Genetic Diversity of *Trypanosoma evansi* in Buffalo based on Internal Transcribed Spacer (ITS) Regions

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ABSTRACT. The nucleotide sequences of 18S rDNA and internal transcribed spacer (ITS) regions were used for studying the relationships of *Trypanosoma evansi* isolate from a buffalo. The sequences were analyzed and compared to 18S rDNA and the ITS regions of the other *Trypanosoma* spp. Maximum likelihood phylogenetic trees were constructed using *Leishmania major* as the outgroup. The tree of 18S rDNA indicated that *T. evansi* (buffalo B18) isolate was closely related to those of Taiwan and *T. brucei* stock. The ITS tree showed the genetic diversity among 32 clones of *T. evansi* (B18) within a single host. This data will be useful for epidemiological and dynamic studies for designing the rational control programs of the disease.
KEY WORDS: internal transcribed spacers (ITS), phylogenetic, small subunit rDNA, *Trypanosoma evansi*.

Trypanosoma spp. consists of a large group of obligated flagellate blood parasites that infect members of every vertebrate class [20]. Members of this genus can be transmitted by various vectors such as blood-sucking invertebrates; primarily arthropods (for species infecting mammals and birds) and primarily hirudinean annelids (for species infecting poikilothermic vertebrates). In Thailand, where *T. evansi* is the only pathogenic trypanosome, it causes considerable loss in productivity in domestic animals such as horses, beef cattle, dairy cattle, and buffalos [5, 8, 31]. Animals which recover from an acute episode of disease are often clinically normal and *T. evansi* isolates can be obtained from them.

In Thailand, buffaloes are one of the most common hosts of *T. evansi*, and are infected via tabanid flies. It has been reported that there are 45 species of tabanids comprising three genera: *Tabanus, Haematopota*, and *Crysops*. *Tabanus rubidus* was found most frequently, distributed in every part of the country and present in 25 provinces. Clinical signs in animals infected with *T. evansi* are variable, depending on the animal species. Chronic cases occur mostly in infected buffaloes and cattle, which sometimes do not show any clinical signs [31]. However, abortion at late pregnancy or early parturition caused by *T. evansi* is known to occur in buffaloes [27]. In addition, stress factors such as fasciolosis, combined with seasonal malnutrition during the dry season, lower the resistance of the animals, causing exacerbation of clinical surra [26].

Generally, classification of trypanosomes has been based on a morphological description of bloodstream trypomastigotes and morphometric parameters. However, identification by means of such descriptions is often very difficult because of extreme morphological variation. Consequently, there is much confusion in the literature [10, 11, 24].

Molecular characterization has produced additional information on the affinities of trypanosomes. Some specific genes have been sequenced, aligned, and analyzed in order to study the phylogenetic relationships among morphologically indistinguishable trypanosomes [19]. Evolutionary events have been addressed by studying the ribosomal RNA (rRNA) genes and their associated spacer regions, collectively called ribosomal DNA [32]. Techniques such as the analysis of small or large subunit ribosomal RNA sequences [23, 35]. Restriction Fragment Length Polymorphisms (RFLPs) in DNA [6, 29, 49], ribosomal DNA restriction [7], the polymorphisms of PCR amplification products [4, 12], and molecular karyotypes have all been successfully used in distinguishing between morphologically similar protozoa [44, 45]. Nevertheless, the aforementioned methods have been unsuccessful in determining the relationships within trypanosome species.

The 18S rDNA gene has been studied because it is among the slowest evolving sequence found extensively throughout living organisms, and has therefore been very useful for examining ancient evolutionary events. The first molecular phylogenetic studies based on comparisons of genes encoding mitochondrial and nuclear ribosomal RNA (rRNA) showed trypanosomes to be a monophyly [14, 28, 38–41] as did studies based on protein-coding genes [1, 3, 15–17, 36, 47]. Doubt has now been cast on this consensus by a re-analysis of SSU rRNA gene sequences, but the previous SSU rRNA gene trees did not adequately prove monophyly of trypanosomes because they either included an inadequate number and selection of taxa, or were rooted inappropriately. In recent SSU rRNA gene trees, trypanosomes and trypanosomatids appear to be a paraphyly [21, 22].

