Molecular Karyotype and Chromosomal Localization of Genes Encoding β-tubulin, Cysteine Proteinase, hsp 70 and Actin in *Trypanosoma rangeli*

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The molecular karyotype of nine Trypanosoma rangeli *strains was analyzed by contour-clamped homogeneous electric field electrophoresis, followed by the chromosomal localization of β-tubulin, cysteine proteinase, 70 kDa heat shock protein (hsp 70) and actin genes. The* T. rangeli *strains were isolated from either insects or mammals from El Salvador, Honduras, Venezuela, Colombia, Panama and southern Brazil. Also,* T. cruzi *CL-Brener clone was included for comparison. Despite the great similarity observed among strains from Brazil, the molecular karyotype of all* T. rangeli *strains analyzed revealed extensive chromosome polymorphism. In addition, it was possible to distinguish* T. rangeli *from* T. cruzi *by the chromosomal DNA electrophoresis pattern. The localization of β-tubulin genes revealed differences among* T. rangeli *strains and confirmed the similarity between the isolates from Brazil. Hybridization assays using probes directed to the cysteine proteinase, hsp 70 and actin genes discriminated* T. rangeli *from* T. cruzi, *proving that these genes are useful molecular markers for the differential diagnosis between these two species. Numerical analysis based on the molecular karyotype data revealed a high degree of polymorphism among* T. rangeli *strains isolated from southern Brazil and strains isolated from Central and the northern South America. The* T. cruzi *reference strain was not clustered with any* T. rangeli *strain.*

Key words: *Trypanosoma rangeli* - karyotype - pulsed field gel electrophoresis - housekeeping genes - genetic variability - phenogram

Trypanosoma rangeli (Kinetoplastida: Trypanosomatidae) is a parasite that can infect both domestic and wild mammals and triatomine insects. Its transmission to the vertebrate host mainly occurs by inoculation of infective metatrypomastigotes formed in the salivary glands of the insects. The geographical distribution of *T. rangeli* is overlapped with *T. cruzi*, the causative agent of Chagas disease, and comprises almost all the countries of South and Central America (Grisard et al. 1999b). Reports of *T. rangeli* in Brazil have been made from the northern region of the Amazon basin to the southern country, precisely in the State of Santa Catarina (Miles et al. 1983, Steindel et al. 1991, Coura et al. 1996). In contrast to *T. cruzi*, *T. rangeli* is considered non-pathogenic to the vertebrate host. However, *T. rangeli* is pathogenic to its insect vectors (D'Alessandro & Saravia 1992). Single or mixed infections in both vertebrate and invertebrate hosts by these two trypanosome species might be expected and reinforces the needs of a correct specific identification.

High levels of cross reactivity between *T. rangeli* and *T. cruzi* observed in different immunological assays have been reported and may lead to misdiagnosis (Cuba Cuba 1998). These species can be distinguished by the morphology of some developmental stages (Vallejo et al. 1988, reviewed by Sousa 1999). The classic method for identification of *T. rangeli* is the finding of typical parasite forms in the haemolymph and/or salivary glands of infected triatomines and its transmission to susceptible hosts through biting (D'Alessandro 1976). In addition, biochemical, immunological and molecular methods have been used to characterize *T. rangeli* strains and to differentiate it from *T. cruzi*, such as isoenzyme electrophoresis (Miles et al. 1983), lectin agglutination (Schottelius et al. 1986),

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monoclonal antibodies (Acosta et al. 1991), polymerase chain reaction (PCR) (Coura et al. 1996) and pulsed field gel electrophoresis (PFGE) (Henriksson et al. 1996b).

A low degree of polymorphism has been shown by isoenzyme analysis of different *T. rangeli* isolates from the Brazilian Amazon basin (Miles et al. 1983), Colombia (Holguín et al. 1987), and Honduras (Acosta et al. 1991). On the other hand, strains from Panama revealed a high polymorphism by the same method (Kreutzer & Souza 1981). Using DNA fingerprinting and RAPD analysis, Macedo et al. (1993) and Steindel et al. (1994) have demonstrated that *T. rangeli* isolated in southern Brazil were genetically distinct from *T. rangeli* isolated in Honduras, Colombia and Venezuela. These results are in accordance with previous results obtained by isoenzyme analysis of the same samples (Steindel et al. 1992). Recently, a review corroborated these findings based on results of indirect immunofluorescence, lectin agglutination, isoenzyme electrophoresis, RAPD, triatomine susceptibility and mini-exon gene analysis (Grisard et al. 1999b).

