

DEVELOPMENT OF NUCLEAR DNA PROBES FOR THE TYPING OF *TRYPANOSOMA CRUZI*

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We have developed and tested a new way of typing Trypanosoma cruzi, namely the use of cloned nuclear DNA fragments as genetic markers. Restriction fragment length polymorphisms were verified on Southern blots hybridized to random probes. Fragment patterns were analyzed and dendrograms constructed. Our results on well characterized laboratory strains correlate well to published isoenzyme studies. Some of the probes were also hybridized to chromosomes separated by pulse field gel electrophoresis and a higher degree of heterogeneity was observed at this level.

Key words: *Trypanosoma cruzi* - random probes - typing - strains — numerical taxonomy

Trypanosoma cruzi is an organism whose great variability can be detected at the level of morphology, virulence, tissue tropism, drug resistance, etc. American trypanosomiasis, the disease it causes, is also characterized by various clinical manifestations that range from asymptomatic to very serious cardiac forms of disease, that can cause sudden death. This variability in the clinical symptoms could be reflecting the heterogeneity of the infective parasite populations. From these facts stems the interest to establish reliable ways to classify these organisms. Many classification techniques have been used so far with variable degrees of success. Among these are schizodeme analysis (Morel et al., 1980), zymodeme analysis (Toye, 1974), and monoclonal antibodies (DeSimone et al., 1987).

Cloned DNA probes have been used to differentiate various parasites at the level of strains, isolates and species. They could determine relationships between *T. brucei* stocks and subspecies (Paindavoine et al., 1986). Cloned ribosomal genes could differentiate schistosomes in species, strains

and sex (McCutchan et al., 1984). Also genomic diversity in *Theileria parva* stocks was detected using DNA probes (Conrad et al., 1987) and randomly cloned DNA fragments can be used in these studies (Paindavoine et al., 1986; Conrad et al., 1987).

Here we demonstrate that cloned nuclear DNA fragments can be used to differentiate strains of *T. cruzi*.

MATERIALS AND METHODS

Growth of T. cruzi — *T. cruzi* strains CL (Brenner & Chiari, 1963), Colombiana (Federici et al., 1964), Dm28 and clone Dm28c (Goldenberg et al., 1984), F (Deane & Kloetzel, 1974), Y (Silva & Nussenzweig, 1953) and y(c12)2 clone (Dr Zigman Brenner, Centro de Pesquisa René Rachou, Belo Horizonte) were grown in LIT medium (Chiari & Camargo, 1984), supplemented with 10% fetal calf serum at 28°C with weekly passages and initial concentrations of 10⁶ cells/ml. The *Leishmania* species were a gift from Dr Gabriel Grimaldi from the Department of Immunology, FIOCRUZ, the *Crithidia* and *T. conorrhini* were a gift from Dr Maria Deane from the Department of Protozoology, FIOCRUZ, and the *Phytomonas* were a gift from Dr Victor Jankevicius, State University of Londrina, PR.

DNA preparation and digestion — *T. cruzi* and plasmid DNAs were prepared as described

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TABLE I
PstI Clones of *Trypanosoma cruzi* (Y) in pBR322

Clones	Insert size (bp)	Clones	Insert size (bp)
pMYP-B19	120	pMYP-P18	2265
pMYP-R18	486	pMYP-P42	2400
pMYP-R2 _a 2 _a	632	pMYP-R22	2406
pMYP-G34	658	pMYP-G47	2384 and 374
pMYP-B14	670	pMYP-R42	2818
pMYP-16/18	901	pMYP-R ₁ 22	2990
pMYP-7	950	pMYP-M2	2570 and 530
pMYP-RN49	1850	pMYP-16N47	3196
pMYP-16N46	1911	pMYP-16N30	1745 and 1524
pMYP-RN11	2056	pMYP-R ₂ 32a	3270
pMYP-P11B	2152	pMYP-P38	2510 and 1012
pMYP-16/20	2208	pMYP-P32a	2650 and 1122
		pMYP-PRO33	3690

(Ozaki & Traub-Cseko, 1984), and digested with restriction enzymes according to instructions from the manufacturers (New England BioLabs, Beverly, MA, Appligene, Strasbourg, and FIOCRUZ, Rio de Janeiro).

Preparation of probes — *T. cruzi* Y strain DNA digested with PstI was cloned into pBR322. Mini-prep (Ozaki & Traub-Cseko, 1984) DNAs were cut with PstI and checked for the presence and size of inserts using 0.8% agarose gels. The probes obtained are given in Table I.

Southern transfers, dot blots and hybridizations — Southern transfers were performed by standard methods (Southern, 1975). The dot blots were prepared by passing 1 µg of sheared DNA in 300 µl water into nitrocellulose through a Schleicher & Schuell minifold, and the filters were treated using a standard protocol (Maniatis et al., 1982). Overnight hybridization to nick translated probes (Rigby et al., 1977) was carried out in 6 X SSC (1 X SSC is 150 mM NaCl, 15 mM Na citrate), 0.2% bovine serum albumin, 0.2% polyvinylpyrrolidone, 0.2% ficoll, 50 mM trisHCl, pH 7.5, 0.1% Na pyrophosphate, 1% sodium dodecyl sulphate (SDS), 100 µg/ml sonicated and denatured herring sperm DNA and approximately 10⁶ cpm of probe ml⁻¹, at 68°C. Filters were washed three times at 65°C with 1XSSC and 0.1% SDS.

