Ann. Soc. belge Méd. trop.
1993, **73**, 101-118

KARYOTYPE POLYMORPHISM AND CONSERVED CHARACTERS IN THE *LEISHMANIA (VIANNIA) BRAZILIENSIS* COMPLEX EXPLORED WITH CHROMOSOME-DERIVED PROBES

by

J.C. DUJARDIN[1], N. GAJENDRAN[2], J. AREVALO[3], A. LLANOS-CUENTAS[3], H. GUERRA[3], J. GOMEZ[3], J. ARROYO[3], S. DE DONCKER[1], D. JACQUET[1], R. HAMERS[2] & D. LE RAY[1]

[1]*Laboratory of Protozoology, Institute of Tropical Medicine «Prince Leopold», Nationalestraat 155, B-2000 Antwerpen 1, Belgium*
[2]*Institute for Molecular Biology, Vrije Universiteit van Brussel, Paardenstraat 65, B-1640 St. Genesius Rode, Belgium*
[3]*Institute of Tropical Medicine «Alexander von Humboldt», Universidad Peruana Cayetano Heredia, AP 5045 Lima 100, Peru*

Summary. — Molecular karyotype of 45 reference populations of Neotropical leishmanias was analyzed with ethidium bromide staining and with 6 chromosome-derived probes selected from a genomic library of *Leishmania* (*Viannia*) *braziliensis*. Size-conserved patterns were identified and found to be specific to subgenus *Viannia* and to its constitutive species. An important issue for epidemiology and clinical investigations was the discrimination between *L.* (*V.*) *peruviana* and *L.* (*V.*) *braziliensis*, 2 species found very similar by other genetic techniques, but responsible for totally different clinical patterns. The suggested existence of genetically distinct demes, or karyodemes, within the group-species might also show to be of importance, as these populations might differ in virulence, host-specificity and clinical manifestations.

KEYWORDS: *Leishmania*; New World; Karyotype; DNA Probe; Polymorphism; Conservation; Specificity

Introduction

New World tegumentary leishmaniasis is represented by a broad spectrum of clinical manifestations ranging from limited, self-healing cutaneous lesions to mutilating, oro-nasal mucosal metastases. A wide set of morphologically similar etiological agents has been identified, initially on clinical grounds, later on according to biological characters, and eventually with molecular criteria.

Accordingly, neotropical leishmanias are classified as species into two complexes, *mexicana* (belonging to subgenus *Leishmania*) and *braziliensis* (erected as subgenus *Viannia*) (16). The most severe clinical manifestations are associated with the subgenus *Viannia*, generally with the species *Leishmania* (*Viannia*) *braziliensis*.

However, clinical risks cannot be assessed by species identification of the parasite only. A variety of lesions results from apparently the same parasite. A major question still pending concerns the respective roles of host and parasite polymorphisms in accounting for the protean nature of clinical manifestations. Furthermore clinical variability may depend also on geographical location, host status, vector challenge, etc. Therefore accurate

Abbreviations: OFAGE, Orthogonal Field Alternated Gel Electrophoresis; SDS, sodium dodecyl sulfate; SSC, sodium chloride, sodium citrate; TBE, Tris, boric acid, EDTA; TE, Tris, EDTA.

identification of parasite populations at both taxonomic and infra-taxonomic levels is becoming necessary for clinical and epidemiological studies.

Various molecular approaches have been successfully developed for characterizing leishmanias: isoenzyme analysis (15), immunolabeling with monoclonal antibodies (22), and DNA analysis by restriction, hybridization, amplification, sequencing, etc. (2, 19, 29, 30). Such approaches seize differences resulting mainly from point mutations.

More recently, molecular karyotyping by pulsed-field gradient gel electrophoresis (8) has given access to another level of information. Karyotype variability has been shown to result mainly from chromosomal rearrangement, an event taking place at a rate distinct from the rate of point mutations (32). In genus *Leishmania*, molecular karyotype displays extensive plasticity as well as conserved characters (18). The latter are of two kinds: chromosomal bands sized on ethidium bromide-stained OFAGE gel (6, 10, 12, 23) and, chromosomes sized following hybridization with probes for house-keeping genes (9-12). In a preliminary karyotype study of New World leishmanias (10), we showed on a limited number of strains that both characters did allow to discriminate the complexes *braziliensis* and *mexicana*. Herein we confirmed on a large series of parasites (45 stocks) the presence of conserved, ethidium bromide-stained chromosomal bands specific to the *braziliensis* and *mexicana* complexes respectively.

The present study was aimed at further evaluation of the discriminatory potential of molecular karyotyping at species and intra-species ranks. We searched for chromosome sequences showing polymorphic hybridization patterns within the *braziliensis* complex. Six chromosome-derived recombinant DNA probes were prepared and hybridized with OFAGE karyotypes from 37 reference strains and stocks representative of constitutive and putative (*L. peruviana*) species in subgenus *Viannia*. Consistent karyotype grouping was evidenced with several probes at various populational levels. Correlation between karyotype groups and existing taxa was analyzed and candidate markers were identified accordingly.

Materials and methods

All reagents were purchased from Merck unless specified.

Parasites and samples. Forty-five leishmanial populations (table 1) were analyzed: 4 reference strains of the *mexicana* complex, 11 reference strains and 30 stocks of the *braziliensis* complex from various areas of South America. Most parasites were previously typed isoenzymatically by the donor laboratories. Clone populations were prepared from 16 strains and stocks. Cloning of reference strains was achieved by the microdrop method (31). Peruvian isolates were cloned through six platings on 1% agar plates containing 15% rabbit blood, with alternate amplification in biphasic blood-agar medium. Promastigotes were amplified in blood agar (27) or in GLSH (17), harvested at early log phase by day 2, and processed for OFAGE according to Van der Ploeg *et al.* (28). For DNA electrophoresis, each slot was loaded with about 22×10^6 organisms.

TABLE 1

Designation and origin of the 45 neotropical *Leishmania* reference strains and stocks studied herein. Donor and identification (ID, enzyme analysis) laboratories are quoted. Sixteen parasite populations (*) were cloned for the present study. Codes are referred to in text and legends.

