ELSEVIER

Molecular and Biochemical Parasitology 73 (1995) 279–283

MOLECULAR AND BIOCHEMICAL PARASITOLOGY

Short communication

Marine fish and ray trypanosomes have large kinetoplast minicircle DNA

Milan Jirků [a,b], Alexander A. Kolesnikov [a,c], Oldřich Benada [d], Julius Lukeš [a,b,*]

[a] *Institute of Parasitology, Czech Academy of Sciences, University of South Bohemia, Branišovská 31, 37005 České Budějovice, Czech Republic*

[b] *Faculty of Biology, University of South Bohemia, Branišovská 31, 37005 České Budějovice, Czech Republic*

[c] *Faculty of Biology, Moscow State University, Moscow, Russia*

[d] *Institute of Microbiology, Czech Academy of Sciences, Prague, Czech Republic*

Received 11 May 1995; accepted 16 July 1995

Keywords: Minicircle DNA; kDNA; Kinetoplast; Digestion; Trypanosome

Fish trypanosomes were the first trypanosomes ever reported [1]. Until recently, they received attention mostly from morphologists who have described more than 200 species parasitizing numerous fish species virtually worldwide. Many of these descriptions are, however, based solely on observation of flagellates in the blood of new fish hosts [2].

The primary aim of our work was to address the problem of species composition of fish trypanosomes. It was shown that some trypanosome strains differ in pathogenicity and host range, while other strains, even from geographically distant localities, did not exhibit any differences [2]. Previous analyses of selected enzymes and surface lectins failed to provide clear measures to differentiate among fish trypanosomes [3,4]. However, length polymorphism of maxicircle kDNA in strains isolated from several freshwater fish species clustered the studied strains into two groups, indicating the existence of at least two trypanosome species. These results have recently been confirmed by sequence analysis of the minicircle conserved regions (CRs) of selected strains (Kolesnikov et al., unpublished data).

Here we present results of the analysis of the minicircular component of the kDNA of selected freshwater and marine fish and ray trypanosomes which revealed unexpected differences in size, sequence heterogeneity and distribution of conserved regions.

Trypanosoma carassii (strain CC-Nem) was isolated from the blood of a common carp *Cyprinus carpio*, Hluboká, Czech Republic. *T. triglae* and *T. boissoni* were isolated from the blood of marine fish *Triglae lineata* and marine ray *Zanobatus atlanticus*, respectively, both Green Cap, Senegal. *T. carassii* and *T. boissoni* were cultivated in L4NHS medium [5] at 25°C, while *T. triglae* cells were grown in SNB-9 medium [6] at 20°C. kDNA was isolated from approx. 5×10^8 cells washed twice in NET50 (50

Abbreviations: kDNA, kinetoplast DNA; CR, conserved region; CSB, conserved sequence block; EM; electron microscopy; PCR, polymerase chain reaction; SD, standard deviation.

* Corresponding author. Fax: (42-38) 47743; e-mail: jula@paru.cas.cz

SSDI 0166-6851(95)00121-2

mM EDTA/100 mM NaCl/10 mM Tris, pH 8.0), lysed by *N*-lauroylsarcosine (Fluka) and pronase E (Merck) at the final concentrations 3% (w/v) and 0.5 mg ml^{-1}, respectively, for 1 h at 4°C. The lysate viscosity was reduced by homogenizer and the lysate was centrifuged at $100\,000 \times g$ for 110 min in a Beckman SW28Ti rotor. The kDNA pellet was deproteinized and spun at $130\,000 \times g$ for 45 min in a Beckman SW60Ti rotor. The pelleted kDNA was precipitated, air dried and resuspended in water. Restriction endonuclease digestion, agarose gel electrophoresis and cloning were performed according to standard protocols [7]. Minicircles isolated from *T. carassii* (strain TT-FR) were linearized with the *Msp*I restriction endonuclease and ligated into the *Acc*I site of pBluescript SK^- (Stratagene).

