

MOLECULAR KARYOTYPE ANALYSIS AND MAPPING OF HOUSEKEEPING GENES TO CHROMOSOMES OF SELECTED SPECIES COMPLEXES OF *LEISHMANIA*

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The molecular karyotypes for 20 reference strains of species complexes of Leishmania were determined by contour-clamped homogeneous electric field (CHEF) electrophoresis. Determination of number/position of chromosome-sized bands and chromosomal DNA locations of housekeeping genes were the two criteria used for differentiating and classifying the Leishmania species. We have established two gel running conditions for optimal separation of chromosomes, which resolved DNA molecules as large as 2,500 kilobase pairs (kb). Chromosomes were polymorphic in number (22-30) and size (200-2,500 kb) of bands among members of five complexes of Leishmania. Although each stock had a distinct karyotype, in general the differences found between strains and/or species within each complex were not clear enough for parasite identification. However, each group showed a specific number of size-concordant DNA molecules, which allowed distinction among the Leishmania complex parasites. Clear differences between the Old and New world groups of parasites or among some New World Leishmania species were also apparent in relation to the chromosome locations of beta-tubulin genes. Based on these results as well as data from other published studies the potential of using DNA karyotype for identifying and classifying leishmanial field isolates is discussed.

Key words: *Leishmania* – chromosome – molecular karyotype – gene localization – PFGE: pulsed-field gel electrophoresis

Kinetoplastid protozoan parasites of the genus *Leishmania* are a biologically diverse group of trypanosomatid causing the various human and animal leishmaniasis. A wide variety of clinical manifestations are associated with different species of *Leishmania* and many of these parasites are capable of producing a spectrum of disease in their human hosts. Because of their differences in virulence and variable responses to chemotherapeutic agents, correct parasite identification is essential in any clinicopathologic study of leishmaniasis. Parasite characterization and comparison of various *Leishmania* stocks isolated from humans, reservoir hosts, and sandfly vectors are also of practical use in descriptive epidemiol-

ogy and for disease control (Grimaldi et al., 1989; Anon., 1990).

It is possible to identify a stock as belonging to an already known taxon by using discriminant characters, provided that these characters have been shown to be constant and that the reference system is clearly defined. The identification and taxonomy of species of *Leishmania* traditionally have been based on phenotypic characters such as the clinical manifestations in humans, epidemiological features, and a variety of biological criteria (Lainson & Shaw, 1987). More precise taxonomic markers for *Leishmania* have resulted from the application of established molecular, biochemical, and immunological techniques (e. g., isoenzyme electrophoresis, analysis of kinetoplast minicircle DNA heterogeneity, and the use of species-specific monoclonal antibodies or DNA probes) which can be used to identify, in comparison to reference strains, the leishmanial isolates (Rioux, 1986). However, new techniques are constantly being developed, and

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many other intrinsic parasite characteristics are currently being tested. Among the new molecular tools are the chromosome separation techniques using PFGE (Schwartz & Cantor, 1984; Carle & Olson, 1984; Chu et al., 1986) which has made possible to establish DNA karyotypes and the genomic organization of parasitic protozoa (Van der Ploeg et al., 1984). Molecular karyotype analysis has demonstrated chromosome size polymorphisms among *Leishmania* stocks which reveal a high degree of plasticity in the genome of these parasites (Spithill & Samaras, 1985; Comeau et al., 1986; Scholler et al., 1986; Bishop & Miles, 1987; Samaras & Spithill, 1987; Pages et al., 1989; Bastien et al., 1990). However, there is evidence showing that *Leishmania* karyotype might be stable, with chromosomal changes occurring only rarely (Giannini et al., 1990). Thus, PFGE is an alternate approach for identifying leishmanial field isolates (Giannini et al., 1986; Dujardin et al., 1989). In order to extend these observations, specific profiles of the chromosomes of 20 reference strains of species complexes of *Leishmania* were analyzed in this study, using PFGE and appropriate gel running conditions for optimal separation of chromosome-sized DNA. The use of cloned gene probes for locations of housekeeping genes was an additional tool for parasite classification.

MATERIALS AND METHODS

Parasites examined and in vitro cultivation

All the strains of *Leishmania* used in this study were the WHO-recommended reference strains (Anon., 1984). Identification of the stocks has been further confirmed in our laboratories by their reactivity with monoclonal antibodies, isoenzyme electrophoresis, and restriction endonuclease fragment patterns of kDNA. A detailed list of these strains is given in the Table. Promastigotes of each strain were grown at 23-24 °C in Schneider's *Drosophila* medium (Hendricks et al., 1978), supplemented with 20% heat-inactivated fetal bovine serum. Parasites in the log phase of growth were harvested by centrifugation (1,500 x g for 10 min at 4 °C) and washed twice in buffer containing 75 mM phosphate buffer, pH 8.0, 65 mM NaCl, and 1% glucose. The final pellet was used for preparation of samples for PFGE.

Preparation of samples and PFG electrophoresis – Intact chromosomal DNA for PFGE was prepared as described for trypanosomes

(Van der Ploeg et al., 1984), except that each agarose sample plug contained 10^7 promastigotes. Following protease digestion, the agarose plugs were kept in lysis buffer at 4 °C until use. PFGE was performed by contour-clamped homogeneous electric field (CHEF) technique, using either a Pulsaphor electrophoresis unit from Pharmacia-LKB (Uppsala, Sweden), or a CHEF DRII System from Bio-Rad (Richmond, CA, U. S. A.). Gels were run in 0.5 x TBE buffer (45 mM Tris, 45 mM boric acid, and 1 mM EDTA, pH 8.0) at 12 °C. Electrophoresis was performed as described in the figure legends. The sizes of the *Leishmania* chromosomal DNA molecules were determined by comparison to yeast (*Saccharomyces cerevisiae*) chromosomes (Bio-Rad) used as size markers. Following electrophoresis, gels were stained with ethidium bromide, prior to photography and processing for hybridization.