The internal transcribed spacers (ITS) are versatile genetic markers and have been used for phylogenetic analysis, evaluation of the evolutionary process, and for the deter-

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mination of taxonomic identities [34]. The ITS are located between the repeating array of nuclear 18S, 5.8S, and 28S ribosomal RNA genes; a locus that has 100–200 copies per genome [18]. In 1999, the phylogenetic analysis of ITS sequences was used for studying the polymorphism of *T. rangeli* strains isolated from different hosts and geographic areas (southern Brazil, Central America, and northern South America) [13].

Previously, the intra-species genetic variation of *Trypanosoma* have been reported in *T. brucei* [2], *T. congolense* [30], and *T. evansi* [33, 46] in different hosts basically revealed by PCR based method. Herein, we investigated the diversity of *T. evansi* in a single host (buffalo) using the nucleotide data of 18S rDNA, 5.8S rDNA, and ITS regions. Phylogenetic trees were constructed and compared to those of other trypanosomes reported in GenBank. The data will be useful for epidemiological and dynamic studies for designing the rational control programs of the disease.

MATERIALS AND METHODS

Parasite collection: *T. evansi* were collected during 1999 from the infected blood samples of a buffalo namely B18 in the Ladkrabang district, Singburi province, Thailand. It was previously screened using the thin blood smear technique. Blood were preserved in the phosphate saline glucose buffer (PSG), pH 8.0 (50 mM $Na_2HPO_4.H_2O$, 2 mM $NaH_2PO_4.2H_2O$, 36 mM NaCl and 1.5% glucose) and stored in the liquid nitrogen tank (–196°C) until use.

Parasite infected blood preservation and isolation: Infected blood in the liquid nitrogen tank was thawed at room temperature and 0.1 ml was injected to a mouse using 1 ml Tuberculin syringe and needle gauge No 27. Parasitemia was daily checked by wet blood smear, starting from the third day after injection. At the highest parasitemia (10^8 cells/ml) which was usually in the 3rd -5th day of injection, blood was withdrawn from euthanized mouse by cardiac punctured. An anion exchange column (DE 52 DEAE cellulose) was used to purify parasite from the horde blood cells according to the method of described by Chao [9]. The eluent with infect protozoa was collected and proceeding to parasite lysis and DNA extraction.

DNA extraction and purification: Pellet of parasites was resuspended in PSG buffer in the presence of SDS (final concentration was 2%) and proteinase K (final concentration was 1 mg/ml). The solution was incubated at 42°C for 14 hr. Parasite DNA was extracted by conventional phenol/chloroform which responsed for deproteinization of the aqueous solution containing the desired nucleic acid. The purified DNAs were precipitated by the addition of 2 vol-

umes of cold absolute ethanol. The pellet was dried, dissolved in sterile distilled water and kept at 4°C until use.

PCR amplification: PCR amplifications of 18S rDNA gene and ITS spacer region were carried out using their specific primers (Table 1). All reactions were performed in 25 μl volume containing 100–200 ng of genomic DNA. The 18S rDNA reaction contained 1X PCR buffer, 1.7 mM of each primer, 200 mM of dNTP, 1.5 mM $MgCl_2$ and 3 units of *Taq* DNA polymerase. Sterile distilled water was used to make volume to 25 μl. The PCR amplification was performed using Peltier Thermal Cycler (MJ Research, PTC-200) for 30 cycles. Each cycle consisted of denaturation at 94°C for 1 min, annealing at 37°C for 1 min and extension at 72°C for 1 min. The PCR amplification of ITS regions contained 1 × PCR buffer, 2 mM of each primer, 400 mM of dNTP, 6 mM $MgCl_2$ and 1.5 units of *Taq* DNA polymerase. Sterile distilled water was used to make volume to 25 μl. A PCR amplification was performed using Peltier Thermal Cycler (MJ Research, PTC-200) for 30 cycles. Each cycle consisted of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min. PCR products were analyzed using electrophoresis in 1.2% agarose gel at 110 volts approximately 35 min prior to staining in ethidium bromide solution and observation.