Agarose gel electrophoresis of kDNAs of *Trypanosoma carassii*, *T. boissoni* and *T. triglae* digested by the restriction endonucleases (Fig. 1A) showed significant interspecific differences in the size of minicircles. The minicircles of *T. carassii* (represented by the strain CC-Nem) formed a single prominent 1.6-kb band. The same size of minicircles was observed in kDNA networks of cultured trypanosomes isolated from several cyprinid fishes (*Abramis brama, Barbus barbus, Blicca bjoerkna, Carassius carassius, C. auratus, Cobitis taenia, Cyprinus carpio, Esox lucius, Gobio gobio, Perca fluviatilis, Rutilus rutilus, Scardinius erythrophthalmus* and *Tinca tinca*) from territories of the Czech Republic, Hungary and Poland.

Electrophoresis of *T. boissoni* kDNA treated with various restriction enzymes revealed the presence of at least two populations of minicircles with different size. The smaller and more abundant minicircles were about 3.7-kb long, while the size of the larger and less abundant minicircles was about 4.1 kb. The analysis of the kDNA of *T. triglae* showed only one population of large minicircles comigrating with 5.2 kb (Fig. 1A and data not shown).

To confirm these unusual lengths of minicircles we prepared the kDNA samples for electron microscopy by the cytochrome-*c* method [8]. Plasmid pBR322 DNA served as an internal standard. The samples were examined with a Philips CM12/STEM electron microscope at 80 kV and a magnification of $15\,000\times$. The contour lengths of the minicircles were measured from prints using the HIPAD digitizing tablet (Houston Instruments). The precise magnification was determined using replica grating (Balzers).

In the sample of *T. carassii* (strain RR-PO), 73% ($n = 94$) of minicircles exhibit mean size of 1.57 kb (SD 0.11 kb) and 20% ($n = 26$) 1.20 kb (SD 0.10 kb) (Fig. 2F,G). As expected, the size pattern determined by EM appeared more complex for the kDNA of *T. boissoni*. A set of 172 circular molecules exhibited a broad size distribution with several peaks. About 77% of minicircles fall into two classes with

