FOLIA PARASITOLOGICA 42: 251–254, 1995

Analysis of kinetoplast DNA of freshwater fish trypanosomes

A. A. Kolesnikov[1], M. Jirků[2], H. Pecková[2], A. Polák[2], D. A. Maslov[1,3], and J. Lukeš[*2]

[1]Department of Molecular Biology, Moscow State University, 119899 Moscow, Russia;
[2]Institute of Parasitology, Czech Academy of Sciences, Branišovská 31, 370 05 České Budějovice, Czech Republic;
[3]Present address: Department of Biology, University of California, Riverside, California 92521, USA

Key words: kinetoplast DNA, *Trypanosoma*, minicircle, conserved region, fish

Abstract. The sequences of 10 conservative regions (CR) of minicircles of 6 selected isolates of freshwater fish trypanosomes have typical organization of this region with high degree of sequence conservation. The comparison with CRs of other trypanosomatids showed that freshwater fish trypanosomes represent a compact separate group within the genus *Trypanosoma*. The alignment of all sequences obtained revealed, however, the existence of 2 types of CRs in sequenced minicircles, with the differences concentrated in a short region. Taxonomic consequences of these results are discussed.

Although a trypanosome in the blood of salmon was the first flagellate described for science (Valentin 1847), the taxonomy of fish trypanosomes has not been settled until recently. Since this observation more than 200 species have been described in the blood of marine and freshwater fish species. In almost all cases the species description was based on the supposed host-parasite specificity. Most "new" trypanosome species were therefore established because they were found in separate fish species (Lom 1979). This concept of strict specificity is, however, in contrast with the transmission experiments performed by several authors with marine (Khan 1977, 1985, Woo and Black 1984) and freshwater fish trypanosomes (Robertson 1911, Lom 1973) which showed that these parasites can be transmitted from one fish species to another either directly by blood inoculation or via leech. The old concept of a single ubiquitous fish trypanosome species (Breindl 1915) represents another extreme attitude to this problem.

However, the failure of several experimental transmissions, e.g. that of trypanosomes from perch into goldfish, and the lysis of these parasites by heterologous serum (Lom, Pecková, pers. commun.) indicate the presence of more than one species in fish hosts. Moreover, there are also more or less pronounced morphological differences between some trypanosomes of fish (Lom 1979). To shed more light on the species composition of freshwater fish trypanosomes we have undertaken the study of kinetoplast DNA of these organisms. Our interests were focused on the restriction enzyme polymorphism analysis of maxicircles and on the sequence analysis of the conservative region of minicircles which can be used as one of several taxonomic criteria in systematics of trypanosomatids (Ray 1989).

MATERIALS AND METHODS

Trypanosomes used in this study were isolated from infected freshwater fishes captured in different localities in South Bohemia and South Moravia, Czech Republic. Their origin is summarized in Table 1. The blood containing flagellates was collected by cardiac punction and immediately inoculated in SNB-9 medium and cultivated as described previously (Zajíček and Pecková 1990). Kinetoplast DNA (kDNA) was isolated from about 10^9 cultured cells, washed twice at 1000 g for 10 min in NET-50 (50 mM EDTA, 100 mM NaCl, 10 mM Tris, pH 8.0), lysed by N-lauroylsarcosin (Fluka) and pronase E (Merck) at the final concentrations of 3 % (w/v) and 0.25 mg/ml, respectively, for 1 hr at 4°C. The lysate viscosity was reduced by shearing and the lysate was centrifuged at 100 000 g for 110 min in Beckman SW-28 Ti rotor. The kDNA pellet was deproteinized and pelleted again at 130 000 g for 45 min in Beckman SW-60 Ti rotor. The pelleted kDNA was precipitated with ethanol, dried and resuspended in water. Digestion with restriction endonucleases (Fermentas, Lithuania), agarose gel electrophoresis, cloning and sequencing were performed according to standard protocols (Sambrook et al. 1989). MspI-linearized minicircles were ligated in the AccI site of pBluescript SK$^-$ vector, transformed into *Escherichia coli* XL-1 competent cells, and sequenced by the dideoxy method using Sequenase 2.0 kit (USB).

