

Review

Molecular phylogenetics of Trypanosomatidae: contrasting results from 18S rRNA and protein phylogenies

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Abstract

Phylogenetic analyses of the family Trypanosomatidae have been conducted using both 18S rRNA gene sequences and a variety of protein sequences. Using a variety of phylogenetic methods, 18S rRNA phylogenies indicate that the genus *Trypanosoma* is not monophyletic. Rather, they suggest that the American and African trypanosomes constitute distinct clades. By contrast, phylogenetic analyses of available sequences in 42 protein families generally supported monophyly of the genus *Trypanosoma*. One possible explanation for these conflicting results is poor taxon sampling in the case of protein coding genes, most of which have been sequenced for only a few species of Trypanosomatidae.

Introduction

The family Trypanosomatidae (Euglenozoa: Kinetoplastida) includes several of the most serious vector-borne protist parasites of humans, numerous species parasitic on non-human vertebrates, and numerous parasites of insects, other invertebrates, and plants. The major human parasites include a number of species in the genera *Leishmania* and *Trypanosoma*. In *Trypanosoma*, the two major human parasites are *T. cruzi*, the causative agent of Chagas' disease, and *T. brucei*, the causative agent of African sleeping sickness. *T. cruzi* belongs to a major grouping within the genus *Trypanosoma* known as the American trypanosomes (or Stercoraria), while *T. brucei* belongs to another major grouping known as the African trypanosomes (or Salivaria).

As with most other single celled organisms, evolutionary relationships within Trypanosomatidae were very poorly known prior to the availability of molecular data because there are few morphological characters documenting relationships within this family. The advent of molecular

sequence data provided many additional characters for phylogenetic analysis, but so far evolutionary relationships within the family remain poorly resolved even by molecular data [1–7]. Here we briefly review some of the major results of previous molecular phylogenetic analyses of Trypanosomatidae and present new analyses based on 42 protein families. In particular, we address the issue of the relationship between American and African trypanosomes and whether or not the genus *Trypanosoma*, as currently recognized, represents a clade or monophyletic group (i.e., whether *Trypanosoma* includes all the descendants of a single ancestral species and only the descendants of that ancestral species).

This question is of more than theoretical interest because *Trypanosoma* includes both African and American trypanosome parasites of humans. If these species are not closely related, it may have important implications for our understanding of these species' basic biology. This in turn may have implications for the development of potential new strategies of prophylaxis and treatment. We will show that

phylogenies based on 18S ribosomal RNA (18S rRNA) genes have provided an answer to this question that appears inconsistent with the results of the majority of phylogenies based on available protein sequences. We then discuss possible explanations for this discrepancy.

18S rRNA Phylogenies

In one of the earliest 18S rRNA phylogenies of trypanosomes, *T. brucei* clustered outside a group that included *T. cruzi*, other American trypanosomes, and members of *Leishmania* and six other genera of Trypanosomatidae [1]. According to this phylogeny, the American and African trypanosomes do not form a monophyletic group. However, sequences from only a relatively small number of species were available at the time of this analysis. In addition, the tree was rooted with sequences from two members of the family Bodonidae, a family of free-living kinetoplastids believed to be closely related to Trypanosomatidae. However, if the family Trypanosomatidae itself is not monophyletic, this rooting might not be valid.

Subsequent studies, including additional 18S rRNA sequences, tended to support the monophyly of the genus *Trypanosoma* [2–6]. However, most of these phylogenies were also rooted with Bodonidae, thus raising questions regarding the validity of the rooting. However, Wright and colleagues [5] rooted their phylogenetic tree with certain species of Euglenida and stramenopiles (Chrysophyceae and Eustigmatophyceae). Since these species are unquestioned outgroups to both Trypanosomatidae and Bodonidae, the phylogeny of Wright et al. [5] provided the strongest support yet for monophyly of *Trypanosoma*. However, this phylogeny included only a small number of species.

In addition to the question of the relationship between American and African trypanosomes, 18S rRNA phylogenies of Trypanosomatidae have addressed the question of the phylogenetic relationships of *Trypanosoma vivax*. *T. vivax* was isolated from a cow in Africa, but its 18S rRNA sequence is divergent from those of other African trypanosomes [8]. In certain phylogenetic analyses, *T. vivax* has clustered with other African trypanosomes [6]; however, Haag and colleagues [3] excluded it from their analysis because they believed that its 18S rRNA gene has evolved more rapidly than those of other *Trypanosoma*. Stevens and Rambaut [8] presented evidence of a high rate of evolution in the 18S rRNA gene of *T. vivax* by comparisons with an outgroup. However, the outgroup these authors used consisted of members of the genera *Crithidia*, *Endotrypanum*, and *Leishmania*, all of which belong to the family Trypanosomatidae. If the genus *Trypanosoma* does not constitute a monophyletic group, this is not a valid outgroup, since some *Trypanosoma* may be closer to these three genera than are others.

Hughes and Piontkivska [7] conducted the most extensive analysis to date of 18S rRNA sequences from Trypanosomatidae and Bodonidae; and they applied several different phylogenetic methods. The phylogenetic trees were rooted with species of Euglenida, which constitute an appropriate outgroup. Although details of the phylogenetic trees differed depending on the methods used, none of the phylogenies supported monophyly of the genus *Trypanosoma*. Support for paraphyly of *Trypanosoma* was strongest in the case of the tree reconstructed by the minimum evolution (ME) method [9], illustrated in Figure 1. In this tree, the African trypanosomes fell outside a clade including the American trypanosomes, along with members of *Leishmania* and seven other genera (Figure 1). The statistical support for the branch establishing this pattern was highly significant (Figure 1).