PFGE-based molecular karyotyping of several protozoan parasites such as *Plasmodium* sp. (Kemp et al. 1987), *T. cruzi* (Henriksson et al. 1990) and *Leishmania* sp. (Cruz et al. 1993) revealed a high degree of intraspecific polymorphism.

Molecular karyotyping associated with gene localization within chromosomes has been used as tools for identification and characterization of isolates and clones of trypanosomatids (Cano et al. 1995, Henriksson et al. 1996a). On this field, Henriksson et al. (1996b) have analyzed a reasonable number of *T. rangeli* samples using molecular karyotyping.

In this work *T. rangeli* strains isolated from distinct vectors, reservoirs and geographical regions of Central and South America were analyzed by contour-clamped homogeneus electric field (CHEF) electrophoresis and hybridized with housekeeping genes probes.

MATERIALS AND METHODS

Parasites - Nine *T. rangeli* strains isolated from different geographical regions of South and Central America and the *T. cruzi* CL-Brener clone were analyzed in this study. The parasite strains, geographical origins and sources are shown in the Table. All strains were maintained at 28ºC in NNN + LIT supplemented with 20% heat-inactivated fetal bovine serum through weekly passages.

Preparation of chromosomal DNA - Parasites were harvested by centrifugation at 1,500 x g at 4ºC. The pellets were washed three times with PSG buffer (75 mM buffer phosphate pH 8.0, 65 mM NaCl, 1% glucose) and resuspended in PSG at 5 x 10^8 cells/ml. An equal volume of 1.5% low melting point agarose in PS buffer (PSG without glucose), pre-warmed to 37ºC, was added to the cells. The cell/agarose mixture was immediately distributed into a block mould generating small blocks of 2 x 5 x 4 mm (at 4ºC), containing approximately 10^7 parasites/block. Once solidified, the blocks were incubated with lysis solution (1% sodium laurylsarcosinate, 1 mg/ml proteinase K, 0.5 M EDTA pH 9.0) at 48ºC for 48 h, and stored in 50 mM EDTA pH 8.0 at 4ºC.

PFGE - Chromosomal DNA was electrophorezed at 12ºC using a CHEF-DRIII® system (Bio-Rad, Richmond, USA). The different running conditions necessary to obtain a fine resolution for each chromosomal size class are described in the legend of Fig. 1.

As molecular size markers, chromosomes from *Saccharomyces cerevisiae* strain YNN295 and *Hansenula wingei* strain YB-4662-VIA (Bio-Rad, Richmond, USA) were used. Gels were stained with 0.5 µg/ml ethidium bromide for 20 min and

TABLE

Strains, hosts, geographical origins and sources of *Trypanosoma cruzi* and *T. rangeli*

Species	Code	Host	Strain/Clone	Origin	Source
T. cruzi	CT-IOC 005	*T. infestans*	CL-Brener clone	Brazil	CT-IOC
T. rangeli	CT-IOC 002	Human	R-1625	El Salvador	CT-IOC
T. rangeli	CT-IOC 174	Human	H-9	Honduras	Dr A Romanha - CPqRR
T. rangeli	CT-IOC 038	Human	H-14	Honduras	CT-IOC
T. rangeli	CT-IOC 273	Human	Macias	Venezuela	Dr M Steindel - UFSC
T. rangeli	CT-IOC 271	*R. prolixus*	Choachi	Colombia	Dr M Steindel - UFSC
T. rangeli	CT-IOC 270	*E. dasithryx*	SC-58	Brazil	Dr M Steindel - UFSC
T. rangeli	CT-IOC 272	*E. dasithryx*	SC-61	Brazil	Dr M Steindel - UFSC
T. rangeli	CT-IOC 158	Human	Pepita Gonzales	Panama	CT-IOC
T. rangeli	CT-IOC 275	Human	San Agustin	Colombia	Dr A Romanha - CPqRR

CT-IOC: Coleção de Tripanosomatídeos, Instituto Oswaldo Cruz, Fiocruz, Rio de Janeiro, Brazil; CPqRR: Centro de Pesquisas René Rachou, Belo Horizonte, Brazil; UFSC: Universidade Federal de Santa Catarina, Brazil

photographed using an UV transilluminator using Polaroid 55 films. DNA was transferred to nylon filters (Hybond N®, Amersham, Sweden) by standard techniques (Southern 1975).