Designation	Origin	Lab		Code
		ID	donor	
Complex *L. (V.) braziliensis*				
L. (V.) braziliensis				
MHOM/BR/75/M 2903	Brazil, Para, Carajas	d	d	1
MHOM/BR/75/M 2904*	Brazil, Para, Carajas	c	c	2
MHOM/BR/84/LTB 300	Brazil, Bahia, Tres Bracos	d	d	3
MHOM/BO/82/LPZ 13*	Bolivia, Yungas	c	c	4
MHOM/BO/83/LPZ 155*	Bolivia, Beni	c	c	5
MHOM/BO/84/LPZ 440*	Bolivia, Alto Beni	c	c	6
MHOM/BO/84/LPZ 595*	Bolivia, Alto Beni	c	c	7
MHOM/BO/84/LPZ 662*	Bolivia, Alto Beni	c	c	8
MHOM/BO/84/LPZ 704*	Bolivia, Alto Beni	c	c	9
MHOM/BO/84/CEN 002	Bolivia, Santa Cruz, Santa Fe	c	f	10
MHOM/BO/85/CEN 003	Bolivia, Santa Cruz, Punta Rieles	c	f	11
MHOM/BO/85/CEN 004	Bolivia, Santa Cruz, Punta Rieles	c	f	12
MHOM/BO/85/CEN 005	Bolivia, Santa Cruz, Marabol	c	f	13
MHOM/BO/85/CEN 006	Bolivia, Santa Cruz, Punta Rieles	c	f	14
MHOM/BO/85/CEN 007	Bolivia, Santa Cruz, Guarayos	c	f	15
MHOM/PE/84/LC 01*	Peru, Madre de Dios, Iberia	c	a	16
MHOM/PE/84/LC 03*	Peru, Madre de Dios, Mazuco	c	a	17
MHOM/PE/84/LC 53*	Peru, Madre de Dios, Rio Colorado	c	a	18
L. (V.) panamensis				
MHOM/PA/71/LS 94	Panama	d	d	19
MHOM/PA/67/M 4037	Panama	d	d	20
MCHO/PA/00/M 4039*	Panama	d	e	21
L. (V.) guyanensis				
MHOM/BR/82/M 1670	Brazil, Para, Monte Alegre	d	b	22
MHOM/BR/69/M 1142	Brazil, Para	d	d	23
MHOM/BR/78/M 5378*	Brazil, Para, Monte Dorado	e	e	24
MHOM/BR/75/M 4147	Brazil, Para, Monte Dorado	d	d	25
MHOM/SR/87/WAR	Surinam, Paramaribo	d	h	26
MHOM/SR/87/KLEI	Surinam, Paramaribo	d	h	27
MHOM/GF/85/LEM 669	French Guyana	i	i	28
MHOM/GF/84/CAYH 166	French Guyana	g	g	29
MCHO/GF/83/CAYA 116	French Guyana	g	g	30
MHOM/GF/85/CAYH 197	French Guyana	g	g	31
MHOM/GF/87/CAYH 293	French Guyana	—	g	32
MHOM/GF/82/CAYH 60	French Guyana	g	g	33
MCHO/BR/80/M6200	Brazil, Para	d	d	34
L. (V.) peruviana				
MHOM/PE/76/SL 2	Peru, Lima, Santa Eulalia	a	b	35
MHOM/PE/76/SL 3	Peru, Lima, Santa Eulalia	a	b	36
MHOM/PE/76/SL 5	Peru, Lima, Santa Eulalia	a	b	37
MCAN/PE/76/D 8	Peru, Lima, Santa Eulalia	a	b	38
MHOM/PE/84/LH 78*	Peru, Ancash, Caraz	a	a	39
MHOM/PE/00/LC 106*	Peru, Lima, Santa Eulalia	a	a	40
MHOM/PE/84/LC 26*	Peru, Ancash, Huayllacayan	a	a	41
Complex *L. (L.) mexicana*				
L. (L.) amazonensis				
MPRO/BR/77/M 1845 (LV 78)*	Brazil, Para	d	b	42
L. (L.) mexicana				
MNYC/BZ/62/M379 (ITMAP 2167)	Belize	d	d	43
L. (L.) aristedesi				
MORY/PA/68/GML3	Panama,Sasardi Darien	—	e	44
L. (L.) garnhami				
MHOM/VE/76/JAP78	Venezuela	—	e	45

(a) Pr J. Arevalo, Laboratory for Trypanosomatidae, Institute of Tropical Medicine «Alexander von Humboldt», Lima, Peru.
(b) Dr M. Chance, Department of Parasitology, Liverpool School of Tropical Medicine, Liverpool, U.K.
(c) Dr Ph. Desjeux, OMS/CTD, Genève, Switzerland.
(d) Dr D. Evans, Department of Medical Protozoology, London School of Hygiene and Tropical Medicine, London, U.K.
(e) Dr J.J. Shaw, Instituto Evandro Chagas, Belem, Brasil.
(f) Dr R. Urjel, Laboratory of Parasitology, CENETROP, Santa Cruz de la Sierra, Bolivia.
(g) Pr J.P. Dedet, Laboratoire d'Ecologie Médicale, Faculté de Médecine, Montpellier, France.
(h) Dr G. Van Eys, Department of Molecular Cell Biology, Faculty of Medicine, Maastricht, Nederland.
(i) Pr J.A. Rioux, Laboratoire d'Ecologie Médicale, Faculté de Médecine, Montpellier, France.

OFAGE. The electrophoresis equipment used in this study was described elsewhere (10). Runs were performed in a $10 \times 12 \times 0.5$ cm agarose gel (from 1 to 1.5 % agarose, BRL) in $0.5 \times$ TBE ($1 \times$ TBE = 0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA; Tris and EDTA were purchased from Janssen Chimica), at 14-18°C, 15 V cm^{-1}, for 24 hours. Resolution of the whole karyotype was achieved with 3 pulses of 45, 65 and 115 s respectively. One reference stock (*L. (V.) braziliensis* M2903 or M2904) was run with each gel in order to calibrate the relative position of chromosomal bands. *Saccharomyces cerevisiae* DNA from strain YPH80 (Biolabs) was used to size DNA molecules from 225 kb to 1,640 kb.

Chromosomal probes. Individual candidate chromosomal bands from *L. braziliensis* M2904 were cut out from preparative gels on a UV transilluminator (TM-40, UVP Inc.), placed in a dialysis bag containing $0.5 \times$ TBE and electro-eluted overnight in conditions similar to the OFAGE's ones. Then, DNA was concentrated with 2-butanol, phenol-extracted, ethanol-precipitated, vacuum-dried and resuspended in 20 μl of TE (10 mM Tris.Cl, pH 7.4 in 1 mM EDTA pH 8.0). From 6×10^8 organisms, 20 μl of DNA was usually recovered and labelled with 32P-dCTP by nick-translation (21).

Southern blotting and hybridization. OFAGE gels were transferred onto Nylon filters (Hybond N, Amersham) and hybridized according to the manufacturer's instructions. Post-hybridization washings were performed as follows: $6 \times$ SSC at room temperature for 15 min, $2 \times$ SSC/0.1 % SDS at 65°C for 30 min, and $0.1 \times$ SSC at 65°C for 10 min unless otherwise stated ($20 \times$ SSC = 3 M NaCl, 0.3 M sodium citrate, pH 7.0).

L. braziliensis genomic library and chromosome-derived clones. High molecular weight DNA isolated from *L. braziliensis* M2904 according to Maniatis (21) and plasmid pUC 18 were both digested with PstI, ligated with T4 DNA ligase and transformed according to kit instructions (Boehringer). Recombinant clones were transferred onto a nylon membrane and screened by hybridization with chromosomal probes. Rapid analysis of the clones by the alkaline lysis plasmid preparation method, and large scale plasmid isolation and purification by centrifugation on a CsCl gradient were performed according to Maniatis (21). Labelling was performed as above. Recombinant DNA was digested with different restriction enzymes (BRL) according to the manufacturer's specifications. Digestion products were fractionated in 0.9 % agarose gel.

Results

Conserved banding patterns

Ethidium bromide-stained karyotype of 23 parasites representative of the two neotropical complexes of *Leishmania* species was analyzed with special attention to conserved banding patterns. Analysis involved 9 reference strains representative of the 4 species of the *braziliensis* complex, 10 Peruvian stocks typed enzymatically either as *L. (V.) braziliensis* or *L. (V.) peruviana* (1), and 4 reference strains of 4 species in the *mexicana* complex.

Size of chromosomal bands (fig. 1) ranged from either 250 kb (*mexicana* complex) or 295 kb (*braziliensis* complex) to more than 1,640 kb. Extensive

variability was observed between karyotype patterns that affected both the size of chromosomal bands and the number of co-migrating chromosomes, the latter being indicated by marked differences in fluorescence intensity among chromosomal bands. Variability was visualized mostly in the region of medium-sized chromosomes (400-800 kb).

Beside this overall polymorphism, some bands showed a rather constant position throughout the karyotype patterns. Whenever their size-variation coefficient was less than 5 %, they were considered as size-conserved bands as proposed by Giannini *et al.* (12).

This conservation was best illustrated by a 1,000-kb band observed in both complexes for all the parasites analyzed herein (fig. 1, dotted band, and fig. 2).

A striking feature was the presence of a similar set of two size-conserved bands in the two complexes: at 405 and 900 kb in the *braziliensis* complex (fig. 1, hatched bands) and at 350 and 930 kb in the *mexicana* complex (fig. 1, shaded bands).

As the position of these size-conserved bands in one complex was not occupied in the other complex, such bands did qualify as candidate markers specific to the corresponding complex. With respect to the *braziliensis* complex, a further band of 295 kb (fig. 1) was confirmed to be diagnostic. The 295-kb position is occupied in some *L. (L.) mexicana* stocks but we showed previously that it does not contain the beta-tubulin genes whose presence is a constant feature in the *braziliensis* complex-specific 295-kb band (10, 11).

Within the *braziliensis* complex, one size-conserved band of 1,150 kb (fig. 1, vertical striped bands; fig. 2, arrowhead) was found to be specifically present in all but one (LC 26) stocks of *L. peruviana.* LC 26 was cloned and retyped enzymatically, and its identity was confirmed.

