Comparisons of molecular karyotype and RAPD patterns of anuran trypanosome isolates during long-term *in vitro* cultivation

Zhao-Rong Lun\(^1\) and Sherwin S. Desser\(^2\)\(^*\)

\(^1\)Department of Biology and Chemistry, City University of Hong Kong, Kowloon, Hong Kong; 
\(^2\)Department of Zoology, University of Toronto, Toronto, Ontario, Canada M5S 3G5

Key words: molecular karyotyping, random amplified polymorphic DNA, anuran trypanosomes, *in vitro* cultivation

Abstract. The patterns of random amplified fragments and molecular karyotypes of 12 isolates of anuran trypanosomes continuously cultured in *in vitro* were compared by random amplified polymorphic DNA (RAPD) analysis and pulsed field gradient gel electrophoresis (PFGE). The time interval between preparation of two series of samples was one year. Changes were not observed in the number and size of sharp, amplified fragments of DNA samples from both series examined with the ten primers used. Likewise, changes in the molecular karyotypes were not detected between the two samples of these isolates. These results suggest that the molecular karyotype and the RAPD patterns of the anuran trypanosomes remain stable after being cultured continuously *in vitro* for one year.

---

Division of bloodstream trypomastigotes, like that recorded for the African trypanosomes, has not been observed in anuran hosts, which probably accounts for the low parasitaemia values recorded for these parasites (Martin 1993). Anuran trypanosomes do, however, undergo extensive cyclic changes and prolific multiplication in their leech hosts and *in vitro* (Diamond 1965, Martin and Desser 1991). Pronounced ultrastructural differences between culture and leech forms of *Trypanosoma fallisii* Martin et Desser, were attributed to environmental factors, such as the use of bovine instead of bufonid blood in culture, the higher nutrient level in culture during the exponential growth phase of the parasites, and the absence of microorganisms in culture as reported by Martin and Desser (1991).

Several recent studies have indicated that changes of genetic characters, including genetic exchange, rearrangement of chromosomes, mutation and loss of infectivity to their hosts, occur in species of *Trypanosoma, Leishmania* and *Plasmodium* during *in vitro* cultivation (Van der Plouw et al. 1984a, 1985, Kemp et al. 1985, Zweygarth et al. 1990, Blaineau et al. 1991, Schweizer et al. 1991). This study was designed to determine whether the molecular karyotype and RAPD patterns of several species and isolates of anuran trypanosomes remain stable during long-term *in vitro* cultivation.

---

**MATERIALS AND METHODS**

*In vitro* cultivation. Details on the isolates of anuran trypanosomes used in the study are shown in Table 1. All isolates used in this study have been cloned (Martin et al. 1992) with exception of the American Type Culture Collection (ATCC) isolate, *Trypanosoma ranarum* Lankester (ATCC 30040). All isolates were continuously cultured in 50 ml Falcon tissue culture flasks containing 5 ml slants of whole citrated sheep blood agar prepared according to a modification of the method of Tobie et al. (1950) using an overlay of 5 ml of Eagle's minimum essential medium, supplemented with 5% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin at 25°C. The highest density of these anuran trypanosome isolates in *in vitro* cultivation was from 4.6–7.1 × 10^7 and the generation time was from 5 to 8.5 hours depending on the isolates. New cultures from the density of 10^7/ml became totally saturated in 3 to 5 days. Each isolate was subcultured weekly with a concentration of 5 × 10^7 trypanosomes/ml.

DNA extraction and amplification. Cultured trypanosomes were collected and washed twice with PBS (pH 7.2) by centrifugation at 1000 g for 10 minutes at 4°C. DNA was extracted as previously described (Medina-Acosta and Cross 1993). The time interval between preparation of two DNA samples was one year, and are hereafter designated as the old and new samples. All DNA samples were frozen at −20°C until use.

