

TAXONOMY OF *LEISHMANIA*. USE OF ISOENZYMES. SUGGESTIONS FOR A NEW CLASSIFICATION

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SUMMARY

The authors propose a new classification for the genus *Leishmania* Ross, 1903 based both on the use of intrinsic and extrinsic characters and on Linnean and Adansonian methods. The type of vertebrate host makes it possible to recognize the genus group: *Leishmania* designates Kinetoplastida parasites of mammals. Neighbouring forms which parasite Reptiles are now grouped in the genus *Sauroleishmania* Ranque, 1973. Characteristics of the intravectorial cycle (supra- and peri-pyloric) are used to define the subgenus group (*Leishmania*, *Viannia* Lainson and Shaw, 1987). The classification uses biochemical, particularly enzymatic, characters. Elementary taxonomic units are made up of all the strains having

the same isoenzyme profile, *i. e.* the zymodeme. The grouping of the zymodemes is usually performed through automatic techniques which lead to bush-like trees (dendrograms) showing either simple affinities between units (phenograms) or their phyletic relationships (cladograms). The branches recognized as being stable are individualized as « zymodeme complexes ». They bear the name of either the previously defined species taxa or that of a specially created one. Two examples of taxonomic constructions, phenetic and cladistic, are presented. Finally, a general classification of the genus is proposed.

RÉSUMÉ : Taxonomie des *Leishmania*. Utilisation des isoenzymes. Propositions pour une nouvelle classification.

Les auteurs proposent une nouvelle classification du genre *Leishmania* Ross, 1903, basée sur l'utilisation de caractères intrinsèques et sur les méthodes linnéennes et adansonniennes. Le type d'hôte vertébré permet d'individualiser le groupe genre : les *Leishmania* désignent des Kinetoplastida parasites de Mammifères. Les formes voisines, intéressant les Reptiles, sont à présent regroupées dans le genre *Sauroleishmania* Ranque, 1973. Les caractéristiques du cycle intravectoriel (supra- et péripylorique) servent à définir le groupe sous-genre : *Leishmania*, *Viannia* Lainson et Shaw, 1987. En deçà, la classification utilise les caractères biochimiques, en particulier enzymatiques. Les unités taxonomiques élémentaires sont

constituées par l'ensemble des souches présentant le même profil enzymatique, le zymodème. Le regroupement des zymodèmes s'effectue habituellement par les techniques automatiques. Il aboutit à des arbres buissonnants (dendrogrammes), traduisant soit les simples affinités entre unités (phénogramme), soit leurs relations phylétiques (cladogramme). Les rameaux reconnus comme stables sont individualisés en tant que « complexes de zymodèmes ». Ils portent le nom des taxons spécifiques antérieurement définis ou créés à cette occasion. Deux exemples de construction taxonomique, phénétique et cladistique, sont présentés. *In fine*, une classification générale du genre est proposée.

Faced with a problem of systematics the parasitologist can adopt one of two attitudes, either that of the physician or epidemiologist for whom the identification of a pathogenic agent is an end in itself, or that of the naturalist-ecologist who attempts to reach biogeographic or phyletic levels of understanding.

The diagnostic approach is based on the search for discriminant characters which allow the individualization of a living object, *i. e.* its identification (« I. D. card »). This procedure can be extended to a group of objects. For organisms, such as *Leishmania*, which are difficult to separate by their morphological characters, the term used is

typing. Typing generally involves intrinsic characters such as metabolic or genetic features. In some clinical forms of cutaneous leishmaniasis, typing of the causative organism should precede any prescription. The identification of *L. braziliensis*, now possible thanks to enzymatic methods, thus requires a prolonged treatment and both a careful clinical and immunological follow-up. In the same way, for cutaneous leishmaniasis around the Mediterranean basin, isolation of the viscerotropic zymodeme *L. infantum* MON-1 should suggest the possibility of spreading and lead *ipso facto* to a parenteral treatment with antimony. Eco-epidemiological analyses of foci of leishmaniasis demand the enzymatic identification of the parasite for the incrimination both of « true » vectors and of « true » reservoirs.

The classificatory approach seeks to define the relationships between objects or groups of objects. Like the diagnostic approach, it first focuses on analyzing the indivi-

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dual characters of elementary units. But it also goes beyond this stage to find those characters common to several group members. A return to base units, however, occurs when hierarchical constructions are used to elaborate dichotomous keys, which are in turn used for identification. It should finally be noted that most classifications include a notion of parental relationships (phylogeny).

I. LINNEAN SYSTEMATICS
OF THE GENUS *LEISHMANIA*.
FROM SPECIES TO COMPLEXES
AND FROM GENERA TO SUB-GENERA

Since the creation of the genus *Leishmania* by Ross, 1903, the number of named species has continually increased. The descriptions of the binomials *L. donovani* Laveran and Mesnil, 1903, *L. tropica* Wright, 1903, *L. infantum* Nicolle, 1908, *L. braziliensis* Vianna, 1911, *L. peruviana* Velez, 1913, *L. major* Yakimoff and Schokhor, 1914, *L. tarentolae* Wenyon, 1921, *L. enriettii* Muniz and Medina, 1948, *L. mexicana* Biagi, 1953, *L. guyanensis* Floch, 1954 mark the first analytic period. Except for some taxa (*L. enriettii*, *L. major*, *L. mexicana*) the criteria used for classification were extrinsic.

At the beginning of the 70s, a systematic regrouping of the taxa was carried out. It was formalized particularly by Lainson and Shaw's important studies on intravectorial cycles. The taxa were placed in three sections : Hypopylaria, Peripyalaria and Suprasyplaria (23, 24). Within these,

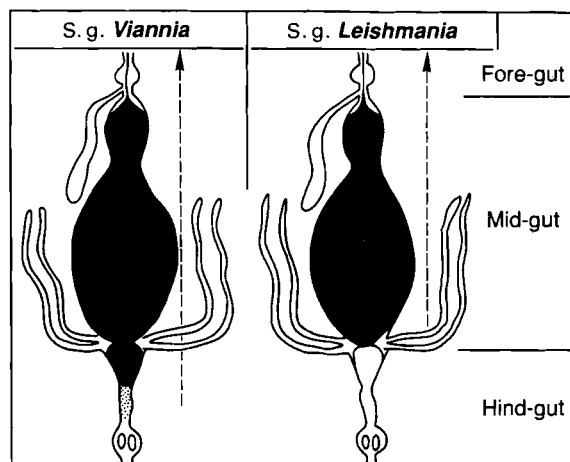


FIG. 1. — Sub-genus *Leishmania* Ross, 1903 and *Viannia* Lainson and Shaw, 1987. Classification based on behaviour in the gut of the sandfly host (according to Lainson and Shaw 1979 and 1987).

and still based on extrinsic characters, the binomials were listed as sub-species (*L. donovani infantum*, *L. braziliensis guyanensis*) which were in turn regrouped into complexes (*L. donovani*, *L. braziliensis*, etc.). In 1987, these same authors created the sub-genus *Viannia* to replace the Peripyalaria section (25). The Suprasyplaria section thus became a synonym for the sub-genus *Leishmania* (fig. 1). Prior to this, Reptile parasites, which had up to this time been placed in all three sections, Hypo-, Peri- and Suprasyplaria,

TABLE I. — Reference strains of *Leishmania* species. Recommended by the Expert Committee on Leishmaniasis of the World Health Organization (Geneva, 1989), these strains are deposited in the main international banks. Some are related to types (*L. arabica*, *L. killicki*), while others were designated at a later date by experts who considered the host and the country of the first description (43). Although they cannot be considered as neotypes, they nevertheless have their essential characters. Thus, the reference strain for *L. infantum* Nicolle, 1908 comes from a child living near Tunis, suffering from visceral leishmaniasis. (CL = cutaneous leishmaniasis; DCL = disseminated cutaneous; MCL = muco-cutaneous; VL = visceral).

