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Phylogeny of trypanosomes as inferred from the small and large subunit rRNAs: implications for the evolution of parasitism in the trypanosomatid protozoa[1]

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Abstract

Sequences of the small rRNA genes and partial sequences of the large rRNA genes were obtained by PCR amplification from a variety of vertebrate trypanosomes. The trypanosome species and hosts included *Trypanosoma avium* from a bird, *T. rotatorium* from an amphibian, *T. boissoni* from an elasmobranch, *T. triglae* from a marine teleost and *T. carassii* from a freshwater teleost. Phylogenetic relationships among these species and other representatives of the family Trypanosomatidae were inferred using maximum likelihood, maximum parsimony and evolutionary parsimony. The trypanosomatid tree was rooted using rRNA sequences from two species from the suborder Bodonina. All methods showed that the mammalian parasite, *Trypanosoma brucei*, constitutes the earliest divergent branch. The remaining trypanosomes formed a monophyletic group. Within this group, the bird trypanosome was grouped with *T. cruzi*, while the elasmobranch trypanosome and the two fish trypanosome species formed a group with an affinity to *T. rotatorium*. Our results provide no evidence for co-evolution of trypanosomatids and their hosts, either vertebrate or invertebrate. This suggests that evolution of trypanosomatids was accompanied by secondary acquisitions of hosts and habitats.

Keywords: Kinetoplastida; *Trypanosoma*; Phylogeny; rRNA; Evolution of parasitism

Abbreviations: EDTA, ethylenediaminetetraacetic acid; LSU rRNA, large subunit ribosomal RNA; PCR, polymerase chain reaction; SSU rRNA, small subunit ribosomal RNA

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[1] *Note*: Nucleotide sequences reported in this work are deposited in the GenBank™ database with the following accession numbers: U39583 (*T. rotatorium* SSU), U39582 (*T. rotatorium* LSU), U39578 (*T. avium* SSU), U39579 (*T. avium* LSU), U39580 (*T. boissoni* SSU), U20785 (*T. boissoni* LSU), U39584 (*T. triglae* SSU), U20784 (*T. triglae* LSU), U20868 (*T. carassii* LSU), U39577 (*P. serpens* SSU), U39581 (*T. borreli* LSU).

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1. Introduction

The problem of the origin and evolution of parasitism in kinetoplastids has attracted substantial attention since the beginning of this century. Two opposing views have been proposed, which disagreed as to the nature of the primary host (vertebrate or invertebrate) and the times and modes of acquisition of the digenetic life cycles [1].

One hypothesis, pioneered by Minchin [2] and further developed by Lavier [3], Wallace [4] and others [1], states that, after initially parasitizing the gut of early aquatic vertebrates, the ancestral kinetoplastids colonized the blood and were subsequently introduced to leeches and hematophagous insects, which in turn continued to spread the parasites among different groups of vertebrates (both aquatic and terrestrial). This theory would explain why trypanosomes are found in all classes of vertebrates, but preferentially occur in hematophagous groups of invertebrates.

The second hypothesis, which dates back to Legér [5] and was elaborated by Grassé [6], Baker [7], Hoare [8] and others [1], proposes that parasites of vertebrates descend from parasites of hematophagous insects and leeches. In this model, parasitism was originally established in ancient invertebrates and the parasites co-evolved with their hosts. Both insects and leeches would have inherited their parasites from a common annelid-like ancestor.

Both of these hypotheses remained within the paradigm which postulated that monogenetic life cycles should precede digenetic life cycles. However, recent attempts to reconstruct kinetoplastid phylogeny using molecular approaches did not support this paradigm [9–14]. Instead, the earliest diverging lineages are represented by the two digenetic parasites of mammals, *Trypanosoma brucei* and *T. cruzi*, while the monogenetic parasites of insects form a more recently diverged group. This phylogenetic tree topology led to the conclusion that the ancestral trypanosomatid was indeed digenetic [12]. The resulting paradox that, at the time of the estimated divergence of the trypanosome lineage from the lineage of a free-living relative (in the Ordovician) [11], hematophagy had probably not yet appeared in evolution, can be resolved by assuming that the digenetic life cycles of trypanosomes evolved independently several times from the trypanosomatids – parasites of insects [11,15].