Probes and hybridization assays - The following probes were used: a 3.8 Kb fragment of the *L. major* β-tubulin gene – clone pLT-1 (Huang et al. 1984), the Lpcys2 cysteine proteinase gene of *L. pifanoi* (Traub-Cseko et al. 1993), a 0.4 Kb EcoRI fragment containing *T. cruzi* hsp 70 coding sequence (De Carvalho et al. 1990) and a sequence of the *T. cruzi* actin gene (Paixão 1995). These probes were radiolabeled with [α-32P]dCTP by the random priming method (Rediprime kit®, Amersham, Sweden). The nylon filters were prehybridized at 42ºC for 3 h in 6 x SSC (1 x SSC = 150 mM NaCl, 15 mM Na3C6H5O7), 5 x Denhardt's solution (0.2% bovine serum albumin, 0.2% polyvinylpyrrolidone, 0.2% Ficoll), 0.5% SDS, 100 µg/ml of sonicated and denatured salmon sperm DNA and 50% formamide. Hibridization was performed overnight at 42ºC. After that, filters were washed three times for 30 min using 1 x SSC, 0.5 % SDS at 42ºC and exposed overnight at -70ºC to Kodak X-Omat® film in intensifying screens.

Numerical analysis - A schematic diagram showing the molecular karyotype of all strains studied was constructed. From these karyotype profiles that could be identified with confidence on the basis of their intensity and individuality, a numerical matrix based on the presence and absence of bands was built.

This data was compared using Jaccard's similarity coefficient to determine the proportion of mismatched bands between strains. Based on the similarity matrix, a dendrogram was built using the unweighted pair-group method (UPGMA). These numerical analysis were performed using the NTSYS-pc® software (Version 1.70, Exeter Software, Setauket, USA).

RESULTS

Molecular karyotype analysis - The result of the molecular karyotype analysis of nine *T. rangeli* strains and one *T. cruzi* strain by CHEF electrophoresis using four distinct conditions is shown in Fig. 1 and represented in Fig. 2. The ethidium bromide stained gels revealed that each strain has a unique karyotype, although the two strains from Brazil (SC-58 and SC-61) show a high similarity between each other. Some features are specific of those strains, as the presence of a small band of 390 Kb (Fig. 1A). This band is smaller than the observed for the other *T. rangeli* strains (450 Kb) as well as for the *T. cruzi* reference strain (550 Kb). A variable absence of bands was noted in each strain, ranging between 750 and 1050 Kb, clearly visible in strains H-9; H-14; Macias and Choachi (Fig.1A). Another variable absence of bands above 1400 Kb is suggested in Fig. 1B and 1C and is evident in Fig.1D in the range of 1400 Kb-2500 Kb in all strains of *T. rangeli*. In the range between 2350 - 2700 Kb we observed a single chromosomal band in all *T. rangeli* strains, except in Pepita Gonzales, while in *T. cruzi* CL-Brener clone we detected two bands in the same range (Fig. 1D). In this Fig., the presence of DNA is noted in the compression zone corresponding to chromosomes larger than 3130 Kb that are not separated by this electrophoretic condition.

Chromosomal localization of genes - The chromosomal localization of β-tubulin, hsp 70, actin and cysteine proteinase genes in *T. rangeli* strains and in the *T. cruzi* CL-Brener clone obtained through Southern blots of CHEF gels is presented on a diagram (Fig. 2). In this diagram hatched boxes indicate those bands that showed hybridization with the four studied genes. The diagram was constructed based on ethidium bromide stained gels and represents the molecular karyotypes containing chromosomes smaller than 2700 Kb.

Chromosomal localization of β-tubulin genes allowed the separation of *T. rangeli* strains in two distinct groups, while cysteine proteinase, hsp 70 and actin genes were useful to differentiate *T. rangeli* from *T. cruzi*.

ß-tubulin - *T. cruzi* CL-Brener clone as well as six out of nine *T. rangeli* strains (R-1625, H-9, H-14, Macias, Choachi and San Agustin) hybridized with the β-tubulin gene probe in the compression region above 1000 Kb (Fig. 3A).

The *T. rangeli* strains SC-58, SC-61 and Pepita Gonzales hybridized with the β-tubulin gene probe in chromosomal bands of 690 and 720 Kb (SC-58); 690 Kb (SC-61) and 650 Kb (Pepita Gonzales) and also showed a weak hybridization in the compression zone (Fig. 3A).