Chromosome separation — Chromosome separation was performed using the OFAGE (Carle & Olsen, 1984) technique in the Pulsaphor System

from LKB. Agarose inclusion and lysis of *T. cruzi* were carried out as described (Van der Ploeg et al., 1984).

RESULTS

Construction of probes and testing on homologous *T. cruzi* DNA — A library of total DNA of *T. cruzi* Y strain was constructed in the PstI site of pBR322. The clones obtained were initially tested on Southern blots of homologous DNA digested with either RsaI or EcoRI. The probes giving rise to clearly individualized bands were considered putative genetic markers capable of identifying different strains and isolates of *T. cruzi*, and further tested. Some of these are shown in Fig. 1. None of the probes obtained contained kDNA sequences.

Probing of *T. cruzi* laboratory strains — Selected probes were used to test *T. cruzi* strains Cl, Colombiana, Dm28, F and Y. DNA was digested with RsaI or EcoRI and gels were hybridized to the indicated probes (Figs 2 and 3). Different patterns of hybridization were observed. In some cases a very simple and conserved pattern was seen, going from the appearance of identical bands in all strains (probes pMYP-7, pMYP-P11b, pMYP-P32a) to slight differences in a few strains (probes pMYP-16N46, pMYP-R42, pMYP-R₁22). In other cases a complex pattern was observed (probes pMYP-B14, pMYP-R2_a2_a, pMYP-P38). The clone Dm28c was also tested with some probes and

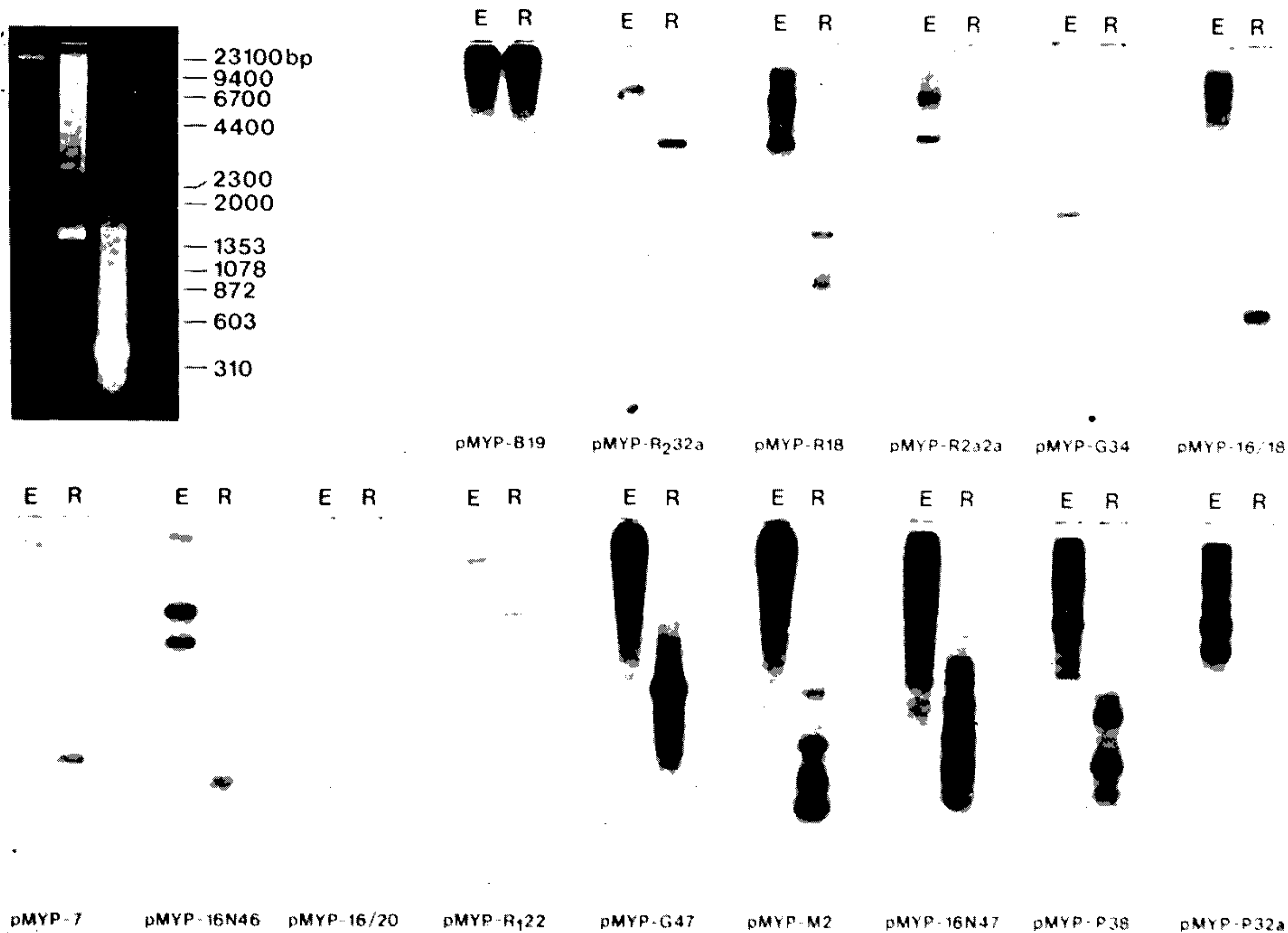


Fig. 1: testing probes on *Trypanosoma cruzi* strain Y DNA digested with RsaI and EcoRI. 5µg of total DNA was digested with 10u of the enzyme and run on a 0.8% agarose gel. A typical gel stained with ethidium bromide is shown. Replicas of the gel were transferred to nitrocellulose and hybridized to the indicated probes. E: EcoRI; R: RsaI.

