A previously unclassified trypanosomatid responsible for human cutaneous lesions in Martinique (French West Indies) is the most divergent member of the genus *Leishmania ss*

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SUMMARY

Two cases of skin lesions similar to those caused by *Leishmania* parasites have been reported from Martinique. Parasites isolated from these lesions were unlike *Leishmania* reference strains by isoenzyme analysis and electron microscopy and were assumed to be monoxenous trypanosomatids which normally only infect invertebrates. Both strains have now been retyped by isoenzyme analysis and found to be identical to each other and distantly related to all other *Leishmania* species. The sequence of the 18S ribosomal RNA gene and partial sequences of the DNA polymerase alpha and RNA polymerase II largest subunit genes were obtained. These sequences indicated that the Martinique parasites clustered with *L. enriettii* and were basal to all other euleishmania. However, support for both the position basal to all euleishmania and the clustering with *L. enriettii* was low. The Martinique parasites may cluster with *L. (Leishmania)* or *L. (Viannia)* or form a novel clade within the euleishmania either with or without *L. enriettii*.

Key words: leishmaniasis, Antilles, Trypanosomatidae, cutaneous leishmaniasis, taxonomy.

INTRODUCTION

There have been occasional reports of leishmaniasis like infections in Martinique since 1917. The first parasites to be isolated and identified by isoenzyme electrophoresis were from an HIV-infected patient. These parasites were found to be quite unlike any other species of *Leishmania* by isoenzyme analysis and electron microscopy (EM) (Dedet *et al*. 1995). The application of EM showed that the morphology of these organisms was more characteristic of monoxenous trypanosomatids. It was consequently concluded that the HIV infection had made the patient susceptible to a monoxenous parasite of invertebrates that would not normally infect humans or other mammals. Subsequently another strain was isolated from a localized cutaneous lesion in an HIV-negative patient, living on the same island of Martinique. This parasite belonged to the same zymodeme as the one that had been isolated from the immunocompromised patient (Boisseau-Garsaud *et al*. 2000). There have also been reports of other cases of human and mammalian infections with what appear to be monoxenous trypanosomatids (Schnur *et al*. 1992; Jimenez *et al*. 1996; Pacheco *et al*. 1998; Sousa *et al*. 1998). Three hypotheses may explain such observations: (l) a novel group or groups of digenetic trypanosomatids exist that can infect humans, (2) some humans are susceptible to occasional infections by normally monoxenous trypanosomatids and (3) the parasites have been incorrectly reported to be a lower trypanosomatid.

In order to test these alternative hypotheses we have classified both of the unusual parasites from Martinique using isoenzymes and one of them with the partial sequences of the RNA polymerase, DNA polymerase and small subunit ribosomal RNA genes. All these methods show that the Martinique parasites are not closely related to any genus of monoxenous trypanosomatid but are highly divergent members of the genus *Leishmania*.

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MATERIALS AND METHODS

Strains studied

The 2 strains obtained from the Martinique patients are MHOM/MQ/92/MAR1 and MHOM/MQ/

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Table 1. Taxa, zymodemes and WHO code numbers of the 49 reference strains used for isoenzyme analysis