Polymorphic hybridization patterns

In order to evaluate further the discriminative potential of molecular karyotyping we searched in the complex *braziliensis* for polymorphic hybridization patterns with chromosomal and recombinant DNA probes.

Chromosomal probes

In a first step, three whole chromosomal bands were tested as hybridizing probes (chromosomal probes) on the karyotypes of 15 to 27 (depending on the probe tested) representatives of the *braziliensis* complex.

The 1,150-kb band specific in position to *L. peruviana* was eluted from an OFAGE karyotype of stock SL3 (band 1,150-SL3) and hybridized. Strong

---

Legend of figure 1 :

Karyotype of neotropical *Leishmania* from ethidium bromide-stained OFAGE gels. Banding patterns range from 250 kb to over 1,640 kb as estimated with a *Saccharomyces cerevisiae* scale.

Size scale is not linear due to runs at three different pulses, but relative position of each band is respected. Larger bands correspond to stronger fluorescence intensity. Position-conserved (coefficient of variation <5 %) bands are observed at 1,000 kb (▒) in all *Leishmania,* at 295, 405 and 900 kb (▨) in the *braziliensis* complex, at 350 and 930 kb (■) in the *mexicana* complex, and at 1,150 kb (▥) in *L. (V.) peruviana.*

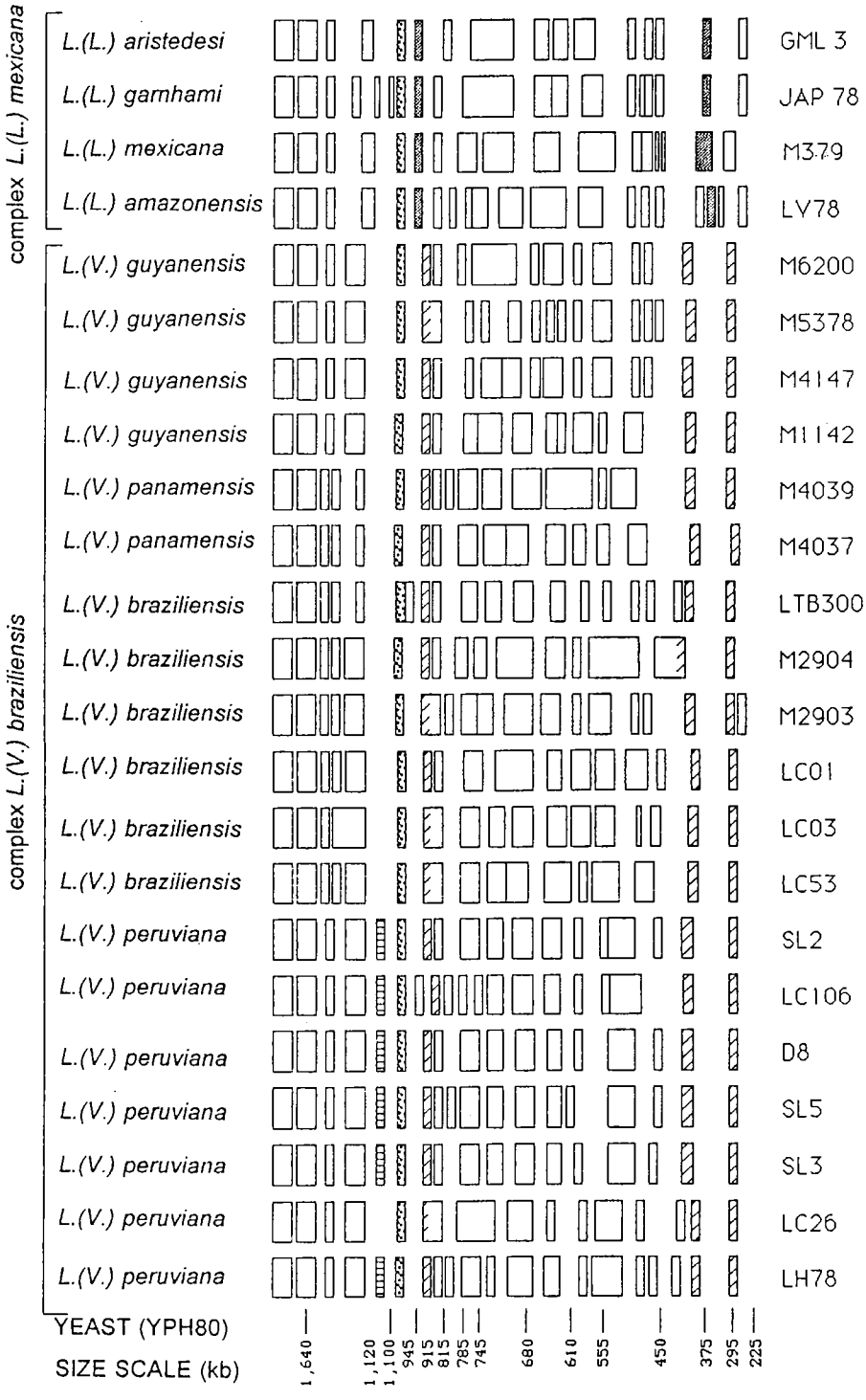
complex L.(L.) mexicana
complex L.(V.) braziliensis
L.(L.) aristedesi
L.(L.) garnhami
L.(L.) mexicana
L.(L.) amazonensis
L.(V.) guyanensis
L.(V.) guyanensis
L.(V.) guyanensis
L.(V.) guyanensis
L.(V.) panamensis
L.(V.) panamensis
L.(V.) braziliensis
L.(V.) braziliensis
L.(V.) braziliensis
L.(V.) braziliensis
L.(V.) braziliensis
L.(V.) braziliensis
L.(V.) peruviana
L.(V.) peruviana
L.(V.) peruviana
L.(V.) peruviana
L.(V.) peruviana
L.(V.) peruviana
L.(V.) peruviana
GML 3
JAP 78
M379
LV78
M6200
M5378
M4147
M1142
M4039
M4037
LTB300
M2904
M2903
LC01
LC03
LC53
SL2
LC106
D8
SL5
SL3
LC26
LH78
YEAST (YPH80)
SIZE SCALE (kb)
1,640
1,120
1,100
945
915
815
785
745
680
610
555
450
375
295
225

Fig. 1.

isologous hybridization did take place with the 1,150-kb band in all but one (LC 26) *L. peruviana* stocks, and bands of 1,300 to 1,400 kb in the other species of the *braziliensis* complex (fig. 3a) and LC26. Weaker hybridization of the 1,150 kb band of *L. peruviana* LH78 was due to a lower quantity of DNA in that band, as revealed by ethidium bromide staining (not shown).

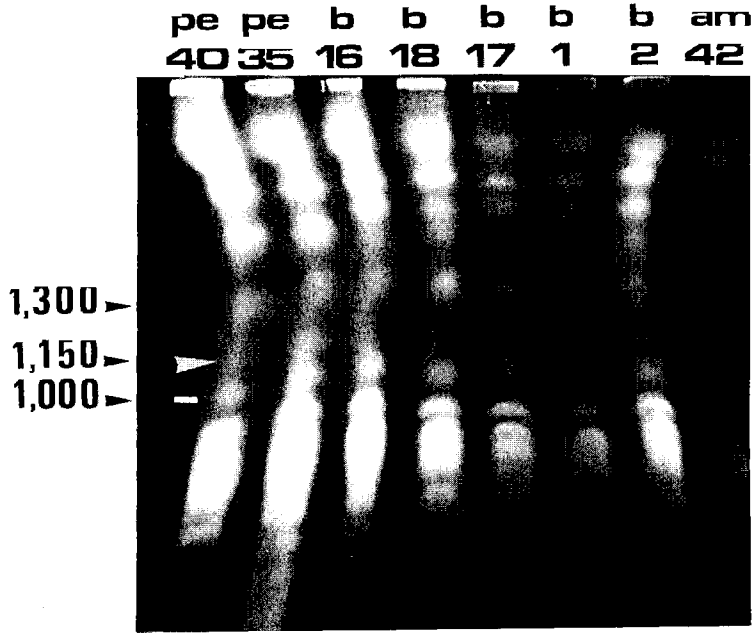

Figure 2

L. (V.) peruviana karyotype (pe, stocks 40 and 35, see table 1 for codes) is characterized by a unique size-conserved band at 1,150 kb (white arrowhead) absent in the other species exemplified here by *L. (V.) braziliensis* (b: stocks 16, 18, 17 from Peru; strains 1 and 2 from Brazil) and by *L. (L.) amazonensis* (am: strain 42). For comparison, note the 1,000-kb band (white stripe) conserved in all *Leishmania*. Pulse 115s, stain ethidium bromide, size in kb.