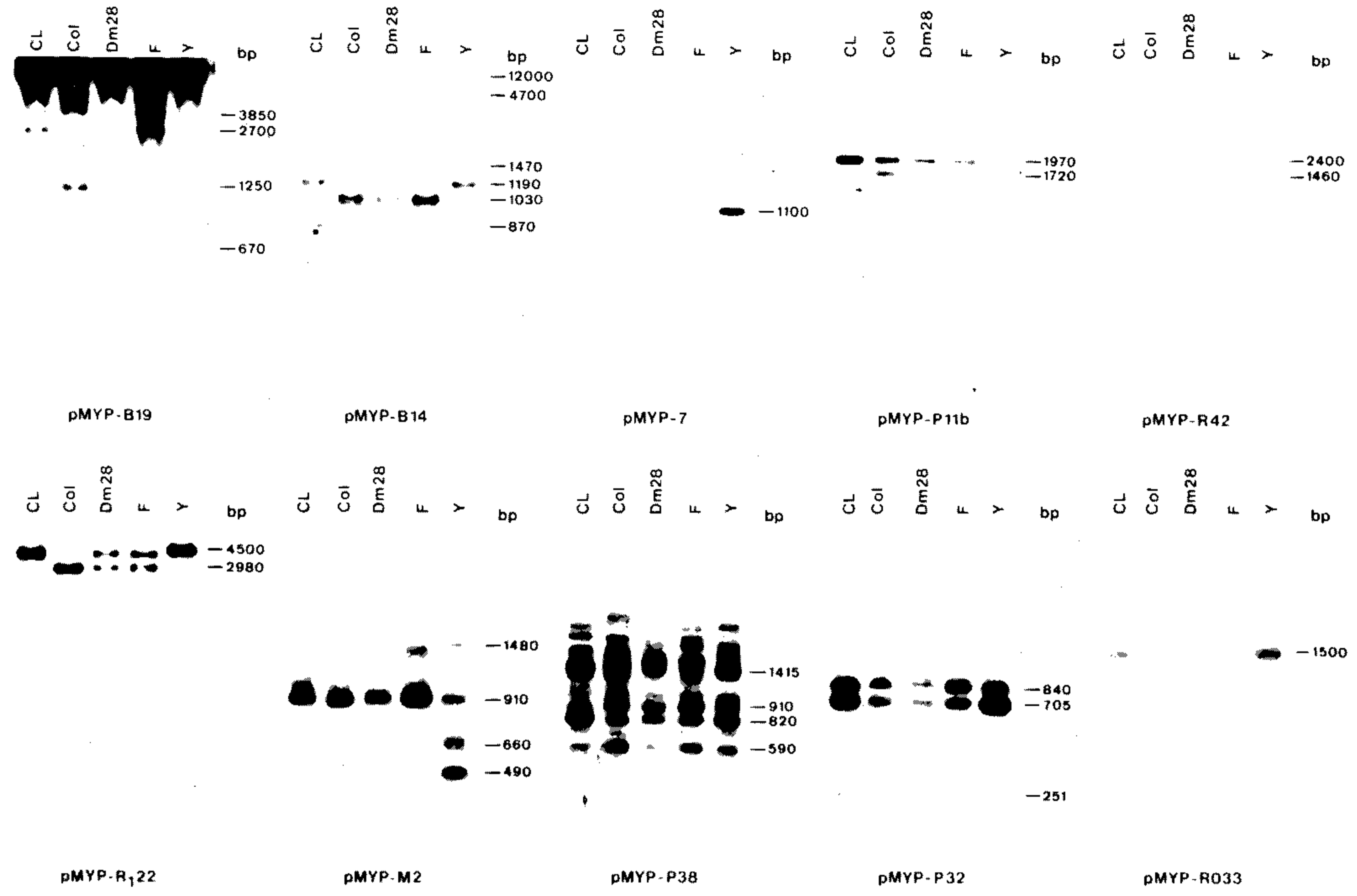


Fig. 2: testing of probes on *Trypanosoma cruzi* laboratory strains. 5µg of *T. cruzi* DNA of the indicated strains was digested with RsaI. Replicas of the gel were transferred and hybridized to the indicated probes.

showed a band pattern identical to the uncloned strain (Fig. 3).

Treatment of data — The strains have been compared using a numerical analysis of the band patterns obtained by hybridization. The patterns observed with 13 probes were compared using Jaccard's similarity index (Jaccard, 1908). Each band was treated as a unit character and band intensities were ignored. Using these indices, a matrix of association (Table II) and a dendrogram were constructed (Fig. 4). The SIM program was used for the dendrogram construction.