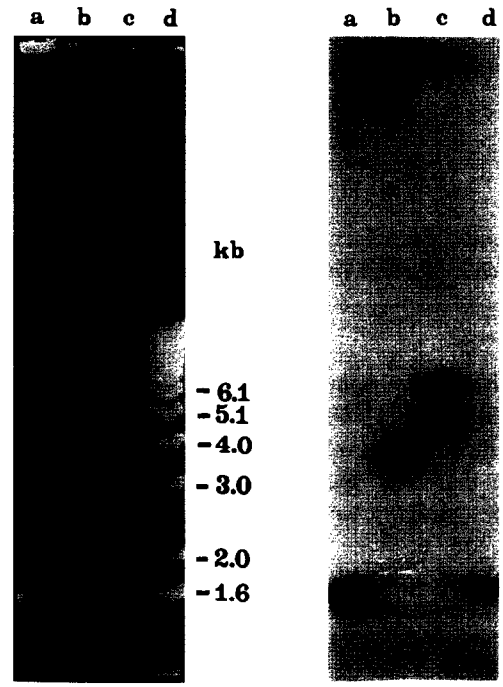
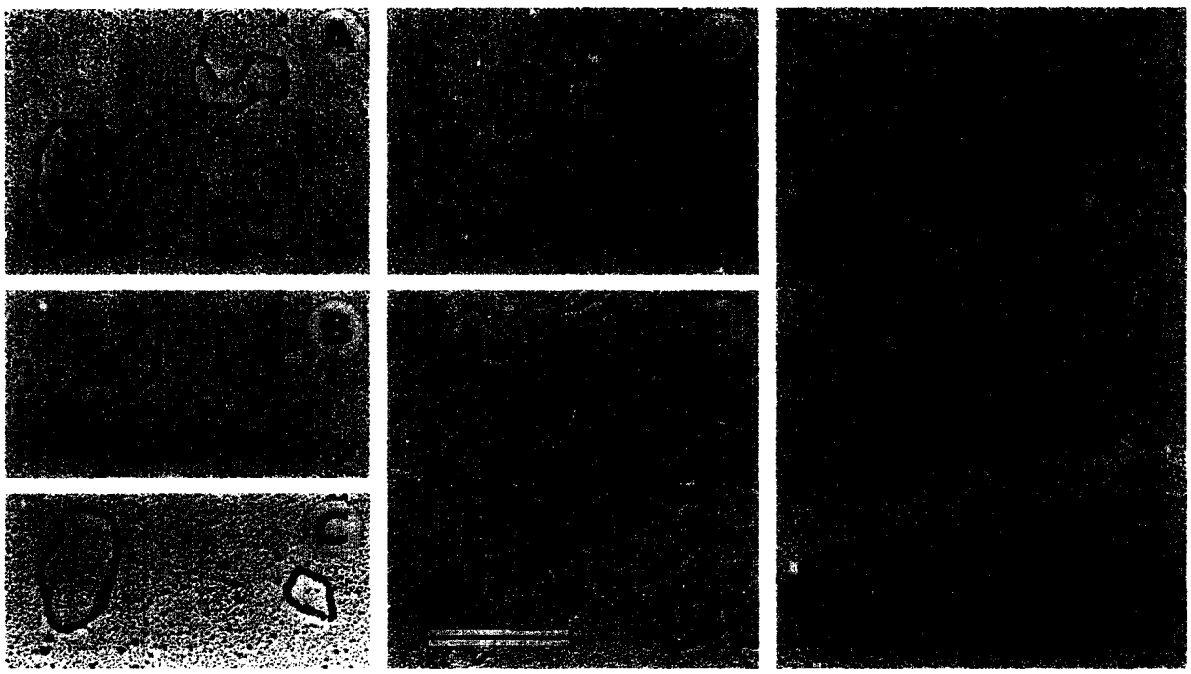
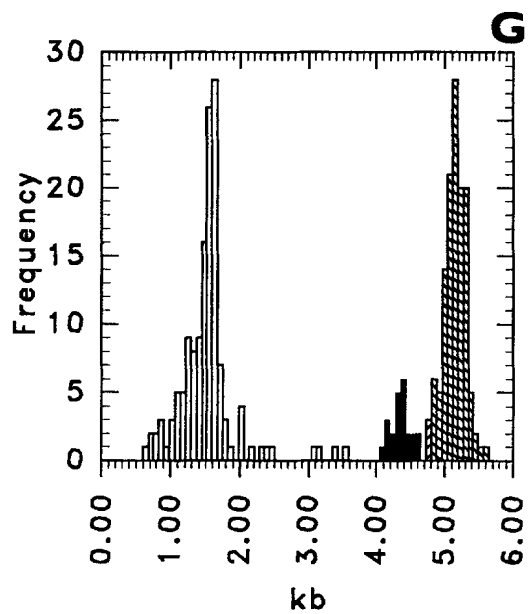


Fig. 1. (A) Photograph of an ethidium-stained gel of kDNAs of *T. carassii* digested with *Hin*dIII (lane a), *T. boissoni* digested with *Xba*I (lane b) and *T. triglae* digested with *Hin*dIII (lane c); lane d, 1-kb ladder. The band co-migrating with 7.5 kb in lane c is most likely a minicircle dimer that disappeared after complete digestion of the kDNA. (B) Autoradiogram of a Southern blot of the gel in (A) hybridized with cloned *T. carassii* minicircle (pTT-FR3) labelled with [α-^{32}P]dATP (3000 Ci $mmol^{-1}$, Amersham). Hybridization was carried out overnight in Na·phosphate solution (0.25 M NaH_2PO_4/0.25 M Na_2HPO_4/7% SDS/1 mM EDTA) at 55°C. Blots (Hybond-N, Amersham) were washed extensively at 55°C in three changes of 6×SSC/0.1% SDS, respectively, and exposed to X-OMAT S film (Kodak). The nature of the 1.6-kb marker band hybridizing with the pTT-FR3 probe is unknown, but may represent contamination of the probe with the pBluescript DNA that probably constitutes part of the 1-kb ladder.