Two further bands were selected and eluted from the most variable region (400 to 800 kb) of the karyotype of *L. braziliensis* M2904, respectively at 430 kb and at 640 kb (bands 430-M2904 and 640-M2904). Here again karyotype hybridization was strong with the homologous band of M2904 and, it was polymorphic in the other representatives of the complex *braziliensis*. In the latter 1 to 2 bands of various size did hybridize, either in the range of 430 to 640 kb with chromosomal probe 430-M2904 (fig. 3b) or at 640 to 780 kb with probe 640-M2904 (not shown).

Recombinant probes

This second step aimed at recombinant DNA probes which would also generate polymorphic hybridization patterns. A genomic library of *L. braziliensis* M2904 was constructed and screened with the 3 chromosomal probes described above. Six recombinant DNA clones hybridizing strongly with these

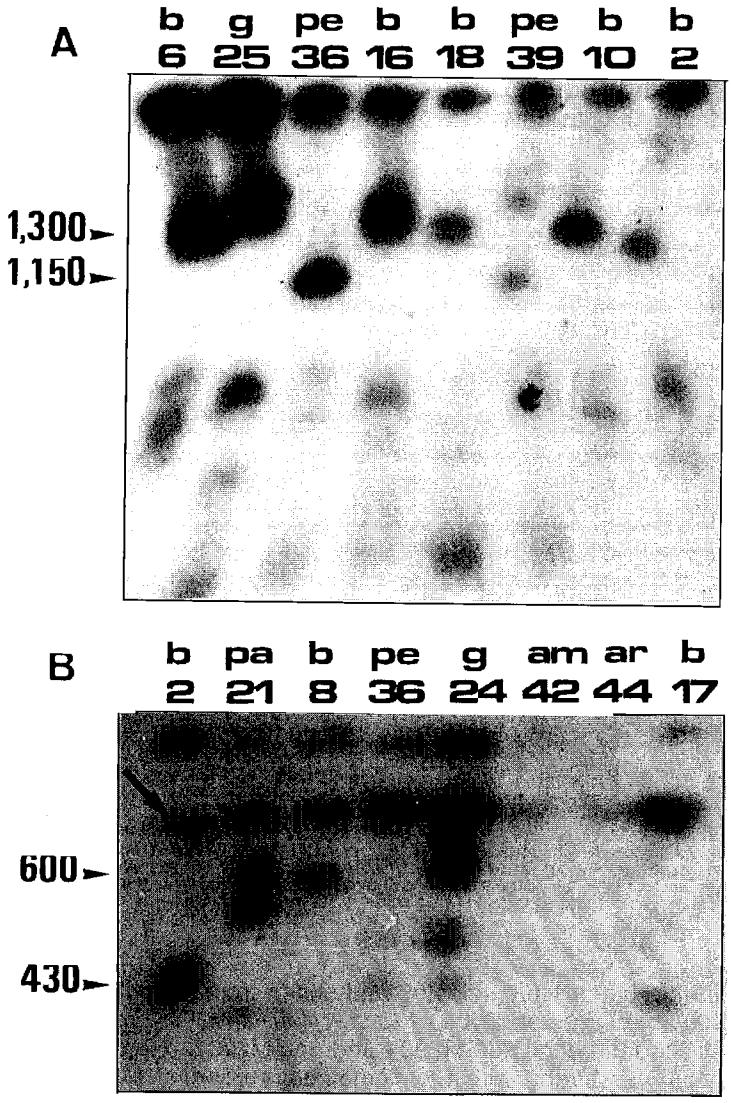

Figure 3

Polymorphic patterns in the *braziliensis* complex following hybridization with electro-eluted chromosomal bands (chromosomal probes) onto OFAGE transfers (molecular size, in kb; codes, as described in table 1):

a) probe 1,150-SL3 (*L. peruviana*): hybridization to a 1,150-kb band in *L. peruviana* (pe) SL 3 (36) and LH 78 (39), and to 1 or 2 major bands at 1,300-1,400 kb in the other species (here *L. braziliensis* (b) Bolivia = lanes 6, 10; Peru = lanes 16, 18; Brazil = lane 2; *L. guyanensis* (g) = lane 25). Pulse 110s.

b) probe 430-M2904 (*L. braziliensis*): on the homologous karyotype (2), strong hybridization to itself (430 kb) and fainter to other bands. In other karyotypes a strong hybridization is observed on bands of different sizes, *e.g.* at 600 kb in *L. braziliensis* (b) LPZ 662 (8), and at both 430 and 600 kb in *L. guyanensis* (g) M5378 (24). Hybridization in the compression zone (arrow) is constant. No significant hybridization was found in the *mexicana* complex (*L. amazonensis* (am), lane 42; *L. aristedesi* (ar), lane 44). Pulse 45s.

probes were derived: (i) pLb-149 selected with the 430-M2904 probe, (ii) pLb-13, pLb-134, pLb-168 and pLb-180 with the 640-M2904 probe, and (iii)

pLb-22 with the 1,150-SL3 *L. peruviana* probe. These chromosome-derived recombinants were then used themselves as hybridization probes (chromosome-derived recombinant DNA probes). All of them hybridized in M2904 with the band used to select them, except for pLb-134 which was found to hybridize to a 700 kb band and larger ones (fig. 4b). Selection of this recombinant clone is probably due to a slight contamination of the 640 kb chromosomal probe with material from the closely migrating 700 kb band.

Hybridization of the recombinant DNA probes on a panel of representatives of the *braziliensis* complex (figs. 4, 5) resulted in 2 types of banding patterns. One pattern was monomorphic: pLb-180 hybridized to one single 640-kb chromosome in all the populations of the *L. braziliensis* complex tested (not shown). All the other patterns were polymorphic in terms of number and/or size of chromosomes: (a) pLb-149 hybridized to 1 to 3 chromosomes ranging from 405 to 640 kb (figs. 4a, 5c); (b) pLb-134 recognized in most isolates 2 to 3 chromosomes located within a size range of 405-800 kb (figs. 4b, 5b); (c) both pLb-168 (figs. 4c, 5b) and pLb-13 (not shown) hybridized generally to one and the same chromosome at 600-700 kb; (d) pLb-22 hybridized to 1 to 2 chromosomes sizing between 1,150 and 1,900 kb (figs. 4d, 5a).

Correlation between hybridization patterns and taxa in the braziliensis complex

Thirty-seven stocks of the *braziliensis* complex were OFAGE-run and hybridized with the 4 chromosome-derived probes pLb-22, -134, -168 and -149 (fig. 5) generating karyotype polymorphism, in order to evaluate the variability of the patterns described hereabove and to check whether such patterns were associated with existing taxonomical units.

Hybridization patterns generated by pLb-149 were highly variable and most organisms had a unique pattern (fig. 5c). At the contrary, comparison of patterns obtained with the other 3 probes evidenced several size-conserved, position-specific, discriminative chromosomes (table 2). These chromosomes had a coefficient of size variation lower than 5% within a given species, their mean size position differed from species to species and, for a given species, was not occupied in the other species but for a few exceptions.