TABLE II

Nuclear DNA fragment pattern relationships within *Trypanosoma cruzi* strains

Strain	CL	Col	DM28	F	Y
CL	0.000				
Col	0.529	0.000			
DM28	0.529	0.694	0.000		
F	0.529	0.694	0.810	0.000	
Y	0.652	0.529	0.529	0.529	0.000

Dot-blots of *T. cruzi* and other trypanosomatids DNA — DNA of the five *T. cruzi* strains and of other trypanosomatids were applied to nitrocellulose and probed with pMYP-B19, pMYP-R₂32a, pMYP-B14, pMYP-16/18, pMYP-16N46, pMYP-RN11, pMYP-P11B, pMYP-M2, pMYP-16N30 and pMYP-P32a. Herring sperm DNA was also used as a control. Most of the probes recognized only *T. cruzi*, with none being strain specific. There were eventual intensity differences indicating quantitative but not qualitative differences. Only a few probes recognized trypanosomatids other than *T. cruzi*. pMYP-16N46 hybridized to *T. conorrhini* and *Crithidia fasciculata*. pMYP-M2 hybridized to *Phytomonas* and to *L. panamensis* (Fig. 5). These results were confirmed by hybridization to the Southern blots of these species, where bands — quite different from the ones observed with *T. cruzi* — were seen (data not shown).

Chromosome separation and hybridization — The OFAGE system of pulse field gel electrophoresis was used to separate chromosome sized DNA molecules of strains Colombiana, Dm28, F and Y. A large heterogeneity of band patterns was observed among the strains in an ethidium bromide stained gel (Figs 6A and B). The gels were trans-

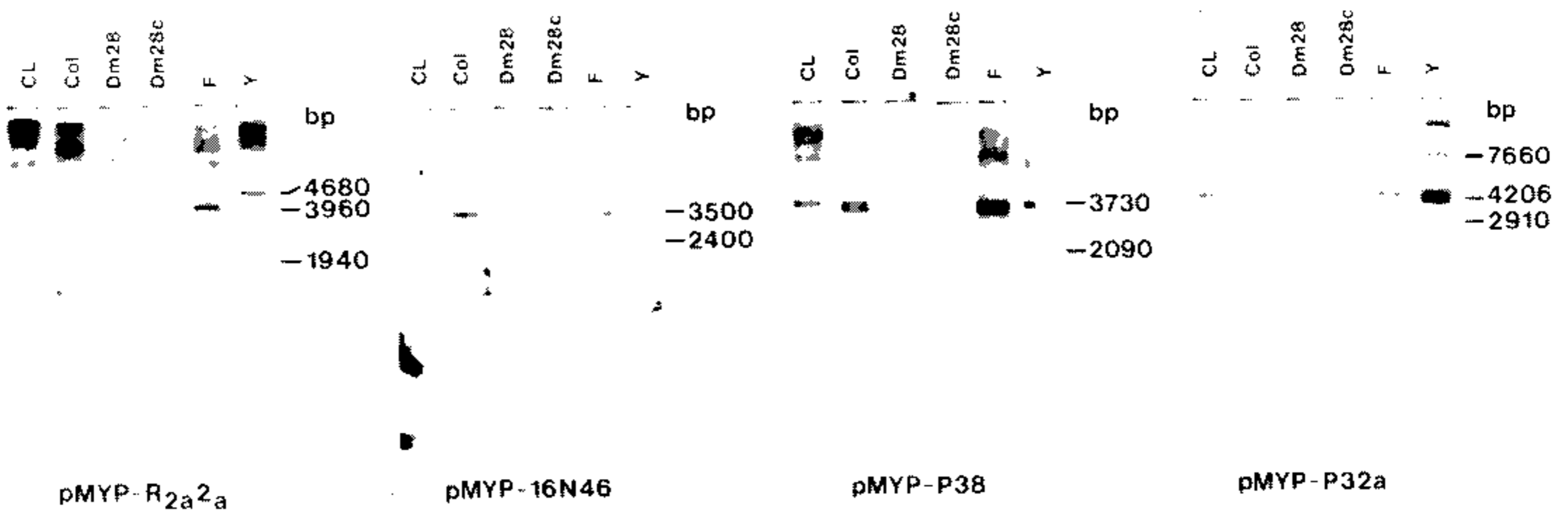


Fig. 3: testing of probes on *Trypanosoma cruzi* laboratory strains. Same as Fig. 2, but DNA was digested with EcoRI.

ferred and hybridized to different probes. Gel A was hybridized first to pMYP-R₁22, that in a Southern blot of RsaI cut DNA gives rise to two bands that are seen in all combinations in the five strains (Fig. 2). This probe hybridized to the chromosome compression zone in all strains and also to a smaller chromosome (800 kb) in the Y strain (Fig. 6A.1). This filter was dehybridized and probed with pMYP-M2, that in a Southern blot gives rise to a rather strong hybridization, that may be indicative of a repetitive sequence, with only strain Y giving rise to a pattern different from the other strains (Fig. 2). The hypothesis of a repetitive sequence was confirmed in the chromosome probing, as there is a very strong hybridization to the compression zone and also to different smaller chromosomes (Fig. 6A.2). Probe pMYP-P32a that hybridizes strongly to the same RsaI bands from all strains (Fig. 2) lights up many heterologous chromosome bands (Fig. 6B.1).