*to whom correspondence should be addressed

Table 1. Origin of fish trypanosome isolates.

	Isolation	Host	Locality
AB-TB	11. 12. 1987	*Abramis brama*	Staňkovský pond, South Bohemia
BaB-PO	12. 9. 1989	*Barbus barbus*	Pohořelice pond, South Moravia
CaC-RA	26. 5. 1989	*Carassius carassius*	Radčice pond, South Bohemia
EL-1	24. 7. 1986	*Esox lucius*	Římov dam, South Bohemia
EL-2	25. 11. 1987	*Esox lucius*	Vltava river, South Bohemia
PF-FR	4. 5. 1987	*Perca fluviatilis*	Frauenvaiger pond, South Bohemia
TT-FR	4. 5. 1987	*Tinca tinca*	Frauenvaiger pond, South Bohemia

RESULTS

The electrophoretic profile of kDNAs of all isolates used in this study cleaved by the restriction enzyme MspI consists of several thin sharp bands in the upper part of the gel that represent maxicircles. The presence of a single thick band of 1.6 kb showed that the majority (if not all) of minicircles are cut once by MspI (data not shown). Moreover, we have found that the whole conserved region (CR) of minicircles can be sequenced in a single run. The 10 CRs studied are generally about 140 bp long and their organization is very similar to that of other trypanosomatids. They contain conserved sequence blocks (CSB) 1–3 inter- spersed by regions which are less conservative. As shown in the Fig. 1, the beginning of the CR could be well identified by a stretch of 30 bp with almost full homology preceding the CSB I. This decamer (ACGGGCGTGC) differs from the consensus decamer of Ray (1989) in the positions 2 and 9. The CR proceeds with 29 bp identical in all fish trypanosomes studied followed with an octamer CCCCGTTC (CSB 2) which is identical with the consensus sequence of kinetoplastids for CSB 2 (Ray 1989). The comparison of all sequenced CRs revealed two types of organization of the following stretch of 24 bp. For both types the consensus sequences have been constructed (Fig. 1). The first type comprises both CRs of minicircle 2 (2a and 2b) of PF-FR strain and CRs of minicircles 12 and 17 of BaB-PO strain. It is characterized by a stretch of five As flanked by a single G (type A). The second type includes CRs of two TT-FR and CaC-RA minicircles, respectively, whereas strains EL-1, and AB-TB are represented in our study by a single CR. This type is characterized by a stretch of six Ts flanked by C (type T). Within both groups of CRs the mentioned motifs exhibit a strong homology. The variable motif is followed by a conservative domain 37 nt long of almost 100 % identity within minicircles studied. It contains the most conserved minicircle region, dodecamer GGGGTTGGTGTA (CSB 3) and is finished by the MspI site.

Sequences of the following region have been obtained only for minicircles of strains PF-FR, EL-1, BaB-PO, and AB-TBU. However, it is obvious that they comprise a variable region with no significant homology neither between minicircles of different strains, nor between individual minicircles of the same strain (data not shown).