mean sizes of 3.70 kb (SD 0.11 kb) and 4.08 kb (SD 0.11 kb), respectively (Fig. 2A–C). The rest of circular molecules can be sorted into three groups with mean sizes of 1.3 kb, 2.3 kb and 3.4 kb (Fig. 2H). The EM of *T. triglae* kDNA confirmed the presence of one major population of single-sized,





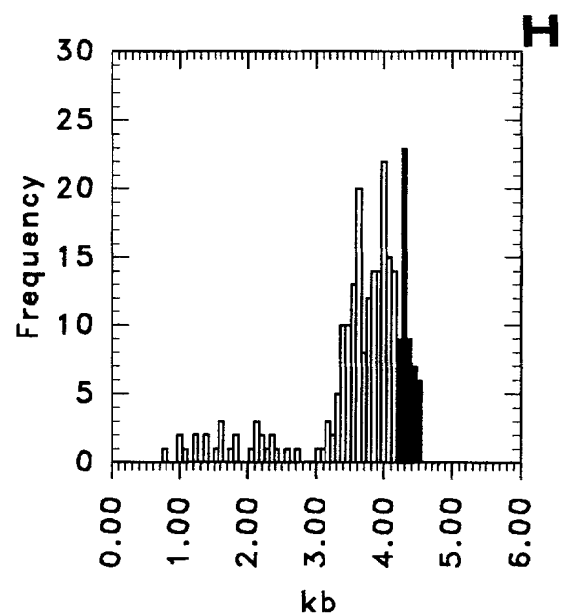


Fig. 2. Electron microscopy of kDNA minicircles. (A–C) minicircles of *T. boissoni*; (D,E) minicircles of *T. triglae*. Three catenated minicircles are shown in D; (F) minicircles of *T. carassii* (CC-Nem). The bar represents 0.5 μm (approx. 1.6 kb). Size distribution of minicircles determined by measuring their length with those of pBR322 (black); (G) *T. carassii* (open) and *T. triglae* (shaded); (H) *T. boissoni* (open).

5.15 kb (n = 126)(SD 0.16 kb) long minicircles (Fig. 2D,E,G).

The theoretical standard deviation (SD) of a homogeneous DNA sample is proportional to DNA length [8]. For 5.15 kb this value is 0.158 kb and is, therefore, in good agreement with SD of the major population minicircles of *T. triglae*. It clearly demonstrates the size homogeneity of minicircles in this kDNA network. Moreover, the EM confirmed the presupposition that the high-molecular-mass bands observed in the agarose gels were minicircle dimers and fragments of maxicircles.

The smallest (0.68 kb) and largest (2.8 kb) minicircles were described in *Leishmania* [9] and *Phytomonas* [10] networks, respectively. The minicircles of *Trypanosoma boissoni* and *T. triglae* are significantly larger than minicircles in other trypanosomatids, the size of which is species specific, ranging between 0.6 kb in *T. vivax* [11] and 2.3 kb in *T. mega* [12].

As judged from the electrophoretic analysis of the kDNA digested by restriction enzymes, the minicircle population appears to be heterogeneous in sequence to different extent in individual trypanosome species. The hexanucleotide-target restriction endonucleases (*Eco*RI, *Bam*HI and *Xba*I) cut usually only once in minicircles, whereas *Msp*I and *Hha*I (recognizing 4-bp targets) cut the minicircles of *T. carassii* at only a few sites while with kDNA of both marine fish trypanosomes the same endonucleases will produce a high number of apparently non-stoichiometric bands (data not shown). Considering their size and sequence heterogeneity, minicircles of marine fish and ray trypanosomes might harbour a high number of different guide RNA genes.

