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Research paper

Further evidence from SSCP and ITS DNA sequencing support *Trypanosoma evansi* and *Trypanosoma equiperdum* as subspecies or even strains of *Trypanosoma brucei*

Yan-Zi Wen [a], Zhao-Rong Lun [a,b,c,d], Xing-Quan Zhu [b], Geoff Hide [c,d], De-Hua Lai [a,*]

[a] Center for Parasitic Organisms, State Key Laboratory of Biocontrol, School of Life Sciences, Sun Yat-Sen University, Guangzhou 510275, PR China
[b] State Key Laboratory of Veterinary Etiological Biology, Key Laboratory of Veterinary Parasitology of Gansu Province, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou, Gansu Province 730046, PR China
[c] Ecosystems & Environment Centre, School of Environment and Life Sciences, University of Salford, Salford M5 4WT, UK
[d] Biomedical Research Centre, School of Environment and Life Sciences, University of Salford, Salford M5 4WT, UK

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ABSTRACT

The subgenus *Trypanozoon* includes three species *Trypanosoma brucei*, *Trypanosoma evansi* and *Trypanosoma equiperdum*, which are morphologically identical and indistinguishable even using some molecular methods. In this study, PCR-based single strand conformation polymorphism (PCR-SSCP) was used to analyze the ribosomal DNA of the *Trypanozoon* species. Data indicate different patterns of ITS2 fragments between *T. brucei*, *T. evansi* and *T. equiperdum* by SSCP. Furthermore, analysis of total ITS sequences within these three members of the subgenus *Trypanozoon* showed a high degree of homology using phylogenetic analysis but were polyphyletic in haplotype networks. These data provide novel nuclear evidence to further support the notion that *T. evansi* and *T. equiperdum* should be subspecies or even strains of *T. brucei.*

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1. Introduction

Kinetoplastid flagellates of the subgenus *Trypanozoon* include three species, *Trypanosoma brucei*, *Trypanosoma evansi* and *Trypanosoma equiperdum*. *T. brucei* is the causative pathogen of Nagana in economically important animals and sleeping sickness in humans. The latter is listed as one of the major tropical neglected tropical diseases by WHO. *T. evansi* causes Surra in most domestic animals while *T. equiperdum* causes Dourine only in equines. The evolutionary relationship of these pathogens is essential for understanding the taxonomy, epidemiology and chemotherapy of the pathogens causing trypanosomiasis (Hide et al., 1994; Lun et al., 2004). The morphology of *T. evansi*, *T. equiperdum* and *T. brucei* is usually very similar, except that *T. brucei* undergoes differentiation into stumpy bloodstream forms while neither *T. evansi* nor *T. equiperdum* does. Therefore, the stumpy form is a key morphological stage that distinguishes *T. brucei* from the other two trypanosomes. However, the stumpy form is not usually easily found, which limits the usage of this criterion. Therefore, a variety of molecular techniques, directly or indirectly aimed at analyzing the trypanosome genome, have been developed to classify the subgenus *Trypanozoon* (Gibson, 2009; Hide and Tait, 2009). These include techniques such as isoenzyme variation (Gibson and Gashumba, 1983; Enyaru et al., 1993; Lun et al., 1992a), RFLP (Hide et al., 1990), random amplified polymorphic DNA (Ventura et al., 2002), amplified fragment length polymorphism (Agbo et al., 2002), multiplex-endonuclease genotyping (Claes et al., 2003), mobile genetic element-PCR (Tilley et al., 2003) and simple sequence repeat-PCR (Li et al., 2005b; Li et al., 2005c). However, none of them can provide accurate taxonomic criteria for classification of the inter-subgenus species. In order to distinguish the species in *Trypanozoon*, Lun et al. (1992b) and Ou et al. (1991) focused on the kinetoplast DNA of these parasites and found heterogeneity between these species. *T. brucei* possesses an intact kDNA maxicircle (~24 kb), while *T. evansi* is completely lacking the maxicircle. Thus, it is potentially easy to distinguish these two trypanosomes by PCR based on the presence or absence of maxicircle DNA. However, detection of the maxicircle DNA is not sufficient to distinguish *T. equiperdum* from *T. brucei* since full length or partial maxicircle molecules exist in *T. equiperdum* (Ventura et al., 2000; Lai et al., 2008). Therefore, molecular methods based on the nuclear DNA or kinetoplast maxicircle analysis have, in investigations conducted so far, failed to unequivocally distinguish these species from each other. To date, the classification of members of the *Trypanozoon* still depends on the mode of transmission, host range and pathogenicity, which is complex and time-consuming to determine (Brun et al., 1998). A rapid, reliable and sensitive approach for such a purpose is urgently needed.

