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

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Abstract

The subgenus *Trypanozoon* includes three species *Trypanosoma brucei*, *T. evansi* and *T. 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*.

Key words: *Trypanozoon*, PCR-SSCP, ITS, phylogenetic tree, haplotype network.

1. Introduction

Kinetoplastid flagellates of the subgenus *Trypanozoon* include three species, *Trypanosoma brucei*, *T. evansi* and *T. 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* are 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 analysing 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. 2005a; Li et al. 2005b). 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. (1992) 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.

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. 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 spacers (ITS), including ITS1 and ITS2, within the rRNA arrays are divergent among species. Consequently, the phylogenetic relationship of the ITS regions have 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 Nano-Drop 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) in 50- μ l volumes using 50 pmol of each primer, 250 μ M of each dNTP, 3.0 mM MgCl₂, and 2U Taq polymerase (Takara, Japan). The following cycling conditions were used: 94 °C for 5 min, followed by 30 cycles of 94 °C for 30

sec, 50 °C for 30 sec and 72 °C for 30 sec, 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. 2005; Li et al. 2006; 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.

2.4. Phylogenetic tree and the haplotype network construction

The PCR products amplified, using primers NC2/NC3, were sequenced from both directions using the same primers as used in the amplification reaction. Sequencing was done by Invitrogen Biotechnology Co., Ltd (Guangzhou, China). The sequences have been lodged with NCBI (Genbank: KU552340 to KU552357). Representative sequences of the ITS regions, from *T. brucei* and *T. evansi*, were downloaded from the NCBI and EUPATHDB databases (Table 1). All of the sequences were aligned using Clustal X (Thompson et al., 1997) and analysed using the MEGA 4.0 program to calculate the inter-specific pairwise distances. The neighbor-joining method within MEGA was used to construct the phylogenetic tree to analyze the relationships among *Trypanozoon* and the other trypanosomes. The phylogenetic relationships among *T. brucei*, *T. equiperdum* and *T. evansi* were further displayed in a haplotype network constructed using the software Dnasp 5.0 and Network.

3. Results

3.1. PCR amplification and single strand conformation polymorphism

Nine representative strains of *Trypanozoon* were selected for PCR-SSCP, three from each species of *T. brucei*, *T. equiperdum* and *T. evansi* (Table 1). Gel electrophoresis was conducted on all amplicons of the 28S rDNA (Fig 1A upper panel) and ITS2 (Fig 1B upper panel) and showed single bands with the expected lengths. No detectable difference was observed between species within the *Trypanozoon* group. No product was amplified from the negative control. The amplicons were then subjected to non-isotopic SSCP analysis to enable the detection of nucleotide sequence variation.

Figure 1A (lower panel) shows the SSCP analysis result for the 28S rDNA gene amplicons. Detection of variation in patterns between strains was not found, indicating that they had identical sequences, as subsequently confirmed by DNA sequencing.

The same approach using the ITS2 region provided higher resolution in the tested strains. Interestingly, the SSCP banding patterns from *T. brucei* were different from those of *T. evansi* and *T. equiperdum* (Fig 1B lower panel). Furthermore, there were two different patterns within the *T. brucei* group, in which two strains (*T. brucei* STIB 940 and STIB 777) showed a smaller band size while STIB 920 had a much larger band size. Further sequencing of the ITS2 revealed that the length of the amplicons from STIB 940 and STIB 777 were 10 bp longer than the other strains (Fig 2). A closer look at the alignment in Figure 2 reveals 59 polymorphic sites. The SNPs with a frequency of lower than 50% were boxed (Fig 2). All *T. brucei* were highly divergent, as between 22-25 boxed SNPs were present, while the *T. evansi* and *T. equiperdum* alignments contained only 3-10 boxed sites.

In summary, PCR-SSCP using ITS2 but not 28S rRNA could distinguish *T. evansi* and *T. equiperdum* from *T. brucei* at least among the strains we used.

3.2. Phylogenetic analysis of the *Trypanozoon* trypanosomes based on the ITS rDNA.

To further investigate the diversity of the internal transcribed spacer of rDNA

among the *Trypanozoon* species, the ITS rDNA fragment composed of ITS1, 5.8S and ITS2 rDNA was amplified from 18 strains of trypanosomes (Table 1). Additionally, other ITS sequences from 7 *Trypanozoon* strains were downloaded from the Eukaryotic Pathogen Database Resource (EuPathDB) or NCBI and were also included in the alignment. Furthermore, the ITS sequences of *Leishmania donovani*, *Leishmania infantum* (from EuPathDB) and *Trypanosoma cruzi* (from NCBI) were used as outgroup species.