Southern blotting and hybridization – The gels were processed for Southern blotting and DNA was transferred from the gel onto nylon membranes (Nytran; Schleicher & Schuell, Inc., Keene, NH, U. S. A.) as described (Maniatis et al., 1982). The probes tested were the coding sequence for the alfa-tubulin gene of *L. enriettii* (Wirth & Slater, 1983), which was provided by Dr D. Wirth, Harvard School of Public Health; the beta-tubulin gene of *L. major* (Spithill & Samaras, 1985) which was obtained from Dr D. McMahon-Pratt, Yale University School of Medicine; and the DHFR-TS (Beverley et al., 1984) and mini-exon (Iovannisci & Beverley, 1989) genes of *L. major* which was a gift from Dr S. Beverley, Harvard School of Medicine. Cloned DNAs were labeled by the random priming method (Feinberg & Vogelstein, 1983), using Random Primer Kit (Boehringer Mannheim, Bromma, Sweden). Hybridization of filters to ^{32}P labelled specific probes were performed according to Takeuchi & Traub-Csekö (1990). Dehybridization was carried out boiling the filters for 30 min in 0.1 X SSC (1 X SSC is 150 mM NaCl, 15 mM Na citrate)/0.5% sodium dodecyl sulphate.

RESULTS

Molecular karyotypes – A comparison of molecular karyotypes from 20 reference strains representing selected species complexes of *Leishmania* (Table) was done by CHEF electrophoresis. All gels were run at 175 V at 12 °C. As shown in Fig. 1, optimal size separa-