Probe pLb-22 (fig. 5a) hybridized at 1,300 - 1,390 kb with the *L. guyanensis* and *L. braziliensis* tested (plus an extra-band at 1,900 kb in

---

Legend of figure 4:

Chromosome size-polymorphism in the complex *braziliensis* as shown by hybridization with chromosome-derived DNA probes (size, in kb; codes, as in table 1; hybridization to the bulk of unresolved chromosomes, arrow):

(a) pLb-149 (pulse 45s) hybridized to 1 to 2 chromosomes in a size range of 405-640 kb, f.i. at 430 kb in *L. braziliensis* (b) M2904 (2) and at 600 and 640 kb in *L. braziliensis* M2903 (1); (g = *L. guyanensis*, pa = *L. panamensis*);

(b) pLb-134 (pulse 65s) shows an hybridization pattern specific to *L. guyanensis* (g, lanes 25 to 29) and involving 2 chromosomes at 600 and 750-760 kb while the size of the smallest hybridizing chromosome was 700 kb (star) in *L. braziliensis* (b, lane 1); note an extra 405-kb band in stocks KLEI (27) and H 166 (29);

(c) pLb-168 (pulse 65s) evidenced an hybridization pattern specific to *L. peruviana* (pe): the probe hybridized to 1 chromosome at 640 kb in *L. braziliensis* (b: lanes 2, 16, 3) and at 710 kb in *L. peruviana* (lanes 35 to 38);

(d) pLb-22 (pulse 115s) showed an hybridization pattern also specific to *L. peruviana* (pe, lanes 40 and 36), with one band at 1,150 kb as compared to the 1,300-1,390-kb band present in the other species of the complex *braziliensis* (b = *L. braziliensis*; g = *L. guyanensis*).

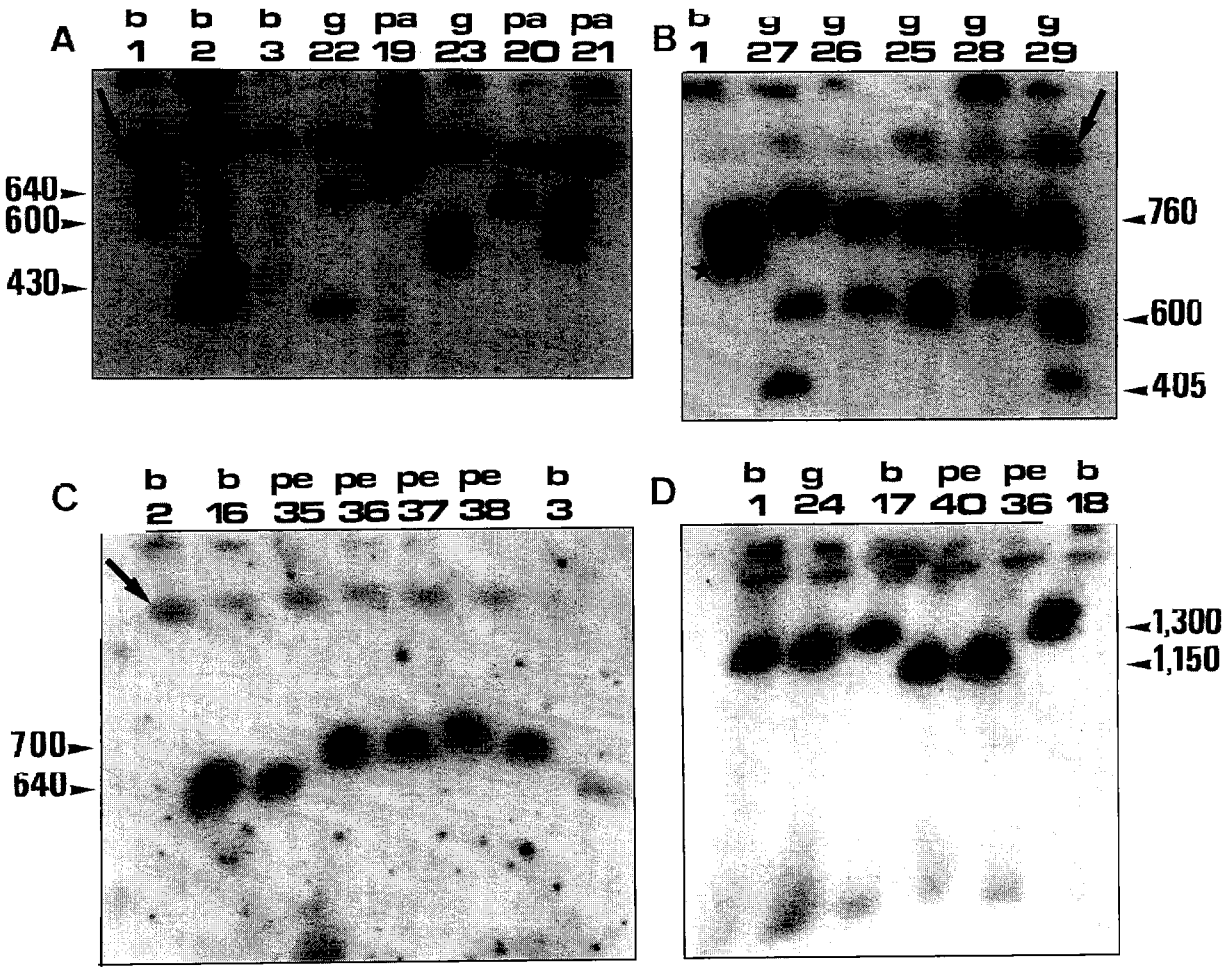
A
b b b g pa g pa pa
1 2 3 22 19 23 20 21
640
600
430
B
b g g g g g
1 27 26 25 28 29
760
600
405
C
b b pe pe pe pe b
2 16 35 36 37 38 3
700
640
D
b g b pe pe b
1 24 17 40 36 18
1,300
1,150

Fig. 4.

M2904) and specifically with a 1,150-kb chromosome in 6 out of 7 *L. peruviana.* This latter specificity was not tested further against *L. braziliensis* and *L. guyanensis* as none isolate of these 2 species did present a 1,150 kb chromosomal band.

TABLE 2

Size distribution (in kb) and average size (in brackets; bold: species-specific position) of discriminative chromosomes among 3 species of the *braziliensis* complex.

	L. b.	*L. pe.*	*L. gu.*
pLb-22	1,300-1,900	1,150-1,390 **(1,150)**	1,300-1,390
pLb-134	640-730 **(700)**	600-660 **(640)**	405-610 **(600)**
pLb-168	630-650 (640)	640-720 **(700)**	580-660 (600-640)

As probe pLb-134 (fig. 5b) hybridized with up to 3 chromosomes, the smallest one was selected for consideration. It was found to be discriminative, with an average size of 700 kb in *L. braziliensis* (16 out of 18 stocks), 680 kb in *L. panamensis* (2/2 stocks) and, specifically, 640 kb in *L. peruviana* (6/7 stocks) and 600 kb in *L. guyanensis* (10/10 stocks, fig. 4b).

With probe pLb-168 (fig. 5b, dotted line) a 700-kb chromosome characterized specifically all the *L. peruviana* (7/7) from *L. braziliensis* (640 kb in 18/18 stocks). In *L. guyanensis,* two discrete size categories of chromosomes were observed at, respectively, 600 kb (7/10 stocks) and 640 kb (7/10 stocks) on the average.

Four exceptions were noted (fig. 5). In *L. braziliensis*, stock LPZ 13 displayed the 640-kb pLb-134 chromosome found in *L. peruviana. L. braziliensis* LPZ 440 shared with *L. panamensis* the 680-kb position. In *L. peruviana,* stock LC 26 had both the 1,300-kb pLb-22 chromosome characteristic of *L. guyanensis* and *L. braziliensis*, and the 600 kb pLb-134 chromosome characteristic in *L. guyanensis*; clone population LH 78 displayed the patterns of both *peruviana* and *braziliensis* with the 3 probes pLb-22, -134 and -168.

Potential existence of intra-specific karyotypical groups was illustrated in *L. guyanensis* hybridized with pLb-168. Three patterns were observed: one single 640-kb chromosome in 3 stocks, a double banding at 640 and 600 kb in 3 other stocks, and a single 600-kb chromosome in the 4 last stocks tested (fig. 5b).

The 4 recombinant probes were tested on several stocks of the *mexicana* complex. No significant hybridization was found but for pLb-134 (data not shown) which did hybridize weakly. Accordingly the corresponding sequences displayed a homology restricted to the *braziliensis* complex.