<i>L. aethiopica</i>	MHOM/ET/72/L100	CL, DCL
<i>L. amazonensis</i>	MHOM/BR/73/M2269	CL, DCL
<i>L. arabica</i>	MPSA/SA/83/JISH220	Not observed in humans
<i>L. archibaldi</i>	Not selected	VL
<i>L. aristedisi</i>	MORY/PA/69/GML3	Not observed in humans
<i>L. braziliensis</i>	MHOM/BR/00/LTB300	CL, MCL
<i>L. chagasi</i>	MHOM/BR/74/M2682	CL, VL
<i>L. deanei</i>	MCOE/BR/74/M2674	Not observed in humans
<i>L. donovani</i>	MHOM/IN/80/DD8	VL
<i>L. enriettii</i>	MCAV/BR/45/L88	Not observed in humans
<i>L. garnhami</i>	MHOM/VE/76/JAP78	CL
<i>L. guyanensis</i>	MHOM/BR/75/M4147	CL
<i>L. gerbilli</i>	MRHO/CN/60/GERBILL1	Not observed in humans
<i>L. hertigi</i>	MCOE/PA/65/C8	Not observed in humans
<i>L. infantum</i>	MHOM/TN/80/IPT1	CL, VL
<i>L. killicki</i>	MHOM/TN/80/LEM904	CL
<i>L. lainsoni</i>	MHOM/BR/81/M6426	CL
<i>L. major</i>	MHOM/SU/73/5-ASKH	CL
<i>L. mexicana</i>	MHOM/BZ/82/BEL21	CL
<i>L. panamensis</i>	MHOM/PA/71/LS94	CL
<i>L. peruviana</i>	Not selected	CL
<i>L. pifanoi</i>	MHOM/VE/57/LL1	DCL
<i>L. tropica</i>	MHOM/SU/74/K27	CL
<i>L. venezuelensis</i>	MHOM/VE/00/H17	CL

were gathered together into the genus *Sauroleishmania* Ranque, 1973 (19). Finally, following the recommendations of the Montpellier Symposium (*Leishmania. Taxonomy and Phylogenesis*, 1984) the trinomial nomenclature was abandoned and most of the taxa described at the beginning of the century were again given their species name (46).

It should be noted that the systematic categories thus defined involve a monothetic procedure and are based only on extrinsic criteria: clinical forms, epidemiological cycles (zoonosis, anthroponosis), host type, intravectorial behaviour, geographical distribution.

II. BIOCHEMICAL ANALYSIS AND ADANSONIAN SYSTEMATICS. NUMERICAL TAXONOMY. PHENETICS AND CLADISTICS

1 — PAST AND FUTURE

In the 70s, the studies on biochemical (enzyme, DNA) and immunological (excreted factor) identification of *Leishmania* already focused on taxonomy (8, 11, 12, 53). They always referred to Linnean systematics, *i. e.* to the categories genus (*Leishmania*), sub-genus (*Sauroleishmania*), species and sub-species (*L. infantum*, *L. tropica*, *L. braziliensis panamensis*, etc.) (35).

From the beginning of the 80s, attempts of automatic classification were made (2, 21, 30, 32, 52). Initially they were simple hierarchical relationships (phenograms), although a certain reference to evolution could be seen in the chronological dendrograms (29). From this period on, it was also recommended to designate reference strains (standard, marker) and to protect them in cryobanks (*table I*).

Continuing the analytical effort, the next stage concentrated on evolution. It involved the application of Hennig's concepts and methods (17) and led to the construction of particular phyletic trees (cladograms). However, the absence of fossil remains made it necessary for the authors to reconsider the group origin (mono- or polyphyletic) and the status of characters (primitive or derived).

The next stage will probably involve molecular genetics. It will be based on the analysis of nuclear (6, 13) and kinetoplastic (3, 5) DNA and ribosomal RNA and will focus on the study of the fundamental mechanisms involved in speciation and taxonomic divergence. It will no doubt be long and difficult as the Trypanosomatidae, like many other Protozoa, represent primitive groups whose evolutionary strategies probably follow genetic rules quite different from those of modern Eukaryotes, particularly multicellular ones (59).

2 — FROM ENZYMATIC CRITERIA TO CHEMOTAXONOMIC UNIT

As we have suggested above, difficulties can be found at two levels. The first one, risky and essential, is the cor-

rect identification of the parasite and consequently the selection of the « right » characters; the second, more easily dealt with today, is the use of automatic classification techniques.

• Individualization of characters.

Electromorphs, isoenzymes and allozymes

In the classificatory model used the characters are represented by enzymatically active proteins called isoenzymes (isozymes). In fact, they represent alleles (alloenzymes, allozymes) generally coded at the same locus (genenzyme). In *Leishmania*, the genenzyme usually generates a single isoenzyme. This particular feature, which can be found in other asexually reproducing systematic groups, has always fascinated researchers. The rarity of heterozygous structures could be explained either by a typical haploidy, or by a diploidy with elimination of heterozygotes or repression of one of the alleles (37, 41, 47).

In practice, the protein extracts are prepared from bulk cultures of promastigotes. The isoenzymes are separated in the form of electromorphs, after electrophoresis on starch gel (36), polyacrylamide gel (27) or cellulose acetate (20). Many difficulties are encountered at this stage with the techniques of migration and of electromorph revelation and identification. In general, it is preferable to use low voltages and extended migration times.

The selected enzymes must be both numerous and diversified. However, their number is often reduced due to difficulties during revelation and to reagent costs. Around 20 enzymes can be used if the selection effort is made 1) *a priori*, to retain the « stable » systems (structural enzymes, low sensibility to selection pressures), 2) *a posteriori*, to select non-redundant polymorphic systems, with, if possible, a phylogenetic relevance (plesiomorphs, synapomorphs). We currently use the following 15 enzymatic systems: malate dehydrogenase, MDH : EC 1.1.1.37; malic enzyme, ME : EC 1.1.1.40; isocitrate dehydrogenase, ICD : EC 1.1.1.42; phosphogluconate dehydrogenase, PGD : EC 1.1.1.44; glucose-6-phosphate dehydrogenase, G6PD : EC 1.1.1.49; glutamate dehydrogenase, GLUD : EC 1.4.1.3; diaphorase NADH, DIA : EC 1.6.2.2; nucleoside purine phosphorylase, NP1 : EC 2.4.2.1 and NP2 : EC 2.4.2.*; glutamate-oxaloacetate transaminase, GOT1 and GOT2 EC : 2.6.1.1, phosphoglucomutase, PGM : EC 2.7.5.1; mannose phosphate isomerase, MPI : EC 5.3.1.8; glucose phosphate isomerase, GPI : EC 5.3.1.9.

The electromorph can only be correctly identified by referring to an isoenzyme marker. This is a strain, or if possible a clone, selected for one or several electromorphs. These markers should be systematically included in the electrophoresis gels. Even more so than for the other strains, their biochemical integrity should be ensured by careful preservation. Currently, only liquid nitrogen fulfills the necessary conditions (cryobank). The protein extracts should

TABLE II. — Enzyme profile of reference strains of Old World Leishmania.