Species complex	Taxon and zymodeme	Zymodeme reference strain (WHO code)
L. (L.) aethiopica	*L. aethiopica* MON-14	MHOM/ET/72/L100
	L. aethiopica MON-69	MHOM/ET/82/101-82
	L. aethiopica MON-70	MHOM/ET/83/68–83
L. (L.) turanica	*L. turanica* MON-21	MRHO/SU/65/VL
	L. turanica MON-64	MRHO/SU/74/95A
L. (L.) major	*L. major* MON-25	MHOM/MA/81/LEM265
	L. major MON-26	MHOM/YD/76/LEM62
L. (L.) tropica	*L. tropica* MON-5	MRAT/IQ/72/ADHANIS
	L. tropica MON-7	MHOM/PK/00/LV691
L. (L.) killicki	*L. killicki* MON-8	MHOM/TN/80/LEM163
L. (L.) donovani	*L. donovani* MON-2	MHOM/IN/00/DEVI
	L. donovani MON-18	MHOM/ET/67/HU3
L. (L.) infantum	*L. infantum* MON-1	MHOM/FR/78/LEM75
	L. infantum MON-30	MHOM/SD/82/GILANI
	L. mexicana MON-40	MYNC/BZ/62/M379
L. (L.) mexicana	*L. mexicana* MON-121	MHOM/MX/89/RIOS
	L. mexicana MON-152	MHOM/MX/85/SOLIS
	L. mexicana MON-156	MHOM/BZ/BEL21
L. (L.) amazonensis	*L. amazonensis* MON-41	IFLA/BR/67/PH8
	L. amazonensis MON-132	MHOM/BR/73/M2269
	L. amazonensis MON-157	IFLA/TT/71-110
	L. aristedesi MON-133	MORY/PA/68/GML-3
L. (L.) enriettii	*L. enriettii* MON-97	MCAV/BR/45/L88
	L. enriettii MON-227	MCAV/BR/95/CUR3
L. (V.) guyanensis	*L. guyanensis* MON-45	MHOM/GF/79/LEM85
	L. guyanensis MON-143	MHOM/EC/90/AR032
L. (V.) panamensis	*L. panamensis* MON-47	MHOM/PA/75/M4037
	L. panamensis MON-51	MHOM/CO/83/REST417
	L. panamensis MON-124	MHOM/CO/88/UA264
L. (V.) shawi	*L. shawi* MON-144	MCEB/BR/84/M8408
L. (V.) braziliensis	*L. braziliensis* MON-43	MHOM/BR/75/M2903
	L. braziliensis MON-44	MHOM/CO/83/LEM469
	L. braziliensis MON-141	MCAN/PE/91/LEM2222
	L. braziliensis MON-164	MHOM/CO/90/1257
	L. braziliensis MON-166	MHOM/BR/83/LTB300
	L. braziliensis MON-167	MHOM/BZ/81/BEL6
L. (V.) peruviana	*L. peruviana* MON-127	MHOM/PE/84/LC26
	L. peruviana MON-128	MHOM/PE/84/UN59
	L. peruviana MON-142	MHOM/PE/84/CE49
	L. peruviana MON-177	MRAT/PE/84/A3
L. (V.) naiffi	*L. naiffi* MON-148	MDAS/BR/79/M5533
	L. naiffi MON-193	MHOM/00/94/CRE54
	L. naiffi MON-254	MHOM/GF/97/CRE88
L. hertigi	*L. hertigi* MON-135	MCOE/PA/65/C8
	L. deanei MON-52	MCOE/BR/74/M5088
	L. deanei MON-134	MCOE/BR/74/M2674
L. (V.) lainsoni	*L. lainsoni* MON-149	MHOM/BR/81/M6426
	L. lainsoni MON-150	IUBI/BR/00/M12025
	L. lainsoni MON-151	MCUN/BR/85/M9342

97/MAR2. For isoenzyme analysis they were both compared to 49 zymodemes chosen from reference strains (Table 1); 14 of which were from the Old World and 35 from the New World.

Isoenzyme analysis

Starch gel electrophoresis was performed according to the method described by Rioux *et al*. (1990) using

the following 13 enzyme systems: malic enzyme (ME), EC 1.1.1.40; isocitrate dehydrogenase (ICD), EC 1.1.1.42; 6-phosphogluconate dehydrogenase (PGD), EC 1.1.1.44; glucose 6-phosphate dehydrogenase (G6PD), EC 1.1.1.49; NADH diaphorase (DIA), EC 1.6.2.2; purine nucleoside phosphorylase1 (NP_1), EC 2.4.2.1; purine nucleoside phosphorylase2 (NP_2), EC 2.4.2*; glutamate oxaloacetate transaminase1 and 2 (GOT_1 and GOT_2), 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; glucose phosphate isomerase (GPI), EC 5.3.1.9. Iso-electrofocussing was also used since it has greater resolving power (Piarroux *et al*. 1994).