Legend of figure 5:

Diagramme of hybridization patterns in the *braziliensis* complex with 4 *L. braziliensis* M2904 chromosome-derived DNA probes: (a) pLb-22, (b) pLb-134, pLb-168 (dotted line) and (c) pLb-149 (size, in kb; n, hybridization not done). Five size-conserved, species-specific chromosomes are indicated: 3 in *L. peruviana* (arrowhead), 1 in *L. guyanensis* (star) and 1 in *L. braziliensis* (arrow).

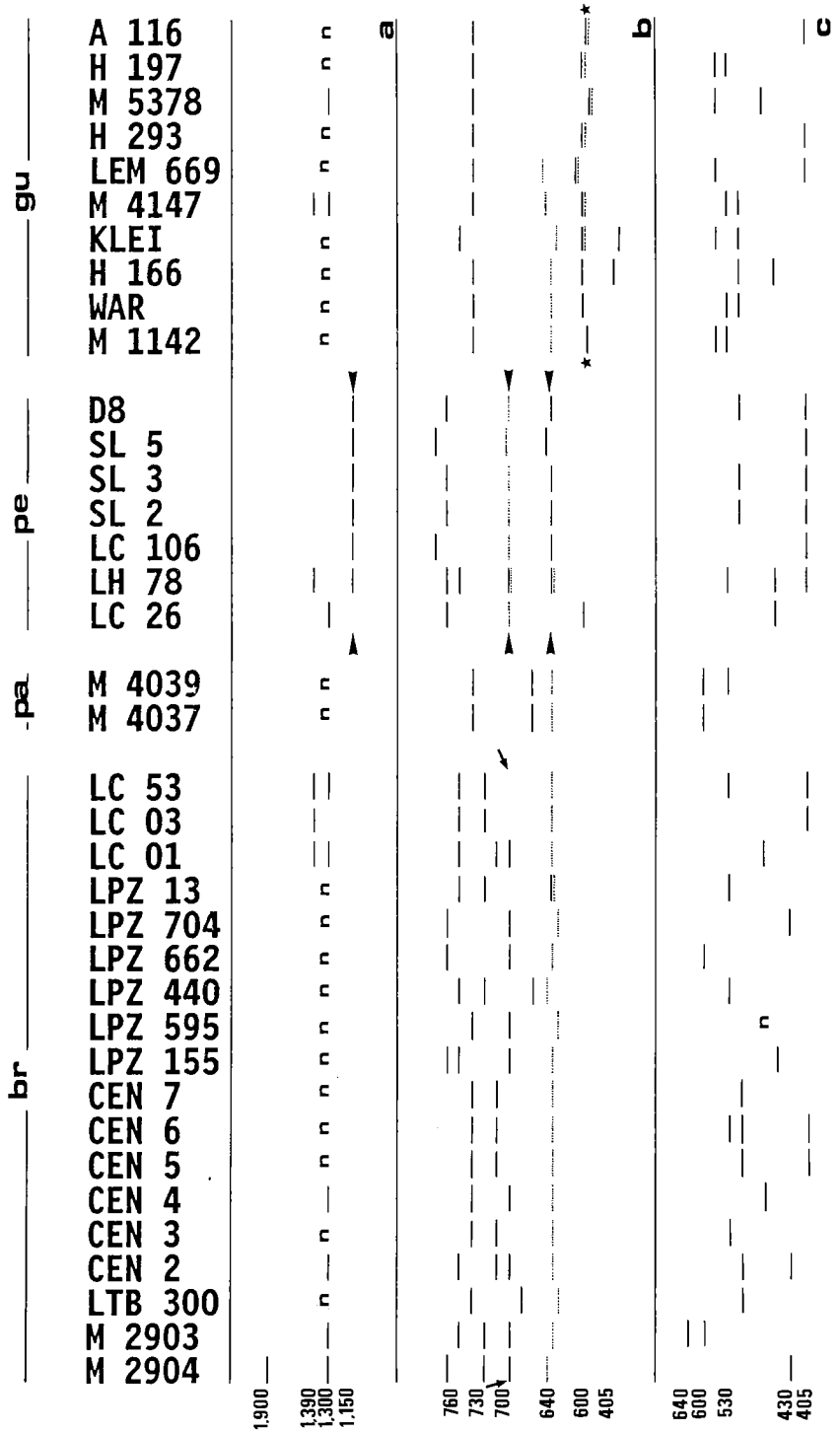
gu
A 116
H 197
M 5378
H 293
LEM 669
M 4147
KLEI
H 166
WAR
M 1142
pe
D8
SL 5
SL 3
SL 2
LC 106
LH 78
LC 26
pa
M 4039
M 4037
br
LC 53
LC 03
LC 01
LPZ 13
LPZ 704
LPZ 662
LPZ 440
LPZ 595
LPZ 155
CEN 7
CEN 6
CEN 5
CEN 4
CEN 3
CEN 2
LTB 300
M 2903
M 2904
a
b
c
1.900
1.390
1.300
1.150
760
730
700
640
600
405
640
600
530
430
405

Fig. 5.

A brief restriction map of clones pLb-22, -134, -149 and -168 is shown in fig. 6b. The Pst I restriction site used for cloning was still present within the insert in 3 recombinants (pLb-149, -134, -168). It could result from either incomplete digestion during cloning, possibly as result of the presence of methylated nucleotides within the original cloned fragment, or simultaneous cloning of 2 fragments not adjacent naturally (chimaeric clone).

The possible chimaeric nature of those clones does not appear to account for their hybridization on different chromosomes from the same parasite. Southern hybridization of Pst I digests from M2904 DNA with the 3 probes evidenced marked differences in hybridization intensity among Pst I fragments (fig. 6a). Similar differences should be observed after hybridization on different chromosomes if the chimaeric nature of the recombinant was responsible for the hybridization on different chromosomes. This is not the case as illustrated by comparison between figs. 4a, b and fig. 6a (lanes 1, 2). Further characterization (subcloning and sequencing) of these recombinants is needed to confirm that suggestion.

Discussion

In this study, molecular karyotype of 45 reference strains and stocks representative of the Neotropical *Leishmania* was analyzed, with special attention paid to the *braziliensis* complex of species or subgenus *Viannia* (16), in order to appreciate the extent of genomic polymorphism, to search for conserved characters and to analyze any correlation of the latters with taxonomical entities.

Chromosomal banding patterns resulting from pulsed field gradient gel electrophoresis were characterized in two ways, by ethidium bromide staining and by hybridization with 6 chromosome-derived recombinant probes. The latters were isolated from a genomic library of *L. braziliensis*, by screening with 3 size-polymorphic chromosomes and by selection according to strong hybridization signal suggestive of sequence repetitivity.

While genomic plasticity of *Leishmania* karyotype is now well documented (3, 5, 23, 26), concomitant existence of conserved karyotype patterns has been considered recently (6, 12, 18), but is still controversial due to differences in techniques as well as in interpretation of the magnitude of chromosome size variations (see review (18)). In the present study, two criteria are proposed for identifying conserved, specific chromosomes: (i) coefficient of size variation $< 5\%$, according to Giannini *et al.* (12, 18) and, (ii) average size position unique to the group of organisms considered.

Accordingly, conserved patterns were identified, they did cluster the populations tested into karyotype groups, and some of these groups correlated with existing taxa at both subgenus and species ranks.