Phenetic complexes	Zymodemes MON-	Enzyme profiles														Reference strains	
		MDH	ME	ICD	PGD	G6PD	GLUD	DIA	NP ₁	NP ₂	GOT ₁	GOT ₂	PGM	FH	MPI		GPI
<i>L. infantum</i>	1	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	MHOM/FR/78/LEM 75
	11	104	100	100	100	105	100	100	130	100	100	100	100	100	100	100	MHOM/FR/80/LEM 189
	24	104	100	100	100	100	100	100	140	100	100	100	100	100	100	100	MHOM/DZ/82/LIPA 59
	27	100	100	100	100	100	100	100	130	100	100	100	100	100	100	100	MHOM/IT/79/ISS 7
	28	104	100	100	100	102	100	100	140	100	100	100	100	100	100	100	MHOM/ES/83/BCN 2
	29	104	100	100	100	105	100	100	140	100	100	100	100	100	100	100	MHOM/ES/81/BCN 1
	30	112	100	100	100	100	100	100	140	100	100	100	100	100	100	100	MHOM/SD/82/GILAN!
	33	104	100	100	100	105	100	100	100	100	100	100	100	100	100	100	MHOM/FR/82/LEM 356
	34	104	100	100	100	100	100	100	100	100	100	100	100	100	100	100	MHOM/FR/84/LEM 538
	72	100	100	100	100	100	100	100	100	100	100	100	109	100	100	100	MHOM/IT/86/ISS 218
	77	100	100	100	100	102	100	100	100	100	100	100	100	100	100	100	MCAN/ES/86/LEM 935
	78	104	100	100	100	100	100	100	140	100	100	100	100	110	100	100	MHOM/MT/85/BUCK
	80	104	100	100	100	100	100	100	130	100	100	100	100	100	100	100	MHOM/DZ/83/LEM 425
	81	112	100	100	100	100	100	100	150	100	100	100	100	100	100	100	MHOM/SD/00/3 S
	98	100	90	100	100	100	100	100	100	100	100	100	100	100	100	100	MHOM/EG/87/RTC 2
	105	100	100	100	100	105	100	100	100	100	100	100	100	100	100	100	MCAN/ES/88/LEM 1355
	108	100	100	100	100	100	100	120	100	100	100	100	100	100	100	100	MCAN/FR/87/RM 1
111	104	100	100	100	100	100	100	140	100	100	100	100	100	120	100	MHOM/IT/85/ISS 175	

<i>L. donovani</i>	2	104	100	100	93	100	100	100	140	100	113	113	100	100	100	100	MHOM/IN/00/DEV 1
	3	104	100	100	100	100	100	100	140	100	113	113	100	100	100	86	MHOM/IQ/73/IF 3
	18	112	100	100	100	100	100	100	140	100	113	113	100	100	100	100	MHOM/ET/67/HU 3
	31	104	100	100	100	100	100	100	140	100	113	113	100	100	110	86	MHOM/SA/81/JEDDAH-KA
	32	104	100	100	100	100	100	100	150	100	113	113	100	100	100	100	MHOM/KE/75/H 9
	35	104	100	100	100	100	150	100	150	100	113	113	100	100	100	86	MHOM/CN/00/WANGJIE-1
	36	104	100	100	100	100	100	100	100	100	113	113	100	100	100	100	MHOM/KE/55/LRC-L 53
	37	104	100	100	100	100	100	100	140	100	113	113	100	100	100	100	IMAR/KE/62/LRC-L 57
	38	100	100	100	100	100	100	100	100	100	113	113	100	100	100	100	MHOM/IN/54/SC 23
	73	104	100	100	100	100	100	100	140	100	113	113	100	110	100	100	MHOM/SU/84/MARZ-KRIM
	82*	112	100	100	100	100	100	100	140	100	110	110	100	100	100	100	MHOM/ET/72/GEBRE 1
	83	104	100	100	100	100	100	100	140	100	113	113	100	98	110	86	MHOM/ET/84/ADDIS 164
	84	112	100	100	100	100	100	100	150	100	113	113	100	100	100	100	MHOM/MT/00/MALTA 33

* *L. archibaldi*

TABLE II. — (following).

Phenetic complexes	Zymodemes MON-	Enzyme profiles															Reference strains
		MDH	ME	ICD	PGD	G6PD	GLUD	DIA	NP ₁	NP ₂	GOT ₁	GOT ₂	PGM	FH	MPI	GPI	
<i>L. tropica</i>	5	112	95	100	93	82	80	100	450	100	135	90	100	110	110	76	MRAT/IQ/72/ADHANIS 1
	6	100	95	100	93	82	95	110	450	100	135	90	100	100	101	76	MHOM/IQ/65/BRAY L 75
	7	112	95	100	93	82	95	110	450	100	135	90	100	100	101	76	MHOM/PK/00/LV 691
	8*	100	100	100	93	82	110	100	300	100	127	90	100	100	110	76	MHOM/TN/80/LEM 163
	13	112	95	100	93	82	95	100	450	100	135	90	100	110	101	76	MHOM/LB/76/LEM 61
	19	100	90	100	105	82	80	100	450	110	140	85	90	100	110	60	MHOM/NA/74/BRANDT
	20	100	100	100	105	82	80	100	450	110	140	85	90	100	110	60	IROS/NA/80/ROSSI 80
	39	100	95	100	95	82	95	100	450	90	135	90	100	100	101	76	MHOM/IQ/65/AV SINAI
	53	112	95	100	95	82	95	110	450	90	135	90	100	100	101	76	MHOM/IR/70/NADIM 3
	54	112	95	100	100	82	80	120	450	90	135	90	100	110	100	76	MHOM/IL/80/SINGER
	55	112	95	100	95	82	95	100	450	90	135	90	100	100	101	76	MHOM/TR/83/ISS 68
	56	118	95	100	93	82	80	120	340	90	135	90	100	110	100	76	MHOM/GR/80/GR-L 35
	57	118	100	100	95	82	80	120	450	90	135	90	100	110	110	76	MHOM/GR/82/SER-L 60
	58	112	95	100	93	82	95	100	450	100	135	90	108	100	101	76	MHOM/AF/82/K 001
	59	112	100	100	93	82	95	100	450	100	135	90	100	100	100	76	MHOM/AF/82/K 006
	60	100	95	100	93	82	95	100	450	100	135	90	108	100	101	76	MHOM/SU/74/K 27
	61	112	95	100	95	82	95	100	450	100	135	90	108	100	101	76	MHOM/SU/66/V
	62	112	95	100	93	82	95	110	450	90	135	90	100	110	101	76	MCAN/IN/71/DBKM
	63	112	95	100	93	82	80	110	450	100	135	90	100	100	101	76	MHOM/ET/00/LV 471
	71	100	100	100	93	82	80	100	450	110	140	85	100	98	110	76	MHOM/YE/86/LEM 955
	75	112	95	100	95	82	95	100	450	100	135	90	100	100	101	76	MHOM/SU/58/OD
76	112	95	100	93	82	95	110	450	100	135	90	108	110	101	76	MHOM/SY/86/LIPA 154	
102	118	95	100	95	82	80	120	450	90	130	70	108	110	100	76	MHOM/MA/88/LEM 1314	
104	100	95	100	95	82	95	110	450	100	135	90	100	100	101	76	0000/00/84/LEM 643	
107	104	80	100	95	85	80	120	420	110	135	90	109	110	110	88	MHOM/MA/88/LEM 1452	
109	118	95	100	108	82	80	120	450	90	130	70	108	110	100	76	MHOM/MA/89/LEM 1591	
112	118	95	100	102	82	80	120	450	90	130	70	108	110	100	76	MHOM/MA/88/LEM 1451	
113	112	95	100	95	82	95	100	450	100	128	90	108	100	101	76	MHOM/MA/89/LEM 1777	

<i>L. aethiopica</i>	14	130	108	100	140	82	100	100	700	120	127	110	123	105	105	53	MHOM/ET/72/L 100
	69	112	108	90	140	87	110	100	700	120	127	110	123	105	105	53	MHOM/ET/82/101-82
	70	130	100	90	140	87	100	100	700	120	127	110	123	105	105	53	MHOM/ET/83/68-83

* *L. killicki*

TABLE II. — (end).