Cloning and DNA sequencing: The PCR fragments were eluted from the gels, purified using TOPO XL PCR cloning kit (Invitrogen). The procedure of purification was performed according to the instruction manual provided by the company. The entire amplified both of 18S rDNA and ITS regions were purified and cloned using the procedures as specified by the manufacturer. After checking the cloned products by colony PCR amplification, 32 clones of ITS region were selected for further DNA sequencing using the Big Dye Terminator Cycle Sequencing procedure. The 300 ng of sample was used for PCR amplification using 25 cycles of denaturation at 96°C for 10 sec, annealing at 50°C for 4 sec and extension at 60°C for 4 min. The sample was dissolved with dye and formamide solution prior to DNA sequencing. The nucleotide sequence data were analyzed using software of ABI PRISM Model 377 version 3.4. The alignment of the sequences was achieved by using Clustal X software Version 1.83 (multiple sequence alignment) program [43] and refined using the manual method. *Leishmania major* and *Trypanosoma congolense* were used as the outgroup to root the 18S rDNA and ITS trees, respectively. Blocks of sequence data containing 2,349 characters for 18S rDNA and 1,125 characters for ITS regions were used for the tree analysis and were indicated by nucleotide position. The phylogenetic trees were constructed by PAUP program version 4.0 [42]. The cladograms were created by using

Table 1.   Oligonucletides used for PCR amplification

Names	Sequences	Description	Ref
A	AAC CTG GTT GAT CCT GCC AGT	5' end of 18S rDNA	37
B	TGA TCC TTC TGC AGG TTC ACC TAC	3' end of 18S rDNA	37
ITS/F	GGT GAT CGG ACC GTC GCT CGT CT	5' end of ITS regions	this study
ITS/R	CCT CTT CGC TCG CCG CTG ACT G	3' end of ITS regions	this study

Table 2.   GenBank accession numbers of 18S rDNA and ITS regions of *Trypanosoma spp.* and other blood parasites

	Parasites names	specific Host/Lab host	GenBank accession numbers
118S rDNA	*Leishmania major*		AC005806.3
	T. brucei		AL929605.1
	T. b. gambiense		AJ009141.1
	T. b. rhodesiense		AJ009142.1
	T. congolense	*Rattus norvegicus*	U22319.1
	T. cruzi		AJ009148.1
	T. eqiperdum		AJ009153.1
	T. evansi		D89527.1
	T. evansi Buffalo (B18)		AY904050
	T. godfreyi		AJ009155.1
	T. rangeli		AJ012416.1
	T. simiae		U22320.1
	T. vivax	*Rattus norvegicus*	U22316.1
ITS region	*T. congolense*	*Rattus norvegicus*	U22319.1
	T. evansi		D89527.1
	T. evansi Buffalo (B18)	Buffalo	AY904050
	T. evansi clone No.2–10	Buffalo	DQ472679-DQ472687
	T. evansi clone No.12–21	Buffalo	DQ472688-DQ472697
	T. evansi clone No.24	Buffalo	DQ472698
	T. evansi clone No.26	Buffalo	DQ472699
	T. evansi clone No.30–34	Buffalo	DQ472700-DQ472704
	T. evansi clone No.36	Buffalo	DQ472705
	T. evansi clone No.38–40	Buffalo	DQ472706-DQ472708
	T. evansi clone No.48–49	Buffalo	DQ472709-DQ472710
	T. brucei	*Homo sapiens*	X05682.1
	T. brucei NW2	*Homo sapiens*	AF306776.1
	T. brucei TH2	*Homo sapiens*	AF306777.1
	T. brucei Da1972	Pig	AF306774.1
	T. brucei B8/18	*Glossina palpalis*	AF306772.1
	T. brucei KP2	Lion	AF306773.1
	T. brucei STIB215	Lion	AF306771.1
	T. brucei H3		AF306770.1
	T. brucei TREU927		AL929605.1