Assuming a polyphyletic origin of digenetic parasites, which has also been proposed by Molyneux [16], a prediction would be the presence of monogenetic parasites of insects as sister lineages to derived lineages of digenetic parasites. In specific taxonomic terms, lineages of the digenetic parasites *Trypanosoma*, *Leishmania* or *Endotrypanum* should have closely related sister lineages belonging to the insect parasite genera, *Crithidia*, *Leptomonas* or *Blastocrithidia*. However, sister lineages of insect parasites have been found only for *Leishmania* and *Endotrypanum*, supporting an origin of these genera from the monogenetic parasites of mosquitoes. Although the absence of closely related insect parasites for the mammalian trypanosomes, *T. brucei* and *T. cruzi*, may have a trivial explanation, it is also consistent with a recent origin of mammalian parasites from more primitive trypanosomes [17]. In this paper we have investigated the phylogenetic position of trypanosomes isolated from aquatic vertebrates and a bird in order to shed more light on the evolution of Trypanosomatidae.

2. Materials and methods

2.1. Strain origin and cultivation conditions

The strain of *Trypanosoma rotatorium* B2I was isolated by D. Martin in 1990 from blood of the frog, *Rana catesbeiana*, in Algonquin Park, Ontario, Canada [18], and was provided by K.-P. Chang. *T. carassii* strain El-ČP was isolated by H. Pecková in 1990 from blood of a pike, *Esox lucius* (order Salmoniformes), in Sobeslav, Southern Bohemia, Czech Republic. *T. triglae* strain ITMAP 2212 was isolated from blood of a sea robin, *Trigla lineata* (order Scorpaeniformes), and *T. boissoni* strain ITMAP 2211 was isolated from the blood of a ray, *Zanobatus atlanticus*, by P. Ranque in 1969 at Green Cape, Dakar, Senegal, and

provided by D. Le Ray. *T. avium* strain A1412 was isolated by J. Kučera in 1979 from the bone marrow of a raven, *Corvus frugilegus*, in Prague, Czech Republic. Total cell DNA of *Phytomonas serpens* strain S isolated from tomato by R. Brazil was provided by O. Fernandes. *Trypanoplasma borreli* Pg-JH (ATCC 50433) was isolated from the leech [19].

T. rotatorium was grown in Difco liver infusion-tryptose medium supplemented with 10 μg ml^{-1} hemin and 10% fetal bovine serum at 26°C, *T. triglae* was cultivated in L4NHS medium at 24°C, *T. carassii* and *T. boissoni* were cultivated in SNB-9 medium at 20°C, and *T. triglae* and *T. avium* were cultivated in the same medium at 24°C [20]. *T. borreli* was grown as described previously [21].

2.2. Isolation of DNA

Cells from stationary phase cultures were pelleted at 4000 rpm and washed with an equal volume of SET (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 100 mM EDTA). The final pellet was suspended in 0.5 ml SET and lysed with 2% Sarcosyl and 0.5 mg ml^{-1} pronase at either 65°C for 30 min or 4°C for 60 min. The lysate was phenol-chloroform extracted and DNA was precipitated with an equal volume of isopropanol. Pellets were rinsed with ethanol, dried and resuspended in TE (10 mM Tris–HCl, pH 8.0, 0.1 mM EDTA). Approximately 10–100 ng of genomic DNA were used for PCR amplification.

2.3. PCR amplification

SSU rRNA genes were amplified with the oligonucleotides S-762, GACTTTTGCTTCCTCTA(A/T)TG, and S-763, CATATGCTTGTTTCAAGGAC, which anneal to the conserved 5′- and 3′-end regions. For LSU rRNA genes the oligonucleotides S-1842, GGGTCTAGAGTAGGAAGACCGATAGC, and S-1843, GTGGTACCGGTGGATTCGGTTGGTGAG, were used which amplify the 5′-end of the gene (LS1 region [22]). Reaction mixtures contained 20 mM Tris–HCl, pH 8.3, 1.5 mM $MgCl_2$, 25 mM KCl, 0.05% Tween 20, 0.1 mg ml^{-1} bovine serum albumin, 250 μM each of dGTP, dATP, dTTP and dCTP, 20 μM of each amplification primer and 2.5 U of Taq DNA polymerase. Conditions were as follows: initial denaturation at 95°C for 5 min followed by five cycles at 95°C for 1 min, 45°C for 30 s, 65°C for 1 min and 35 cycles at 95°C for 1 min, 50°C for 30 s, 72°C for 1 min and a final extension at 65°C for 30 min.