Ten decamer oligonucleotide primers (Kit I) from Operon Technologies Inc. (USA) which had been found to give
Table 1. Natural hosts and geographical origin of the isolates and species of anuran trypanosomes.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Species</th>
<th>Natural host</th>
<th>Locality</th>
<th>Year of isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC* 50294</td>
<td>T. chattoni</td>
<td>Rana pipiens</td>
<td>Minnesota</td>
<td>1992</td>
</tr>
<tr>
<td>C2</td>
<td>T. fallisi</td>
<td>Bufo americanus</td>
<td>Ontario</td>
<td>1988</td>
</tr>
<tr>
<td>F II</td>
<td>T. fallisi</td>
<td>B. americanus</td>
<td>Ontario</td>
<td>1988</td>
</tr>
<tr>
<td>M IV</td>
<td>T. fallisi</td>
<td>B. americanus</td>
<td>Ontario</td>
<td>1988</td>
</tr>
<tr>
<td>ATCC 30038</td>
<td>T. mega</td>
<td>B. regularis</td>
<td>Africa</td>
<td>1970</td>
</tr>
<tr>
<td>ATCC 30040</td>
<td>T. ranarum</td>
<td>Rana pipiens</td>
<td>Minnesota</td>
<td>1970</td>
</tr>
<tr>
<td>Lou I</td>
<td>T. rotatorium-like</td>
<td>R. clamitans</td>
<td>Louisiana</td>
<td>1970</td>
</tr>
<tr>
<td>Lou II</td>
<td>T. rotatorium-like</td>
<td>R. clamitans</td>
<td>Louisiana</td>
<td>1970</td>
</tr>
<tr>
<td>ATCC 50295</td>
<td>Trypanosoma sp.</td>
<td>R. sphenoecephala</td>
<td>Florida</td>
<td>1992</td>
</tr>
<tr>
<td>ATCC 30641</td>
<td>Trypanosoma sp.</td>
<td>R. esculentum</td>
<td>Yugoslavia</td>
<td>1977</td>
</tr>
</tbody>
</table>

* ATCC = American Type Culture Collection.

desirable amplification of the anuran trypanosome DNA (Lun and Desser 1996) were used in this study. The primer sequences (5' to 3') were as follows: OPI-2, GGAG-GAGAG; OPI-3, CAGAAGCCA; OPI-6, AAGGCGCGCAG; OPI-7, CAGCGACAAG; OPI-10, ACAACCGAG; OPI-11, ACATGCGCTG; OPI-12, AGAGGCGACA; OPI-14, TGACGGCCTG; OPI-17, GTGATTGTATG; OPI-20, AAAGTGCGGG. Each 25 μl amplification reaction was carried out using 1.25 units of Taq DNA polymerase (Promega), 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM MgCl₂, 0.001 % gelatine, 200 μM of each deoxynucleotide triphosphate, 1 μM primer and 20 ng of template DNA. Amplification was performed in a Perkin-Elmer Cetus thermal cycler for 40 cycles of 1 minute at 94°C, 1.5 minutes at 40°C, and 2 minutes at 72°C. DNA samples of isolates to be compared were simultaneously amplified in the same reaction and the products run on the same gel. Each isolate/primer combination was repeated at least twice and 10 μl of the amplification reaction was electrophoresed in a 1.5 % agarose gel. Products were detected by UV illumination after staining with ethidium bromide.

Karyotype analysis. Samples for pulsed field gel electrophoresis (PFGE) were lysed and deproteinized in situ in low melting agarose blocks as previously described (Schwartz and Cantor 1984, Van der Ploeg et al. 1984b, Lun and Desser 1995). PFGE was carried out using a CHEF-DR II system (Bio-Rad). As with the DNA samples, the time interval between preparation of the old and new agarose blocks was one year. The run time was 24 h at 6 V/cm (200 V) with a 60-90 second switch time ramp at an included angle of 120°. Gels were stained with ethidium bromide after electrophoresis and viewed using UV illumination.