Cysteine proteinase - Hybridization with the cysteine proteinase gene probe revealed bands of approximately 500 Kb in R-1625, H-9, H-14, Macias, Choachi, SC-58, SC-61 and San Agustin *T. rangeli* strains. All *T. rangeli* strains and *T. cruzi* hybridized with this probe at the compression region (Fig. 3B).

Hsp 70 - The presence of the hsp 70 genes was observed in *T. rangeli* strains in two chromosomal bands very close to each other, ranging from 950 Kb in Pepita Gonzales strain to 1200 Kb in R-1625 strain. In *T. rangeli* H-14 and SC-61 strains the hsp 70 genes were detected in an unique chromosomal band of 1125 and 1050 Kb, respectively. In *T. cruzi*, those genes were observed in a single 1500 Kb chromosomal band (Fig. 3C).

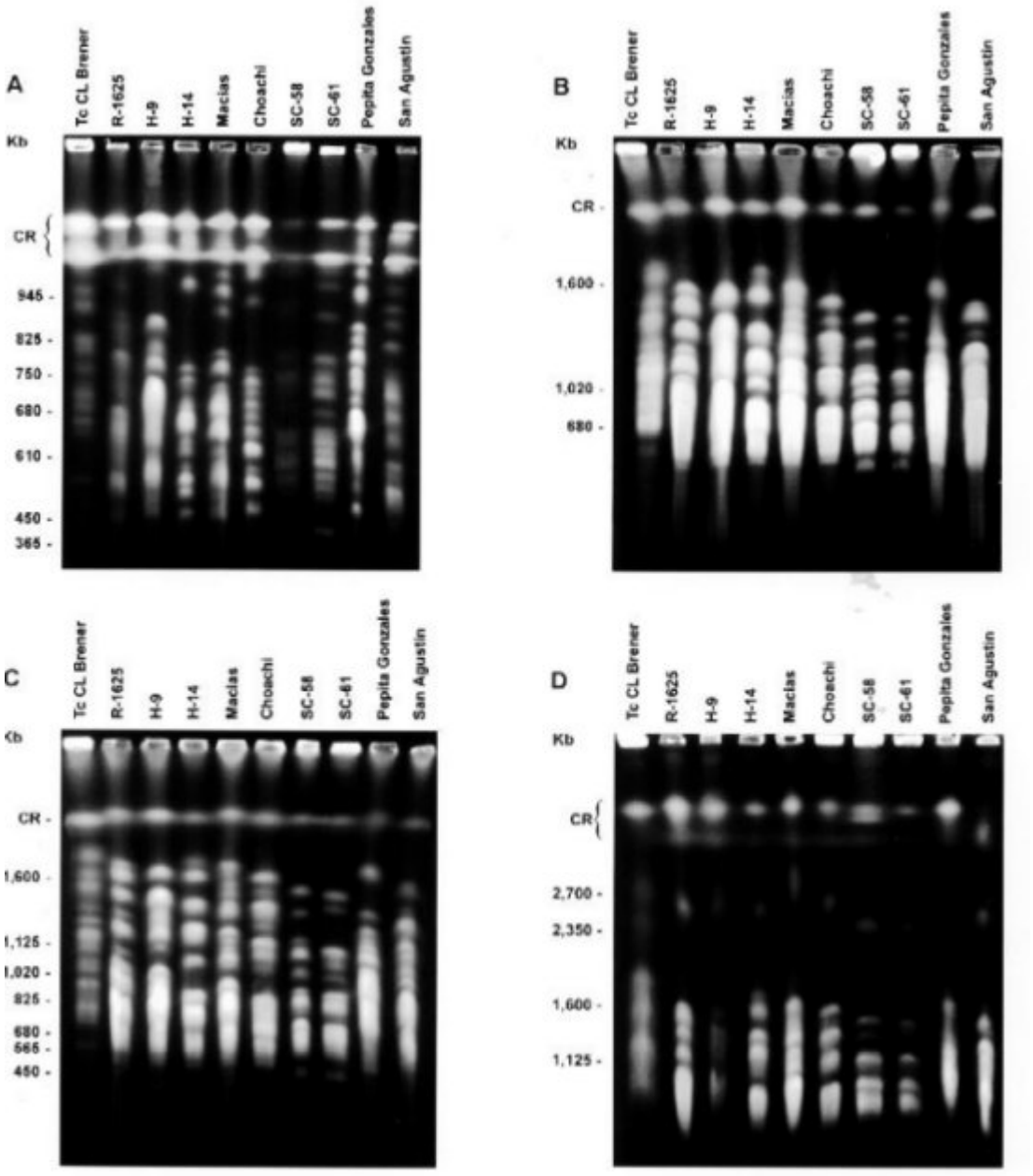

Fig. 1: molecular karyotype of *Trypanosoma rangeli* and *T. cruzi* isolates revealed after ethidium-bromide staining of contour-clamped homogeneous electric field electrophoresis gel. Separation of chromosomes smaller than 1000 Kb was achieved using a 1.5% agarose gel in 0.5x TBE (45 mM Tris/45 mM boric acid/1 mM EDTA pH 8.0), pulse ramp: 100-50 s running time: 40 h field strength: 5.5 V/cm field angle: 120º (A). For chromosomes between 1000 and 2200 Kb two distinct conditions were used: a 