maximum likelihood and maximum parsimony methods for 18S rDNA gene and ITS region, respectively. Bootstrap values were replicated 100 times and computed by PAUP. The *T. evansi* sequences were compared to those of other trypanosomes reported in Genbank as indicated in Table 2.

RESULTS

This study used the molecular phylogenetics of 18S rDNA to identify the species of trypanosome isolated from a buffalo in Thailand. The PCR product of 18S rDNA was approximately 2.1 kb in size. The phylogenetic tree of 18S rDNA sequences from different *Trypanosoma spp.* stocks confirmed the phylogenetic position of *T. evansi* in the *Trypanosomatids* family and suggested that *T. evansi* stocks from buffalo in Thailand belong to a single genus receiving highly significant support when using *Leishmania major* as the outgroup (Fig. 1). According to the tree, Salivaria, except for *T. vivax*, formed a cluster which was supported by a highly significant internal branch falling outside the highly significantly supported cluster of Stercoraria and outgroup. The tree also revealed that *T. evansi* strain B18 was homol-

ogous to *T. evansi, T. brucei, T. b. gambiense, T. b. rhodesiense*, and *T. eqiperdum* stock submitted to GenBank. This larger cluster was supported by a highly significant internal branch.

Sequence analysis of the ITS region containing ITS-1, 5.8S rDNA, and ITS-2 from 32 clones of *T. evansi* B18 isolates was performed. The 5.8S rDNA sequences showed no significant differences among 32 clones of *T. evansi* against *T. brucei*, whereas the ITS-1 and ITS-2 genes exhibited single nucleotide polymorphisms (SNPs) characterized by base alterations, insertions, or deletion. Along with probable insertion/deletion events, several perfect and imperfect microsatellite repeats with variable motifs (TA, AT, GG, GT, TT, and CTT) and length were mainly observed in the sequences. A comparative analysis of the sequence alignments carried out by ClustalX (Version 1.83) revealed that 180 and 312 variable sites were observed in ITS-1 and ITS-2 regions, respectively.

The phylogenetic tree based on ITS-1 and ITS-2 regions (Figs. 2 and 3) of 32 clones from *T. evansi* B18 stock showed that most of them were separated from *T. brucei* when *T. congolense* was used as the outgroup. However,
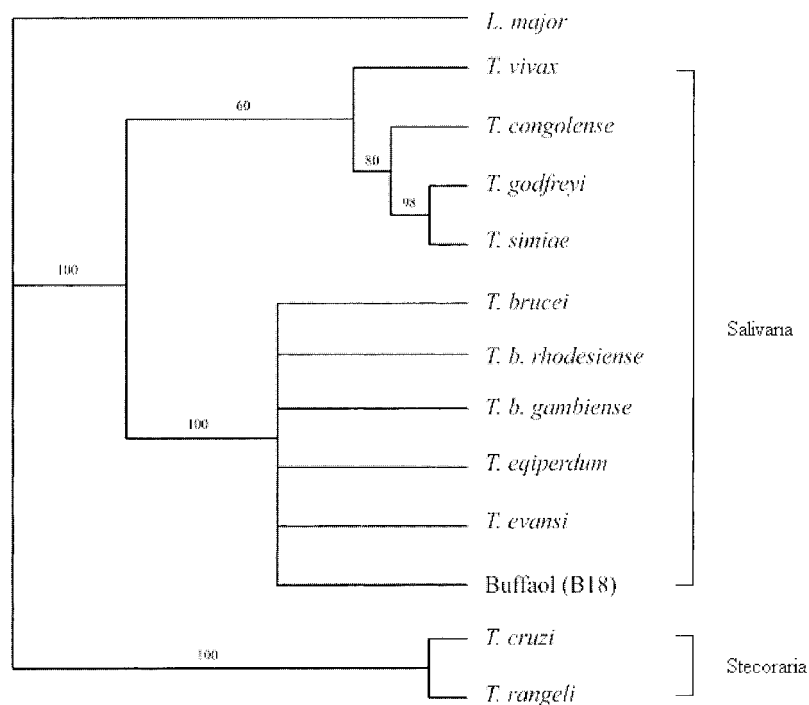
Fig. 1. Phylogenetic relationship of 18S rDNA obtained from the likelihood methods among *Trypanosoma* spp. using *L. major* as the outgroup. The tree was constructed based on the heuristic search using PAUP version 4.0 and inferred from complete 18S rDNA gene sequences using a bootstrap resembling of the data set. The numbers at the nodes represent the percentage of times the group occurred out of 100 trees. Branches with less than 50% bootstrap support were collapsed. No significant relation was placed on the lengths of the branches connecting to the species.