* Corresponding author.
E-mail address: laidehua@mail.sysu.edu.cn (D.-H. Lai).

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Although PCR is a common and sensitive molecular method, polymorphisms based on amplicon length ignore minor changes in the DNA sequences. To make up for this deficiency, additional DNA sequencing of amplicons would be helpful but involves time-consuming processes including cloning and running sequencing reactions. An alternative approach is the use of PCR-based single strand conformation polymorphism (SSCP) analysis that utilizes differences in DNA sequence between two single strand conformations to analyze the fragment amplified by PCR (Hayashi, 1991). Based on the sensitivity and feasibility, this approach has been widely and successfully used for investigating genes from organisms as distinct as prokaryotes and humans. Specifically, this approach has been used to analyze genes important in defining disease (Ullah et al., 2015; Saha et al., 2015).

It is well known that the ribosomal RNA genes are highly conserved from protozoans to metazoans but microheterogeneity has been found among different species. Therefore, they have been widely used to analyze the genetic diversity among the eukaryote organisms. For instance, the 28S rRNA gene has been used to characterize *Trypanosoma grosi* but never to identify the homology with *Trypanozoon* (Sato et al., 2005). The internal transcribed spacer (ITS), including ITS1 and ITS2, within the rRNA arrays are divergent among species. Consequently, the phylogenetic relationship of the ITS regions has been widely used and shown to be useful in classifying many different kinds of organisms.

Here, we have investigated the possibility of employing 28S rDNA and ITS2 as molecular markers to distinguish *T. evansi*, *T. brucei* and *T. equiperdum* by PCR-SSCP. The whole ITS fragment was also sequenced to further analyze the diversity among these species.

2. Materials and methods

2.1. Trypanosome strains and DNA preparation

The strains of *T. evansi*, *T. brucei* and *T. equiperdum*, used in this study, are shown in Table 1. Trypanosomes were isolated from the blood of infected mice by DEAE cellulose (DE-52) as described by Lanham and Godfrey (1970). Mice were treated under the protocols approved by the National Institute for Communicable Disease Control and Prevention and the Laboratory Animal Use and Care Committee of Sun Yat-Sen University under the license 2010CB53000. DNA was released from the parasites by proteinase K digestion, extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated with ethanol. Pellets were suspended in TE buffer and the DNA was quantified using a NanoDrop spectrophotometer (Thermo, USA). The quality of the genomic DNA was examined on a 0.8% ethidium bromide stained agarose gel.

2.2. Genomic DNA amplification

Oligonucleotide primers were designed to evolutionarily conserved sequences that flank the variable region of the target genes. A fragment of 28S rDNA (300 bp) was amplified by PCR with primer set of wy2f (5′-GAG AGT GAC ATA GAA CCT GA-3′) and wy2r (5′-TTG GTC CGT GTT TCA AGA CG-3′) from the DNA templates. A fragment of ITS2 (347 bp) was amplified by the primers of ITS2F (5′-TGT CAC GCA TAT ACG TGT GTG -3′) and ITS2R (5′-TAC ACA CAT ACA CAC TAT CCG -3′). A joining fragment (1110 bp) of ITS1, 5.8S and ITS2 was amplified by primers of NC2 (5′-TTA GTT TCT TTT CCT CCG CT -3′) and NC5 (5′-GTA GGT GAA CCT GCG GAA GGA TCA TT -3′). PCR was performed in a thermal cycler (Biometra, Germany) in 50-μl volumes using 50 pmol of each primer, 250 μM of each dNTP, 3.0 mM $MgCl_2$, and 2 U Taq polymerase (Takara, Japan). The following cycling conditions were used: 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s, and a final extension of 72 °C for 5 min. As a negative control, the template was replaced by distilled water. PCR products were examined on a 0.8% agarose gel, stained with ethidium bromide, and photographed using a gel documentation system (UVITEC, Germany).