Phenetic complexes	Zymodemes MON-	Enzyme profiles															Reference strains
		MDH	ME	ICD	PGD	G6PD	GLUD	DIA	NP ₁	NP ₂	GOT ₁	GOT ₂	PGM	FH	MPI	GPI	
<i>L. major</i>	4	160	88	90	122	94	200	100	400	90	110	110	118	79	150	77	MRHO/SU/59/P-strain
	21	160	108	130	140	94	220	100	500	110	110	110	118	70	110	77	MRHO/SU/65/VL
	23	160	88	90	122	94	200	100	400	100	110	110	118	79	150	65	MMER/IN/73/GTBM
	25	150	88	100	122	94	200	100	400	90	110	120	118	87	150	77	MHOM/MA/81/LEM 265
	26	160	88	100	122	94	200	100	400	90	110	110	118	79	150	77	MHOM/YD/76/LEM 62
	64	160	100	130	150	94	220	100	500	120	110	110	118	70	150	77	MRHO/SU/74/95 A
	65	160	108	130	140	94	220	100	500	120	110	110	118	70	150	77	MMEL/SU/79/Mel
	66	160	88	100	130	94	200	100	400	90	110	110	110	79	150	77	MHOM/IL/83/IL 24
	67	160	88	100	130	94	200	100	400	90	110	110	118	79	150	77	MHOM/IL/83/IL 53
	68	160	88	100	122	94	200	100	400	110	110	110	110	79	150	77	MHOM/IL/83/IL 32
	74	160	88	100	122	94	200	90	400	90	110	110	118	79	150	77	MCAN/EG/00/ASC-D 1
103	160	88	100	122	94	200	100	400	100	110	110	118	79	150	77	MPSA/JO/87/JP-s-G	
<i>L. gerbilli</i>	22	104	95	120	130	94	250	100	350	120	150	80	118	79	130	55	MRHO/CN/60/Gerbilli
<i>L. arabica</i>	99	150	93	100	140	87	220	100	350	100	120	30	118	60	160	50	MPSA/SA/83/JISH 220

TABLE III. — Enzyme profile of reference strains of the New World Leishmania.

Phenetic complexes	Zymodemes MON-	Enzyme profiles													Reference strains
		ME	ICD	PGD	G6PD	DIA	NP ₁	NP ₂	GOT ₁	GOT ₂	PGM	FH	MPI	GPI	
<i>L. braziliensis</i>	43	A ³	B ¹	C ⁴	D ⁴	E ²	F ³	G ⁴	H ⁴	I ⁵	J ²	K ²	L ²	M ⁴	MHOM/BR/75/M 2903
	44	A ²	B ¹	C ⁴	D ⁴	E ²	F ³	G ⁴	H ⁴	I ⁵	J ²	K ²	L ²	M ⁴	MHOM/CO/83/LEM 469
<i>L. guyanensis</i>	47	A ²	B ²	C ²	D ⁵	E ³	F ¹	G ³	H ⁵	I ³	J ¹	K ³	L ¹	M ⁴	MHOM/PA/75/M 4037
	51	A ²	B ²	C ³	D ⁶	E ³	F ¹	G ³	H ⁵	I ³	J ¹	K ³	L ¹	M ⁴	MHOM/CO/83/REST 417
	100	A ²	B ²	C ²	D ⁶	E ³	F ¹	G ³	H ⁵	I ³	J ¹	K ³	L ¹	M ⁴	MHOM/CO/83/487
	101	A ²	B ²	C ¹	D ⁶	E ³	F ¹	G ³	H ⁵	I ³	J ¹	K ³	L ¹	M ⁴	MHOM/CO/86/UA 126
	45	A ²	B ²	C ⁵	D ⁶	E ³	F ⁵	G ²	H ⁵	I ³	J ¹	K ³	L ¹	M ⁴	MHOM/GF/79/LEM 85
<i>L. mexicana</i>	40	A ⁴	B ³	C ⁷	D ²	E ⁴	F ²	G ²	H ¹	I ²	J ⁴	K ⁴	L ⁴	M ¹	MNYC/BZ/62/M 379
	41	A ⁴	B ⁵	C ⁷	D ²	E ⁵	F ¹	G ²	H ²	I ²	J ¹	K ⁴	L ⁴	M ²	IFLA/BR/67/PH 8
<i>L. enriettii</i>	97	A ¹	B ⁴	C ⁷	D ³	E ¹	F ¹	G ⁵	H ³	I ⁴	J ¹	K ¹	L ⁵	M ³	MCAV/BR/45/L 88
<i>L. hertigi</i>	52	A ⁵	B ³	C ⁶	D ¹	E ²	F ⁴	G ¹	H ⁴	I ¹	J ³	K ⁴	L ³	M ²	MCOE/BR/78/M 5088

nevertheless be used rapidly (< 12 months). The recognition of a new electromorph needs special care, particularly for slight differences in migration distances: it is often necessary to repeat the migrations to confirm the authenticity of the electromorph in question. At this stage, modifications of pH, voltage and migration time can be useful.

Any identification of a strain should be based on the complete list of its electromorphs (e. g. G6PD 100, MDH 112, etc.). Along these lines a relevant synthesis of notation and designation modes for enzymes and their isoenzymes was published by Chance (7). This author particularly recommends using a standard reference strain which allows the calculation of the mobility of the different enzymes according to the formula: relative mobility of the *i* band = (distance from the start of the *i* band/distance from the start of the reference band) × 100. For the past several years, this recommendation has been rigorously followed by the Montpellier Center. The standard strain's international code is MHOM/FR/78/LEM75. The corresponding extracts must be continually available (continuous bulk culture). It should be noted that no enzymatic changes in this strain have been found since its isolation (10 years in cryopreservation along with continuous culture).

- Definition of the taxonomic unit, the zymodeme

The enzymatic characters thus identified allow the individualization of the zymodeme, a concept proposed by Godfrey (14). By definition, the zymodeme corresponds to all the strains with the same enzymatic profile. The zymodeme thus constitutes a taxon whose systematic level is undefined, *i. e.* one that does not refer to a « category » recognized by the Nomenclature Code. It is an elementary phenon as defined by Sokal and Sneath (57). This position has been adopted by most researchers since, at the available evidence, the mode of evolution in *Leishmania* is still considered as clonal. Then the zymodeme would represent the true evolutionary unit. In the light of recent findings, it appears nevertheless possible that sexual (or parasexual) phenomena occur (see below). If these phenomena were essential, some groups of finely differentiated zymodemes (« small variants ») would simply represent the « population expression » of the same biological entity (38). However, the present evidence does not allow to draw such conclusions.

The choice of the zymodeme as a unit raises another difficulty: the use of additional enzymes may lead to the break up of the zymodeme into two or several new units. Consequently, for each zymodeme, it is essential to designate one strain (clone if possible) as a reference which will remain attached to it (*tables II and III*). This clone then will allow, at least partially, the recognition of synonymies by the Centers of enzymatic identification which often use non totally overlapping techniques. In order to avoid any confusion,

each Center should also preferably use an independent logo (LON—for London, MON—for Montpellier) (50).

3 — FROM ADANSONIAN CONCEPTS TO AUTOMATIC CLASSIFICATIONS

The essential notions of numerical taxonomy were anticipated two centuries ago by the French botanist Adanson (1). He imagined the concomitant use of numerous characters (polythetism), rather than the successive use of some of them (monothetism). The development of computers has made it possible to put Adanson's concepts in practice. Thanks to North-American and British researchers, this method has today reaped its just reward (56).

It should be recalled that this type of taxonomy requires a « large number » of characters (variables, descriptors, « signifiers ») and a « large number » of units (objects, phenons, « signified »). The numerical classification of *Leishmania* is no exception. The characters, called unitary, are represented by isoenzymes; the operational taxonomic units (OTUs) are represented by the zymodemes. The affinities, or resemblances, between units are calculated using distance coefficients chosen according to the objective of the study (Jaccard index, Nei index, Manhattan index, Euclidian distance, etc.). After coding the characters, several classificatory techniques can be used. The agglomerative hierarchical methods are best adapted to enzymatic data analysis (54). To perform this, several algorithms are available using simple linkage, group average linkage or complete linkage (34, 51). The result is a bush-like tree diagram (phenetic dendrogram or phenogram) which expresses the affinities between OTUs and OTU groups. The authenticity in terms of objective divisions (9, 54) of the branches obtained can be verified by other procedures, such as the moving center analysis or ordination techniques (multidimensional analysis).