1% agarose gel in 0.5x TBE, pulse ramp: 200-100 s running time: 24 h field strength: 5.5 V/cm field angle: 120º (B) and a 1% agarose gel in 0.5 x TBE, pulse ramp: 200-50 s running time: 24 h field strength: 5.5 V/cm field angle: 120º (C). For chromosomes larger than 2200 Kb a 0.8% agarose gel in 1x TAE (40 mM Tris/40 mM glacial acetic acid/1 mM EDTA pH 8.0), pulse ramp: 500 s running time: 48 h field strength: 3.0 V/cm field angle: 106º (D) was used. The identities of samples are indicated on top of the gels, the *T. cruzi* sample is indicated with Tc, CR is compression region and the sizes of *Saccharomyces cerevisiae* and *Hansenula wingei* chromosomes are indicated on the left in kilobases pairs (Kb).

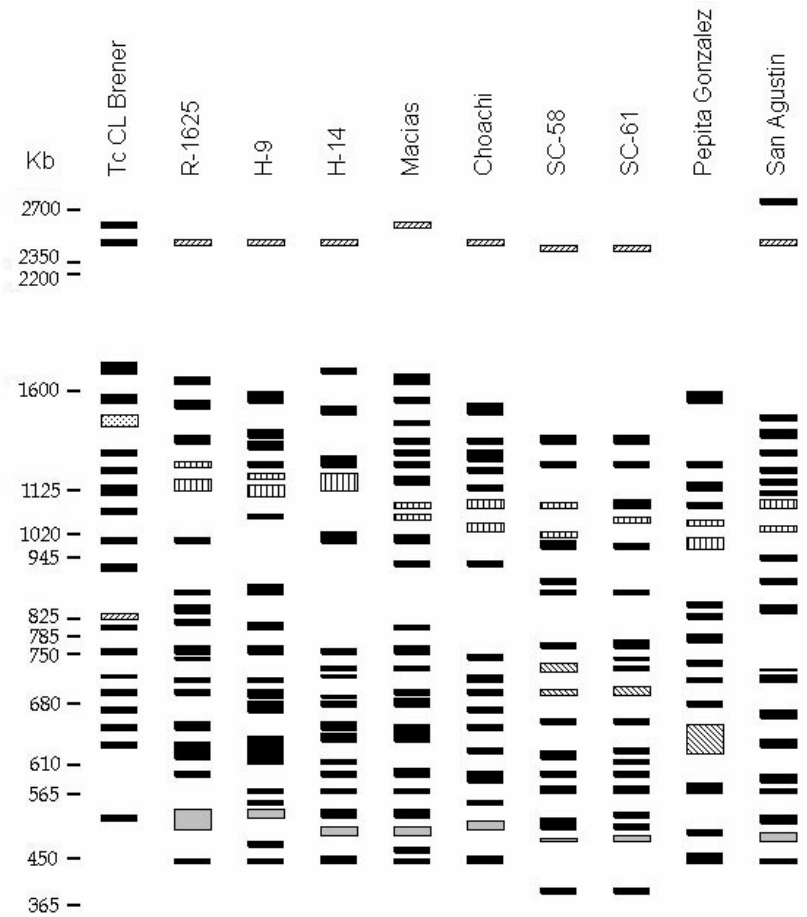

Fig. 2: diagrammatic representation of *Trypanosoma rangeli* and *T. cruzi* karyotypes. The identities of samples are indicated on top of the Figure. The *T. cruzi* sample is indicated with Tc. Chromosomes of *Saccharomyces cerevisiae* and *Hansenula wingei* were used as references and are indicated on the left in kilobases pairs (Kb). Chromosomal localization of housekeeping genes studied on this paper are shown by hatched boxes: β-tubulin; cysteine proteinase; hsp 70; actin; hsp 70 and actin (the same localization).