Firstly, ethidium-bromide staining (fig. 1) discriminated two groups of organisms corresponding to the complexes *braziliensis* and *mexicana*: both were characterized by a unique set of size-conserved chromosomal bands positioned respectively at 295, 405 and 900 kb in the complex *braziliensis*

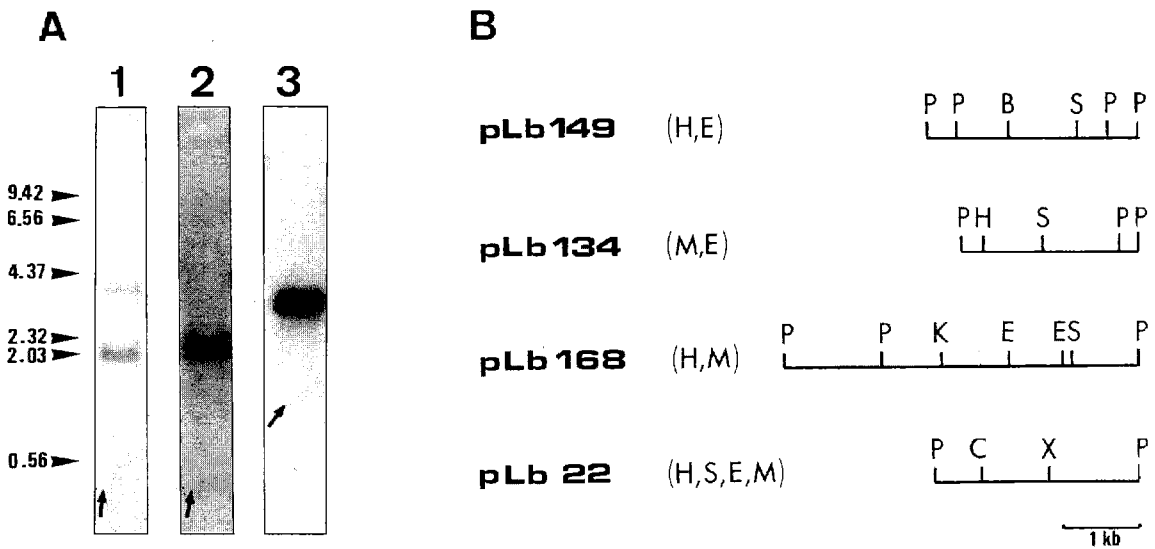

Figure 6

Characterization of chromosome-derived recombinant clones: (a) Southern analysis of Pst-I digested *L. braziliensis* M2904 genomic DNA by hybridization with chromosome-derived DNA probes (1) pLb-149, (2) pLb-134 and (3) pLb-168 (the smallest digests homologous to the small Pst I fragments of the respective recombinants were hardly visible, arrow; size in kb); (b): restriction maps of the respective inserts and that of pLb-22 (B, BamHI; C, ScaI; E, EcoRI; H, HindIII; K, KpnI; M, SmaI; P, PstI; S, SalI; X, XhoI; enzymes in bracket did not cut).

and at 350 and 930 kb in the complex *mexicana.* Our results confirm and expand previous observations of conserved banding patterns made on smaller series of New World (10) and Old World (6, 12, 18) parasites. Within the complex *braziliensis*, the putative species *L. peruviana* did segregate on the basis of a unique 1,150-kb band (see below). It has been recently shown (18) that size-conserved chromosomes also have a high degree of sequence homology. This point is further illustrated here by the *braziliensis* complex-specific 295-kb chromosome : its position is occupied in some *L.(L.) mexicana* representatives but it harbours beta-tubulin genes in the *braziliensis* complex only (10).

Secondly, recombinant DNA probes selected with size-polymorphic chromosomes did evidence size-conserved, consensus hybridization patterns specific to the constitutive species of the *braziliensis* complex. Following karyotype hybridization with 6 chromosome-derived probes, one hybridization pattern (pLb-180) was monomorphic throughout the *braziliensis* complex; another pattern (pLb-149) was highly polymorphic and almost strain-specific; the sequence recognized by one probe (pLb-13) was associated constantly to another one (pLb-168), but found to be different by restriction analysis (not shown). The last three probes (pLb-22, -134, -168) showed conserved patterns specific to *L. braziliensis* (pLb-134), to *L. guyanensis* (pLb-134) and to *L. peruviana* (pLb-22, pLb-134 and pLb-168) (fig. 5, table 2) and they did not hybridize significantly with the *mexicana* complex. Since the probes were selected for discriminating groups within the complex *braziliensis* as they did, the possible chimaeric nature of some of them was considered not to interfere with this goal.

Consensus patterns displayed by *L. peruviana* are most interesting as the status of this organism is unclear. *L. peruviana* is the only leishmania present at high altitude (1,500-2,500 m). It is responsible for the less severe cutaneous leishmaniasis of Latin America, known as «uta» or Andean cutaneous leishmaniasis. Despite this major clinical difference (1, 13, 16, 20) uta parasites were undistinguishable from *L. braziliensis* (1, 19, 24, 25) until recently when 2 enzyme loci (MPI, MDH) were found to be discriminative (1). Herein the distinct status of *L. peruviana* is confirmed with 3 different DNA probes, in agreement with enzyme data.

Grade of size variation in the proposed conserved patterns did differ sometimes from species to species within the $< 5\,\%$ - coefficient of variation. The pLb-134 conserved pattern in *L. braziliensis* displayed more variation than the other patterns, it might be related to the larger eco-geographical distribution of the populations tested as compared to the *L. peruviana* and *L. guyanensis* samples. This fact requires further sampling as well as quantitative data processing.

Our results suggest the existence of genetically distinct demes — here karyodemes — within taxa of Neotropical *Leishmania,* as illustrated by the three conserved patterns found with pLb-168 in 10 *L. guyanensis* beside the species-specific pLb-134 pattern and, as suggested to a lower extent by the variation of the pLb-134 conserved pattern in *L. braziliensis.* Intra-species clusters have been described in *L. infantum* (6). Our karyodeme hypothesis is currently being tested on a large collection of *L. peruviana.* Validation of the existence of populational structuration within the group-species would allow epidemiological applications of molecular karyotyping as suggested

elsewhere (18), including correlation studies with ecological and clinical data. It would address also the evolutionary significance of karyodemes (7).

Another avenue to be explored is the molecular and genetic understanding of chromosomal polymorphism and conservatism. Genomic plasticity in *Leishmania* results mainly from chromosomal rearrangements through amplification and deletion of repeated sequences in subtelomeric regions (14, 18, 23) (reviewed in (4, 18)).

With respect to possible genetic exchanges accounting for part of the polymorphism reported herein, our present broad sampling should be followed by a study of strictly sympatric isolates as conducted recently for *L. infantum* (6) before the question could be addressed. Sympatric sampling is presently in progress in two foci in Peru and in Bolivia. In the present study, possible genetic exchange is documented — for the first time to our knowledge in Latin America — by the hybrid pattern *peruviana - braziliensis* displayed by Peruvian clone population LH 78 identified enzymatically as *L. peruviana.*

Finally, as dispersion of digenetic leishmanias is compulsory dependant on vectorial transmission, entomological and genetic data on phlebotomine vectors are strongly needed for documenting properly parasite genetics data.

Acknowledgments. — *We would like to thank Drs J. Arevalo, M. Chance, Ph. Desjeux, J.P. Dedet, D. Evans, J.A. Rioux, J.J. Shaw, R. Urjel and G. Van Eys for reference strains and field isolates. We are also most grateful to Drs J.P. Dujardin and G. Van Eys for reviewing this manuscript, and to Dr G. Matthyssens for its guidance at the early stage of the work. This investigation received financial support from EEC (Contracts TS2-CT90-0315 and TS3-CT92-0129), and FGWO (Nationale Loterij, grants 346/1990 and 9.0024.90).*

Exploration du polymorphisme caryotypique et des caractères conservés dans le complexe *Leishmania (Viannia) braziliensis* au moyen de sondes dérivées de chromosomes.

Résumé. — Le caryotype moléculaire de 45 populations de référence de leishmanies Néotropicales a été analysé après coloration au bromure d'éthidium et à l'aide de 6 sondes dérivées de chromosomes et sélectionnées à partir d'une librairie génomique de *Leishmania (Viannia) braziliensis*. Des patterns de taille conservée ont été identifiés et trouvés spécifiques du genre *Viannia* et de ses espèces constitutives. Un résultat important pour l'épidémiologie et la recherche clinique a été la discrimination entre *L. (V.) peruviana* et *L. (V.) braziliensis,* 2 espèces considérées comme très semblables par d'autres techniques génétiques, mais responsables de patrons cliniques totalement différents. L'existence suggérée de dèmes génétiquement distincts, ou caryodèmes, à l'intérieur du groupe-espèce pourrait aussi être importante, car ces populations pourraient différer du point de vue de leur virulence, spécificité d'hôte et manifestations cliniques.

Exploratie van het karyotypen polymorfisme en de geconserveerde kenmerken in het *Leishmania (Viannia) braziliensis* complex bij middel van door chromosomen afgeleide proben.