some of them were classified within the same clade as *T. brucei*. Based on the ITS-1 tree (Fig. 2), the data revealed that these clones were diverse and separated into two groups, group 1 and group 2, which consisted of 29 and 3 taxa, respectively. Referring to the ITS-2 tree (Fig. 3), the clones were separated into four groups containing 3, 18, 9, and 2 taxa, respectively. The phylogram showed intermediate significant bootstrap supported clusters and the variation of nucleotides in the ITS-2 region showed the variation of *T. evansi* in buffalo.

DISCUSSION

The nucleotide sequence alignment of 18S rDNA from *T. evansi* showed a sufficiently high degree of homology to demonstrate that nucleotide misincorporation may occur while using regular *Taq* DNA polymerase in PCR; the consistency of these polymorphisms was assessed by separate analysis of each clone sequence. Nucleotide alterations on the consensus sequences of all clones were also observed. The 18S rDNA data clearly indicated that *T. evansi* B18 stock was relatively straightforward to include under the *T. evansi* clade.

Analysis of the ITS regions revealed that mononucleotide insertion/deletion or transition and transversion occurred,

whereas the 5.8S rDNA was completely conserved among all of selected clones. This indicated that the ITS regions were more informative than the 5.8S rDNA. When phylogenetic trees were separately constructed using the nucleotide data of either ITS-1 or ITS-2, it was clear that the ITS-2 tree was more informative than the ITS-1 tree. However, both ITS regions of *T. evansi* B18 isolate revealed intra-species genetic diversity; at least 2–4 groups within one host were observed. Previously, the genetic diversity of *T. evansi* has been reported using MGE-, SSR-, RAPD-PCR profiling, and expression-site-associated genes (ESAG 6) [25, 48]. These studies indicated that there are at least 3–7 different patterns of *T. evansi* with different hosts. However, the data showed no genetic diversity of *T. evansi* within a single host.