Actin - Actin genes were detected in 825 Kb and 1500 Kb chromosomal bands in *T. cruzi* and also in the compression region above 2200 Kb for all *T. rangeli* strains (Fig. 3D). Using electrophoretic conditions that separate the chromosomes larger than 2200 Kb we detected actin genes in an unique chromosomal band with sizes between 2350 and 2700 Kb in *T. rangeli* strains, except for Pepita Gonzales (data not shown) (Fig. 2).

Our results revealed the presence of hsp 70 and actin genes in the same 1500 Kb band in *T. cruzi* CL-Brener clone (Fig. 2), but despite the size we could not affirm that they are in the same chromosome.

Numerical analysis of molecular karyotypes - Among different coefficients of similarity tested, Jaccard's coefficient showed to be the most adequate. Using data from the similarity matrix a dendrogram was constructed using UPGMA (Fig. 4). This phenetic tree showed that *T. rangeli* strains and *T. cruzi* CL-Brener clone were clearly clustered in different branches, indicating that *T. cruzi* and *T. rangeli* are genetically very distant. Among *T. rangeli* strains two distinct groups were formed: one containing the Colombian strains, Choachi and San Agustin (sub-branch 1) and another containing the other *T. rangeli* strains (sub-branch 2).

Sub-branch 2 was divided in other two subgroups: sub-branch 2A, including *T. rangeli* R-1625, H-9, H-14, Macias and Pepita Gonzales strains and sub-branch 2B formed by *T. rangeli* strains isolated from the State of Santa Catarina, Brazil (SC-58 and SC-61), which showed similar molecular karyotypes (Fig. 1).

Except for *T. rangeli* strains isolated from southern Brazil (SC-58 and SC-61), the molecular karyotype analysis among all *T. rangeli* strains revealed low similarity.

DISCUSSION

The molecular karyotype of nine *T. rangeli* strains was analyzed by CHEF electrophoresis using four different electrophoretic conditions and revealed the presence of a great variability in the number and size of chromosomal bands. Henriksson et al. (1996b) detected chromosomal polymorphism in several *T. rangeli* strains, including some used in this work. The authors suggested that more than 20 chromosomal bands may be present in the different strains. Tanaka et al. (1994)