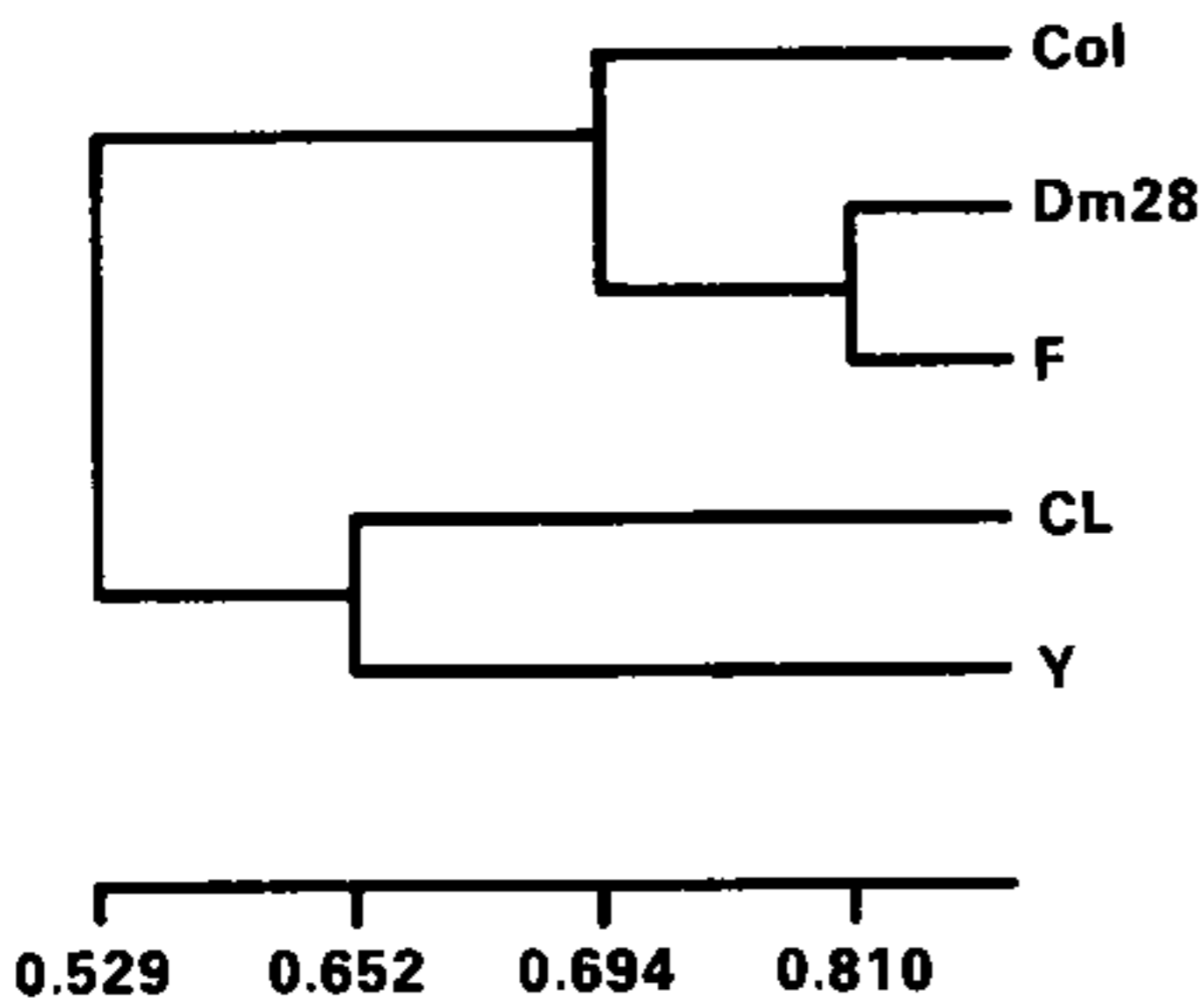


Fig. 4: dendrograms of DNA banding pattern similarities among the *Trypanosoma cruzi* strains.

DISCUSSION

Random probes generated by cloning PstI fragments of *T. cruzi* Y strain DNA recognized discrete bands on Southern blots of five laboratory strains of this parasite. The band patterns went from very simple and conserved to slightly different and finally to very complex patterns. By a numerical analysis of the data we could construct a similarity matrix and a dendrogram. These results agree with some published zymodeme studies. For instance, the so called polar strains CL and Y belong to zymodemes 2a and 2d respectively (Tibayrenc & LeRay, 1984), and also cluster closer together to each other than to the other strains as characterized by our probes. Strain Colombiana be-