2.4. Cloning and sequence analysis

PCR products were purified on 1% agarose gels and isolated by electroelution. DNA was extracted with phenol-chloroform and precipitated with ethanol. Cloning was performed using pT7Blue vector (Novagen) and DH5α competent cells (Gibco BRL). Both strands were sequenced using either the Sequenase kit (version 2.0, U.S. Biochemical) or the *fmol* DNA Sequencing System (Promega) and a set of primers designed to match the regions of SSU and LSU genes which are conserved among all kinetoplastids. For the first strand of SSU genes the following primers were used (listed in the order of occurrence in the gene):

S-823, CGAA(T/C)AACTGC(C/T)CTATCAGC;

S-713, CCGCGGTAATTCCAGCTCC;

S-825, ACCGTTTCGGCTTTTGTTGG;

S-827, GATTAGAGACCATTGTAGTC;

S-757, TCAGGGGGGAGTACGTTCGC;

S-828, CAACAGCAGGTCTGTGATGC.

The second strand was sequenced with the primers:

S-829, GCATCACAGACCTGCTGTTG;

S-714, CGTCAATTTCTTTAAGTTTC;

S-662, GACTACAATGGTCTCTAATC;

S-826, CCAACAAAAGCCGAAACGGT;

S-755, CTACGAACCCTTTAACAGCA;

S-824, GCTGATAG(A/G)GCAGTT(A/G)TTCG.

For the LS1 region of the LSU genes, we used the following oligonucleotides. For the first strand:

S-1845, A(A/C)ATAGA(A/C)CCTGAA(G/A)TCGTGACAA;

S-1846, AAATGGGGTG(C/T)C(C/T)(C/T)-CACCCGTCTT;

S-1847, GAGTGTGCCTGTTTGGACCC-GAAA;

S-1848, GCGGTAAAGCAAATGATTAGAGG;

S-1849, CTT(A/G)TGGGCTCATTCTCGATA-CGTG.

For the second strand:
S-1850, CACGTATCGAGAATGAGCCCA(T/C)AAG;

S-1851, CCTCTAATCATTTGCTTTACCGC;

S-1852, TTTCGGGTCCAAACAGGCACACTC;

S-1853, AAGACGGGTGAAGACACCC-CATTT;

S-1854, TTGTCACGATTTCAGGGTCTATGT.

2.5. Phylogenetic reconstructions

The original alignment, which contained the published SSU sequences of *Crithidia fasciculata* (GenBank™ accession number X03450), *Leptomonas* sp. (X53914), *Leishmania tarentolae* (M84225), *Endotrypanum monterogei* (X53911), *Trypanosoma brucei* (M12676), *T. cruzi* (M31432) and *Bodo caudatus* (X53910), was retrieved from the Ribosomal Database Project [23]. SSU sequences of *Herpetomonas muscarum* (L18872), *Crithidia oncopelti* (L29264), *Blastocrithidia culicis* (U05679), *Trypanoplasma borreli* (L14840), *T. carassii* (L14841), as well as the sequences obtained within this project, were added and necessary realignments were made by eye using an interactive sequence alignment program [24]. After removal of all sites with ambiguous alignments, the SSU alignment contained 1806 characters (with gaps). For a subset of species, a combined data set was analyzed which included both SSU and LSU sequences and contained 2666 unambiguously aligned characters (with gaps). Besides the LSU sequences determined in this work, we used the LSU sequences from *Leptomonas* sp. (L19409), *Leishmania donovani* (L19408), *Phytomonas* sp. (L19410), *B. culicis* (L19404), *T. cruzi* (L19411), *T. brucei* (X14553) and *B. caudatus* (L19405). Both alignments are available upon request. Maximum parsimony and evolutionary parsimony [25] analyses were performed using the PAUP program for the Macintosh (version 3.1.1) [26]. Maximum likelihood analysis [27] was performed with the fastDNAml program [28] on a VAX 4000.