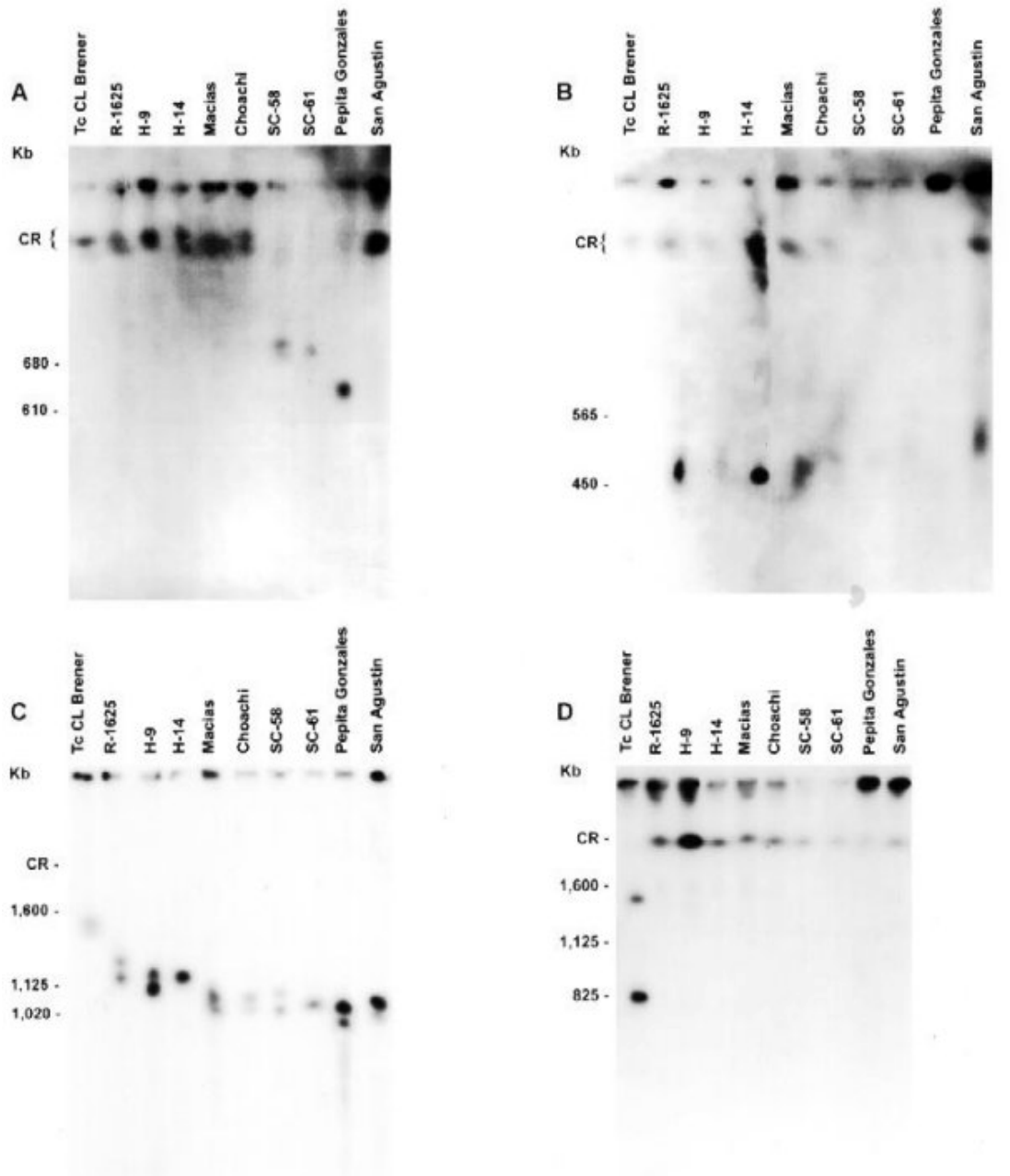

Fig. 3: chromosomal localization of β-tubulin, cysteine proteinase, hsp 70 and actin genes in *Trypanosoma rangeli* and *T. cruzi*. Southern blot hybridization with β-tubulin probe (A), cysteine proteinase probe (B), hsp 70 probe (C) and actin probe (D). The identity of samples, the chromosomes sizes and the electrophoretic conditions are described in the legend of Fig. 1.

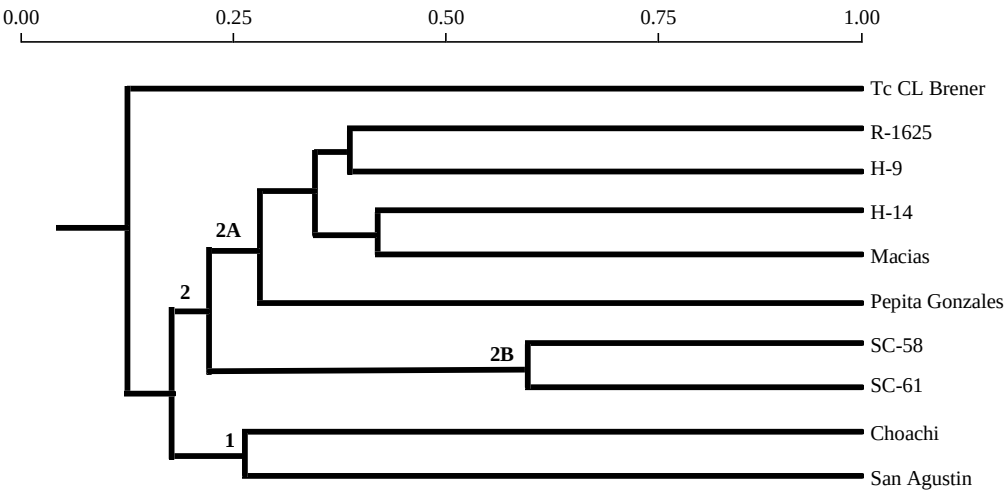

Fig. 4: phenogram produced by the unweighted pair-group method and Jaccard's similarity coefficient based on the molecular karyotype of *Trypanosoma rangeli* strains and the *T. cruzi* CL-Brener clone. For strains description, please refer to Table.

observed 14 chromosomal bands in a single *T. rangeli* strain studied.

The nine *T. rangeli* strains studied in the present work showed a variable number of chromosomal bands, from 16 to 24, with sizes ranging from 390 Kb to 3130 Kb. Also, chromosomal bands over 5700 Kb were observed as previously reported (Henriksson et al. 1996b) (data not shown). The absence of such large chromosomes in the *T. cruzi* reference clone is in agreement with other authors findings (Cano et al. 1995, Henriksson et al. 1995).