Cladistic analyses

For the taxonomic study, 50 operational taxonomic units (OTU) were used which represented a comprehensive range of the most important zymodemes. Thirty-five zymodemes were from the phylogenetic complexes of New World as defined by Rioux & Lanotte (1993): 6 *L. guyanensis*, 10 *L. braziliensis*, 3 *L. naiffi*, 3 *L. hertigi*, 3 *L. lainsoni*, 4 *L. mexicana*, 4 *L. amazonensis* and 2 *L. enriettii*. Fourteen zymodemes were from the phylogenetic complexes of the Old World: 3 *L. aethiopica*, 2 *L. turanica*, 2 *L. major*, 2 *L. tropica*, 1 *L. killicki*, 2 *L. donovani*, 2 *L. infantum*, the zymodeme MON-229, corresponding to the enzyme profile of the 2 strains of the Martinique island.

The cladogram construction was based on Hennig's (1965) principles and used the MIX program in PHYLIP (Felsenstein, 1988).

Molecular phylogeny

DNA was prepared by proteinase K, SDS lysis, phenol–chloroform extraction and ethanol precipitation. The 18S rDNA alignment was compiled using published sequences from GenBank with the addition of the sequences of *L. hertigi* and MAR1. The 18S rDNA of *L. hertigi* and MAR1 were amplified by PCR and the PCR products were directly sequenced in both directions using the primers described by Maslov *et al*. (1996) on an ABI 377 using Big Dye chemistry (Advanced Biosystems). The sequences were aligned using the PILEUP program in GCG and adjusted by eye to remove ambiguities. Sequence regions that were present in only a minority of species or that could not be aligned with confidence were deleted to give an overall aligned sequence length of 1811 bp.

RNA polymerase II largest subunit sequences of MAR1 and *L. enriettii* were amplified by PCR and directly sequenced using the primers of Croan, Morrison & Ellis (1997) (RPOF1 5′GTAAGCGAGCCAGGTGT; RPO-R1 5′GCAGCCGCACAATGCGCT; the sequence of RPO-F1 given here is different from that of Croan *et al*. (1997) where it was incorrectly reported.) The following 3 combinations of sequencing primers were also used (the position of the primers in the sequence of the *L. donovani* RNA polymerase gene GenBank accession number AF126254 is indicated after the sequence of each primer). (1) PRO-1F (GACACAGCCGTCAAGAC; > 2512–2528); RPO-2R (CTGCAGCTCCCGCAC < 3051–3031); (2) RPO-3F (CAC(G/A)AC(G/A)ATGGGTAAGC > 2966–2983); RPO-4R((A/G)AT(A/G)AACTGCTG(C/T)GCCTC < 3504–3487); (3) ROP-5F (CAGCAGTC(C/A)CTCATCACC > 3253–3270); ROP-6R (GCAGCCGCACAATGC < 3800–3814).

This strategy yielded the full length of the RNA polymerase II fragment used by Croan *et al*. (1997) of *L. enriettii* but failed to amplify the 5′ end of the MAR1 sequence. Consequently the RNA polymerase II alignment corresponds to positions 491–1248 of the RNA polymerase II alignment used by Croan *et al*. (1997). The complete sequence of the DNA polymerase fragment used by Croan *et al*. (1997) was obtained for both *L. enriettii* and MAR1. The DNA polymerase gene was amplified with primers DPO1 (5′ AACGAGCGCGCACTGCT) and DPO2 (5′ GCCGAGGCAGCCATACAT) (Croan *et al*. 1997) and sequenced with these primers and internal sequencing primers L1023-F (AACCTGTGGAGCCGTAC 400–416 in AF009141) and L1023-R(GTAATGAACTT(A/G)AG(A/G)TCGTGG 488–468 in AF009141). The sequence of primer DPO1 given here is different from that reported by Croan *et al*. (1997) where it was incorrectly reported. The new sequences were aligned with the sequences of homologous genes submitted to GenBank by Croan *et al*. (1997) using the Clustal V option in the Megalign program (Higgins, Desmond & Sharp, 1989; Saitou & Nei, 1987). Regions that were not present in all taxa were deleted. The alignments of the RNA (758 bp) and DNA (915 bp) polymerase genes were combined to give a single data set for analysis as per Noyes *et al*. (2000) and a phylogeny was compiled using the DNAML maximum likelihood program in PHYLIP (Felsenstein, 1985).