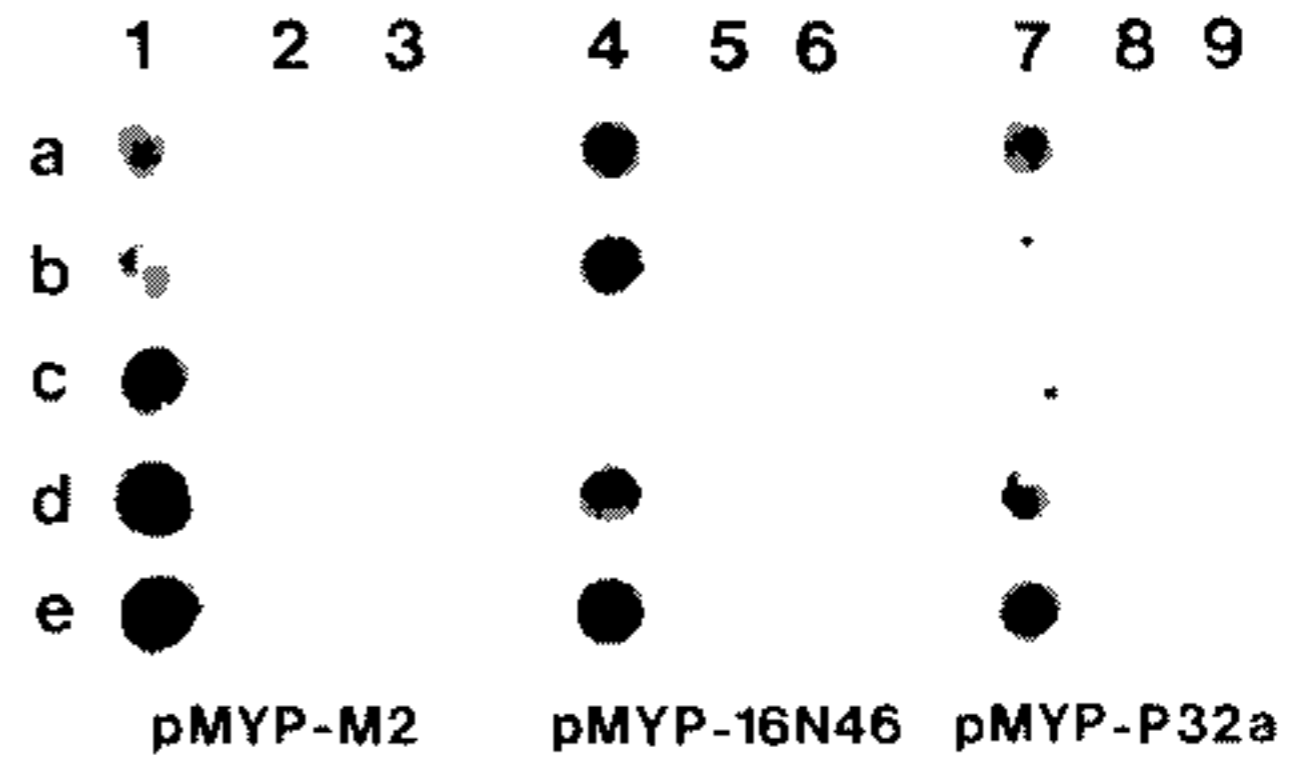


Fig. 5: dot blots of various strains of *Trypanosoma cruzi* and species of trypanosomatids. 1 µg of DNA from the indicated strains and species were applied to nitrocellulose filters and hybridized to the indicated probes. The parasites are: 1, 4 and 7, *T. cruzi* strains CL (a), Col (b), Dm28 (c), F (d), Y (e); 2 (a-e) are respectively *L. pifanoi*, *L. amazonensis*, *L. guyanensis*, *L. panamensis*, *Phytomonas sp.* (1TD); 3 (a-e) are *Phytomonas sp.* (1G), *Phytomonas sp.* (30T), *C. fasciculata*, *C. culicis*, control; 5 and 8 are *T. conorrhini* (a), *T. rangeli* (b), *C. fasciculata* (c), *Phytomonas* 1TD (d), 1G (e); 6 and 9 are *Phytomonas* 30T (a), *L. guyanensis* (b), *L. amazonensis* (c), *L. panamensis* (d), control (e).

longs to type III (Andrade et al., 1983) that correlates to zymodeme I (Miles et al., 1980), and it is expected that Dm28 (an opossum isolate from Venezuela) should also fall within this zymodeme, for the vast majority of sylvatic and human isolates in this country belong to Z1 (Miles et al., 1981). They also cluster quite closely in the dendrogram constructed.

These probes were in most cases specific for *T. cruzi* and in only a few cases recognized other trypanosomatids. These might represent repetitive sequences or conserved genes, and may be useful in phylogenetic studies. Cloned nuclear DNA fragments have been used in evolutionary studies in the genus *Leishmania* (Beverley et al., 1987). We found no strain specific probes.

To test the usefulness of these probes on the characterization of uncloned populations of parasites, we compared Dm28 to clone Dm28c. With four probes tested the patterns were identical between them. Strain Y was also compared to a clone, and no differences were seen (data not shown).

When we tested some of the probes on chromosomes separated by pulse field gel electrophoresis, we observed a more complex pattern of hybridization than that observed on the DNA fragments obtained by restriction enzymes. This was at first unexpected, but comes to confirm mounting evidence

