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Environmental kinetoplastid-like 18S rRNA sequences and phylogenetic relationships among Trypanosomatidae: Paraphyly of the genus *Trypanosoma*

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Abstract

Using kinetoplastid-like sequences from deep-sea environmental samples as an outgroup, we applied phylogenetic analysis to 18S rRNA sequences of the families Trypanosomatidae and Bodonidae (Eugelenozoa: Kinetoplastida). The monophyly of the genus *Trypanosoma* was not supported by a number of different methods. Rather, the results indicate that the American and African trypanosomes constitute distinct clades, therefore, implying that the major human disease agents *T. cruzi* (cause of Chagas' disease) and *T. brucei* (cause of African sleeping sickness) are not as closely related to each other as they were previously thought to be. Likewise, the results did not support monophyly of the genera *Leishmania*, *Leptomonas*, *Bodo* and *Cryptobia*.
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1. Introduction

Members of one of the protist order Kinetoplastida (phylum Eugelenozoa) are characterized by the presence of kinetoplast, a DNA-containing mitochondria-like organelle and a flagellum [1,2]. Traditional morphology-based classification separates kinetoplastids into two suborders, Bodonina and Trypanosomatina [3,4]. The former suborder includes species with two flagella and a large kinetoplast, while the latter is composed of obligatory parasitic species with single flagellum and a smaller kinetoplast.

The family Trypanosomatidae, belonging to the suborder Trypanosomatina, includes several of the most serious vector-borne parasites of humans, including members of the *Leishmania* and *Trypanosoma*, numerous species parasitic on non-human vertebrates, and numerous parasites of insects, other invertebrates, and plants [5]. Within the genus *Trypanosoma*, traditional classification distinguished two major groupings [5–7]: (1) the section Stercoraria, also known as the American trypanosomes, that includes *T. cruzi*, the causative agent of Chagas' disease, along with other parasites of mammals and leech-transmitted parasites of aquatic vertebrates and (2) the section Salivaria, also designated as the African trypanosomes, that includes *T. brucei*, the causative agent of African sleeping sickness, along with other African parasites of mammals.

Because of the medical importance of the obligatory parasites in the family Trypanosomatidae, relationships of these organisms have been studied extensively using 18S rRNA nucleotide sequences [5–11]. Less attention has been paid to the related free-living organisms, including members of the family Bodonidae. Thus, although there have been numerous attempts to resolve relationships among trypanosomes, skewed taxonomic sampling in many of these studies, due to under-representation of the free-living species, has yielded controversial results. Among other possible reasons for lack of resolution of deep divergences within the

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family Trypanosomatidae has been the utilization of inappropriate outgroup species [12]. In addition, there have been several attempts to reconstruct trypanosomatid phylogeny using protein-coding genes [13–15]. The results generally supported monophyly, but these studies suffered from biased taxonomic sampling to an even greater extent than did those based on 18S rRNA.

A comprehensive recent study [11] including 75 kinetoplastid species did not support monophyly of the genus *Trypanosoma*. Rather, the American and African trypanosomes formed separate clusters in the tree [11]. In addition, several other genera of kinetoplastids were shown to be paraphyletic, including *Leishmania* from the family Trypanosomatidae and *Bodo* and *Cryptobia* from the family Bodonidae. In this study, two euglenid species were used as an outgroup [11]. However, euglenids, while representing the same phylum as kinetoplastids, still constitute fairly distant outgroup. In order to avoid artifacts related to long branch-attraction [16,17], it is important to include sequences of intermediate divergence when a distantly related outgroup it is used to root.