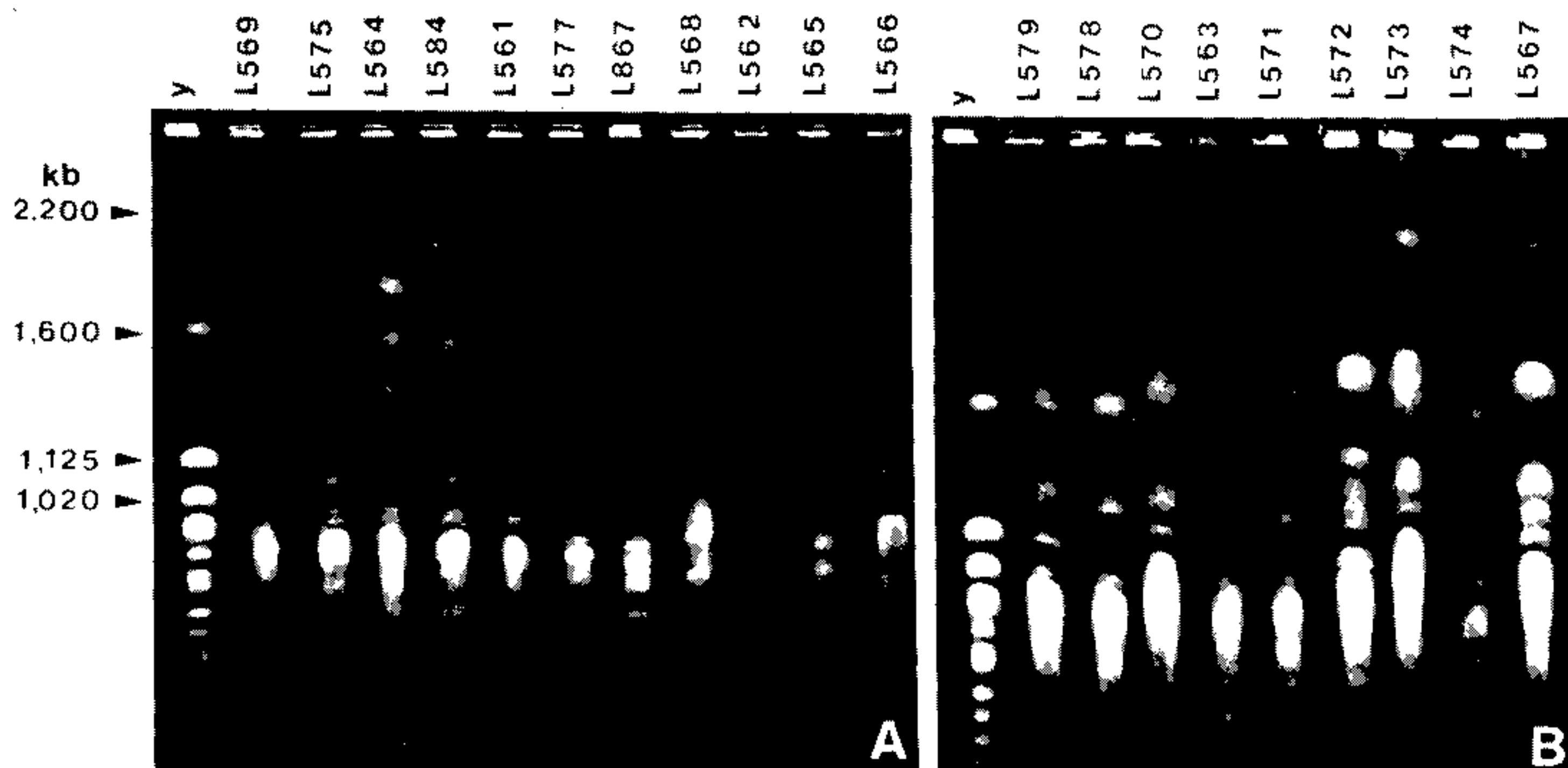


Fig. 1: CHEF electrophoresis separation in the high size range (above 1,000 kb) of chromosome-sized DNA molecules from reference strains of species complexes of *Leishmania*. These 1% agarose gels were run at 175 V for 24 hr (A) or 30 hr (B), at 12 °C, with a 200-100 s pulse ramp (using a CHEF DRII System from BioRad) and then stained with ethidium bromide and photographed under UV transillumination. The stock codes of the strains are indicated above the lanes; information on their origin is given in the Table. The apparent sizes of DNA molecules are estimated relative to yeast chromosomes used as markers.

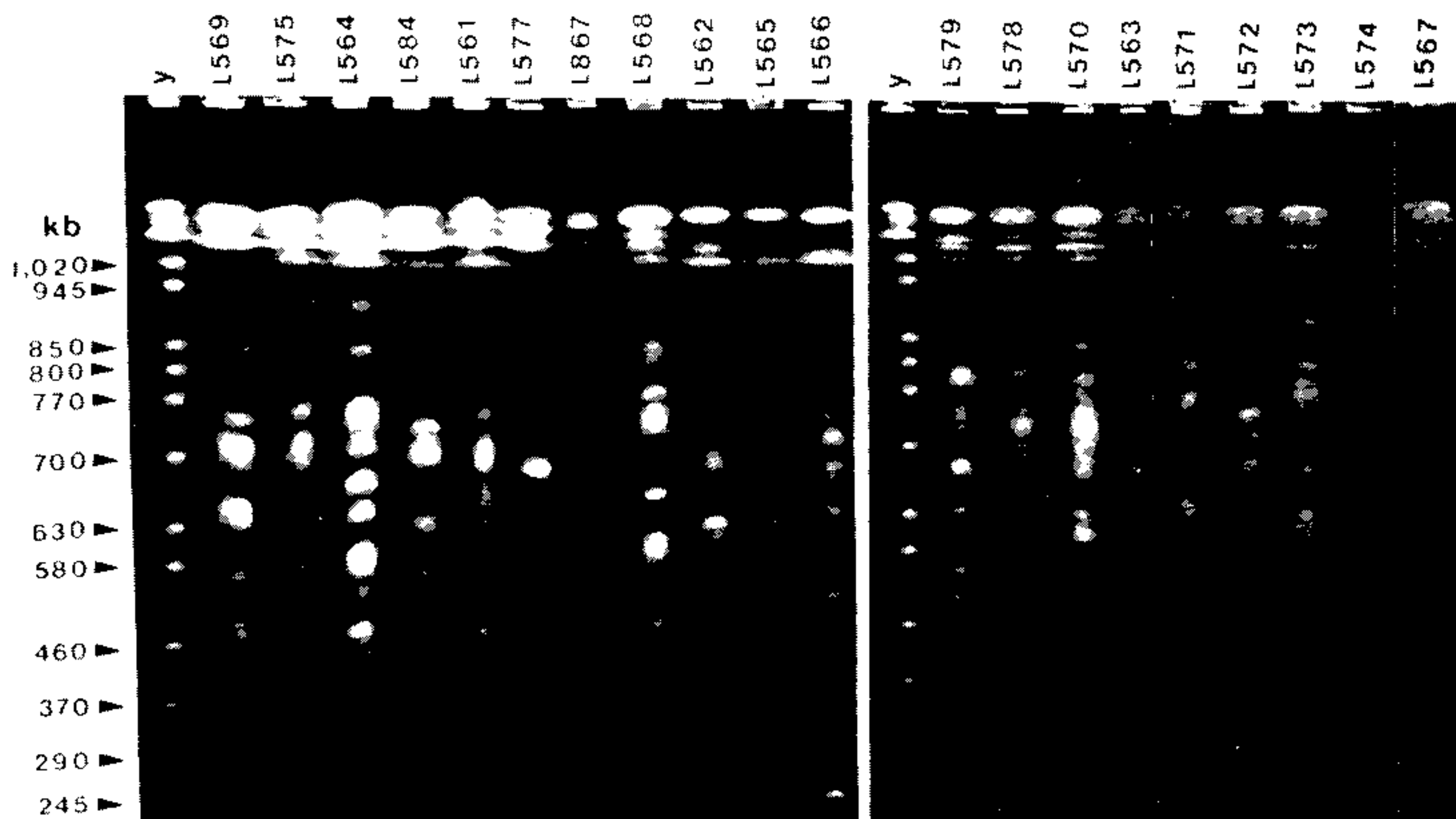


Fig. 2: CHEF electrophoresis separation of chromosome-sized DNA molecules under 1,000 kb from reference strains of species complexes of *Leishmania*. These 1.5% agarose gels were run at 175 V with a 100-50 s pulse ramp, for 40 hr (using a CHEF DRII System from BioRad). The stock codes of the *Leishmania* strains analyzed are indicated above the lanes, and their origins are shown in the Table.