Thus, it is proposed that the ITS-2 nucleotide sequence data represent a useful genetic marker for classification within the group of *T. evansi* which can be applied in epidemiological and dynamic studies for designing the rational control programs of the parasites.
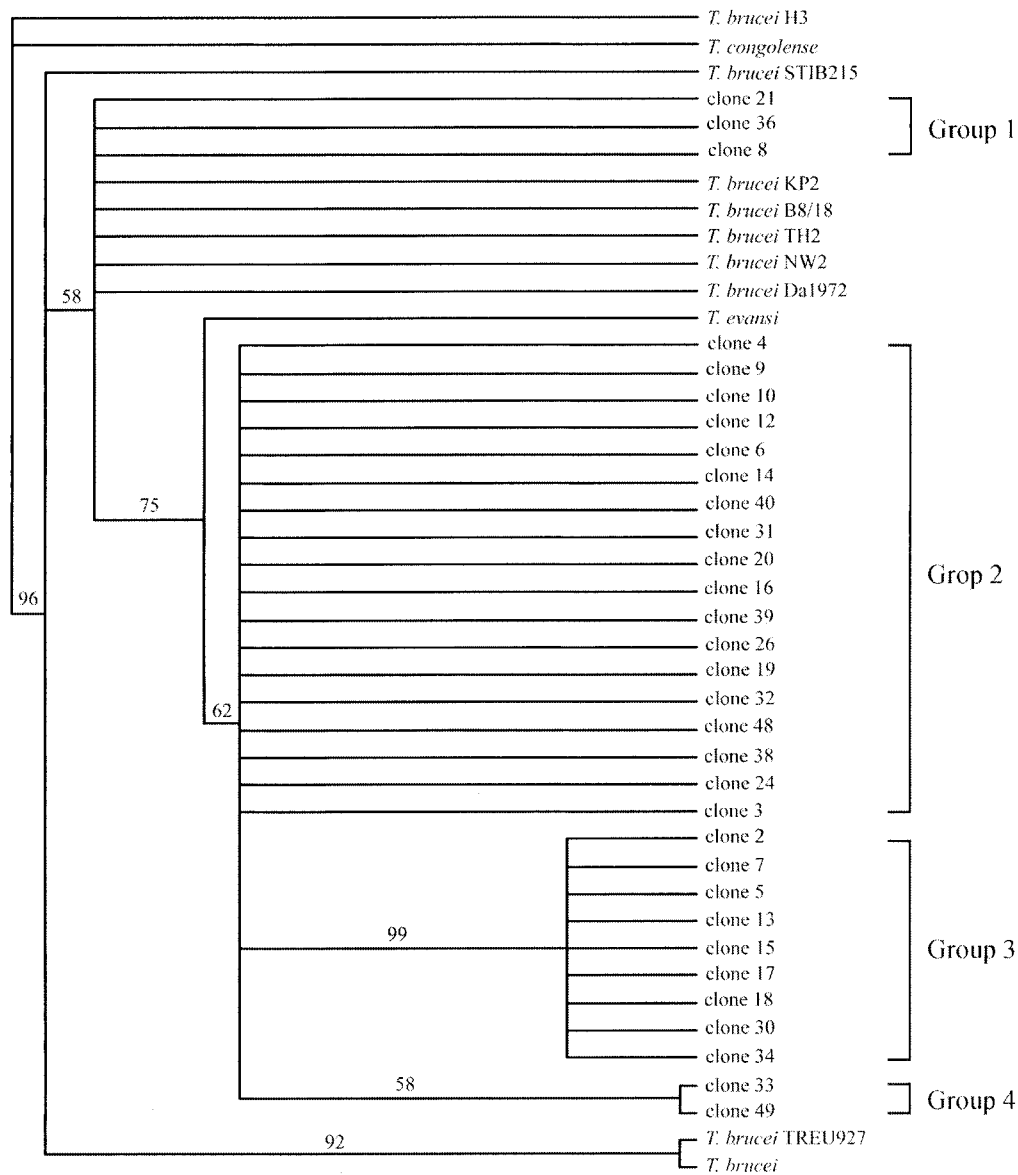
Fig. 2. A maximum parsimony methods of ITS-1 among 32 clones of *T. evansi* B18 isolates and *T. brucei,* outgroup with *T. congolense*, produced by the heuristic search of PAUP version 4.0 and inferred from complete ITS region using a bootstrap resembling of the data set. The numbers at the nodes represent the percentage of times the group occurred out of 100 trees. Branches with less than 50% bootstrap support were collapsed. No significance is placed on the lengths of the branches connecting the species.