3. Results

3.1. Analysis of the SSU data set

The trypanosomatid subtree of the entire kinetoplastid phylogenetic tree was rooted using outgroup organisms from the suborder Bodonina: *Trypanoplasma borreli* (a cryptobiid) and *Bodo caudatus* (a bodonid). With both the parsimony and likelihood methods, the tree was rooted at the *T. brucei* lineage (Fig. 1).

Both parsimony and likelihood analyses indicated that *T. avium* is the closest relative of *T. cruzi* and *T. rotatorium* is the closest relative of fish trypanosomes. These trypanosomes formed a monophyletic group. Interestingly, the two lineages of bony fish trypanosomes (*T. triglae* and *T. carassii*) are not closest relatives to each other. Instead, the two species from marine vertebrates found in the same locality (*T. triglae* and *T. boissoni*) are more closely related, even though their hosts (a teleost and an elasmobranch) are separated by a greater evolutionary distance.

Bootstrap analysis supported a *T. cruzi*, bird, fish, elasmobranch and amphibian trypanosome clade only in 57% of pseudoreplicates. Within this clade, the monophyly of *T. rotatorium* and fish and elasmobranch trypanosomes is not well supported, while the monophyly of fish and elasmobranch trypanosomes, as well as their internal branching order are supported at a high level. The low bootstrap value for the *T. cruzi*–*T. avium* group (63%) probably results from the unstable position of the *T. rotatorium* lineage (see also below). Evolutionary parsimony supports the above conclusions, although it suggests that the *T. rotatorium* lineage is closer to the *T. cruzi*–*T. avium* clade than to the fish trypanosomes (data not shown).

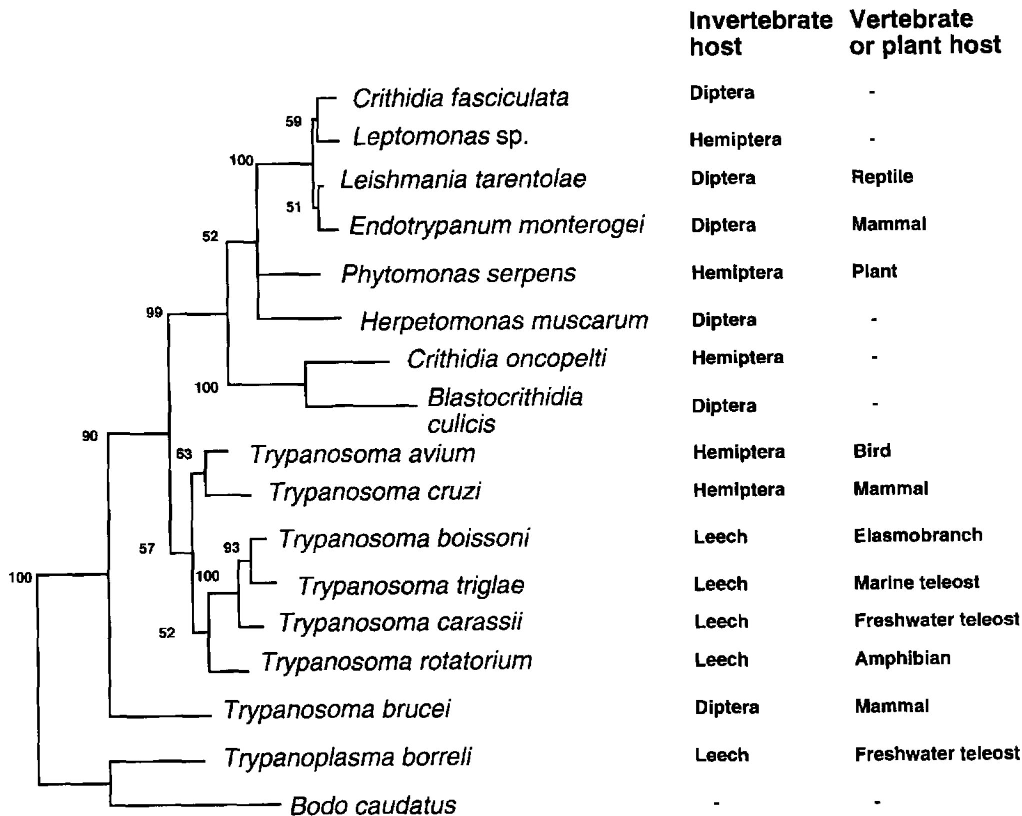

Fig. 1. Majority consensus (50%) parsimony tree of kinetoplastid species constructed from the SSU rRNA dataset. Bootstrap analysis was performed with 200 replicates. The frequency of occurrence of each clade is given at the internal nodes. An indication of the invertebrate host, and the vertebrate or plant host for the digenetic species is shown on the right.