2.3. Non-isotopic SSCP analysis of amplicons

SSCP assay has been described for the phylogenetic analysis of parasites previously (Li et al., 2005a; Li et al., 2006a; Lin et al., 2007). Briefly, 7 μl of each PCR product was mixed with 13 μl of loading buffer containing 10 mM NaOH, 95% formamide, 0.05% bromophenol blue and 0.05% xylene cyanol. After denaturation at 94 °C for 5 min and snap-cooling on a freeze-block (−20 °C), 20 μl of each sample was loaded onto a 0.5 × MDE (Mutation Detection Enhancement) gel and subjected to electrophoresis in a Mini-Protean 3 Cell (Bio-Rad, USA) at 120 V and 20 °C for 5 h using 0.5 × TBE as the buffer. After electrophoresis, gels were stained with ethidium bromide for 1 h and photographed using ultraviolet transillumination.

Table 1
The strains of *T. evansi*, *T. brucei and T. equiperdum* used in this study.

Stock	Species	Host origin	Geographical origin	Year of isolation	Haplotype number	ITS data sources
STIB 815	*T. evansi*	Horse	China, Guangdong	1964	H_6	KU552349
STIB 807	*T. evansi*	Water buffalo	China Guangdong	1979	H_17	KU552344
STIB 817	*T. evansi*	Mule	China, Guangdong	1964	H_8	KU552351
STIB 810	*T. evansi*	Water buffalo	China, Zhejiang	1985	H_2	KU552346
STIB 812	*T. evansi*	Water buffalo	China, Yunnan	1987	H_7	KU552348
STIB 816	*T. evansi*	Camel	China, Xinjiang	1978	H_9	KU552350
STIB 811	*T. evansi*	Water buffalo	China, Hunan	1982	H_4	KU552347
STIB 780	*T. evansi*	Camel	Kenya	1982	H_5	KU552341
STIB 821	*T. evansi*	unknown	unknown	unknown	H_10	KU552353
CPO GZ	*T. evansi*	Water buffalo	China, Guangdong	2005	H_16	KU552357
STIB 808	*T. evansi*	Water buffalo	China, Jiangsu	1985	H_1	KU552345
STIB 804	*T. evansi*	Water buffalo	China, Guangdong	1981	H_15	KU552343
STIB 942	*T. brucei*	unknown	unknown	unknown	H_25	KU552356
STIB 920	*T. brucei*	Hartebeest	Tanzania	1971	H_23	KU552355
STIB 777	*T. brucei*	Tsetse fly	Uganda	1971	H_24	KU552340
STIB 818	*T. equiperdum*	Horse	China, Beijing	1979	H_18	KU552352
STIB 841	*T. equiperdum*	unknown	South Africa	unknown	H_20	KU552354
STIB 784	*T. equiperdum*	unknown	unknown	unknown	H_3	KU552342
Guangxi	*T. evansi*	Water buffalo	China, Guangxi	unknown	H_22	FJ416612
Hubei	*T. evansi*	Water buffalo	China, Hubei	unknown	H_11	FJ416613
NW2	*T. brucei*	Human	Uganda	1992	H_12	AF306776
Lister 427	*T. brucei*	Buffalo	Tanzania	1956	H_19	EuPathDB
DAL972	*T. brucei*	Human	Daloa, Ivory Coast	1978	H_13	EuPathDB
TREU927	*T. brucei*	Tsetse	Kenya	1970	H_21	EuPathDB
STIB 805	*T. evansi*	Water buffalo	China, Jiangsu	1985	H_14	EuPathDB

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