RESULTS

Isoenzyme analysis

The 2 Martinique strains MAR1 and MAR2 studied presented the same enzyme profile, corresponding to a zymodeme numbered MON-229: ME^{45}, ICD^{95}, PGD^{87}, $G6PD^{78}$, DIA^{30}, NP_1^{00}, NP_2^{85}, GOT_1^{170}, GOT_2^{00}, PGM^{104}, FH^{65}, MPI^{13}, GPI^{52}. With the exception of 2 isoenzymes (NP_1 and NP_2), all the electromorphs are unique to the Martinique strains, demonstrating the extreme divergence of this zymodeme from all other *Leishmania* zymodemes.

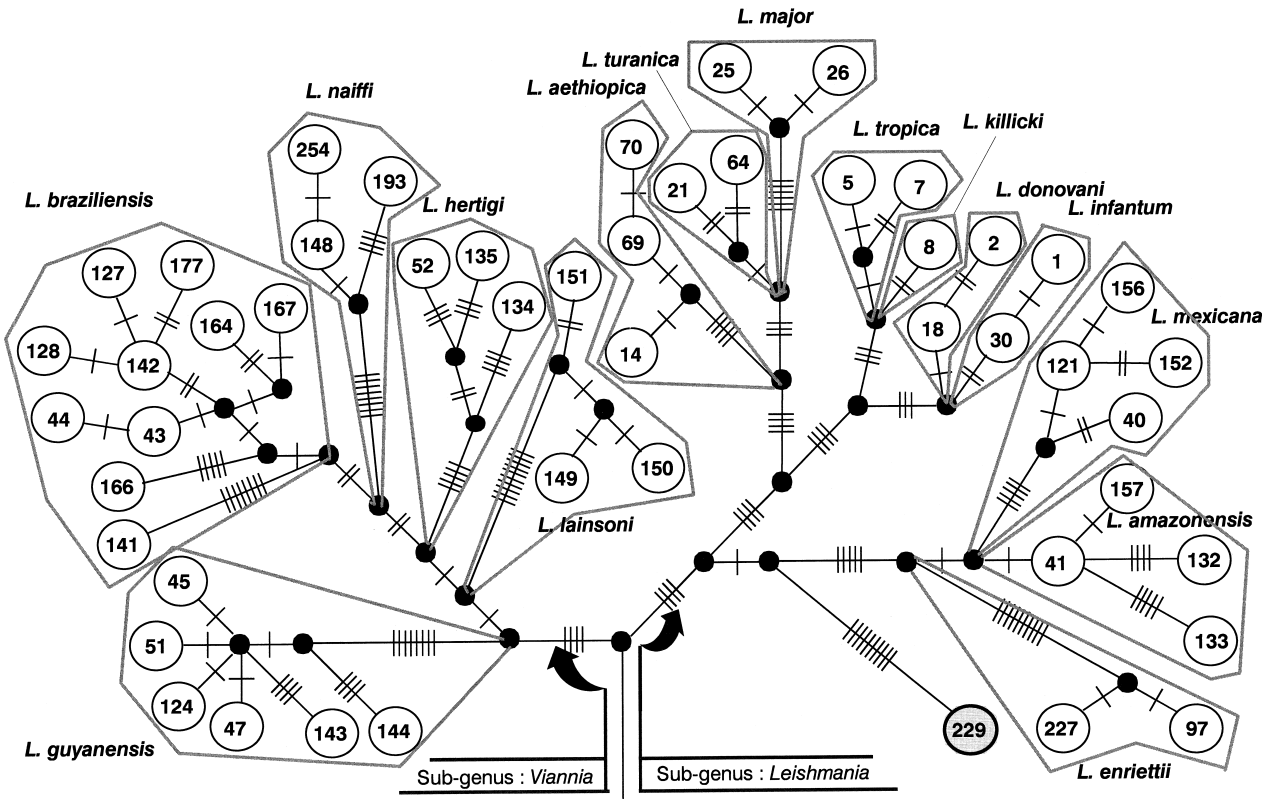

Fig. 1. Cladogram that includes 50 operational taxonomic units, corresponding to 35 New World zymodemes, 14 Old World zymodemes and the Martinique zymodeme MON-229. MON-229 appears clearly distant from other groups and is located at the base of the New World *Leishmania (Leishmania)* clusters. The numbers in the cladogram are the zymodeme numbers the names of species that are represented by zymodemes are indicated in Table 1.

The most parsimonious cladgram obtained with the 50 OTU has 213 evolutionary steps (Fig. 1). It is consistent with the cladogram obtained by Thomaz Soccol *et al.* (1993), and clearly shows the 2 subgenera *L. (Leishmania)* and *L. (Viannia)*. The OTU MON-229 is located on the *Leishmania* subgenus branch, and more precisely at the base of the New World *Leishmania* cluster. It appears very distant from other groups of this cluster, including *L. enriettii*, *L. mexicana*, and *L. amazonensis*.

18S rRNA sequence phylogeny

A phylogeny of the 18s rRNA gene compiled using the parsimony program DNAPARS in the PHYLIP package showed the separation of the *Leishmania* species into 2 distinct clades. These 2 clades have been informally named (i), the paraleishmania which contain *Endotrypanum monterogeii* and *L. hertigi*, *L. colombiensis*, *L. equatoriensis* and *L. herreri*; and (ii) the euleishmania, containing representatives of the *L. (Leishmania)*, *L. (Viannia)* and *L. (Sauroleishmania)* subgenera (Cupolillo *et al.