

Phylogenetic position of the freshwater fish trypanosome, *Trypanosoma ophiocephali* (Kinetoplastida) inferred from the complete small subunit ribosomal RNA gene sequence

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Abstract The complete small subunit rRNA (SSrRNA) gene sequence (2,142 nucleotides) of the freshwater fish trypanosome *Trypanosoma ophiocephali* Chen (1964) was determined. The phylogenetic analysis deduced using neighbor-joining, maximum parsimony, and Bayesian methods demonstrated the existence of an “aquatic clade”. *T. ophiocephali* was revealed to be a member of the freshwater fish trypanosomes and form the sister species with *Trypanosoma siniperca* and *Trypanosoma* sp. Carpio with high bootstrap values (98% MP, 100% NJ, 100% Bay). The high similarity of SSrRNA gene sequences and morphometric characters showed that *T. ophiocephali*, *T. siniperca* and *T. sp. Carpio* probably were the same species. The phylogenetic trees further suggested that Chinese freshwater fish trypanosome might be paraphyletic, and fish trypanosomes should have low host specificity.

Introduction

Piscine trypanosomes, transmitted by various bloodsucking aquatic leeches, are widespread parasites of fish (Lom

1979; Letch 1979; Gibson et al. 2005). Since Valentin first discovered a trypanosome from the blood of *Salmo fario*, more than 100 fish trypanosome species have been described worldwide, mostly on the basis of “one host–one species” paradigm, according to which a new species was assigned for every “new” fish host (Lom 1979). However, clear-cut evidence of strict host specificity is lacking, while several successful cross-transmission experiments have proved that particular trypanosome species are not restricted to the host species from which they were originally recovered (Lom 1973; Letch 1979; Woo and Black 1984). In addition, Karlsbakk et al. (2005) found a trypanosome species transmitted by the marine leech from Northern Norway showed large disparities in morphology and aspects during different infection time and Gu et al. (2007b) found at least two genotype trypanosomes occurred in *Pseudobagrus fulvidraco* from Chinese Niushan Lake. Moreover, most of the early descriptions are simple and inadequate according to current morphological standards (Smit et al. 2004; Karbowiak et al. 2005; Gu et al. 2006a, 2007a, b) and can hardly be used for a safe determination of species. Therefore, the validity of most species should be questioned, and the taxonomic status and phylogenetic relationships of these species need to be revised. Many taxa are considered nomina dubia, and the taxonomic status of piscine trypanosomes remains generally controversial.

Molecular methods, in particular, the determination of small subunit rRNA (SSrRNA) sequences, have been commonly used to re-evaluate the phylogenetic relationships of trypanosomes in recent years (Jakes et al. 2001; Sehgal et al. 2001; Martin et al. 2002; Votypka et al. 2002, 2004; Gibson et al. 2005; Davies et al. 2005; Karlsbakk and Nylund 2006; Gu et al. 2007b). These studies provided many results inconsistent with those from traditional morphological and

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ontogenetic characters (Sehgal et al. 2001; Davies et al. 2005; Karlsbakk and Nylund 2006; Gu et al. 2007b).

Trypanosoma ophiocephali was previously described from the blood of *Ophiocephalus argus* captured from Liaoho River, Liaoning Province, and Hwa-ma Lake, Hubei Province, China, by Chen (1964) and Chen and Hsieh (1964), respectively. Recently, Gu et al. (2006a) redescribed this parasite from *O. argus* and *Odontobutis obscura* in Niushan Lake, Hubei Province, China, according to current morphological standards (Smit et al. 2004; Karbowiak et al. 2005; Gu et al. 2007a). As a comprehensive analysis of fish trypanosome phylogeny is being carried out in the author's group, we have recently sequenced the SSrRNA gene of the trypanosomes from the blood of *O. argus* for the first time in order to determine its systematic position with molecular biological methods and provide more information on phylogenetic relationships within the fish trypanosomes. The results are supplied and discussed here.

Materials and methods

As described previously (Gu et al. 2006a, 2007a), host fishes were collected from Niushan Lake Fishery in Wuhan, Hubei Province (30°19'N, 114°31'E), China, in May 2007, and transported live to the laboratory. Fish samples were anesthetized, and whole blood was gained from the fish caudal vein. Then the flagellates were collected after centrifugation at 3,000×g for 10 min. Observations on living cells were carried out with light microscopy at ×200 magnification. The Giemsa staining method according to Karbowiak et al. (2005) was used to reveal the morphometric characters. Our result showed that these flagellates were morphologically identical to *T. sp. III* of *T. ophiocephali* described recently (Gu et al. 2006a). The terminology of morphometric parameters used in the present paper is the same as previously reported by Gu et al. (2006a).