All methods of analysis consistently showed that other mono- and digenetic parasites form a recently diverging monophyletic group (with 99% occurrence in the bootstrap pseudoreplicates), although there was a disagreement about the internal branching order of the clade. Maximum likelihood and evolutionary parsimony indicated that the insect parasite, *Herpetomonas*, and the digenetic plant parasite, *Phytomonas*, form a separate clade within this group (data not shown), while maximum parsimony showed that they branch off sequentially, although the bootstrap support for this is low (Fig. 1). The most recently diverged clade within this group contains the digenetic parasites of mammals and reptiles, *E. monterogei* and *L. tarentolae*, and the monogenetic parasites of insects, *Leptomonas* sp. and *C. fasciculata*. Bootstrap support for this assemblage is 100%. The specific branching order of these lineages was resolved differently by the parsimony and maximum likelihood methods and had a low bootstrap support in the parsimony analysis. On the maximum likelihood tree, the lineage of *Leptomonas* sp. branched off earlier, followed by the separation of the *Leishmania–Endotrypanum* dichotomy and the *C. fasciculata* lineage (data not shown). It is important, however, that both methods agree that the two digenetic parasites represent a sister clade to the lineages of the insect parasites.

3.2. Analysis of the combined data set

To evaluate the effect of the addition of the LSU sequences on the robustness of the tree, the combined (SSU and LSU) dataset was also analyzed. The LSU data alone proved to be insufficient to produce a resolved tree: a polychotomy of the trypanosome branches was observed on a bootstrap majority consensus tree (data not shown). Since LSU data are not available for all species, a limited number of organisms was included in this analysis (Figs. 2 and 3).

Monophyly of the trypanosome clade (without *T. brucei*) was confirmed with both maximum parsimony (Figs. 2 and 3) and maximum likelihood analyses. Parsimony analysis with bootstrapping (Fig. 2) showed that the ray and fish trypanosomes and *T. rotatorium* are monophyletic with *T. cruzi* and *T. avium* in 97% of replicas, which is a significantly higher value than that obtained with the SSU dataset (57% of replicates). The combined dataset also statistically supported monophyly of the ray and fish parasites (100% of replicates) and the closer relatedness of the two marine trypanosomes rather than the two bony fish trypanosomes (84%). This value was, however, somewhat lower than that obtained using the SSU dataset alone (93%).

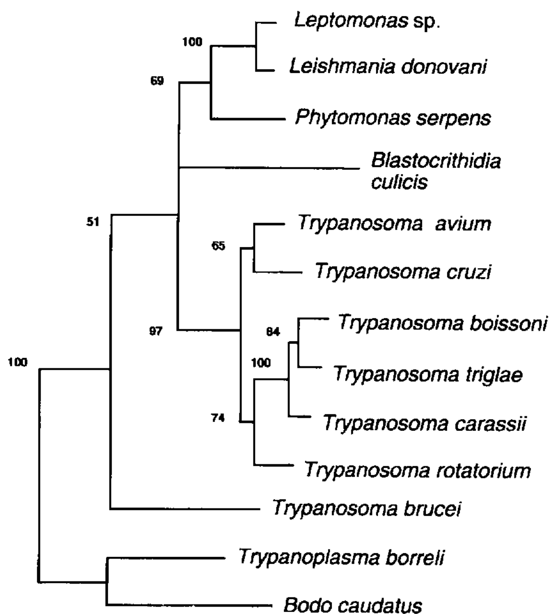

Fig. 2. Majority consensus (50%) parsimony tree derived from the combined (SSU + LSU) rRNA dataset. Bootstrap analyses were performed with 200 replicates.

Addition of the LSU dataset increased support for the monophyly of *T. rotatorium* with the fish trypanosomes from 52 to 74%. In addition, the bootstrap value of the *T. cruzi–T. avium* relatedness increased insignificantly from 63 to 65%. Interestingly, the elimination of *T. rotatorium* from the analysis increased the bootstrap support for the *T. cruzi–T. avium* group to 82% (data not shown).