* 2000). The sequence of the Martinique parasite MAR1 clustered in the euleishmania (*Leishmania ss*) clade (Fig. 2A).

The bootstrap support for the euleishmania in this phylogeny is only 60%. A distance tree had the same topology but with slightly higher bootstrap support (68%) for the euleishmania. Although the 18S gene is not suitable for resolving relationships within the genus *Leishmania* this gene does suggest that MAR1 clusters with this genus of digenetic parasites.

RNA and DNA polymerase phylogeny

The maximum likelihood phylogeny of the combined RNA and DNA polymerase data set had a topology identical to the published phylogeny of the DNA polymerase gene (Croan *et al.* 1997) (Fig. 2B).

L. enriettii and MAR1 cluster together in 100% of bootstrap replicates and this clade was basal to all other euleishmania in 64% of bootstrap replicates; however, in 34% of bootstrap replicates they clustered with the *L. (Leishmania)*/*L. (Sauroleishmania)* clade. They also clustered with *L. (Viannia)* in 2% of trees. The distance matrix derived from the DNA and RNA polymerase genes (Table 2) gives some indications of why the position of the MAR1/*L. enriettii* clade in the maximum likelihood phylogeny is not highly supported. *L. enriettii* and MAR1 were more distant from each other (Kimura distance 0·1110) than any other pair of *L. (Leishmania)* and *L. (Viannia)* species (0·0048–0·0915). Mar1 is also the most divergent of all the euleishmania strains. It is because of the relatively large distances separating *L. enriettii* and MAR1 from all other euleishmania species that these 2 parasites cluster together and not because they are