RESULTS

Comparison of molecular karyotypes. Isolates C2, F II and M IV of Trypanosoma fallist from the same species of host, the American toad (Bufo americanus Holbrook), and from the same locality showed at least 13 to 15 chromosome-sized DNA molecules ranging in size from 510 kilobases (kb) to 2.0 megabases (Mb) (Fig. 1). The number and size of chromosomes of isolates F II (lanes 3, 4) and M IV (lanes 5, 6) were similar. Changes of the patterns and size of chromosomes were not found between the old (lanes 3, 5) and new (lanes 4, 6) samples. Although isolate C2 (Fig. 1, lanes 1, 2) was also obtained from the same species of host and from the same locality as isolates F II and M IV, it showed a different molecular karyotyping pattern compared to these. However, the karyotype between the new and old samples of isolate C2 appeared the same with the exception of overloaded DNA in the agarose block of the old sample (Fig. 1, lane 1). Trypanosoma mega Dutton et Todd, isolated from Bufo regularis Reuss from Africa contains at least 15 chromosome-sized DNA molecules ranging from 500 kb to 1.9 Mb (Fig. 1, lanes 7, 8). The karyotype of T. mega appeared different from molecular karyotypes of other isolates, but the karyotypes between the old and new samples of this isolate were the same. In Fig. 2, T. chattoni Mathis et Leger (lanes 1, 2) isolated from Rana pipiens Schreber from Minnesota, USA and ATCC 50295 (lanes 3, 4) isolated from R. sphenoecephala Cope from Florida, USA showed a similar molecular karyotype. At least 15 chromosome-sized DNA molecules ranging from 530 kb to 1.8 Mb were found in these two isolates. Changes of the number and size of chromosomes between the old and new samples of the two isolates were not found. At least 14 chromosome-sized DNA molecules ranging from 500 kb to 2.0 Mb were detected from ATCC 30040 isolated from R. pipiens from Minnesota, USA (Fig. 2, lanes 5, 6). The karyotype of
Fig. 1. Molecular karyotypes of *Trypanosoma fallisii* C2 (lanes 1, 2), F II (lanes 3, 4), M IV (lanes 5, 6) and *T. megag* (lanes 7, 8). M, *Saccharomyces cerevisiae* chromosome size marker. Lanes 1, 3, 5, 7 are the old samples and lanes 2, 4, 6, 8 are the new samples.

Fig. 2. Molecular karyotypes of *Trypanosoma chattonii* (lanes 1, 2), ATCC 50295 (lanes 3, 4), ATCC 30040 (lanes 5, 6) and ATCC 30641 (lanes 7, 8). M, *Saccharomyces cerevisiae* chromosome size marker. Lanes 1, 3, 5, 7 are the old samples and lanes 2, 4, 6, 8 are the new samples.
Fig. 3. Molecular karyotypes of *Trypanosoma rotatorium*-like trypanosomes B2 I (lanes 1, 2), B2 II (lanes 3, 4), Lou I (lanes 5, 6) and Lou II (lanes 7, 8). M, *Saccharomyces cerevisiae* chromosome size marker. Lanes 1, 3, 5, 7 are the old samples and lanes 2, 4, 6, 8 are the new samples.

Fig. 4. RAPD analysis of amplification fragments of DNA from *Trypanosoma fallisi* C2 (lanes 1, 2), F II (lanes 3, 4), M IV (lanes 5, 6) and *T. mega* (lanes 7, 8) using arbitrary primer OPI-12. M, DNA molecular size markers. Lanes 1, 3, 5, 7 are the old DNA samples and 2, 4, 6, 8 are the new DNA samples.
Fig. 5. RAPD analysis of amplification fragments of DNA from *Trypanosoma chattoni* (lanes 1, 2), ATCC 50295 (lanes 3, 4), ATCC 30040 (lanes 5, 6) and ATCC 30641 (lanes 7, 8) using arbitrary primer OPI-12. M, DNA molecular size markers. Lanes 1, 3, 5, 7 are the old DNA samples and 2, 4, 6, 8 are the new DNA samples.