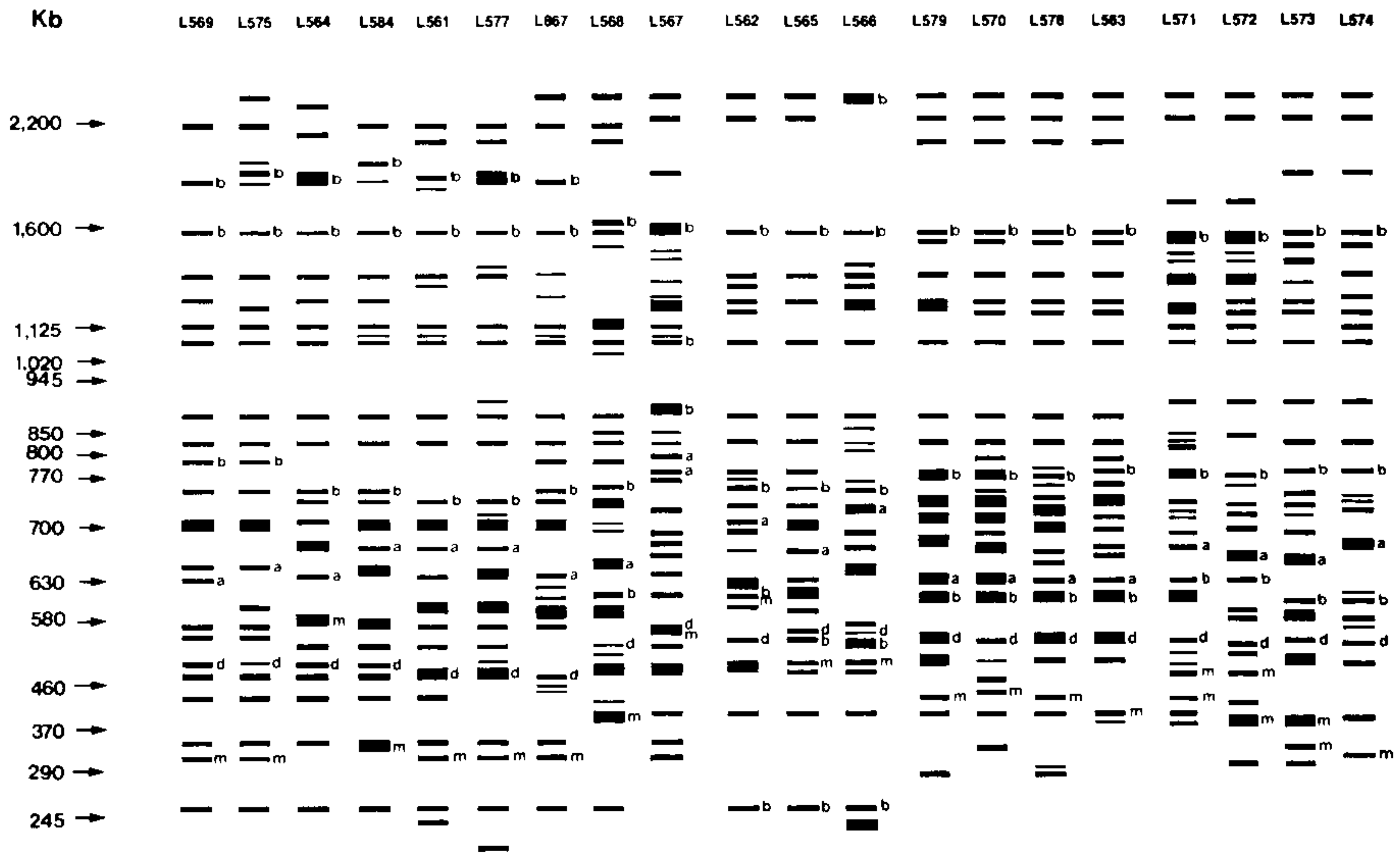


Fig. 3: diagrammatic representation of the karyotypes of 20 reference strains of species complexes of *Leishmania*, based on what is seen on several gels. The stock codes of the *Leishmania* strains analyzed are indicated above the lanes, and their origins are shown in the Table. The 245-2,200 kb scale at the left of the figure is drawn from yeast chromosome-sized DNA molecules. The location of the housekeeping genes on chromosomes is indicated as: a = alpha-tubulin; b = beta-tubulin; m = mini-exon; and d = DHFR-TS.

TABLE

Origin and identification of *Leishmania* reference strains which were characterized by molecular karyotype analysis in this study

Stock code	Stock designation ^a	Clinical form ^b	Geographic origin	Species
L561	MHOM/BZ/82/BEL21	CL	Belize, Belize	<i>L. mexicana</i>
L562	MHOM/PA/71/LS94	CL	Canal Zone, Panama	<i>L. panamensis</i>
L563	MHOM/ET/67/L82	VL	Humeria, Ethiopia	<i>L. donovani</i> s.l.
L564	MORY/PA/68/GML #	VL	Darien, Panama	<i>L. aristidesi</i>
L565	MHOM/BR/75/M4147	CL	Pará, Brazil	<i>L. guyanensis</i>
L566	MHOM/BR/75/M2903	CL	Pará, Brazil	<i>L. braziliensis</i>
L567	MCAV/BR/45/L88	CL	Paraná, Brazil	<i>L. enriettii</i>
L568	MHOM/VE/74/PM-H3	CL	Lara, Venezuela	<i>L. venezuelensis</i>
L569	MHOM/BR/73/M2269	CL	Pará, Brazil	<i>L. amazonensis</i>
L570	MHOM/IN/80/DD8	VL	Bihar, India	<i>L. donovani</i>
L571	MHOM/SU/58/str. OD	CL	Azerbaijan, USSR	<i>L. tropica</i>
L572	MHOM/SU/74/K27	CL	Azerbaijan, USSR	<i>L. tropica</i>
L573	MHOM/IL/67/Jer. II	CL	Jericho, Israel	<i>L. major</i>
L574	MRHO/SU59/nEAL P	CL	Uzbekistan, USSR	<i>L. major</i>
L575	IFLA/BR/67/PH8	—	Pará, Brazil	<i>L. amazonensis</i>
L577	MNYC/BZ/62/M379	CL	Cayo, Belize	<i>L. mexicana</i>
L578	MHOM/TN/80/IPT1	VL	Monastir, Tunisia	<i>L. infantum</i>
L579	MHOM/BR/74/PP75	VL	Bahia, Brazil	<i>L. chagasi</i>
L584	MHOM/VE/76/JAP78	CL	Merida, Venezuela	<i>L. garnhami</i>
L867	MHOM/VE/60/LtRod ^c	DCL	Venezuela	<i>L. pifanoi</i>