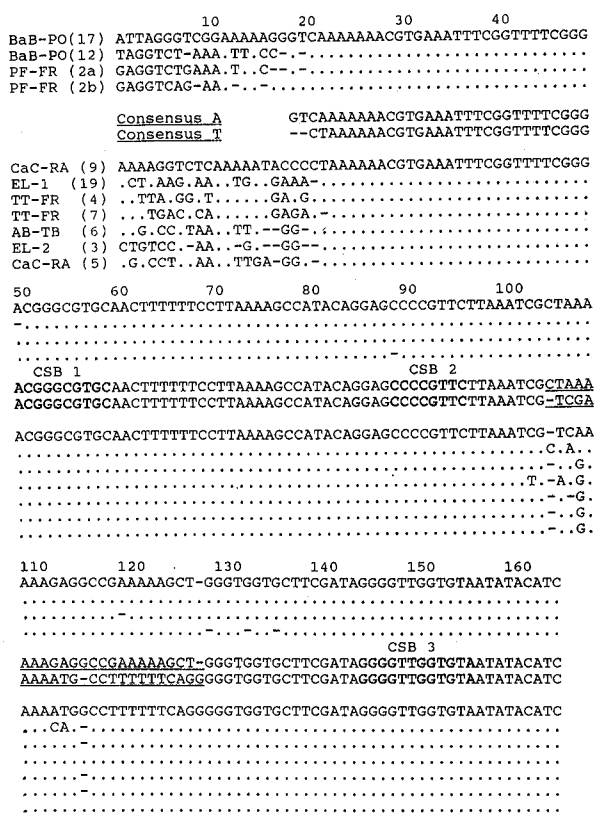

Fig. 1. Sequence alignment of CRs form different minicircles of various freshwater fish trypanosome isolates. Consensus A is deduced from upper 4 CRs, consensus T is deduced from lower 7 CRs. Points indicate homology, deletions required for optimal alignment are represented by dashes, the region in which sequences are different is underlined. CSBs 1–3 are in bold.

DISCUSSION

Only recently, the phylogenetic position of the fish trypanosome *Trypanosoma carassii* (isolate E1-CP) has been proposed. On the basis of both 18S rRNA sequence alignment and the comparison of RNA editing patterns of transcripts of selected maxicircle-located genes, this fish trypanosome fell in the branch of *Trypanosoma cruzi* (Maslov et al. 1994). Our aim was to get an insight in the species composition of trypanosomes isolated from various fish species captured in South Bohemian and South Moravian ponds and rivers. We have used 16 morphologically indistinguishable isolates which were cultivated under the same conditions. The question of whether the isolates represent a single species or more separate species should be answered because there is a suspicion that trypanosomes can be introduced into the fisheries by feral fishes (Lom 1979). The populations of leeches *Piscicola geometra* and *Hemiclepsis marginata* can acquire the infection from low-level parasitemias of these fishes and have the potential to spread the infection within commercially bred carp fingerlings in which the massive infections cause serious pathological changes (Becker 1977, Khan 1985). The biochemical approach based on the enzyme polymorphism data of selected strains resulted in the construction of a dendrogram that did not show any correlation between the enzyme patterns and host species or transmission experiments (Zajíček 1991) and has been distorted by the malate dehydrogenase patterns which were later shown to be the result of the sexual processes (Zajíček 1992). The study of surface saccharides using lectins revealed high degree of similarity of the studied isolates (Zajíček and Pecková 1990). Two marine fish trypanosomes (*Trypanosoma boissoni* and *T. triglae*) could be discerned from freshwater fish isolates only by the interaction with the HPA lectin (Zajíček and Lukeš 1992).

We resorted to the sequence analysis of CR of minicircles from selected strains because a large set of sequence data of this part of minicircles revealed their species-specific structure (reviewed by Ray 1989). Although the presupposition based on the restriction analysis that MspI which linearized the majority of minicircles cuts in the CR was not correct (see below), this restriction enzyme was found to be useful for cloning minicircles with the aim of sequencing the CR. Its target sequence is located on the border of the CR, about 8 nt away from the most conserved dodecamer (CSB 3). The 1599 nt sequence of a single full-length minicircle of fish trypanosome derived from PF-FR strain showed that it contains two almost identical CRs located 180° apart on the minicircle (L. I. Baron, V. Yurchenko and A. A. Kolesnikov, unpubl. results). These results have recently been confirmed for various isolates of fish trypanosomes by the polymerase chain reaction using primers matching the portions of CRs (Jirků et al. 1995). Similar organisation of the minicircles has hitherto been described in *Crithidia fasciculata* (Sugisaki and Ray 1987, Maslov et al. 1988), *Trypanosoma lewisi* (Ponzi et al. 1984) and *Phytomonas serpens* (Sa-Carvalho et al. 1993).