Fig. 6. RAPD analysis of amplification fragments of DNA from *Trypanosoma rotatorium*-like trypanosomes B2 I (lanes 1, 2), B2 II (lanes 3, 4), Lou I (lanes 5, 6) and Lou II (lanes 7, 8) with primer OPI-12. M, DNA molecular size markers. Lanes 1, 3, 5, 7 are the old DNA samples and 2, 4, 6, 8 are the new DNA samples.
the old and new samples appeared to be similar with the exception of the lanes containing overloaded DNA in the agarose block of the old sample. ATCC 30641 (Fig. 2, lanes 7, 8) isolated from R. esculenta Linnaeus from Yugoslavia exhibited at least 18 chromosome-sized DNA molecules ranging from 450 kb to 1.9 Mb. The karyotyping pattern and number of chromosomes of this isolate were different from other species and easily distinguished from them. Fig. 3 shows the molecular karyotypes of four isolates (B2 I, B2 II, Lou I and Lou II) of T. rotatorium-like trypanosomes. Although B2 I and B2 II were isolated from the same species of host, R. catesbeiana Shaw, from Ontario, Canada, they showed different molecular karyotypes and different numbers of chromosome-sized DNA molecules (Fig. 3, lanes 1–4). At least 14 chromosome-sized molecules ranging from 450 kb to 2.0 Mb were found in B2 I, while at least 18 chromosome-sized molecules ranging from 500 kb to 2.2 Mb were detected in B2 II. Isolates Lou I and Lou II from R. clamitans Lateville from Louisiana, USA showed similar molecular karyotypes (lanes 5–8). About 14 chromosome-sized DNA molecules ranging from 600 kb to 2.0 Mb were observed in these two isolates. Changes of molecular karyotyping pattern and size of chromosomes were not found between the old and new samples among these isolates. Minichromosomes ranging from 25 kb to 150 kb were not detected in any of the anuran trypanosome isolates investigated here.

Comparisons of the RAPD patterns. In all ten primer/isolate combinations, four to 14 amplified fragments, ranging from 0.19 kb to 2.0 kb were observed. Figure 4 shows the results of the old and new DNA of isolates C2 (lanes 1, 2), F II (lanes 3, 4), M IV (lanes 5, 6) of T. fallisi and of T. mega (lanes 7, 8) amplified with OPI-12. Ten sharp and reproducible fragments ranging from 0.22 kb to 1.9 kb were found in the C2 isolate. Eleven reproducible amplified fragments ranging from 0.3 kb to 1.9 kb were observed in isolates F II and M IV. Eight fragments, ranging from 0.25 kb to 0.75 kb, showing obviously different patterns to those of isolates of T. fallisi isolated from American toads from Canada, were detected in the isolate of T. mega. Figure 5 shows the amplified fragments of the old and new samples from isolates of T. chattoni (lanes 1, 2), ATCC 50295 (lanes 3, 4), ATCC 30040 (lanes 5, 6) and ATCC 30641 (lanes 7, 8) with primer OPI-12. Eleven reproducible fragments ranging from 0.19 kb to 1.4 kb were shared between T. chattoni and ATCC 50295. Twelve reproducible amplified fragments ranging from 0.19 kb to 1.6 kb were found in ATCC 30040 and 11 amplified fragments ranging from 0.23 kb to 1.70 kb were observed in isolate ATCC 30641. Figure 6 shows the amplified results of the old and new DNA samples from isolates of B2 I, B2 II, Lou I and Lou II with primer OPI-12. Five reproducible fragments ranging from 0.14 kb to 0.49 kb were shared between isolates B2 I and B2 II. Seven fragments ranging from 0.15 kb to 0.75 kb were found in isolates Lou I and Lou II. Different RAPD patterns were not detected between these two isolates and changes in the amplified fragments between the old and new samples were not detected.

DISCUSSION

Molecular karyotype analysis has been widely used to determine the genetic characteristics of African and American trypanosomes (Van der Ploeg et al. 1984a, b, c, Aymerich and Goldenberg 1989, Gibson and Garside 1991). Although the molecular karyotype and the relationship of isolates within species of anuran trypanosomes have been investigated recently using PFGE and RAPD analysis respectively (Lun and Desser 1995, 1996), data on the extent of genetic diversity based on the comparisons of molecular karyotype and RAPD patterns of anuran trypanosomes during long-term in vitro cultivation have not been reported.