To learn more about sequence similarity between the studied minicircles, we performed cross-hybridizations under various conditions using the full-size minicircle of *T. carassii* (pTT-FR3) as a probe. Under high-stringency conditions (65°C), no cross-hybridization was detected between the minicircles of *T. carassii* and those of *T. triglae* and *T. boissoni*. At 55°C, pTT-FR3 hybridized with minicircles released from catenates of all three examined species (Fig. 1B).

To establish the number and distribution of CRs in minicircles of the studied species we designed primers AGTTGCACGCCCGTCCCGA and TGGTGCTTCGATAGGGGTTGG matching the portions of CRs located at their 5′- and 3′-ends (CSBs I and III of Ray [13]), the orientation of primers being directed with their 3′ ends outside the CR. Polymerase chain reactions (PCR) were performed using either 10 to 100 ng kDNA, or 1 to 10 ng DNA of individual cloned minicircles. A Techne thermal cycler was programmed for 30 cycles of 94°C 1 min, 60°C 90 s, 74°C 2 min. PCR amplifications using kDNAs of *T. triglae* and various strains of *T. carassii* as templates yielded a single product of 0.75 kb. A similar size product was obtained from reactions with individual minicircles of the latter species cloned in pBluescript. The invariant amplification of several distinct bands (0.2, 0.32, 0.45 and 0.75 kb) in the PCR reactions with *T. boissoni* kDNA may reflect the minicircle size heterogeneity (data not shown).

The predicted presence of two CRs located 180° apart on the *T. carassii* minicircle has recently been confirmed by the determination of the primary structure of one entire minicircle (Baron, Yurchenko and Kolesnikov, unpublished data). Two CRs have also been found in minicircles of *T. rangeli* [14], *T. lewisi* [15], *Crithidia fasciculata* [16], *Proteomonas brevicola* (Fu and Kolesnikov, unpublished data), and *Phytomonas serpens* [17]. Considering six equidistant CRs in one minicircle, then 6×0.75 kb (total length of variable regions) + 6×0.12 kb (total length of CRs) combined is 5.2 kb, which approximately equals the size of the *T. triglae* minicircles.

The phylogenetic tree of kinetoplastid protozoans based on 18S rRNA sequences [18] placed *Trypanosoma* sp. E1-CP isolated from the pike *Esox lucius* (in the Czech Republic) in the same branch with *T. cruzi*. It should be mentioned that if the preliminary inclusion of fish trypanosomes in the Stercorarian group is correct, all hitherto studied members of this group of species (*T. cruzi* [19], *T. rangeli* [14], *T. carassii*, *T. boissoni* and *T. triglae*) contain more than one CR in their minicircles.

According to the hypothesis of Wallace [20] parasitism of trypanosomes first occurred in the gut and then the blood of vertebrates. Fishes and rays belong to the most primitive vertebrates and it would therefore be interesting to establish the phylogenetic position of fish trypanosomes within the Kinetoplastida. These studies are under way (Maslov et al., unpublished data).

Acknowledgements

The excellent technical assistance of Antonín Polák and the cultivation of trypanosomes by Hana Pecková are acknowledged. We are indebted to Prof. Dominique Le Ray (Prince Leopold Institute of Tropical Medicine, Antwerpen, Belgium) for providing *T. boissoni* and *T. triglae*. This work was funded by the Grant Agency of the Czech Republic, grant No. 204/94/0285 to J.L.

References

[1] Valentin (1847) Archiv of J. Muller. Quoted in Laveran, A. and Mesnil, F. (1912) Trypanosomes and trypanosomiasis, p. 999. Masson, Paris.

[2] Lom, J. (1979) Biology of the trypanosomes and trypanoplasms of fish. In: Biology of the Kinetoplastida (Lumsden, W.H.R. and Evans, D.A., eds.), Vol. 2, pp. 269–337. Academic Press, London.