The organisation of CR of fish trypanosomes is similar to that of other trypanosomatids (Ray 1989). The sequence of CSB 1 is specific for fish trypanosomes by the presence of C in the position 2. As expected, the two regions that exhibited strong interspecific homology are CSB 2, and especially CSB 3. The comparison of all 10 CRs derived from various isolates originating from six fish species showed that the fish trypanosomes represent a well-defined group within Trypanosomatidae. Homology of the CRs of their minicircles with other trypanosomatids is, with a single exception (see below), limited to the CSBs only. However, when compared with each other, all 146 bp long CRs exhibit a very strong homology (87 %) not observed in CRs of strains belonging to other species. Moreover, all differences are concentrated into a single region of 24 bp which divides the CR into two large domains with absolute homology. This variable sequence exhibits two obviously different types of organisation: first type with a poly-T stretch, the second one with a poly-A stretch. With the exception of *Trypanosoma cruzi* where the poly-T stretch is lacking (Degrave et al. 1988), in other species its position within CR is conserved. Along with CSB 1–3 this sequence motif is considered to be presumably essential for minicircle function and maintenance (De Bruin and Barker 1992). We can only speculate whether the poly-A type arose by a single inversion or by a recombination event. Although there are some point differences or deletions within CRs falling into both types, this part of CR enables a clear separation of all isolates studied into two groups. Comparisons of CRs of leishmanias belonging to various species (Schoone et al. 1991, De Bruin and Barker 1992) showed that there are interspecific differences spread almost irregularly in the spacing regions between the CSBs. Considering that the sequence of CR is the same for all minicircles of a given isolate, the existence of two conserved types of CR may reflect the presence of at least two species of trypanosomes within the freshwater fish population studied by us. Our results should be confirmed by further studies. However, using the list of species description of fish trypanosomes published by Lom (1979) we propose to assign the isolates PF-FR from perch and BaB-PO from barbel to the species *Trypanosoma percae* (Brumpt, 1906). Similarly, trypanosomes isolated from pike (EL-1), tench (TT-FR), common bream (AB-TB), and crucian carp (CaCRA) could be assigned, according to

the des cription priority, to the species *Trypanosoma carassii* (Mitrophanow, 1883).

Acknowledgements. The authors kindly acknowledge Dr. Pavel Zajíček (Škoda-Volkswagen group, Mladá Boleslav) for starting this study. Thanks are expressed to Drs. Michal Žurovec and František Sehnal (Institute of Entomology) for much advice and Dr. Jiří Lom (Institute of Parasitology) for fruitful discussions and continuous support. This study was supported by the grants No. 3265013–214 and No. 3265043–286 and by the travelling grants to A. A. K. and D. A. M.

REFERENCES

BECKER C. D. 1977: Flagellate parasites of fish. In: J. P. Kreier (Ed.), Parasitic Protozoa. Vol. 1. Academic Press, London-New York, pp. 357–416.

BREINDL V. 1915: The studies on blood parasites of freshwater fishes. Král. Česká Spol. Nauk (Praha), pp. 1–25. (In Czech.)

DE BRUIN M. H. L., BARKER D. C. 1992: Diagnosis of New World leishmaniasis: specific detection of species of the *Leishmania braziliensis* complex by amplification of kinetoplast DNA. Acta Tropica 52: 45–58.

DEGRAVE W., FRAGOSO S. P., BRITTO C., VAN HEUVERSWYN H., KIDANE G. Z., CARDOSO M. A. B., MUELLER R. U., SIMPSON L., MOREL C. M. 1988: Peculiar sequence organization of kinetoplast DNA minicircles from *Trypanosoma cruzi*. Mol. Biochem. Parasitol. 27: 63–70.