a: code: Host (M = Mammalia; CAV = *Cavia porcellus*; HOM = *Homo sapiens*; NYC = *Nyctomys sumichrasti*; ORY = *Oryzomys capito*; and RHO = *Rhombomys* sp.; I = *Insecta*; FLA = *Lutzomyia flaviscutellata*)/country of origin/year of isolation/original code.

b: CL = cutaneous leishmaniasis; DCL = diffuse cutaneous leishmaniasis; and VL = visceral leishmaniasis.

c: as this strain was found to be phenotypically similar to reference strains of *L. major* it has also been named as "L. major-like" (Hashiguchi et al., 1991).

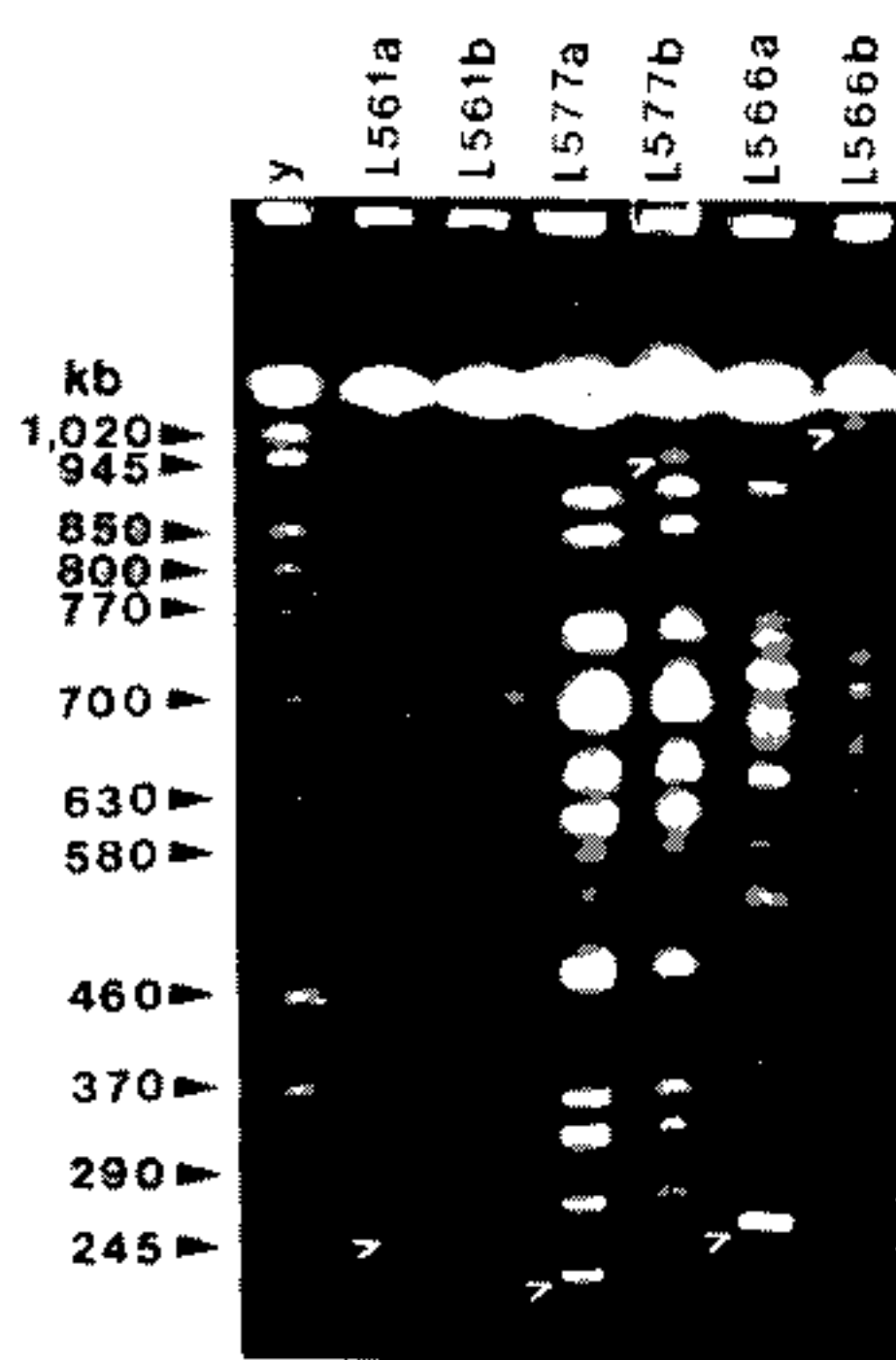


Fig. 4: occurrence (arrowheads) and disappearance of large and small linear DNAs in distinct stocks* of cloned *L. braziliensis* and *L. mexicana* reference strains. Chromosomes from the indicated stocks (see Table for their origins) were resolved by CHEF electrophoresis, using 1.5% agarose gels (170 V, pulse times of 120s for 3 hr, 90 s for 30 hr and 60 s for 15 hr), using a Pulsaphor electrophoresis unit from Pharmacia-LKB. Molecular weights are indicated in kb at the left of the figure. *Stocks L561b, L577a and L566b were kindly provided by Dr D. MacMahon-Pratt, Yale University School of Medicine.