Samenvatting. — Het moleculaire karyotype van 45 referentiepopulaties van Neotropische leishmanias werd geanalyseerd na ethidiumbromide kleuring en met 6 van chromosomen afgeleide proben, geselecteerd uit een genomische bank van *Leishmania (Viannia) braziliensis.* Er werden konservatieve patronen geïdentificeerd die specifiek zijn voor het subgenus *Viannia* en voor de species die erin thuishoren. Een belangrijk resultaat inzake epidemiologie en klinische navorsing was de onderscheiding tussen *L. (V.) peruviana* en *L. (V.) braziliensis,* 2 species die geheel gelijk waren bevonden met andere genetische technieken, maar verantwoordelijk voor totaal verschillende klinische patronen. Het gesuggereerde bestaan van genetisch verschillende demen, of karyodemen, binnen de groep-species, zou ook belangrijk kunnen zijn, want deze populaties zouden in virulentie, gast-specificiteit en klinische manifestaties kunnen verschillen.

Received for publication on April 21, 1993.

REFERENCES

1. Arana M, Evans DA, Zolessi A, Llanos-Cuentas A, Arevalo J: Biochemical characterization of *L.(V.) braziliensis* and *L.(V.) peruviana* by isoenzyme electrophoresis. Trans. Roy. Soc. Trop. Med. Hyg., 1990, **84**, 526-529.
2. Barker DC: Molecular approaches to DNA diagnosis. Parasitol., 1989, **99**, 5125-5146.
3. Bastien P, Blaineau C, Taminh M, Rioux JA, Roizès G, Pagès M: Interclonal variations in molecular karyotype in *Leishmania infantum* imply a «mosaic» strain structure. Mol. Biochem. Parasitol., 1990, **40**, 53-62.
4. Bastien P, Blaineau C, Pagès M: Leishmania: Sex, Lies and Karyotype. Parasitol. Today, 1992, **8**, 174-177.
5. Bishop RP, Miles MA: Chromosome size polymorphism in *Leishmania donovani*. Mol. Biochem. Parasitol., 1987, **24**, 263-272.
6. Blaineau C, Bastien P, Pagès M: Multiple forms of chromosomes I, II and IV in a restricted population of *Leishmania infantum* contrasting with monomorphism in individual strains suggest haploidy or automixy. Mol. Biochem. Parasitol., 1992, **50**, 197-204.
7. Bush GL, Case SM, Wilson AC, Patton JL: Rapid speciation and chromosomal evolution in mammals. Proc. Natl. Acad. Sci. USA., 1977, **74**, 3942-3946.
8. Carle GF, Olson MV: Separation of chromosomal DNA molecules from yeast by orthogonal-field-alternation gel electrophoresis. Nucleic Acids Res., 1984, **12**, 5647-5664.
9. Comeau AM, Miller SI, Wirth DF: Chromosome location of four genes in *Leishmania*. Mol. Biochem. Parasitol., 1986, **21**, 161-169.
10. Dujardin JC, Gajendran N, Hamers R *et al.*: Leishmaniasis in the Lowlands of Bolivia. VII. Characterization and identification of Bolivian isolates by PFG karyotyping. *in* Leishmaniasis: the first centenary (1885-1985). New strategies for control (Ed. D Hart). New York, Plenum Press, NATO ASI series A, 1987, **163**, 137-148.
11. Giannini SH, Schittini M, Keithly JS, Warburton PW, Cantor CR, Van der Ploeg LHT: Karyotype analysis of *Leishmania* species and its use in classification and clinical diagnosis. Science, 1986, **232**, 762-765.
12. Giannini SH, Curry S, Tesh R, Van der Ploeg LHT: Size-conserved chromosomes and stability of molecular karyotype in cloned stocks of *Leishmania major*. Mol. Biochem. Parasitol., 1990, **39**, 9-22.
13. Guerra H: Distribution of *Leishmania* in Peru. *in* Research on control strategies for the Leishmaniases (Eds. BC Walton, PM Wijeyaratne, F Modabber). Ottawa, IDRC, 1988, 135-145 (IDRC-MR, 184e).
14. Iovannisci DM, Beverley SM: Structural alterations of chromosome 2 in *Leishmania major* as evidence for diploidy, including spontaneous amplification of the mini-exon array. Mol. Biochem. Parasitol., 1989, **34**, 177-188.
15. Kreutzer RD, Souraty N, Semko ME: Biochemical identities and differences among *Leishmania* species and subspecies. Am. J. Trop. Med. Hyg., 1987, **36**, 22-32.
16. Lainson R, Shaw JJ: Evolution, classification and geographical distribution. *in* The Leishmaniases in Biology and Medicine (Eds. W Peters, R Killick-Kendrick). London, New York, Academic Press, 1987, 2-104.
17. Le Ray D: Structures antigéniques de *Trypanosoma brucei* (Protozoa, Kinetoplastida). Analyse immunoélectrophorétique et étude comparative. Ann. Soc. Belge Méd. Trop., 1975, **55**, 158-160.
18. Lighthall GK, Giannini SH: The chromosomes of *Leishmania*. Parasitol. Today, 1992, **8**, 192-199.
19. Lopez M, Montoya I, Arana M *et al.*: The use of nonradioactive DNA probes for the characterization of *Leishmania* isolates from Peru. Am. J. Trop. Med. Hyg., 1988, **38**, 308-314.
20. Lumbreras H, Guerra H: Leishmaniasis in Peru. *in* Leishmaniasis. (Eds. KP Chang, R Bray). New York, Elsevier, 1985, **1**, 297-311.
21. Maniatis T, Fritsch EF, Sombrook J: Molecular cloning. A laboratory manual. New York, Cold Spring Harbour Laboratory, 1982.
22. McMahon-Pratt D, Bennet E, David JR: Monoclonal antibodies that distinguish subspecies of *Leishmania braziliensis*. J. Immunol., 1982, **129**, 926-927.
23. Pagès M, Bastien P, Veas F *et al.*: Chromosome size and number polymorphisms in *Leishmania infantum* suggest amplification/deletion and possible genetic exchange. Mol. Biochem. Parasitol, 1989, **36**, 161-168.
24. Reiner NE, Lo R, Llanos-Cuentas A, Guerra H, Button L, McMaster WR: Genetic heterogeneity in Peruvian *Leishmania* isolates. Am. J. Trop. Med. Hyg., 1989, **41**, 416-421.
25. Romero GG, Arana M, Lopez M *et al.*: Characterization of *Leishmania* species from Peru. Trans. Roy. Soc. Trop. Med. Hyg., 1987, **81**, 14-24.
26. Scholler JK, Reed SG, Stuart K: Molecular karyotype of species and subspecies of *Leishmania*. Mol. Biochem. Parasitol., 1986, **20**, 279-293.

27. Tobie EJ, Von Brand T, Mehlman B: Cultural and physiological observations on *Trypanosoma rhodesiense* and *Trypanosoma gambiense.* J. Parasitol., 1950, **36**, 48-54.
28. Van der Ploeg LHT, Cornelissen WWCA, Michels PAM, Borst P: Chromosome rearrangements in *Trypanosoma brucei.* Cell, 1984, **32**, 213-221.
29. Van Eys GJJM, Schoone GJ, Ligthart GS, Alvar J, Evans DA, Terpstra W: Identification of «Old World» *Leishmania* by DNA recombinant probes. Mol. Biochem. Parasitol., 1989, **34**, 53-62.
30. Van Eys GJJM, Schoone GJ, Kroon NCM, Ebeting SB: Sequence analysis of small subunit ribosomal RNA genes and its use for detection and identification of *Leishmania* parasites. Mol. Biochem. Parasitol., 1992, **51**, 133-142.
31. Van Meirvenne N, Janssens PG, Magnus E: Antigenic variation in syringe passaged populations of *Trypanosoma (Trypanozoon) brucei.* I. Rationalization of the experimental approach. Ann. Soc. Belge Méd. Trop., 1975, **55**, 1-23.
32. Wilson AC, Sarich VM, Maxson LR: The importance of gene rearrangement in evolution: evidence from studies on rates of chromosomal, protein and anatomical evolution. Proc. Natl. Acad. Sci. USA, 1974, **71**, 3028-3030.