Comparisons of the old and new samples revealed that the molecular karyotyping patterns of anuran trypanosomes of the same isolate remained relatively stable following in vitro cultivation for at least one year. Although the overloading of trypanosome DNA in the agarose blocks of the old samples of some isolates resulted in the slow migration rate during electrophoresis (for example in Fig. 2, lanes 3, 5), differences in molecular karyotyping patterns were not observed between the old and new sample of the same isolate. The stability of the molecular karyotyping pattern in a cloned population was not surprising, however, the same phenomenon observed in the uncloned isolate of T. ra-narum (ATCC 30040) was unexpected. It appears that a subpopulation of the isolates had been selected during adaptation to culture conditions, resulting in a clone-like population that would exhibit similar karyotyping in both the old and new isolates. Different karyotyping patterns of cloned populations of T. brucei and T. cruzi were observed when they were continuously cultured in vitro or continuously passaged in experimental hosts (Aymerich and Goldenberg 1989, Schweizer et al. 1991). Schweizer et al. (1991) suggested that this observation might be a result of genetic exchange during the in vitro cultivation whereas Van der Ploeg et al. (1984a) stated that it might be the result of chromosome rearrangement. The stability of karyotyping patterns of anuran trypanosomes observed in the present study suggests that genetic exchange and chromosome rearrangement do not occur. Our results from the PFGE analysis are consistent with the findings of Waiumba and Young (1994), which indicated stable molecular
karyotyping patterns for African isolates of *T. evansi*. While Martin et al. (1992) used isoenzyme analysis in an attempt to distinguish species of trypanosome isolates on the basis of point mutations, Lun and Desser (1995) demonstrated that molecular karyotyping by PFGE to study amplification/deletion was a more discriminating and sensitive approach. Likewise, the separation of chromosome-sized DNA molecules provided a finer distinction among *T. evansi* isolates than the analysis of kinetoplast DNA (Lun et al. 1992, Waitumbi and Young 1994) and isoenzymes (Lun et al. 1991).

Fragments of random amplified polymorphic DNA (RAPD) represent anonymous regions distributed randomly throughout the genome of the organism and provide a fingerprint of the isolate or species being investigated (Steindel et al. 1994). RAPD analysis has been widely used for studying genetic variation, especially for isolates and individuals within the same species (Welsh and McClelland 1990, Williams et al. 1990, Tibayrenc et al. 1993) and was used by Lun and Desser (1996) to investigate the genetic relationships among isolates within anuran trypanosome species. In this study, although some amplified fragments in the old sample had a higher yield than in the new sample (for example, in Fig. 4, lanes 5 and 6, the 1.4 kb fragment), the RAPD patterns of the old and new samples from the same isolate remained similar. In fact, changes of the amplified fragment patterns between the old and new samples were not observed in any isolates during long-term in vitro cultivation and with the ten random primers used in this study, the genetic characteristics in the sequences corresponding to the amplified products were stable.

In our previous studies of anuran trypanosomes (Lun and Desser 1996), conclusions based on RAPD data were consistent with those from isoenzyme (Martin et al. 1992), and riboprinting analysis (Clark et al. 1995). Likewise, Tibayrenc et al. (1993) demonstrated by RAPD analysis that the characteristics of six parasitic protozoans were consistent with those obtained by multilocus enzyme electrophoresis. RAPD analysis, in conjunction with molecular karyotyping, is therefore well suited for the examination of genetic stability as well as the genetic variation of anuran trypanosomes during the course of long-term in vitro cultivation.

**Acknowledgements.** We thank Henry Hong, Todd Smith and Chongxie Xiao for their critical review of this manuscript. This research was supported by a Natural Science and Engineering Research Council of Canada (NSERC) Grant No. 6965 to SSD and an NSERC International Fellowship to ZRL.

**REFERENCES**


Received 22 August 1995

Accepted 5 September 1996