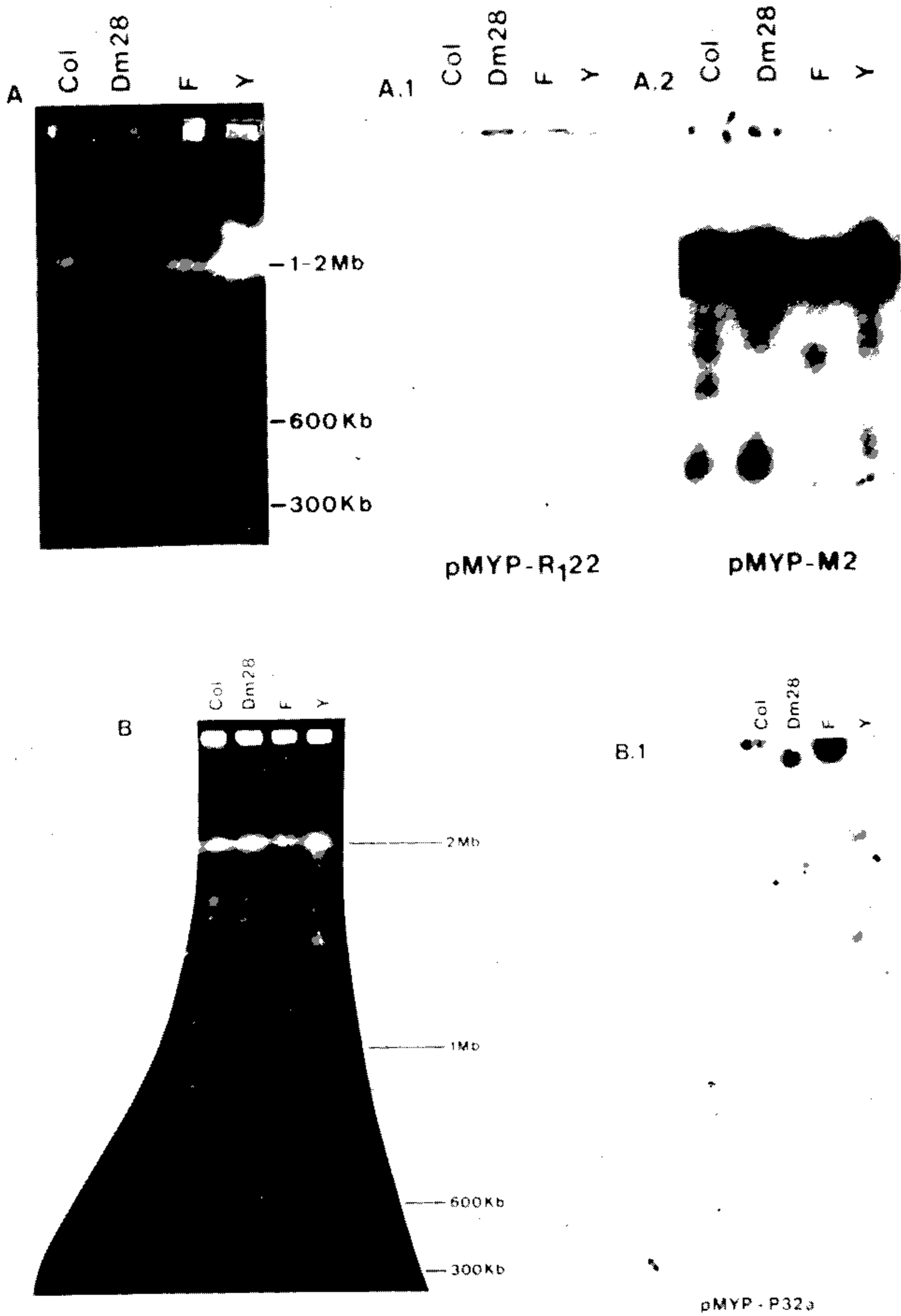


Fig. 6: size fractionation of *Trypanosoma cruzi* chromosomes separated by OFAGE. A – Pulse times: 150 sec for 16 h, 100 sec for 16 h and 50 sec for 16 h. Gel was transferred and hybridized to: A.1 – pMYP-R₁22 and A.2 – pMYP-M2. B – Pulse times: 240 sec for 24 h, 180 sec for 24 h and 120 sec for 24 h. Gel was transferred and hybridized to B.1 – pMYP-P32a.

for a large plasticity of trypanosomatid chromosomes (Engman et al., 1987). Variability seen at the chromosome level seems to be more at the level of schizodeme variability, and for that reason restriction fragment length polymorphisms might be more useful in taxonomical studies of these parasites.

The fact that comparisons between the use of cloned DNA probes and zymodeme analysis seem to agree, is another evidence for the accuracy of the proposed clonal derivation of populations of *T. cruzi* (Tibayrenc et al., 1986).

Having established the usefulness of cloned DNA probes in the characterization of *T. cruzi* strains, we are presently applying these probes to the analysis of isolates from different geographical origins, provenient from various hosts, including patients with the various forms of Chagas' disease.