Compared to *L. donovani*, *L. infantum* and *T. cruzi*, all the strains of *Trypanozoon* were very closely related and clustered together in one sub-branch of the phylogenetic tree (Fig. 3A). The phylogenetic relationships among *T. brucei*, *T. equiperdum* and *T. evansi* were also displayed as a haplotype network (Fig 3B). Overall, the three species were found to intermingle in the network. There were no separate clades for each of the three species. Although, the haplotypes were generally interdispersed and there were two clear clades containing the *T. evansi* stocks. Interestingly, in Clade I, *T. equiperdum* STIB 784 was also closely linked to the majority of the *T. evansi* strains. Additionally, three *T. evansi* strains (STIB 804, STIB 807, and CPO GZ) were grouped in a different clade. This clade (Clade II) contained stocks that were isolated at a different time from water buffalo in the Guangdong Province of China.

4. Discussion

PCR-SSCP takes advantage of the fact that the mobility of single stranded nucleic acids is determined not only by their fragment length but also by their sequence-dependent secondary structures. It has been effectively used for the identification of protozoan parasites, for instance *Plasmodium*, *Leishmania*, *Trichomonas* and *T. cruzi* (De Leon et al., 1998; Tashakori et al., 2006; Higo et al. 2007; Stothard et al., 2007; Matini et al., 2012; Keluskar et al., 2014) and metazoan parasites such as Strongylid and Ascarid nematodes (Gasser et al., 1997). Therefore, we investigated the use of PCR-SSCP as a tool, using the 28S ribosomal DNA and ITS2 fragments, for analyzing the species of *Trypanozoon*. Unfortunately, detected difference was not observed in the 28S rDNA among these three species (Fig 1A).

This indicates that the genetic relationship among *T. brucei*, *T. equiperdum* and *T. evansi* is very close using this marker. Nonetheless, the ITS2 genes of *T. evansi* and *T. equiperdum* share the same single strand conformation, while *T. brucei* shows only a slight difference (Fig 1B). These studies demonstrate that PCR-SSCP can be successfully used as a tool to evaluate the genetic relationship in closely related trypanosomes. Recently, there has been a growing interest in less studied species of trypanosomes (e.g. *T. lewisi* (Lin et al 2016); *T. pestanai* (Ideozu et al. 2015)) and PCR-SSCP could have the potential as a generic tool to be used on a wider range of trypanosomes.

The genetic similarities and differences, identified by the use of PCR-SSCP on the 28S and ITS2 genes, suggests that *T. evansi* and *T. equiperdum* could have a relatively recent common ancestor. All these current data further support the contention that *T. equiperdum* and *T. evansi* are subspecies or even strains of *T. brucei* based on the analysis of kinetoplast DNA and related proteins (Lai et al., 2008; Lun et al, 2009).

Interestingly, the SSCP derived conformation of the ITS2 gene in *T. brucei* STIB 920 was also distinct from the other two strains of *T. brucei* (STIB 940 & STIB 777). We speculate that the diversity of base pair conformations in the different strains of *T. brucei* might be due to the frequent occurrence of genetic mutation, which occurs spontaneously and frequently in *T. brucei*. Furthermore, the identical patterns, seen in *T. equiperdum* and *T. evansi*, suggests that these strains we tested may descend from a direct ancestor with one specific type of ITS2 conformation. However, we did not find this type of ITS2 conformation in the three *T. brucei* strains used in our study.

Therefore, it remains unknown as to whether the direct ancestor of *T. evansi* and *T. equiperdum* is a typical *T. brucei*. The wide diversity of *T. brucei* strains (Gibson et al. 1980) suggests that such a typical ancestral *T. brucei* might be difficult to identify or not exist. Since our sample size of three is very unrepresentative, we would not necessarily expect our sample to contain such an example. It is likely that the timing of events of the ITS2 mutations might occur earlier than the divergence of *T. equiperdum* and *T. evansi*. Additionally, all the conformations in the distinct strains of *T. equiperdum* and *T. evansi* are the same and supports the hypothesis that *T.*

equiperdum and *T. evansi* have recent origins. This is in accordance with previous discoveries that even in the akinetoplastic status (kDNA free), there are dozens of nuclear encoded proteins remaining that would be involved in kDNA replication, transcription and editing despite now being redundant due to the lack of kDNA (Lai et al., 2008). As *T. equiperdum* and *T. evansi* strains are dyskinetoplastic or akinetoplastic, it indicates that the concurrent loss of the kDNA is recent in the evolution of the *Trypanozoon*. A limitation on the detection of descent of other *T. equiperdum* and *T. evansi* strains from the other ITS2 variants, was the relatively small sample size of three strains from each species.