- Phenetics, chronistics and cladistics

Numerical taxonomy leading to the development of tree diagrams fulfills three objectives:

1 — *Simple hierarchical classification, i. e.* without reference to time or to evolution = phenetic tree. The characters are given the same weight and no hypothesis is formulated as to the independence of the sub-groups identified by the dendrograms (*fig. 2 and 4*).

2 — *Calculation of divergence time* between phenons or phenon groups = chronistic tree. This method considers the genetic significance of the characters. The new distance, called genetic distance, expresses the number of replacements of internal nucleotides at each site (mutation). In every case, it presupposes enzyme neutrality and constant mutation rate. Nei, the promoter of this method, has generalized it to asexual organisms (42).

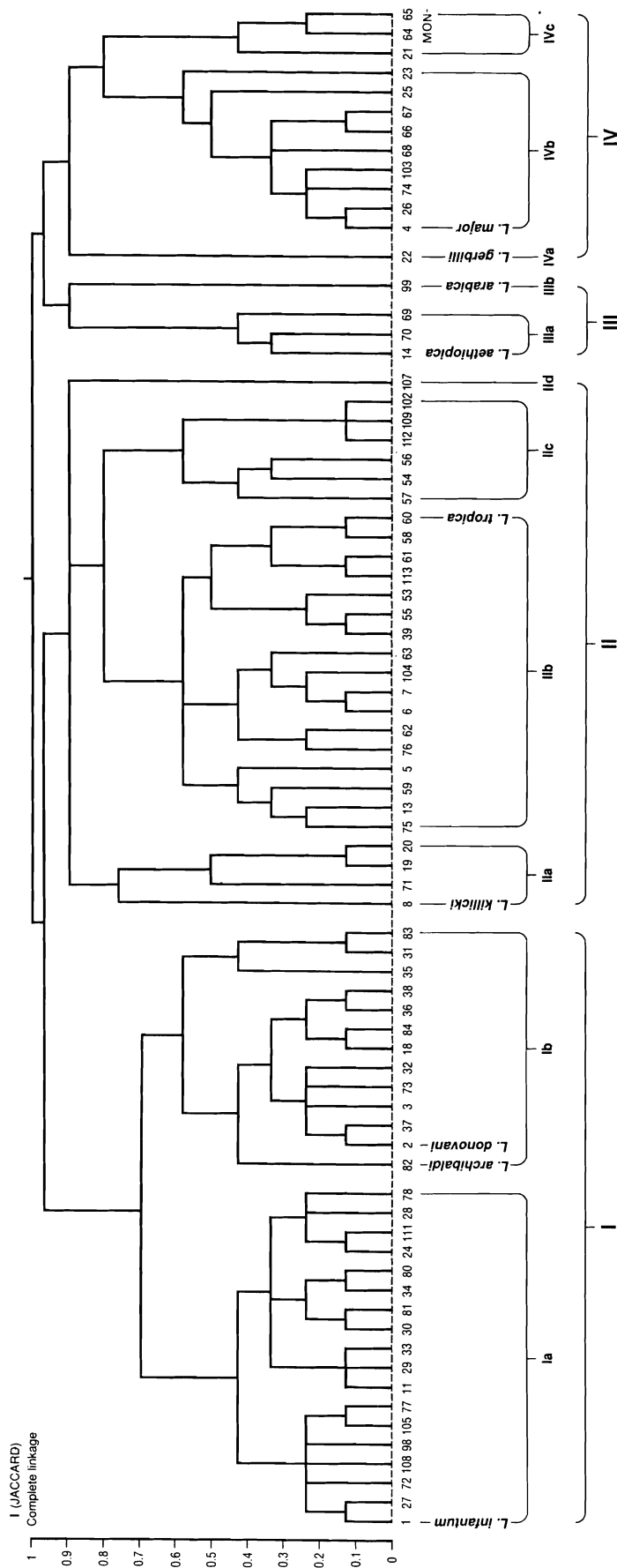


FIG. 2. — Phenogram of the Old World *Leishmania*. This dendrogram was created using 76 Operational Taxonomic Units (zymodemes) and 115 characters (electromorphs), which were in turn obtained from the enzymatic analysis of 752 strains. Several similar constructions are possible. In all cases, four main groups, *L. donovani* s. l. (I), *L. tropica* s. l. (II), *L. aethiopia* (III) and *L. major* (IV) are present. Within group I, *L. infantum* (Ia) is always separated from *L. donovani* s. l. (Ib). But *L. archibaldi* (MON-82) can either be in Ia or in Ib. Within the group II, cluster IIa formed by four zymodemes, MON-8, i. e. *L. killicki* (49), MON-71 from Yemen, and MON-19 and MON-20 from Namibia, and cluster IIb formed by 17 zymodemes, are clearly distinct. Other zymodemes originating from proximal western Asia (MON-54, MON-56, MON-57) and from Morocco (MON-102, MON-109, MON-112) have a clear tendency to regrouping (IIc), but in some phenograms they cannot be separated from sub-group IIb. Still within the *L. tropica* group, the sub-group IIId, represented by zymodeme MON-107, does not take a definite position when several phenograms are compared. Within group III, *L. arabica* (IIIb) is distinct from the closely related zymodemes of *L. aethiopia* s. l. (IIIa), whatever phenogram is considered. Within group IV, three sub-groups are stable: *L. gerbilli* (IVa), clearly separated from the two others, one sub-group enclosing the *L. major* s. s. zymodemes (IVb), and the cluster formed by MON-21, MON-64 and MON-65 (IVc), three zymodemes from USSR. Within each of the stable sub-groups, i. e. the sub-groups always formed by the same unities, the relative positions of zymodemes may nevertheless vary from one phenogram to another. This « instability » reflects the existence of an identical number of links between several zymodemes giving them the same index value (« ex-aequo »).

3 — *Phyletic analysis* emphasized since Hennig. This author's considerations made many fruitful contacts possible between evolutionists and pheneticians. A new science was born, cladistics, which allows the elaboration of « evolutionary dendrograms » (cladograms). Cladistics thus definitely advanced the phyletic understanding of many groups (15). Concepts such as monophyletism, parsimony, succession and non-convergence of the characters are largely responsible for its spread. In the genus *Leishmania*,

this type of construction is always based on the zymodeme, *i. e.* the OTU used in phenetics (fig. 3 and 5). However, the characters here are genenzymes and not allozymes. The latter are reduced to simple « character states » (4). A presumed ancestor, the hypothetical taxonomic unit (HTU), is placed at each branching. The lowest HTU corresponds to the common ancestor of the studied set of OTUs. In *Leishmania*, as in most parasitic Protozoa, the absence of fossil remains, of a living ancestor or of a recognized