tion of chromosomal bands in the high size range was achieved using 1.0% agarose gels which were run with a 200-100 sec pulse ramp, for 24 hr. This procedure allowed the fractionation of DNA molecules from 1,020 kb to at least 2,500 kb pairs in size. As at this long pulse time smaller molecules (< 1,020) migrate with the same apparent M. W. due to reptation (Van der Ploeg et al., 1984), these molecules appeared compressed. However, optimal size separation of DNA molecules of M. W. under 1,000 kb (Fig. 2) was obtained using 1.5% agarose gels which were run with a 100-50 sec pulse ramp, for 40 hr. This procedure did not allow the fractionation of chromosome-sized DNA larger than 1,000 kb, thus giving rise to the compression zone shown at the top of the gel.

Based on results as those shown on Figs 1, 2, we have constructed a diagrammatic representation (Fig. 3) of the total molecular karyotype for each reference strains of the *Leishmania* complexes species. A high proportion (approximately, three-fifths) of the chromosomes of parasites within the genus *Leishmania* ranges between 300 and 950 kb in size, and two bands

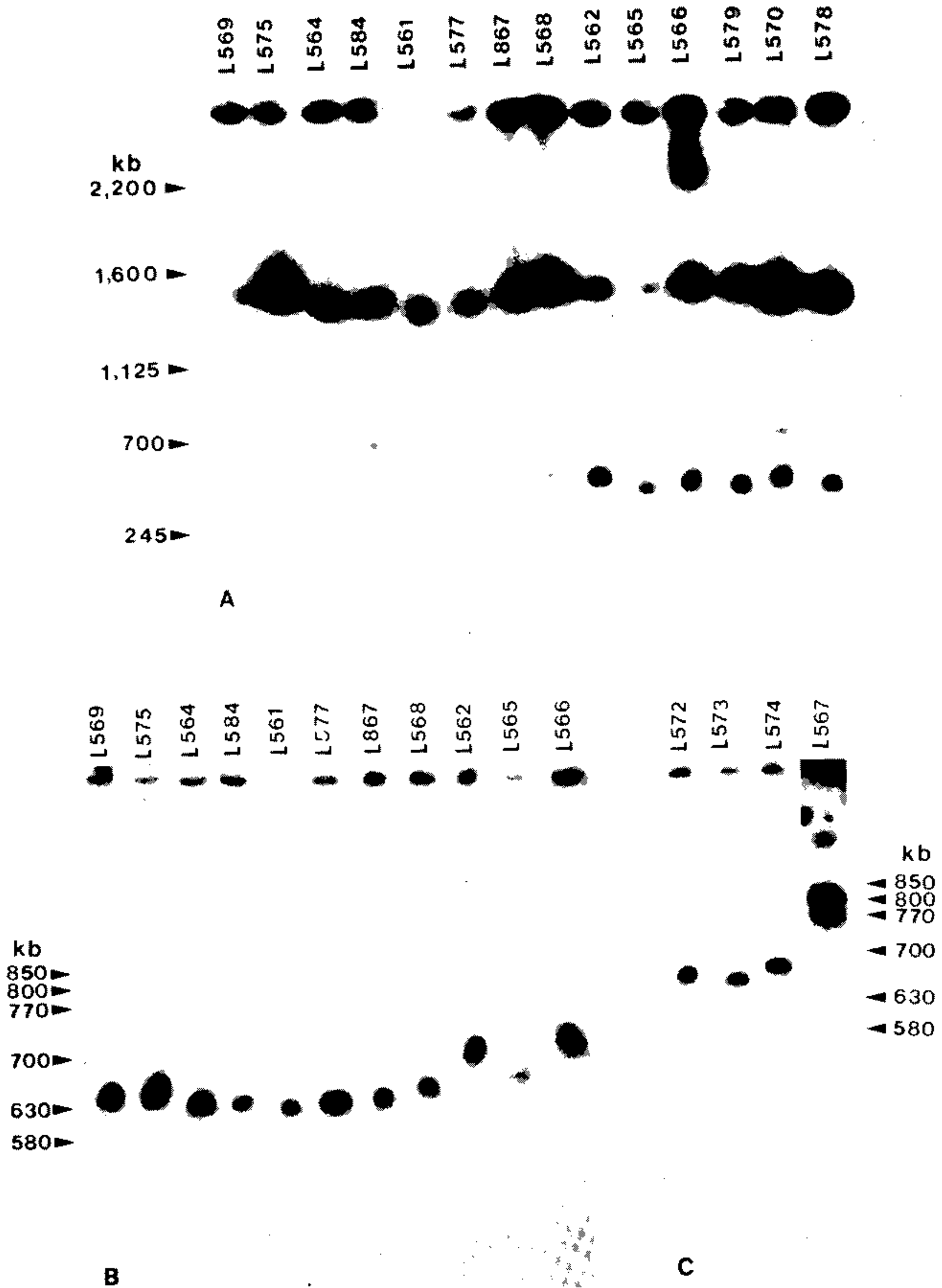


Fig. 5: autoradiograms after Southern hybridization of ³²P-labelled specific probes for the locations of beta-tubulin (A) and alpha-tubulin (B, C) genes to chromosomes of selected species complexes of *Leishmania*. Chromosome-sized DNA molecules from the indicated stocks (see Table for their origins) were resolved by CHEF electrophoresis, using the same running conditions as those in legend to Fig. 1 (A) or Fig. 2 (B and C).

of 1,600 and 1,050 kb in size were common in all stocks.