In the absence of *C. oncopelti* in the combined data set, *B. culicis* is found to represent a sister lineage to *T. brucei* on one of the two most parsimonious trees (Fig. 3B). However, by the length of their branches, *B. culicis* and *T. brucei* are fast evolving species, and in such cases 'branch attraction' artifacts caused by unequal rates of substitution are known to occur [29]. The monophyly of these two lineages or any alternative position of *B. culicis* is not supported statistically in the combined data set; and the majority consensus tree shows this branch as a part of a trichotomy (Fig. 2). Another most parsimonious tree contains the *B. culicis* lineage branching off much later, prior to the separation of *Phytomonas* (Fig. 3A). Maximum likelihood analysis, which is less prone to the above artifact, supported a late separation of the *B. culicis* lineage after the divergence of the *Phytomonas* lineage (data not shown). We conclude that the early divergence of the *B. culicis* lineage on one of the most parsimonious trees (Fig. 3B) is an artifact caused by the absence of the *C. oncopelti* lineage. The correct position for this species is probably better described by the trees in Figs. 1–3A, and also by trees described previously [13,14,30] which place it among the more recently diverging lineages of the insect parasites.

4. Discussion

In this paper we have analyzed the phylogenetic position of trypanosomes isolated from various vertebrate hosts in an attempt to shed more light

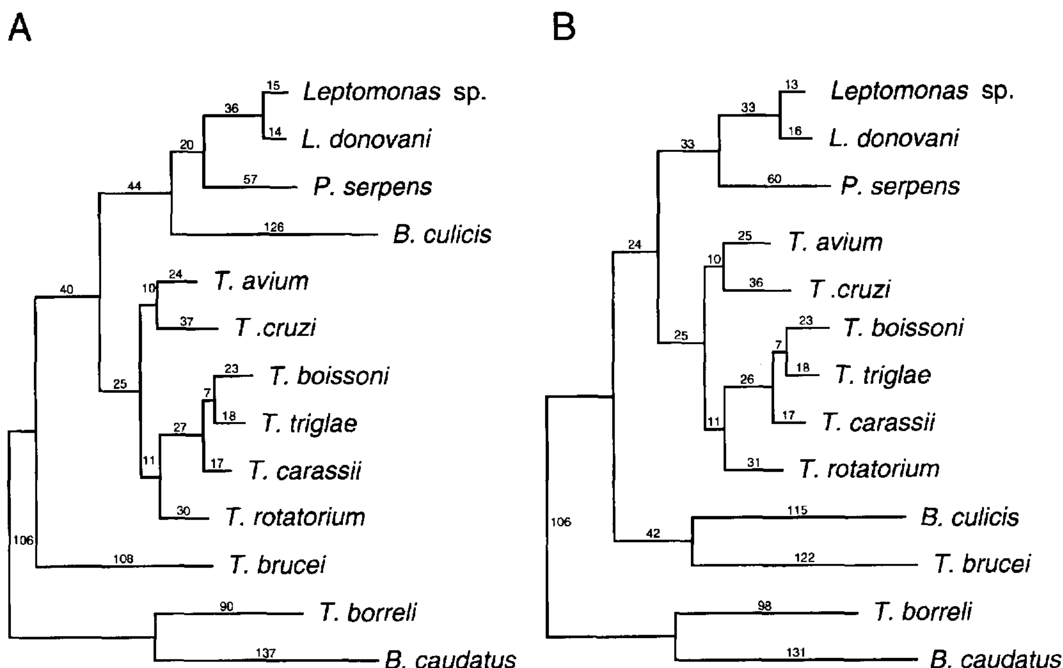

Fig. 3. Two most parsimonious trees obtained from the combined dataset. The branch length (number of substitutions) is indicated above each branch.

on the origin and evolution of parasitism among trypanosomatid protozoa. In any such study, the cautionary note should be made that strain mislabeling is always a possibility, and in the absence of an analysis of multiple independent isolates of a single species, which is technically difficult, the taxonomic designation of a particular species or strain must be in some cases a tentative one. However, a unique molecular phylogenetic position of the specific isolate analyzed is always a good independent criterion for the taxonomic designation.