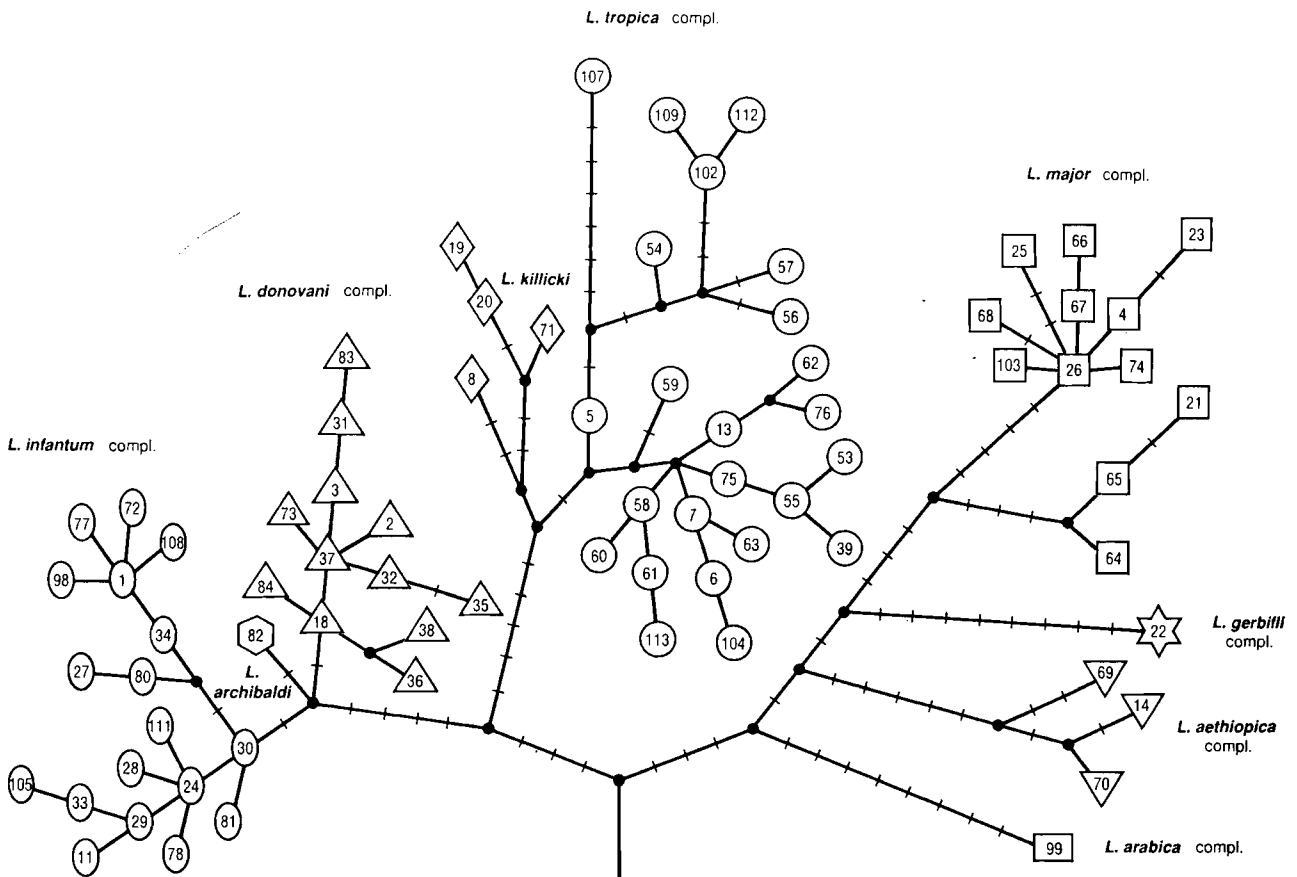


FIG. 3. — Cladogram of Old World *Leishmania*. This was constructed using 15 characters (genenzymes) and 115 states (allozymes) for 76 Operational Taxonomic Units (zymodemes). The present tree is the most parsimonious found. The groups appearing are similar to those on the phenogram. Nevertheless, as it takes into account not only the number but also the intrinsic value of the common characters states, it allows certain details to be clarified, in particular the separation, just-above the root, of two evolutionary groups: one including *L. infantum*, *L. donovani* and *L. tropica*, the other *L. arabica*, *L. aethiopica*, *L. gerbilli* and *L. major*. Moreover, the cladogram visualises the distances between the different OTUs. Hence, following groups are better individualized: 1) *L. arabica*; 2) *L. gerbilli*; 3) the USSR sub-group (MON-21, MON-64, MON-65), up to now included in the *L. major* s. s. group; 4) the cluster formed with zymodemes from western Asia and Morocco (MON-54, MON-56, MON-57, MON-102, MON-107, MON-109, MON-112), within the *L. tropica* group; 5) the *L. killicki* cluster (MON-8, MON-19, MON-20, MON-71). It should be noted that this cluster is ancestral in relation to the other zymodemes of *L. tropica* and that the Namibian zymodeme MON-19 affects Hyracoïds as well as Man. This observation argues in favor of a zoonotic origin for the *L. tropica* complex.

The cladogram brings forward other precisions. Thus, MON-107 is located near the Moroccan group (MON-102, MON-109, MON-112), originating from the same geographical area. MON-82 is distinct from the *L. infantum* and *L. donovani* « bushes », giving rise to a trifurcated structure. The structure thus formed by *L. infantum*, *L. archibaldi* (MON-82) and *L. donovani*, is directly dependent on the glutamate-oxaloacetate transaminases (GOT1 and GOT2) variation. It should be emphasized that MON-82 (*L. archibaldi*), as well as the ancestral zymodeme (MON-18) of the *L. donovani* group and the ancestral zymodeme (MON-30) of the *L. infantum* group, are originating from Sudan.

Lastly, the cladogram shows better than the phenogram the ambiguity of the relations between zymodemes, within most of the groups: numerous zymodemes as ancestors, numerous zymodemes issued from the same ancestor (« rake » or « bunch » pattern).

« sister group » makes it impossible to identify the root of the tree, *i. e.* to orientate it. It is then necessary to

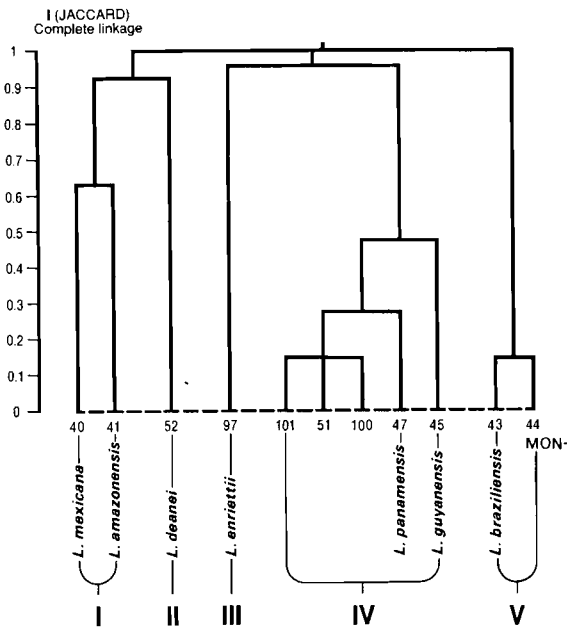


FIG. 4. — Phenogram of New World *Leishmania* created using 11 zymodemes, 13 enzymes and 65 electromorphs. Five main groups are present. Group I formed by species of the *Leishmania* sub-genus (*L. mexicana*, *L. amazonensis*) appears distant from groups IV (*L. guyanensis*, *L. panamensis*) and V (*L. braziliensis*) belonging to the *Viannia* sub-genus, themselves very distinct. The groups II (*L. deanei*) and III (*L. enriettii*), isolated from Vertebrates other than Man, are linked to other groups (respectively group I and group IV) at a very low level of affinity, which does not allow a valid confirmation of the clustering.

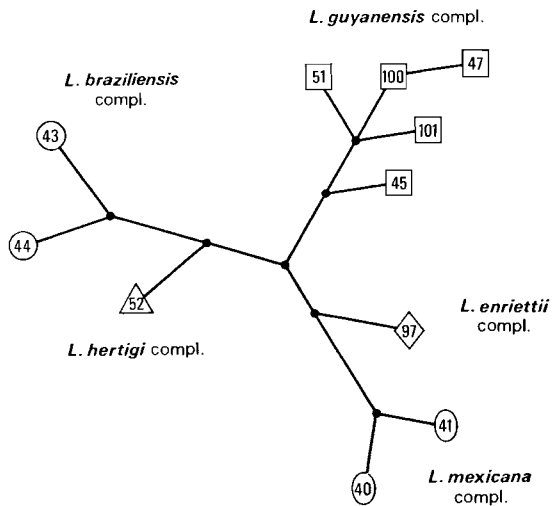


FIG. 5. — Network constructed using the same data than the phenogram of figure 4. The little number of zymodemes and the rare electromorphs in common between the different complexes (1 to 3/13) do not allow a great precision regarding their relative positions. Nevertheless, a unique shortest network can be constructed showing three main branches.

first construct a network whose center holds the common ancestor or a nearby unit (*fig. 5*). The construction of the definitive cladogram is performed by optimization. The most parsimonious tree, issued from the most parsimonious network, *i. e.* the construction containing the least number of reversions, is the one selected (56, 60). Its congruence with other classification systems, *e. g.* Linnean systems, further supports its usefulness. The advantage of the cladistics is to maintain character states until the end of the construction and thus to test their phylogenetic value. It is recommended to superimpose the character states on to the OTUs and the HTUs so as to test the compatibility of the different enzymatic systems between each other (56); also, it is easier to recognize the plesiomorph, synapomorph or autapomorph states. Finally, the method is closer than any other to « natural » taxonomy. The correct interpretation of cladograms cannot in fact be made without an in-depth consideration of the group origin and the phylogenetic meaning of its branches. We can thus speak of true evolutionary systematics (31).