As shown in Fig. 4, comparison between stocks of the same cloned strain, but obtained

from different laboratories, showed karyotype changes as revealed by the appearance/disappearance of small DNA molecules (< 245 kb). In addition, in both L577b and L566b strains, in which the small DNAs were missing, it was

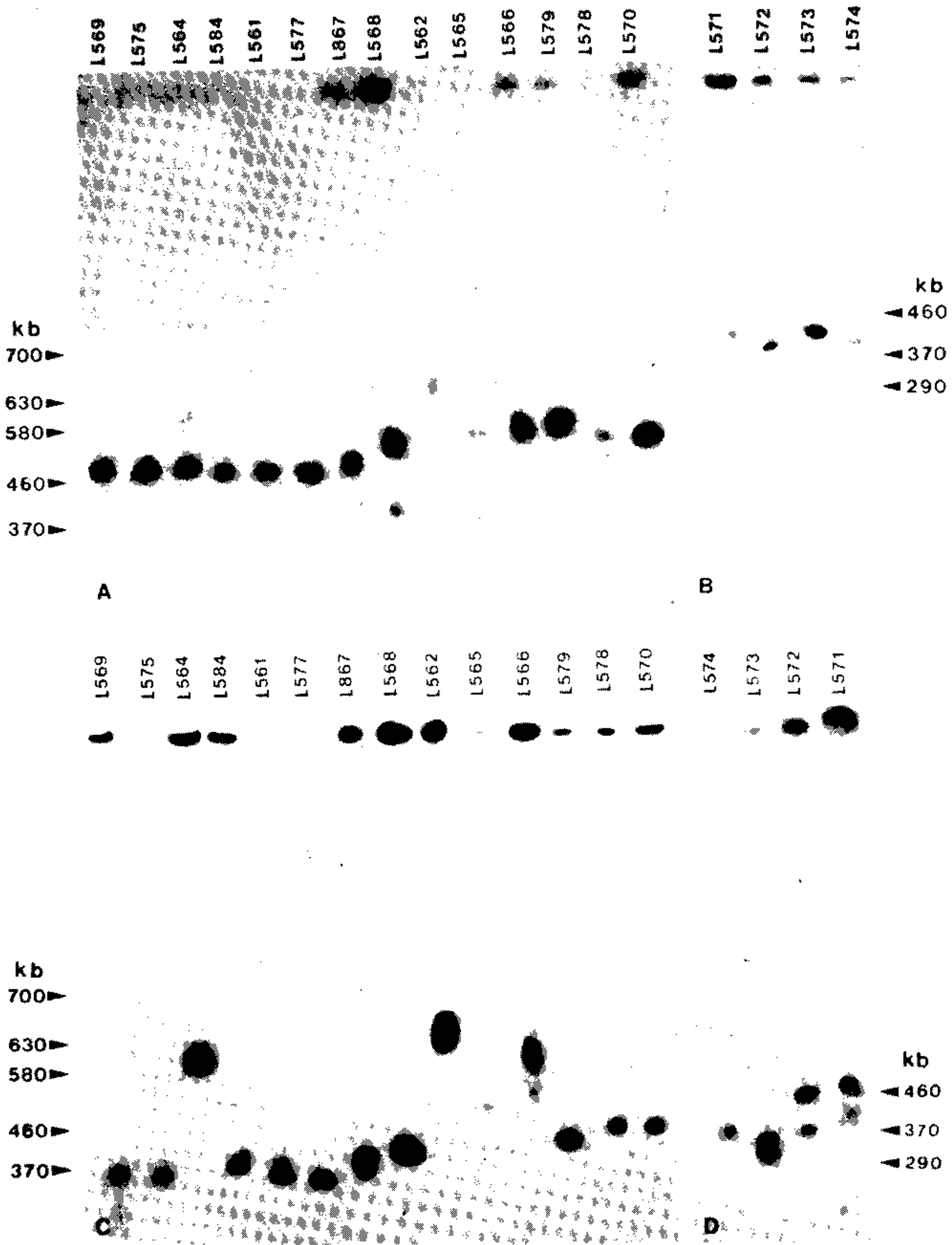


Fig. 6: chromosome location of DHFR-TS and mini-exon genes. Chromosome-sized DNA molecules derived from the indicated stocks were separated by CHEF electrophoresis, blotted to nylon membrane and probed with the DHFR-TS gene (A, B) and mini-exon gene (C, D) specific probes. The running conditions for the gels were as those described in legend to Fig. 2.

demonstrated an extra chromosomal band of approximately 900 kb and 1000 kb in size, respectively, that might represent a translocation.

Comparison of karyotypes among/within complexes of Leishmania – As shown in Fig. 3, a total of 22-30 chromosomal DNA bands ranging in size between 200 and 2,500 kb were

resolved, depending on the strain of *Leishmania*. Polymorphisms were most apparent among the DNA molecules in the low size range, but karyotypic differences among species complexes of *Leishmania* were more evident at the higher molecular weight zone (1,000-2,500 kb). Unique chromosome banding pattern was obtained for each reference strain, based on both the size of specific chromosomes and the overall pattern of ethidium bromide staining. Similarities in the karyotypes of members of the same *Leishmania* complex were great enough to distinguish them from the other groups. For instance, strains of the *L. braziliensis* complex could be distinguished by the characteristic absence of any chromosome band ranging in size between 1,600 and 2,200 kb in this group. Parasites of the *L. donovani* complex also showed a group-specific polymorphism of chromosomes at the M. W. range between 650 and 800 kb. Chromosomes larger than this have a size homology close to 100%. In contrast, although only reference strains were examined, the inter- and intraspecific variability in the number/size of DNA bands were not always clear enough to distinguish parasites within a given species group (Figs 1-3).