Trypanosomes were isolated from host blood cells by centrifugation in Percoll density gradient and filtration through DEAE cellulose as described by Gu et al. (2006b). DNA was extracted as described by Lynn and Sogin (1988). Primers S-762 and S-763 (Malsov et al. 1996) were used for amplifying the trypanosome SSU rRNA gene. The PCR reaction steps, cloning, and sequencing of the SSrRNA gene with a set of M13 primers and “walking” primers were performed as described previously (Gu et al. 2007a).

The nucleotide sequences in the present paper are available from the GenBank/EMBL databases under the following accession numbers: *Trypanosoma siniperca* DQ494415, *Trypanosoma cobitis* AJ009143, *Trypanosoma sp.* EI-CP L14841, Ts-Se-BL AJ620550, Ts-Ab-Tb AJ620556, *Trypanosoma granulosum* UK AJ620551, Marv AJ620549, *T. granulosum* Portugal AJ620552, CLAR

AJ620555, Ts-Tt-HOD AJ620553, R6 AJ620554, *Trypanosoma boissoni* U39580, *Trypanosoma triglae* U39584, *Trypanosoma sp.* K&A leech AJ009167, *Trypanosoma chelodinae* AF297086, *Trypanosoma binneyi* AJ132351, *T. sp.* Carpio EF375882, *Trypanosoma sp.* Pseudobagri EF375883, *Trypanosoma sp.* EF375884. *Trypanosoma lewisi* AJ223566, *Trypanosoma theileri* AB007814, and *Trypanosoma avium* U39578 were selected as the outgroup species.

All sequences were globally aligned with other SSrRNA gene sequences using the Clustal-X (Thompson et al. 1997) with default settings and then examined by eye. The postulated gaps and ambiguously aligned regions were excluded from phylogenetic analyses. As described previously (Gu et al. 2007a, b), phylogenetic trees were constructed by the neighbor-joining method, the maximum parsimony (MP) method and the Bayesian method, which implemented in the MEGA3 computer package (Kumar et al. 2004), PAUP* computer package (Swofford 2003) and MrBayes version 3.0b4 (Huelsenbeck and Ronquist 2001), respectively.

Results

The complete SSrRNA gene sequence of *T. ophiocephali* contained 2,142 nucleotides. The G+C content is 49.4%, and it is in the same range as other fish trypanosomes. The sequences obtained from *T. ophiocephali* and *T. siniperca* were nearly identical (structural similarity 99.99%), with the exception of one variable site and one gap between them. The sequence of *T. ophiocephali* differed in four nucleotides from the sequence of *T. sp. Carpio*, a trypanosome from carp from China (structural similarity 99.82%), while a total of 23 sites were different from that in *T. sp. Pseudobagri*, a slender form of trypanosome from *P. fulvidraco* (structural similarity 98.97%).

Phylogenetic analyses were based on the final edited alignment which was 2,226 bp in length and contained 25 taxa. The neighbor-joining analysis confirmed the clustering patterns of the maximum parsimony analysis and Bayesian analysis in several cases with different bootstrap values (Fig. 1). SSrDNA neighbor-joining tree strongly supported the existence of an “aquatic clade” (Gibson et al. 2005) consisting of trypanosomes from aquatic vertebrates, transmitted by bloodsucking aquatic leeches. All the fish trypanosome isolates fell in the aquatic clade. Four subclades were supported in the aquatic clade: (1) freshwater fish trypanosomes, (2) an unknown species from an aquatic leech (*Piscicola geometra*) from UK (*T. sp.* K&A leech), (3) a clade containing two species from an aquatic tortoise (*T. chelodinae*) and duck-billed platypus (*T. binneyi*), and (4) marine fish trypanosomes containing two species from Senegal (*T. boissoni* and *Trypanosoma senegalense*) and two species from Norway (*Trypanosoma*