[3] Zajíček, P. and Pecková, H. (1990) The interaction of fish trypanosome culture forms with some lectins. Folia Parasitol. 37, 1–8.

[4] Zajíček, P. (1991) Enzyme polymorphism of freshwater fish trypanosomes and its use for strain identification. Parasitology 102, 221–224.

[5] Baker, J.R., Green, S.M., Chaloner, L.A. and Gaborak, M. (1972) *Trypanosoma* (*Schizotrypanum*) *dionisii* of *Pipistrellus pipistrellus* (Chiroptera): intra and extracellular development in vitro. Parasitology 65, 251–273.

[6] Diamond, L.S. and Herman, C.M. (1954) Incidence of trypanosomes in the Canada goose as revealed by bone marrow culture. J. Parasitol. 40, 195–202.

[7] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning. A Laboratory Manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

[8] Fergusson, L. and Davis, R.W. (1978) Quantitative electron microscopy of nucleic acids. In: Advanced Techniques in Biological Electron Microscopy II (Koehler, J.K., ed.), pp. 123–171. Springer, Berlin, Heidelberg.

[9] Barker, D.C., Gibson, L.J., Kennedy, W.P.K., Nasser, A.A.A.A. and Williams, R.H. (1986). The potential of using recombinant DNA species-specific probes for the identification of tropical *Leishmania*. Parasitology 91, S139–174.

[10] Riou, J.F., Dollet, M., Ahomadegbe, J.-Ch., Coulaud, D. and Riou, G. (1987) Characterization of *Phytomonas* sp. kinetoplast DNA. FEBS Lett. 213, 304–308.

[11] Borst, P., Fase-Fowler, F., Weijers, P., Barry, J., Tetley, L. and Vickerman, K. (1985) Kinetoplast DNA from *Trypanosoma vivax* and *T. congolense*. Mol. Biochem. Parasitol. 15, 129–142.

[12] Borst, P., Fase-Fowler, F., Steinert, M. and van Assel, S. (1977) Maxi-circles in the kinetoplast DNA of *Trypanosoma mega*. Exp. Cell Res. 110, 167–173.

[13] Ray, D.S. (1989) Conserved sequence blocks in kinetoplast minicircles from diverse species of trypanosomes. Mol. Cell. Biol. 9, 1365–1367.

[14] Recinos, R.F., Kirchhoff, L.V. and Donelson, J.E. (1994) Characterization of kinetoplast DNA minicircles in *Trypanosoma rangeli*. Mol. Biochem. Parasitol. 63, 59–67.

[15] Ponzi, M., Biragio, C. and Battaglia, P. (1984) Two identical symmetrical regions in the minicircle structure of *Trypanosoma lewisi* kinetoplast DNA. Mol. Biochem. Parasitol. 13, 111–119.

[16] Sugisaki, H. and Ray, D.S. (1987) Kinetoplast DNA minicircles of the insect trypanosomatid *Crithidia fasciculata* contain two nearly identical 173 bp and 177 bp sequences involved in DNA replication. Mol. Biochem. Parasitol. 23, 253–264.

[17] Sa-Carvalho, D., Perez-Morga, D. and Traub-Cseko, Y.M. (1993) Characterization of kinetoplast DNA from *Phytomonas serpens*. J. Euk. Microbiol. 40, 317–322.

[18] Maslov, D.A., Avila, H.A., Lake, J.A. and Simpson, L. (1994) Evolution of RNA editing in kinetoplastid protozoa. Nature 368, 345–348.

[19] Macina, R.A., Sanchez, D.O., Gluschankof, D.A., Burrone, O.R. and Frasch, A.C.C. (1986) Sequence diversity in the kinetoplast DNA minicircles of *Trypanosoma cruzi*. Mol. Biochem. Parasitol. 26, 25–32.

[20] Wallace, F.G. (1966) The trypanosomatid parasites of insects and arachnids. Exp. Parasitol. 18, 124–193.