JIRKŮ M., KOLESNIKOV A. A., BENADA O., LUKEŠ J. 1995: Marine fish and ray trypanosomes have large kinetoplast minicircle DNA. Mol. Biochem. Parasitol. 73: 279–283.

KHAN R. A. 1977: Susceptibility of marine fish to trypanosomes. Can. J. Zool. 55: 1235–1241.

KHAN R. A. 1985: Pathogenesis of *Trypanosoma murmanensis* in marine fish of the northwestern Atlantic following experimental transmission. Can. J. Zool. 63: 2141–2144.

LOM J. 1973: Experimental transmission of freshwater fishes with blood flagellates. J. Protozool. 20: 537.

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

MASLOV D. A., REZEPKINA L. A., KOLESNIKOV A. A. 1988: Some sequence elements of minicircle DNA from the kinetoplast of the trypanosomatid *Crithidia fasciculata*. Mol. Biol. (Moscow), 22: 1482–1490. (In Russian.)

MASLOV D. A., AVILA H. A., LAKE J. A., SIMPSON L. 1994: Evolution of RNA editing in kinetoplastid protozoa. Nature 368: 345–348.

PONZI M., BIRAGO C., BATTAGLIA P. A. 1984: Two identical symmetrical regions in the minicircle structure of *Trypanosoma lewisi* kinetoplast DNA. Mol. Biochem. Parasitol. 13: 111–119.

RAY D. S. 1989: Conserved sequence blocks in kinetoplast minicircles from diverse species of trypanosomes. Mol. Cell. Biol. 7: 1365–1367.

ROBERTSON J. L. 1911: Transmission of flagellates living in the blood of certain freshwater species. Phil. Trans. Royal Soc. (London), 202: 29–50.

SA-CARVALHO D., PEREZ-MORGA D., TRAUB-CSEKO Y. M. 1993: Characterization of kinetoplast DNA from *Phytomonas serpens*. J. Euk. Microbiol. 40: 317–322.

SAMBROOK J., FRITSCH E. F., MANIATIS T. 1989: Molecular cloning. A laboratory manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

SCHOONE G. J., VAN EYS G. J. J. M., LIGTHART G. S., TAUB F. E., ZAAL J., MEBRAHTU Y., LAYWER P. 1991: Detection and identification of *Leishmania* parasites by *in situ* hybridization with total and recombinant DNA probes. Exp. Parasitol. 73: 343–353.

SUGISAKI H., RAY D. S. 1987: DNA sequence of *Crithidia fasciculata* kinetoplast minicircles. Mol. Biochem. Parasitol. 23: 253–263.

VALENTIN 1847: Archiv of J. Muller. Quoted in Laveran A. and Mesnil F. (1912). Trypanosomes and trypanosomiasis. Masson, Paris, 999 pp.

WOO P. T. K., BLACK G. A. 1984: *Trypanosoma danilewski*: Host specificity and host's effects on morphometrics. J. Parasitology 70: 788–793.

ZAJÍČEK P. 1991: Enzyme polymorphism of freshwater fish trypanosomes and its use for strain identification. Parasitology 102: 221–224.

ZAJÍČEK P. 1992: *Trypanosoma* cf. *carassii*: the combination of malic enzyme patterns supporting the theory of diploidy in trypanosomes. Int. J. Parasitol. 21: 753–755.

ZAJÍČEK P., PECKOVÁ H. 1990: The interaction of fish trypanosomes culture forms with some lectins. Folia Parasitol. 37: 1–8.

ZAJÍČEK P., LUKEŠ J. 1993: The differentiation between freshwater and marine fish trypanosomes by lectin agglutinability. Folia Parasitol. 39: 195–200.

Received 30 June 1995

Accepted 31 August 1995