The karyotype variability observed in *T. rangeli* is characterized by the differences in both number and size of chromosomal bands among the different strains.

Thick chromosomal bands observed in all karyotypes may represent chromosomes of closely related size, which was not separated under our CHEF running conditions. Therefore, the total chromosome number may be higher than the observed in this study.

Another evidence of the complexity of the *T. rangeli* karyotype is the absence of chromosomes in some karyotype's regions which vary according to the strain analyzed. This observation can be clearly seen when comparing the karyotypes of *T. rangeli* strains isolated from Honduras (H-9 and H-14), Venezuela (Macias) and Colombia (Choachi).

Housekeeping genes fragments or anonymous markers have been used as probes to map *T. cruzi* chromosomes (Cano et al. 1995). Some of these DNA probes have shown to be useful markers to distinguish *T. cruzi* from *T. rangeli* strains (Henriksson et al. 1996b). In the present work the chromosomal localization of cysteine proteinase, hsp 70 and actin genes allowed us to distinguish *T. cruzi* from *T. rangeli* strains.

The localization of β-tubulin genes separated *T. rangeli* strains in two distinct groups: one comprising the Brazilian strains (SC 58 and SC 61) and the Pepita Gonzales strain from Panama, and another comprising strains isolated from Honduras, El Salvador, Venezuela and Colombia. In the first group (SC-58, SC-61 and Pepita Gonzales strains) β-tubulin genes were detected in chromosomal bands from 650 to 720 Kb, while in the second one, β-tubulin genes were present in chromosomal bands above 1000 Kb. A weak hybridization with this gene probe was observed in the compression zone of gels (above 1000 Kb) in *T. rangeli* SC 58, SC 61 and Pepita Gonzales strains, that may represent a small number of gene copies in a larger chromosome. This polymorphism observed among *T. rangeli* strains is in agreement with early reports from Macedo et al. (1993) using DNA fingerprinting, Steindel et al. (1994) using isoenzyme electrophoresis and RAPD, and Grisard et al. (1999a) using the mini-exon gene sequence analysis.

Cysteine proteinase genes were detected in *T. cruzi* CL-Brener clone only in large chromosomes (above 1000 Kb). In contrast, we observed these genes in *T. rangeli* in large chromosomes and also in a 500 Kb chromosomal band, as previously described by Tanaka et al. (1994). One exception was Pepita Gonzales strain that showed these genes only in large chromosomes above 1000 Kb. This variation in terms of chromosomal localization of cys-

teine proteinase genes in different *T. rangeli* strains has already been pointed out by Martinez et al. (1995).

The hsp 70 genes were localized in *T. rangeli* strains in one or two chromosomal bands varying from 950 to 1200 Kb, whereas in the *T. cruzi* CL-Brener clone these genes were detected in a single 1500 Kb chromosomal band. Most of *T. rangeli* strains hybridized with the hsp 70 gene probe in two bands very close to each other.

The same was observed with the β-tubulin probe in a *T. rangeli* strain. These hybridization assays suggest that there are homologous chromosome pairs with different sizes in *T. rangeli*. As observed in *T. cruzi* and *Leishmania* sp., size polymorphisms between homologous chromosomes seems to be a very frequent phenomenon among trypanosomatids (Cruz et al. 1993, Henriksson et al. 1995).

T. rangeli actin genes are localized in a chromosomal band ranging from 2350 to 2700 Kb, while in *T. cruzi* CL-Brener clone they were detected in two bands of 825 Kb and 1500 Kb.

Although phenetic analysis of *T. rangeli* strains revealed a clear individuality, strains from the same or close geographical areas showed a greater similarity coefficient. Our results are in agreement with previous data based on DNA fingerprinting, RAPD and mini-exon gene sequences analysis that showed that *T. rangeli* populations from Southern Brazil are distinct from *T. rangeli* from Colombia, Venezuela and Honduras (Macedo et al. 1993, Steindel et al. 1994, Grisard et al. 1999a, Stevens et al. 1999).

Natural geographic barriers such as the Amazon rain forest and the Andes, as well as different triatomine vector species may have contributed for the polymorphism observed among strains isolated from Santa Catarina in Southern Brazil and the other *T. rangeli* strains from northern South and Central America (Macedo et al. 1993).

Since sexual reproduction is not known in these species other studies using a representative number of strains and as several markers as possible will be necessary in order to better understand the genetic variability of *T. rangeli*.

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