- Cladistics and mode of evolution:
clonality or/and sexuality

As mentioned above, the use of zymodemes as independent OTUs presumes the absence, or at least the low frequency of genetic exchange. Whether under haploid or diploid conditions, the rare heterozygotes observed can be considered to be « fixed hybrids » similar to those observed in some multicellular organisms with agamous reproduction (42). In other words, the hypothesis of a clonal evolution constitutes the basis of the numerical classifications used here.

Actually however, the existence of sexual processes in *Leishmania* now appears plausible. A number of arguments supporting this hypothesis have accumulated in recent years. Thus, in the same geographical area, electromorphs have been found that might reflect a sexuality: for example, in Israel, strains with PGD 110/118, others with PGD 110 and others with PGD 118 (33); in Spain (Catalonia), strains with either NP1 100/140 or NP1 100 or NP1 140 have been isolated from the same small focus (45). Moreover, among these sympatric strains, pulsed field gel electrophoresis has allowed the repeated finding of particular size of chromosomes whose presence might be explained by a segregation process between daughter cells (44). Likewise, a putative natural hybrid of *L. major* and *L. arabica* has recently been reported (10). Finally, a cytogamy phenomenon has been observed in *L. infantum* and *L. tropica* (28).

Nevertheless, even if the existence of genetic exchange is confirmed in *Leishmania* as in the closely related species *Trypanosoma brucei* (58), it is unlikely that this will greatly affect the cladogram. On the one hand, whether this genetic exchange involves a classical Mendelian sexuality or a conjugation-type parasexuality, it is not certain that, they

play the same role in evolution in these primitive flagellates as in upper Eukaryotes. On the other hand, the only branches of the cladogram which might be affected are the terminal ones, where « rake » or « network » effects are most often observed. The main branches, characteristic of the complexes, remain remarkably stable in most of the phenetic or cladistic trees. In the hypothesis of a sexual evolution, this stability could be explained by the absence or the rarity of exchanges at this level, due to the ancient nature of species divergence and to the presence of ecological or geographical barriers.

In any case, until further clarification of these points, we have, in our constructions, considered the rare heterozygous structures as autonomous electromorphs.

III. SUGGESTIONS FOR A NEW CLASSIFICATION OF THE GENUS *LEISHMANIA*

1 — MONOPHYLETISM OR POLYPHYLETISM OF THE GENUS *Leishmania*?

Even if the reptile parasites (genus *Sauroleishmania*) are excluded, the use of enzymes in the taxonomy of *Leishmania* does not allow confirmation or rejection of the monophyletic origin of the group: for the moment, the sub-genera *Viannia* and *Leishmania* appear to have few or no isoenzymatic similarities. Moreover, the neotropical complex, *L. mexicana* (s. g. *Leishmania*) seems only distantly related to its Old World homologs. At present, the collected data does not allow to question the systematic divisions proposed by Lainson and Shaw (25). Having essentially studied the Old World zymodemes (fig. 2 and 3) and very little the New World (fig. 4 and 5), we have not yet attempted to construct a phyletic tree including zymodemes of both Worlds.

2 — RETURN TO BINOMINAL NOMENCLATURE AND THE NOTION OF PHENETIC COMPLEXES

Since the 1984 International Symposium on *Leishmania* (46), the scientific community appears to agree to rehabilitate the binominal nomenclature (16). This position appears to be well adapted to the taxonomical situation of *Leishmania*: in the absence of evidence for a classical Mendelian system in the genus, it avoids any formalisation in that sense, notably by doing away with the notion of sub-species. Moreover, the category « species » can be used without giving to this word the value of mixiological biological species. It should be noted that many animal or plant species known as sexual are in the same situation, due to the lack of sufficient genetic analysis and detailed crossing experiments.

The term « leishmanial complex », introduced by Lainson and Shaw (23), in the Linnean sense of groups of species

or sub-species, is reused here in its Sokalian phenetic sense, that of group of OTUs. According to our definition, the phenetic complexes are made of closely related zymodemes, i. e. zymodemes both sufficiently linked together and sufficiently separated from the other clusters to form « true » groups. The technique of moving centres (strong forms) and the correspondence analysis make it possible to confirm this relationship. At this stage, cladistics adds its contribution: the complexes then appear as autonomous phyla due to strong synapomorphies.

Once these precisions have been added, the reintegration of the old binominal classification can be made simply through the heads of new complexes. The corresponding reference strains (50) act as enzymatic markers. For example, *L. infantum* Nicolle, 1908, zymodeme MON-1, strain from Tunis (MHOM/TN/80/IPT1) becomes the marker for the *L. infantum* complex.

As we have emphasized, in the genus *Leishmania* the monothetic Linnean method is not recommended to individualize and *a fortiori* to describe a taxon whose level is less than that of the sub-genus. The fragility of extrinsic characters should be stressed, whether they are clinical, epidemiological or biogeographic. Thus, in the Mediterranean basin, the *L. infantum* complex groups dermatotropic and viscerotropic zymodemes. Moreover, one of them, MON-1, has been found to be responsible for cutaneous and visceral forms (48). Likewise, the Latin American taxon *L. chagasi* Cunha and Chagas, 1937 cannot be distinguished from *L. infantum* MON-1 on the basis of its isoenzyme profile (41).

As recommended at the 1984 Symposium (46), numerical taxonomy demands a high degree of rigidity, notably:

« 5.3.3. At the time of first publication, the diagnosis must be accompanied by designation of the geographical locality of isolation (« type locality ») and the original host (« type host ») and by designation of the deposition in a recognized bank of one, and only one reference strain.

5.3.4. It is a wise precaution to send this strain or, if possible, a clone of this strain, to all the cryobanks likely to be able to protect and preserve it. Homologous strains from the same locality and the same host, if they exist, should also be deposited.

5.4. In the case of an old taxon for which no live material exists, it is recommended that a new strain be officially designated. This strain should correspond to the geographical, epidemiological and clinical characteristics of the original description ».

In addition, special attention should be paid to priority rules. For example, enzymatic analyses have shown that *L. guyanensis* Floch, 1954 and *L. panamensis* Lainson and Shaw, 1972 zymodemes are phenetically related and, on the contrary, are both different from *L. braziliensis* Vianna, 1911 (40). If a new phenetic complex is proposed to regroup, *L. guyanensis* and *L. panamensis*, it would be

necessary to use *L. guyanensis* as head of the new complex. In any other case, the « inventor » is a free man and systematics is an open science (46).

3 — THE SANDFLY-*Leishmania* « COUPLE ».

CONGRUENCE OF BOTH SYSTEMATICS

For the past several years, biochemical techniques for *Leishmania* identification have greatly advanced the screening of « true vectors ». In the Old World, enzymatic typing of strains isolated from natural sanfly populations has led to some suspected vectors being shown not to be true vectors. But without a doubt the most important aspect of this research is the proof of a high correlation between leishmanial complexes and the sub-genera of the genus *Phlebotomus*. Thus, all the zymodemes of *L. major* isolated until now were so from the sub-genus *Phlebotomus* (*P. duboscqui*, *P. papatasi*). The same observation can be made for *L. infantum*, isolated from the sub-genus *Larroussius* * (*P. ariasi*, *P. perfiliewi*, *P. perniciosus*) and for *L. tropica* always isolated from *Paraphlebotomus* (*P. sergenti* s. l.). In phyletic terms, this observation leads to the conclusion that the parasite's evolution has generally followed a parallel pathway to that of the vector (co-evolution) (18). Thus, this sanfly-*Leishmania* correlation strengthens the value of the dendrogram made from units obtained from intrinsic characters, *i. e.* the zymodeme.