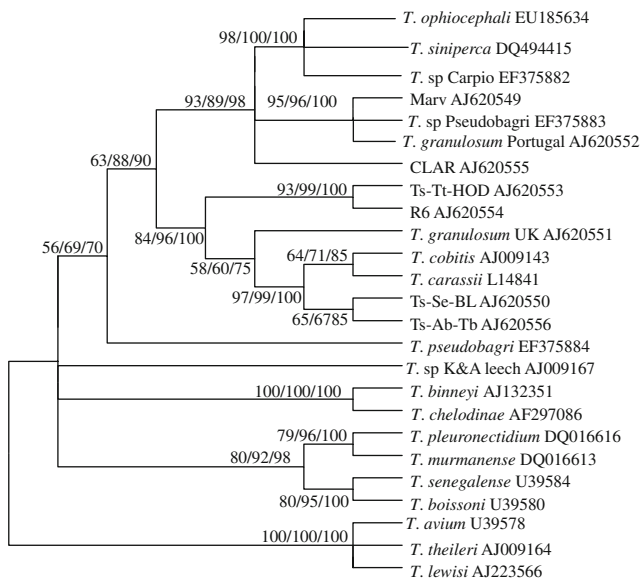


Fig. 1 The neighbor-joining tree of aquatic trypanosomes constructed from complete small subunit ribosomal RNA (SSrRNA) sequences indicating the systematic position of *T. ophiocephali* and phylogenetic relationships among the aquatic trypanosomes whose sequences are available. *T. lewisi*, *T. theileri*, and *T. avium* are taken as the outgroup. Bootstrap values are shown for the maximum parsimony/neighbor-joining/Bayes analyses

pleuronectidium and *Trypanosoma murmanense*). *T. ophiocephali* was placed within the subclade of freshwater fish trypanosomes and formed a sister group with *T. siniperca* and *T. sp. Carpio* with high bootstrap values (98% MP, 100% NJ, and 100% Bay).

Discussion

As demonstrated in the present and many other papers (e.g., Gibson et al. 2005; Karlsbakk and Nylund 2006; Gu et al. 2007a, b), the existence of the aquatic clade was strongly supported, and all freshwater fish trypanosomes grouped in a single clade. In our phylogenetic trees, freshwater fish trypanosomes were subdivided into two main groups, and those from China formed a subgroup, excepting that *T. sp. Pseudobagri*, a larger-form trypanosome from *P. fulvidraco* was placed at the bottom of freshwater fish trypanosome group and became a single isolate. This result was in consistent with the notion of Gu et al. (2007a, b) and revealed that Chinese freshwater fish trypanosome may be paraphyletic (Fig. 1). In addition, Karlsbakk and Nylund (2006) found *T. pleuronectidium* and *T. murmanense* from Norway and *T. senegalense* and *T. boissoni* from Senegal did not group together but formed respective clade, and both formed sister clades with freshwater fish trypanosomes. In the present study, all marine fish trypanosomes, though divided into two subclades, however, clustered

together and then formed sister clade with freshwater fish trypanosomes (Fig. 1). Analyzing the different results between two papers, we thought the main reason was that a combination of insufficient taxa and an imbalance in the spread of included taxa, especially the absence of Chinese trypanosome species, affected the topology structure of trees in the research of Karlsbakk and Nylund (2006).

In our phylogenetic analysis based on the SSrRNA gene sequence, *T. ophiocephali* from the blood of *O. argus* fell within the freshwater fish trypanosome group and formed the sister species with *T. siniperca* and *T. sp. Carpio* with high bootstrap values (98% MP, 100% NJ, 100% Bay). Gu et al. (2007a, b) had compared the morphological characters of *T. siniperca* with *T. ophiocephali* and *T. sp. Carpio*, respectively, and found they shared many similar characteristics. Moreover, *T. siniperca* and *T. sp. Carpio* were considered to be a same species according to a comprehensive analysis of morphological and molecular data (Gu et al. 2007b). In this paper, we observed their movements, summarized the mensural morphometric values of blood trypomastigotes and found that there were greatly significant similarities among *T. ophiocephali*, *T. siniperca*, and *T. sp. Carpio* such as body length and width, nucleus length and width, PN KN distances. Furthermore, the SSrRNA gene sequences obtained from these trypanosomes were nearly identical (structural similarity 99.99%). Therefore, we concluded that *T. ophiocephali*, *T. siniperca*, and *T. sp. Carpio* may be the same species from same region and different hosts.

So far, more than 30 species of trypanosomes have been reported and described from Chinese freshwater fishes according to the traditional taxonomy (Chen and Hsieh 1964, Zhao 1998), but it is believed the actual number of species is much lower, and the freshwater fish trypanosome species richness in China may be exaggerated. Although we cannot make any conclusive statements about the levels of diversification of Chinese freshwater fish trypanosomes, the data in this study do provide the first molecular-based evidence that a single morphospecies of *T. ophiocephali* can infect at least three fish species. In addition, our previous study also provided the molecular evidence that a single fish species could be infected with more than one species of trypanosome (Gu et al. 2007b). This phenomenon can be caused by infections of leeches with different trypanosomes; but so far, no infected leech has been collected in freshwater from China.

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