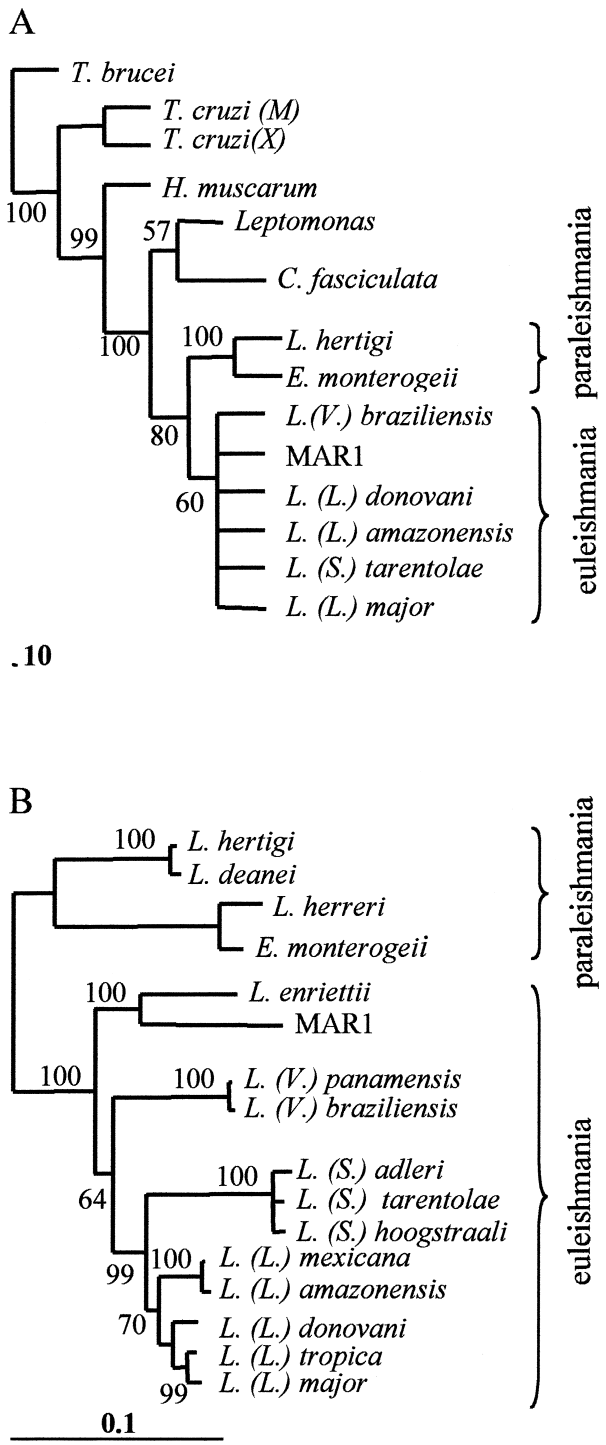

Fig. 2. (A) Unrooted tree produced from the 18S-rDNA sequence alignment by the DNAPARS programme in PHYLIP. GenBank accession number *Leishmania (L.) major* (X53915); *L. (L.) amazonensis* (X53912); *L. (S.) tarentolae* (X53916); *L. (L.) donovani* (X07773); *L. (V.) braziliensis* (M80292); *L. hertigi* (U59492); MHOM/MQ/92MAR1 (AF303938); *Endotrypanum monterogeii* (X53911); *Crithidia fasciculata* (X03450); *Leptomonas* spp. (X53914); *Herpetomonas muscarum* (L18872); *Trypanosoma cruzi* (X53917); *T. cruzi* (M31432); *T. brucei* (M12676). (B) Phylogenetic relationships among combined DNA polymerase Alpha catalytic subunit (915 nucleotides) and RNA polymerase II largest subunit (758 nucleotides) sequences from *Endotrypanum* and selected species of *Leishmania*. The branch lengths are drawn proportional to the evolutionary distances. Sequences are taken from Croan *et al*. (1997) except for the *L. enriettii* sequences (GenBank Accession Nos. AF151728 (DNA polymerase) and AF151727 (RNA polymerase)) and MAR1 sequences (GenBank accession numbers AF326982 (RNA polymerase) and AF326983 (DNA polymerase)). The numbers at the nodes show the bootstrap support for that node, based on 100 bootstrap replicates.

closely related to each other. In the distance matrix *L. enriettii* was slightly more closely related to the *L. (Leishmania)* subgenus (0·1008–0·1078) than to MAR1 (0·1110). The low bootstrap support for the position of MAR1/*L. enriettii* clade relative to other euleishmania subgenera in the polymerase gene phylogeny is a consequence of this anomaly, which may be caused by different evolutionary rates in the different clades. Exclusion of *L. enriettii* had no effect either on the position of MAR1 or the bootstrap support for the other euleishmania.