A recent survey of 18S rRNA diversity in deep-sea environmental samples [18] supplies a set of kinetoplastid-like sequences that appear to be basal to other kinetoplastids [19]. These sequences are derived from free-living kinetoplastids (prokinetoplastids) outside the family Trypanosomatidae and thus represent kinetoplastid lineages that have been poorly sampled in previous analyses. In this study, we include these 18S rRNA sequences in a phylogenetic analyses of 18S rRNA sequences of Trypanosomatidae and Bodonidae in order to further address the phylogenetic relationships between American and African trypanosomes and whether or not the genus *Trypanosoma*, as currently recognized, represents a monophyletic group. In particular, we revisit the phylogenetic position of *Trypanosoma vivax*, which previously was believed to be a distant member of Salivaria clade [5], but in the phylogenetic analysis of Hughes and Piontkivska [11] occupied a separate basal position relative to other Trypanosomatidae. We also examined relationships within the family Bodonidae, and in particular, in the genus *Bodo*.

2. Materials and methods

Phylogenetic analyses were based on the following 18S rRNA sequences: the data set of 68 species previously used by Hughes and Piontkivska [11], representing 9 genera of Trypanosomatidae and 6 genera of Bodonidae; the 2 euglenid species used as an outgroup by Hughes and Piontkivska [11]; 3 kinetoplastid-like (prokinetoplastids) and 4 bodonid-like sequences collected from deep-sea samples [18]; and sequences representing 2 other prokinetoplastid and 5 euglenid species collected from the GenBank database (Supplementary Table 1).

The ClustalW program was used to align the nucleotide sequences [20]. The alignment is available upon request. Any site at which the alignment postulated a gap was excluded from the phylogenetic analysis so that a comparable set of sites was used for each comparison. Phylogenetic trees were reconstructed by four methods: (1) the minimum evolution (ME) method [21], as implemented in the MEGA2 computer package [22]; (2) the quartet maximum likelihood (QML) method [23], as implemented in the TREEPUZZLE 5.0 program; (3) the maximum parsimony (MP) method, using heuristic search, as implemented in the PAUP* computer package [24]; and (4) the Bayesian method, as implemented in MRBAYES Version 2.01 [25].

ME trees were constructed using the Kimura-two-parameter distance [26], which takes into account unequal rates of transition and transversion, and the Tamura–Nei distance [27], which takes into account both transitional and GC content biases. In the QML analysis, the Tamura–Nei model was used. The QML tree was also reconstructed using the Tamura–Nei model with a gamma distribution which takes into account the rate heterogeneity across sites, with the gamma parameter estimated from the data; however, the results were essentially the same as those from Tamura–Nei model. MP trees were generated using a heuristic search option with 10 random stepwise-addition replicates that were followed by nearest-neighbor-interchange branch swapping to completion. To estimate relative branch support of ME and MP trees, bootstrap analysis [28] with 1000 bootstrap replicates was conducted. Reliability of clustering patterns in the QML analysis was assessed by the proportion of 25,000 puzzling steps that supported a given pattern.

In the Bayesian analysis, the general time reversible (GTR) + gamma + proportion of invariant sites nucleotide substitution model was used [29]. An uninformative prior was used. Metropolis-coupled Markov chain Monte Carlo sampling was performed with four chains (one cold and three incrementally heated, with temperature parameter value = 0.2). These chains were run for 1,000,000 generations, with trees sampled every 100 generations from the last 500,000 generations, and 5000 sampled trees were used for inferring the Bayesian tree.

All trees were rooted using the group of seven euglenid species: *Euglena viridis*, *Euglena gracilis*, *Phacus splendens*, *Khawkinea quartana*, *Petalomonas cantuscygni*, *Peranema* sp. BIPA2001 and *Peranema trichophorum* as an outgroup.

3. Results

Fig. 1 illustrates the topology of the ME tree based on the Kimura-two-parameter distance (essentially the same topology was obtained with Tamura–Nei distance, data not shown). Because of high level of sequence similarity between 18S rRNA sequences from relatively closely related species, many of the terminal branches were quite short, therefore, the topology only is illustrated here and not the branch lengths. In the ME tree, a group of prokinetoplastids clustered in intermediate position between the other kinetoplastids and the euglenids (Fig. 1). One cluster included two environmen-

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