4 — MIXED LINNEAN-ADANSONIAN CLASSIFICATION

Bringing together the dual Linnean-Adansonian approaches suggested throughout this paper, we propose a new classification.

The classification remains Linnean for the higher levels in the taxonomic hierarchy, from kingdom to sub-genus. The amended genus *Leishmania* Ross, 1903 no longer includes the sub-genus *Sauroleishmania*, raised to the genus level (19). It is sub-divided, however, into two sub-genera, *Leishmania* Ross, 1903 and *Viannia* Lainson and Shaw, 1987.

The classification becomes Adansonian in so far as binomials, today revalidated, are used only to « mark » complexes which now proceed from numerical analysis. Sub-specific trinomials are naturally eliminated. Finally, the concept of complex is not used in the Linnean sense of species or sub-species groups, but in the Sokalian sense

* An exception concerns *L. infantum* MON-1 transmitted in Latin America by *Lutzomyia longipalpis* under the binomial *L. chagasi*. In the hypothesis of a transoceanic migration of *L. infantum*, this would be an example of « capture » phenomenon.

of sub-groups of OTUs, here zymodemes. Any complex must bear the name of its « marker » binomial when one exists. If not, the oldest zymodeme is used. It is thus raised to the level of a « species » and described as a new binomial.

*
* *

In conclusion, we want to briefly recall the spirit of the proposed classification:

The new taxonomy uses enzymatic criteria and is based on numerical techniques for taxonomic units up to but excluding the sub-genus. The characters (or the character states) are represented by electromorphs, *i. e.* isoenzymes. They are considered simultaneously at every partition level. The point of departure is the zymodeme (OTU), which is made up of a group of strains with the same enzymatic profile. From this point on, the binomial loses its biological significance and is only applicable to a particular zymodeme, a simple elementary unit whose essential role is to act as a marker for a complex. In the phenetic approach, the aggregation of zymodemes and zymodeme clusters up to the complex is performed using a so-called agglomerative hierarchical non-overlapping clustering technique.

The phylogenetic reconstruction (4) is performed using the cladistic method. Here the isoenzymes are character states of the character represented by the enzyme. In practice, this system uses the same OTUs and demands the prior elaboration of networks (60). Wherever possible, the cladistic technique should be used. It takes into account the phylogenetic significance of character states and units better than any other method. In particular, it makes it possible to think in terms of evolution, *i. e.* in terms of genesis and separation of different taxonomic levels.

Above the complex, the traditional categories are once again used: (i) that of sub-genus to designate parasites having a suprapyloric (*Leishmania*) or peripyloric (*Viannia*) development in the vector; (ii) that of genus (*Leishmania*) to designate the Trypanosomatidae of mammals having a dimorphous cycle: amastigote in reservoir, promastigote in vector.

A REVISED CLASSIFICATION OF THE WORLD *Leishmania*

Sub-Kingdom Protozoa Goldfuss, 1817.

Phylum Sarcomastigophora Honigberg and Balamuth, 1963.

Sub-Phylum Mastigophora Diesing, 1886.

Class Zoomastigophores Calkins, 1909.

Order Kinetoplastida Honigberg, 1963.

Sub-Order Trypanosomatina Kent, 1880.

Family Trypanosomatidae Döflein, 1901.

Genus *Leishmania* Ross, 1903.

A. Sub-genus *Leishmania* Ross, 1903.

1. *L. donovani* phenetic Complex¹ (13 zymodemes MON-).
 - . *L. donovani* (Laveran and Mesnil, 1903)².
 - . *Locus classicus* India.
 - . *L. archibaldi* Castellani and Chalmers, 1919. *Loc. class.* Sudan.
2. *L. infantum* phenetic Complex (18 zymodemes MON-).
 - . *L. infantum* Nicolle, 1908. *Loc. class.* Tunisia (syn. *L. chagasi* Cunha and Chagas, 1937).
3. *L. tropica* phenetic Complex (28 zymodemes MON-).
 - . *L. tropica* (Wright, 1903). *Loc. class.* USSR.
 - . *L. killicki* Rioux, Lanotte and Pratlong, 1986. *Loc. class.* Tunisia.
4. *L. major* phenetic Complex (12 zymodemes MON-).
 - . *L. major* Yakimoff and Schokhor, 1914. *Loc. class.* Turkestan, USSR.
5. *L. gerbilli* phenetic Complex (1 zymodeme MON-).
 - . *L. gerbilli* Wang, Qu and Guan, 1973. *Loc. class.* China.
6. *L. arabica* phenetic Complex (1 zymodeme MON-).
 - . *L. arabica* Peters, Elbihari and Evans, 1986. *Loc. class.* Saudi Arabia.
7. *L. aethiopica* phenetic Complex (3 zymodemes MON-).
 - . *L. aethiopica* Bray, Ashford and Bray, 1973. *Loc. class.* Ethiopia.
8. *L. mexicana* phenetic Complex³ (3 zymodemes MON-).
 - . *L. mexicana* Biagi, 1953. *Loc. class.* Mexico.
 - . *L. amazonensis* Lainson and Shaw, 1972. *Loc. class.* Brazil. (syn. *L. garnhami* Scorza *et al.*, 1979).
 - . *L. venezuelensis* Bonfante-Garrido, 1980. *Loc. class.* Venezuela.
9. *L. enriettii* phenetic Complex (1 zymodeme MON-).
 - . *L. enriettii* Muniz and Medina, 1948. *Loc. class.* Brazil.
10. *L. hertigi* phenetic Complex (2 zymodemes MON-).
 - . *L. hertigi* Herrer, 1971. *Loc. class.* Panama.
 - . *L. deanei* Lainson and Shaw, 1977. *Loc. class.* Brazil.

B. Sub-genus *Viannia* Lainson and Shaw, 1987⁴.

11. *L. braziliensis* phenetic Complex (2 zymodemes MON-).
 - . *L. braziliensis* Viannia, 1911⁵. *Loc. class.* Brazil.
 - . *L. peruviana* Velez, 1913. *Loc. class.* Peru.
12. *L. guyanensis* phenetic Complex (5 zymodemes MON-).
 - . *L. guyanensis* Floch, 1954. *Loc. class.* French Guyana.
 - . *L. panamensis* Lainson and Shaw, 1972. *Loc. class.* Panama.

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1. As said in the text, the term complex is used here in the sense of a subset of OTUs. The *L. gerbilli*, *L. arabica* and *L. enriettii* phenetic complexes formed by only one unit are considered as subsets of the type single. Although fundamental differences exist between the notion of complex as used here and that established by Lainson and Shaw, 1972, a congruence of the two classifications should be noted.

2. First species described in the complex. *L. donovani* is also the type species of nominal genus *Leishmania* and of nominal sub-genus *Leishmania*.

3. According to Lainson and Shaw, 1979, *L. pifanoi* Medina and Romero, 1959 and *L. aristediti* Lainson and Shaw, 1979, should be put in the *L. mexicana* complex. We did not have the opportunity to study them enzymatically.

4. Three new species have recently been described in this sub-genus, but we did not have the opportunity to study them enzymatically. Therefore, they are not (yet) included in this classification. They are: *L. lainsoni* Silveira, Shaw, Braga and Ishikawa, 1987. *Loc. class.* Brazil (55), *L. naiffi*, Lainson and Shaw, 1989. *Loc. class.* Brazil (26) and *L. shawi* Lainson, Braga, de Souza, Povoá, Ishikawa and Silveira, 1989. *Loc. class.* Brazil (22).

5. Type species of nominal sub-genus *Viannia*.

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