DISCUSSION

The parasites in zymodeme MON-229 were isolated from 2 patients from Martinique Island. They were initially tentatively described as monoxenous trypanosomatids, since electron micrographs showed the presence of presumed opisthomastigote stages. This identification was supported by the fact that the isoenzymes show a parasite distinct from all known Old and New World *Leishmania* zymodemes (Dedet *et al*. 1995). The 18S rRNA and DNA and RNA polymerase phylogenies clearly indicate that MAR1 is a member of the genus *Leishmania*. All other members of this genus are either known or believed to be digenetic parasites of sandflies and mammals or reptiles, it is therefore likely that MAR1 is also a digenetic parasite of sandflies and vertebrates.

In the light of the 18S rRNA, DNA polymerase and RNA polymerase data, the MON-229 zymodeme was incorporated into a cladistic analysis of the whole genus. The isoenzyme analyses presented here show that the MON-229 zymodeme is clearly distinct from all other *Leishmania* taxa. On the cladogram, it is located within the *L. (Leishmania)* subgenus. Within this subgenus, it shares a common origin with the other New World taxa, from which it follows a long divergent evolution. This long divergent evolution makes it difficult to be confident about the precise relationship of MON-229 with other clades. In principle a more slowly evolving marker such as the DNA and RNA polymerase genes would be expected to give a more robust phylogeny over these longer evolutionary distances. *L. enriettii* and MAR1 are the most basal members of the euleishmania in the DNA and RNA polymerase phylogenies. However, these gene phylogenies may

Table 2. Kimura distances between the taxa shown in Fig. 2B compiled using DNADIST in PHYLIP

	E. monterogeii	*L. herreri*	*L. hertigi*	*L. deanei*	*L. braziliensis*	*L. panamensis*	*L. adleri*	*L. hoogstraali*	*L. tarentolae*	*L. amazonensis*	*L. mexicana*	*L. donovani*	*L. tropica*	*L. major*	*L. enriettii*
E. monterogeii	0·0000														
L. herreri	0·0323	0·0000													
L. hertigi	0·1422	0·1511	0·0000												
L. deanei	0·1457	0·1518	0·0078	0·0000											
L. braziliensis	**0·1845**	**0·1894**	**0·1505**	**0·1557**	**0·0000**										
L. panamensis	0·1845	0·1893	0·1499	0·1550	0·0048	0·0000									
L. adleri	0·2004	0·2070	0·1722	0·1753	0·1207	0·1242	0·0000								
L. hoogstraali	0·1925	0·1975	0·1683	0·1714	0·1185	0·1220	0·0151	0·0000							
L. tarentolae	**0·1936**	**0·2017**	**0·1707**	**0·1737**	**0·1208**	**0·1243**	**0·0139**	**0·0108**	**0·0000**						
L. amazonensis	0·1712	0·1820	0·1530	0·1565	0·0888	0·0902	0·0963	0·0949	0·0950	0·0000					
L. mexicana	0·1672	0·1780	0·1499	0·1535	0·0874	0·0888	0·0915	0·0901	0·0902	0·0066	0·0000				
L. donovani	**0·1666**	**0·1789**	**0·1461**	**0·1526**	**0·0881**	**0·0915**	**0·0882**	**0·0882**	**0·0856**	**0·0392**	**0·0379**	**0·0000**			
L. tropica	0·1642	0·1764	0·1409	0·1474	0·0834	0·0868	0·0888	0·0875	0·0862	0·0430	0·0398	0·0212	0·0000		
L. major	0·1650	0·1757	0·1490	0·1555	0·0841	0·0867	0·0867	0·0841	0·0841	0·0436	0·0404	0·0249	0·0120	0·0000	
L. enriettii	0·1950	0·2014	0·1554	0·1575	0·1174	0·1152	0·1443	0·1392	0·1408	0·1078	0·1028	0·1009	0·1008	0·1028	0·0000
MARI	0·2091	0·2189	0·1810	0·1832	0·1337	0·1338	0·1568	0·1508	0·1511	0·1217	0·1211	0·1195	0·1176	0·1154	0·1110

