this species has survived with the virtual elimination of the wild mammals of Hispaniola. Only two have survived, the insectivorous solenodon, that has venomous saliva and the rodent hutia that inhabit the forest of rough ravines.²⁷ It is likely that one of them is the natural reservoir and if this is proven it is remarkable that a *Leishmania* has survived in such a reduced reservoir population. It also suggests that controlling human cutaneous leishmaniasis by reservoir management may be very difficult.

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