So far, there are only a few strains of *T. equiperdum* available in the laboratory, and most of those are limited to historical collections and information. Claes and colleagues suggested that *T. equiperdum* should be divided into *T. evansi* and *T. brucei* *equiperdum* based on the data from collected on the history, serological analysis and molecular characterization (Claes et al., 2005). However, Li et al. (2006) raise doubts about this hypothesis according to the different complexes of kinetoplast DNA among the *Trypanozoon*. The first reason is that several *T. equiperdum* strains (STIB 818, STIB 841, STIB842) classified by Claes et al., when compared to *T. evansi*, retain the maxicircles which are not found in any *T. evansi* strains. In fact, the presence of maxicircle kDNA is the key feature that distinguishes *T. evansi* from *T. equiperdum* (Schnauffer et al., 2002; Brun et al., 1998; Gibson, 2007). On the other hand, compared to the complexity of minicircle DNA in *T. brucei*, *T. equiperdum* and *T. evansi* have a very low diversity with only a few minicircle sequence classes. Therefore, the question as to whether *T. equiperdum* is a separate species and the position of *T. equiperdum* within the *Trypanozoon* group is still a matter for debate. Our phylogenetic tree based on the ITS shows that *T. brucei*, *T. equiperdum* and *T. evansi* are intermingled suggesting both recent divergence from each other and the possibility of frequent generation of the *T. evansi* and *T. equiperdum* (akinetoplastic/dyskinetoplastic/minicircle limited) strains from the parent species *T. brucei* (kinetoplastic/diverse minicircles). Our data are consistent with the previous

results that *T. equiperdum* is still not able to be clearly distinguished from *T. brucei* and *T. evansi*. Our previous results have demonstrated that the maxicircle was absent in *T. equiperdum* STIB 784 (Lai et al., 2008). As there is a lack of the historical information of STIB 784, it was speculated that this strain might be historically mistaken or that the maxicircles are occasionally lost by drug treatment or by long term laboratory cultivation. Nevertheless, our current data indicate that STIB 784 is much more closely clustered with the other 11 *T. evansi* strains in the Clade I of the ITS haplotype network. Based on the evidence from the ITS sequence and the maxicircle absence, it is highly possible that the STIB 784 strain has been mistakenly identified and should be a strain of *T. evansi*.

Interestingly, our data show that the Clade II of the haplotype network consists of all *T. evansi* strains isolated from water buffalo in Guangdong Province, including STIB 804, STIB 807 and CPO GZ. In contrast, the other strains of *T. evansi* isolated from other animals in Guangdong (STIB 815, STIB 817) or from water buffalo in other provinces (STIB 810, STIB 812, STIB 811, STIB 808, STIB 805) are genetically separated and are in Clade I. This genetic similarity suggests that *T. evansi* has been stably transmitted in water buffalo in Guangdong province, without other strains being involved, for more than two decades. This indicates that *T. evansi* in this area might have established their own transmission cycles in the local water buffalo population.

More and more evidence is accumulating that shows that the genomic relationships among the *Trypanozoon* species are very close. In fact, even the genome of *T. evansi* has recently been reported to have extensive similarities to *T. brucei* (Carnes et al, 2015). There have been suggestions that *T. evansi* and possibly *T. equiperdum* are cancerous variants of *T. brucei* due to the similarity with mammalian cancer cells that have lost the ability to differentiate and have lost the ability to regulate cell proliferation (Lun et al. 2015). Here, we have found a high degree of homology in the ribosomal DNA of the species of *Trypanozoon*. However, the ITS2 of *T. brucei* displays distinct characteristic differences from *T. equiperdum* and *T. evansi* in the pattern of PCR-SSCP although phylogenetic analysis of the entire ITS fragments of

these three species are monophyletic. In addition to existing kinetoplast data, our current results now provide evidence from nuclear genes to support the notion that *T. evansi* and *T. equiperdum* should be subspecies or even strains of *T. brucei*. However, the species names of *T. evansi* and *T. equiperdum* have been widely used to describe the pathogens causing Surra and Dourine, respectively. Therefore, we suggest that we should follow the current system, proposed by Votýpka and colleagues (2015), that we should keep the subspecies/strains concept in evolutionary studies but maintain the use of *T. evansi* and *T. equiperdum* for medical and veterinary disciplines.

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Figure legends

Figure 1. PCR-SSCP profiles of fragment of and 28S rDNA (A) and ITS2 (B) from the genomic DNA of *T. evansi*, *T. brucei*, *T. equiperdum* strains.

Figure 2. The alignment of sequences of the ITS2 fragment.

Polymorphic sites in the ITS2 fragment (54-397) are indicated with an asterisk (*). The SNPs with a frequency of lower than 50% are boxed.

Figure 3. The phylogenetic tree (A) and Haplotype networks (B) of the *Trypanozoon* strains at the ITS2 locus. The strains are indicated by the nodes (yellow: *Trypanosoma brucei*, blue: *T. equiperdum* and red: *T. evansi*). The light blue shade represents 2 clades. The strains corresponding to the haplotype number are listed in Table 1. In figure 3A, Ldon, Linf and Tcru represent *Leishmania donovani*, *L. infantum* and *Trypanosoma cruzi* respectively.