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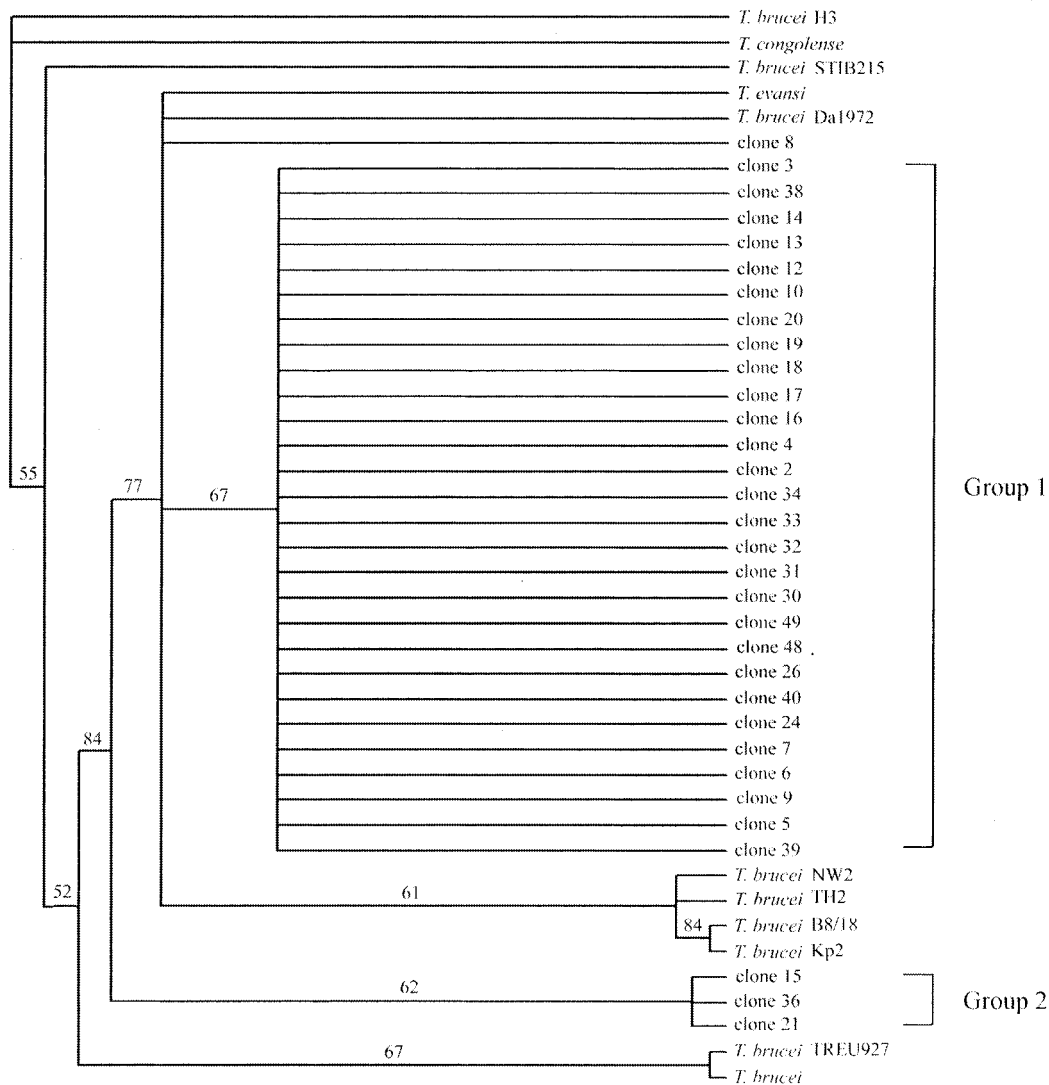
Fig. 3.   A maximum parsimony of ITS-2 among 32 clones of *T. evansi* B18 isolated and *T. brucei*, outgroup with *T. congolense*, produced by the heuristic search of PAUP version 4.0 and inferred from complete ITS region using a bootstrap resembling of the data set. The numbers at the nodes represent the percentage of times the group occurred out of 100 trees. Branches with less than 50% bootstrap support were collapsed. No significance is placed on the lengths of the branches connecting the species.

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