not reflect the parasites true phylogenies either, since the bootstrap support for this basal position is low. The position of the *L. enriettii*/MON-229 clade may be an artifact of Long Branch attraction and not a consequence of evolutionary history (Felsenstein, 1988). Although the classification of MON-229 remains uncertain it is clearly the most genetically divergent strain of euleishmania (*Leishmania ss*) that has been described to date. The distance matrices and the isoenzyme data indicate that both these parasites are most closely related to the *L. (Leishmania)* subgenus. Isoenzymes may be evolving too fast for the accurate classification of the *L. enriettii*/MON-229 clade within the genus *Leishmania*; however, the DNA and RNA polymerase genes may be evolving too slowly. A phylogeny of other genes that are evolving faster and that can be classified on the basis of amino acid sequence will be necessary to classify both *L. enriettii* and MON-229 parasites.

Other MON-229 like parasites may have been found

Prior to these 2 observations, 3 cases of autochthonous cutaneous leishmaniasis were reported from Martinique (Stevenel, 1917; Fouche & Montestruc, 1951; Courmes *et al*. 1966). The parasitological diagnosis of these cases was based on the detection of amastigotes in skin smears. None of these parasites have been classified by modern methods that might indicate their relationships to other *Leishmania* species. The parasites were assumed to belong to the *Leishmania* genus on the basis of the clinical features of the lesions and the presence of amastigotes within the lesions. In the light of our observations on MAR1 and MAR2 it is possible that some or all of these cases are also due to zymodeme MON-229 like parasites.

Ecology of MON-229

The large genetic difference in the polymerase genes and the large phenotypic difference in the isoenzymes of MAR1 from other euleishmania are sufficient to warrant separate species status for these parasites. However, the authors believe that it is premature to describe a new species until more is known of the biology and ecology of MON-229 strains. At present nothing is known of the presumed reservoir hosts or sandfly vectors of MON-229 parasites. *Lutzomyia atroclavatus* is the only species of sandfly to have been reported from Martinique and is therefore a candidate vector (Fauran, Courmes & Mille, 1966). All the indigenous mammalian fauna of Martinique is now extinct with the exception of bats. Bats are not known as reservoirs of *Leishmania* elsewhere so the most likely reservoir is perhaps an imported rodent. Leger reported finding a *Leishmania like* parasite in a lizard in Martinique in 1918. However, there are

no proven records of lizard *Leishmania* from the New World despite some heroic searches and consequently this record has been dismissed by later reviewers (Telford, 1995). Therefore it seems unlikely that MAR1 is related to Leger's *L. henrici* or that lizards are the reservoir. The sequence data indicate that MAR1 is not closely related to Old World lizard *Leishmania*.

It has been shown that MAR1 and MAR2 produce disseminated but well-tolerated infections in Balb/C mice indicating that these parasites may be a valuable model for subclinical infections (Garin *et al*. 2001).

The other presumed monogenetic trypanosomatids that have been isolated from humans and other mammals have not been systematically classified. They have been presumed to be monogenetic parasites because they could not be positively identified as either *Leishmania* or *Trypanosoma* species. The data presented here show that it is essential to classify an unidentified parasite strain using an appropriate gene sequence, such as the 18S gene, since the genus *Leishmania* appears to be more genetically diverse than had been anticipated.

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