In accordance with a number of previous works [11–14], we found that trypanosomes are paraphyletic, with *T. brucei* representing the earliest diverging lineage and other trypanosomes forming a monophyletic clade. This does not agree with the results of Berchtold et al. [31], which support a monophyly of *T. brucei* with *T. cruzi*. The reasons for this discrepancy are not clear, but may be related to a possible insufficiency of the rRNA data set. In our analysis, as well as in the previous works [11–14], bootstrap values for the topology which excludes *T. brucei* from the clade of all other trypanosomatids, are consistently lower than the usually accepted level of high statistical significance (95%). In addition, as has been shown for the SSU and LSU combined data set, the long branch of *T. brucei* is prone to producing an artifactual topology with the parsimony algorithm. Analysis of glyceraldehyde-3-phosphate dehydrogenase by Wiemer et al. [32] also supported the monophyly of *T. brucei* and *T. cruzi*. However, at least one member of this gene family in kinetoplastids has been implicated in horizontal transfer [32]. We believe that inclusion of additional lineages of Salivarian trypanosomes and some other phylogenetic markers in the analysis is required to produce a tree with a more stable topology.

Our results provide no evidence for a long-term co-evolution of trypanosomatids and their hosts, either vertebrate or invertebrate. If leech-parasitizing trypanosomatids diverged from insect parasites at the time when their host lineages separated in the Precambrian [7], this split would

be present on the tree as the deepest divergence between the leech-transmitted and the insect-transmitted groups of parasites. However, the tree topology in Fig. 1 provides no evidence for this model. Additionally, within the insect-borne parasites, the lineages of parasites transmitted by Hemiptera are intermingled with the lineages of parasites transmitted by Diptera, thus suggesting the absence of co-evolution with insects.

The absence of parasite co-evolution with vertebrates is illustrated by the fact that, in the absence of strain misidentification, the lineages of two mammalian trypanosomes, two fish trypanosomes, an elasmobranch trypanosome, an amphibian trypanosome and a bird trypanosome branch in an order which does not reflect the evolutionary history of their hosts (Fig. 1). It is especially evident with the trypanosomes of aquatic vertebrates, in which the most closely related organisms are parasites from an elasmobranch and a teleost, and not those from two teleosts. Our data, however, cannot exclude a possibility of a limited (short-term) co-evolution of hosts and parasites.

These observations indicate that evolution of trypanosomatids was accompanied by multiple changes of hosts and habitats. Both 'digeneity first' [12] and 'monogeneity first' [11,15] hypotheses represent plausible evolutionary scenarios compatible with our new tree topologies. It may be impossible to solve this dilemma without accurate measurements of the nucleotide substitution rate in trypanosomatids.

Without reconstruction of the ancestral state, it may be premature to speculate on the history of host acquisitions in particular branches of the tree. However, it is evident that within the monophyletic clade which includes *T. cruzi*, a bird trypanosome, an amphibian trypanosome, an elasmobranch trypanosome and fish trypanosomes, there were multiple changes of both vertebrate and invertebrate hosts.

The position of the leech-transmitted trypanosomes as a single group surrounded by multiple lineages of the insect-transmitted parasites indicates that acquisition of leech hosts occurred secondarily. This may have occurred through the infection of amphibians with either digenetic terrestrial trypanosomes or monogenetic insect parasites (depending on the resolution of the above evolutionary dilemma) by mosquitoes occasionally feeding on amphibians. A subsequent divergence of freshwater fish trypanosomes may indicate that leeches spread trypanosomes from amphibians to freshwater fishes. A late divergence of the marine fish and elasmobranch trypanosomes suggests that these hosts became infected relatively recently by marine leeches feeding on migratory fishes. The affinity of *T. brucei* and leech-transmitted reptilian trypanosomes [17] may also be explained by the secondary acquisition of leech vectors after occasional inoculation of Salivarian trypanosomes into reptiles by tsetse flies.

The evolutionary history of Kinetoplastida still contains many unsolved problems. To shed more light on these problems, this phylogenetic analysis should be extended to include additional trypanosome lineages, as well as more representatives of the suborder Bodonina. In addition, the derivation of an accurate time scale for the phylogenetic tree may help to discriminate between the alternative scenarios of trypanosome evolution.

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