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REFERENCES

- ANDRADE, V.; BRODSKYN, C. & ANDRADE, S. G., 1983. Correlation between isozyme patterns and biological behavior of different strains of *Trypanosoma cruzi*. *Trans. R. Soc. Trop. Med. Hyg.*, **7**: 796-799.
- BEVERLEY, S. M.; ISMACH, R. B. & MCMAHON-PRATT, D., 1987. Evolution of the genus *Leishmania* as revealed by comparisons of nuclear DNA fragment patterns. *Proc. Natl Acad. Sci. USA.*, **84**: 484-488.
- BRENER, Z. & CHIARI, E., 1963. Variações morfológicas observadas em diferentes amostras de *Trypanosoma cruzi*. *Rev. Inst. Med. Trop. São Paulo*, **5**: 220-224.
- CARLE, G. F. & OLSON, M. V., 1984. Separation of chromosomal DNA molecules from yeast by orthogonal-field-alteration gel electrophoresis. *Nucl. Acids Res.*, **12**: 5647-5664.
- CHIARI, E. & CAMARGO, E. P., 1984. Culturing and cloning of *Trypanosoma cruzi*, p. 23-26. In C. M. Morel, *Genes and Antigens of Parasites, A Laboratory Manual*. 2nd ed. UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases, FIOCRUZ, Rio de Janeiro, Brasil.
- CONRAD, P. A.; IAMS, K.; BROWN, W. C.; SOHANJAL, B. & OLE-MOI YOI, O. K., 1987. DNA probes detect genomic diversity in *Theileria parva* stocks. *Mol. Biochem. Parasitol.*, **25**: 213-226.
- DEANE, M. P. & KLOETZEL, J., 1974. Lack of protection against *Trypanosoma cruzi* by multiple doses of *Trypanosoma lewisi* culture forms. A discussion of some strains of "lewisi". *Exp. Parasitol.*, **35**: 406-410.
- DE SIMONE, G.; CARVALHO, S.; OLIVA, L. C. P.; ANDRADE, S. G. & GALVÃO-CASTRO, B., 1987. *Trypanosoma cruzi* strain-specific monoclonal antibodies: identification of Colombian strain flagellates in the insect vector. *Trans. R. Soc. Med. Hyg.*, **81**: 750-754.
- ENGMAN, D. M.; REDDY, L. V.; DONELSON, J. E. & KIRCHHOFF, L. V., 1987. *Trypanosoma cruzi* exhibits inter and intra-strain heterogeneity in molecular karyotype and chromosomal gene location. *Mol. Biochem. Parasitol.*, **22**: 115-123.
- FEDERICI, E. E.; ABELMAN, W. B. & NEVA, F. A., 1984. Chronic and progressive myocarditis and myositis in C₃H mice infected with *Trypanosoma cruzi*. *Am. J. Trop. Med. Hyg.*, **13**: 272-280.
- GOLDENBERG, S.; CONTRERAS, V. T.; SALLES, J. M.; FRANCO, M. P. A.; BONALDO, M. C.; VALLE, D.; GONÇALVES, A. M. & MOREL, C. M., 1984. Perspectives of vaccination against Chagas' disease through biotechnology. II. Gene expression in *Trypanosoma cruzi* trypomastigotes and cell-free translation of mRNA coding for relevant surface antigens, p. 442-459. G. L. Ada & G. Torrigiani (eds) in *New Approaches to Vaccine Development*.
- JACCARD, P. 1908. Nouvelles recherches sur la distribution florale. *Bul. Soc. Vaudoise Sci. Natur.*, **44**: 223-270.
- MANIATIS, T.; FRITSCH, E. F. & SAMBROOK, J., 1982. *Molecular cloning, a Laboratory Manual*. Cold Spring Harbor Laboratory, N. Y., 315p.
- MACCUTCHAN, T. F.; SIMPSON, A. J. G.; MULLINS, J. A.; NASH, T. E.; LEWIS, F. & RICHARDS, C., 1984. Differentiation of schistosomes by species, strain and sex using cloned DNA markers. *Proc. Natl Acad. Sci. USA.*, **81**: 889-893.
- MILES, M. A.; CEDILLOS, R. A.; PÓVOA, M. M.; SOUZA, A. A.; PRATA, A. & MACEDO, V., 1981. Do radically dissimilar *Trypanosoma cruzi* strains (zymodemes) cause Venezuelan and Brazilian forms of Chagas' disease? *Lancet* **1**: 1338-1340.
- MILES, M. A.; LAHAM, S. M.; SOUZA, A. A. & POVOA, M., 1980. Further enzymic characters of *Trypanosoma cruzi* and their evaluation for strain identification. *Trans. R. Soc. Trop. Med. Hyg.* **74**: 221-237.
- MOREL, C. M.; CAMARGO, E. P.; MATTEI, D. M.; ROMANHA, A. J. & SIMPSON, L., 1980. Strains and clones of *Trypanosoma cruzi* can be characterized by patterns of restriction endonuclease products of kinetoplast DNA minicircles. *Proc. Natl Acad. Sci. USA.* **77**: 6810-6814.
- OZAKI, L. S. & TRAUB-CSEKO, Y. M., 1984. Genomic DNA cloning and related techniques. p. 65-185. In C. M. Morel, *Genes and Antigens of Parasites. A Laboratory Manual*. 2nd ed. UNDP/World

- Bank/ WHO Special Programme for Research and Training in Tropical Diseases, FIOCRUZ, Rio de Janeiro, Brasil.
- PAINDAVOINE, P.; PAYS, E.; LAURENT, M.; GELTMAYER, Y.; LERAY, D.; MEHLITZ, D. & STEINERT, A., 1986. The use of DNA hybridization and numerical taxonomy in determining relationships between *Trypanosoma brucei* stocks and subspecies. *Parasitol.*, 92: 31-50.
- RIGBY, P. W. J.; DECKMAN, M.; RHODES, C. & BERG, P., 1977. Labelling of deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. *J. Mol. Biol.*, 113: 237-251.
- SILVA, L. H. P. DA & NUSSENZWEIG, V., 1953. Sobre uma cepa de *Trypanosoma cruzi* altamente virulenta para o camundongo branco. *Fol. Clin. Biol.*, 20: 191-208.
- SOUTHERN, E., 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.*, 98: 503-517.
- TIBAYRENC, M. & LERAY, D., 1984. General classification of the isoenzymic strains of *Trypanosoma (Schizotrypanum) cruzi* and comparison with *T. (S) C. marinkellei* and *T. (Herpetosoma) rangeli*. *Ann. Soc. Belge Méd. Trop.*, 64: 239-248.
- TIBAYRENC, M.; WARD, P.; MOYA, A. & AYALA, F. J., 1986. Natural populations of *Trypanosoma cruzi*, the agent of Chagas disease, have a complex multiclonal structure. *Proc. Natl Acad. Sci. USA*, 83: 115-119.
- TOYÉ, P. J., 1974. Isozyme variation in isolates of *Trypanosoma cruzi*. *Trans. R. Soc. Trop. Med. Hyg.*, 68: 147.
- VAN DER PLOEG, L. M. T.; SHWARTZ, D. C.; CANTOR, C. R. & BORST, P., 1984. Antigenic variation in *Trypanosoma brucei* analyzed by electrophoretic separation of chromosome-sized DNA molecules. *Cell*, 37: 77-84.