Chromosomal DNA locations of housekeeping genes – The chromosomal locations of genes for beta-tubulin, alpha-tubulin, DHFR-TS and mini-exon are shown in Figs 3, 5, 6. The gene probes hybridized with several chromosomal bands ranging in size between 250 and 2,500 kb. Whereas the sequences for beta-tubulin genes were located on chromosomes of similar sizes among Old World species complexes of *Leishmania*, clear differences in their locations were apparent in the New World *Leishmania* species (Figs 3, 5a) where hybridization was found in three to five bands. The alpha-tubulin genes were localized to a single band in all species with the exception of *L. enriettii* (Figs 3, 5b, c). The DHFR-TS and mini-exon genes were generally found on single chromosomal bands, ranging from 290 to 580 kb among distantly related groups (Figs 3, 6). However, the mini-exon genes were localized in two bands of varying size in strains from both the *L. tropica* and *L. major* complexes (Fig. 6d).

DISCUSSION

Previous studies revealed distinctive molecular karyotypes when *Leishmania* stocks from either the Old or New World were analyzed by PFGE (Giannini et al., 1986; Scholler

et al., 1986; Bishop & Miles, 1987; Samaras & Spithill, 1987; Dujardin et al., 1989). Chromosome polymorphisms occur in *Leishmania* such that each strain or clone of a given species has a unique karyotype (Pagès et al., 1989; Bastien et al., 1990). Indeed, this technique has been used for typing unidentified clinical isolates by comparison with *Leishmania* reference strains (Giannini et al., 1986; Dujardin et al., 1989). Confirming previous data (Scholler et al., 1986; Giannini et al., 1990), our analysis showed that significant karyotype concordances were found among closely related strains than among more distantly related groups. As a possible exception, we should refer to the apparent poor karyotypic relationship between *L. enriettii* and the other species within the *L. mexicana* group (Fig. 3). However, distinct relationships of this parasite and other members of the genus *Leishmania* have been demonstrated, as revealed by quantitative comparisons of nuclear DNA fragment patterns (Beverley et al., 1987), which correspond to those predicted from previous molecular and taxonomic studies using isoenzymes and specific monoclonal antibodies (Momen & Grimaldi, unpublished data). In contrast, clear distinction among parasites within a given species group was generally less evident. For instance, on the basis of the observed molecular karyotypes the *L. amazonensis* strains were as distinct to one another as they were to some other species of the *L. mexicana* complex. This finding suggests that molecular karyotyping is good for classifying *Leishmania* stocks as a group but its potential usefulness for identifying the parasite species in field surveys is less clear. However, the method still remains to be tested in large and geographically widespread epidemiologic and taxonomic studies before any conclusion can be drawn on this matter. Work is in progress in our laboratories to better evaluate the natural variation of these molecular markers under different conditions by examining *Leishmania* isolates from distinct endemic foci in widely separated geographic areas.

As described previously in *L. braziliensis* (Scholler et al., 1986), *L. mexicana* (Samaras & Spithill, 1987), *L. donovani* (Bishop & Miles, 1987) and *L. major* (Beverley & Coburn, 1990), we also observed the appearance of a small chromosome band in each karyotype of the two first species. Its greater intensity compared to the other DNA bands suggests a higher copy number (Scholler et al., 1986; Beverley & Co-

burn, 1990). It may represent replicating circular DNAs of episomal or viral origin, appearing as either an anomalously small band or integrated into one of the DNA chromosomal bands of the *Leishmania* (Gajendran et al., 1989; Hamers et al., 1989; Stuart et al., 1989). Alternatively, recurrent emergence of new small linear chromosomes (ranging in size from 180 to 220 kb) may occur in *Leishmania*, either spontaneously during routine serial passage in vitro or under methotrexate pressure (Beverley & Coburn, 1990). The possibility of the L577 strain being exposed to anti-leishmanial drugs prior to establishment *in vitro* seems unlikely, given the fact that it was isolated from wild reservoir host. Assuming that this as well as the other stocks analyzed in this study have not been exposed to any experimental drug pressure, the small chromosomes may have emerged spontaneously. Work is now in progress to better define the relationship between these small DNA molecules and those previously described.

Our results are generally consistent with previous data (Spithill & Samaras, 1985; 1987; Comeau et al., 1986; Giannini et al., 1986; Scholer et al., 1986; Samaras & Spithill, 1987; Dujardin et al., 1989) on the chromosomal locations of housekeeping genes among different species of *Leishmania*. It is possible that the discrepancy existing in the hybridization patterns of some gene probes obtained in this study and those from other reports is due to either strain variation or different running conditions. Relative mobilities of chromosome-sized DNA molecules can be considerably affected by several parameters for separation (e. g., different voltage and pulse frequency, temperature and ionic strength of the electrophoresis buffer, and type or concentration of agarose) (Van der Ploeg et al., 1984). As the beta-tubulin genes were found on different chromosome size classes, showing some group- or species-specific variations of hybridization patterns, we concluded that these molecular markers may also be useful for parasite classification.

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