In the same tree, *T. vivax* clustered apart from the other African trypanosomes and indeed outside all other Trypanosomatidae and Bodonidae (Figure 1). However, statistical support for this pattern was weak (Figure 1). The phylogenetic tree also did not support monophyly of the genus *Leptomonas* (Trypanosomatidae) and did not support monophyly of several genera in Bodonidae (Figure 1).

Figure 2 shows a phylogeny of the same 18S rRNA sequences reconstructed by the quartet maximum likelihood (QML) method [10]. In this case, the deeper branches of the phylogeny were largely unresolved. *T. vivax* clustered with the African trypanosomes, but the American and African trypanosomes did not cluster together (Figure 2). Thus, the QML analysis also did not support monophyly of the genus *Trypanosoma*. As in the ME tree, monophyly of *Herpetomonas* was not supported in the QML analysis (Figure 2). Similarly, maximum parsimony (MP) [11] and Bayesian [12] analysis did not support monophyly of *Trypanosoma* or *Herpetomonas* [7].

The 18S rRNA phylogeny suggests that the evolution of host specificity in Trypanosomatidae has been complex. It seems a plausible hypothesis that the ancestors of kinetoplastids were free-living. Subsequently, it seems plausible that parasitism on invertebrates evolved, followed by more complex life cycles involving both an invertebrate host and either a vertebrate or a plant host. However, the phylogenies (Figures 1 and 2) suggest that life cycles involving a vertebrate host have evolved more than once independently. The ME tree strongly supports (with statistically significant internal branches) the hypothesis that a life cycle involving a vertebrate host may have evolved independently in the American trypanosomes, and in the African trypanosomes (Figure 1).

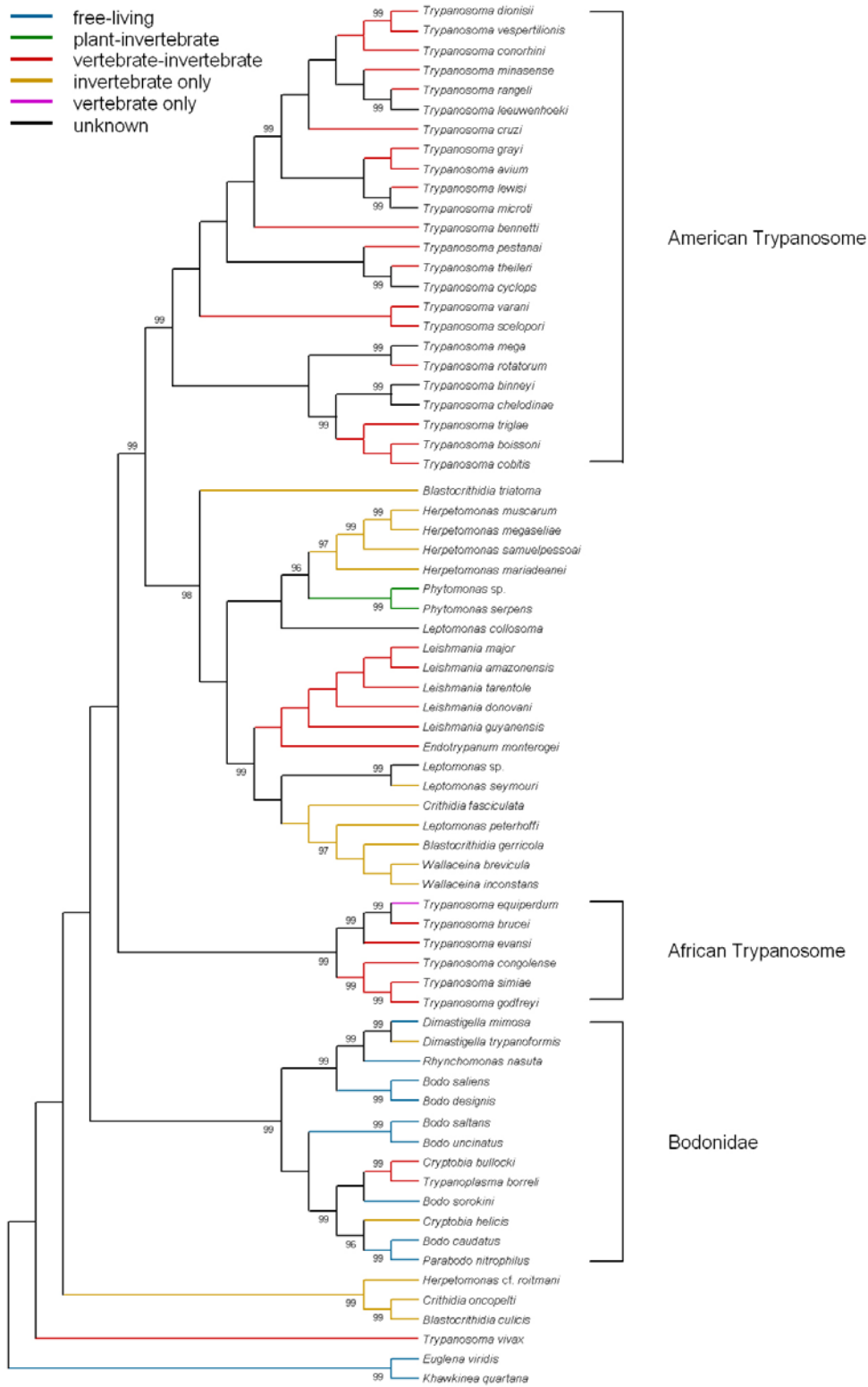


Figure 1
 Minimum evolution (ME) tree of 18S rRNA sequences from Trypanosomatidae and Bodonidae based on the Tamura-Nei [20] distance at 1431 aligned nucleotide sites. Numbers on the branches are significance levels of the standard error test of the branch lengths; only values \geq to 95% are shown.

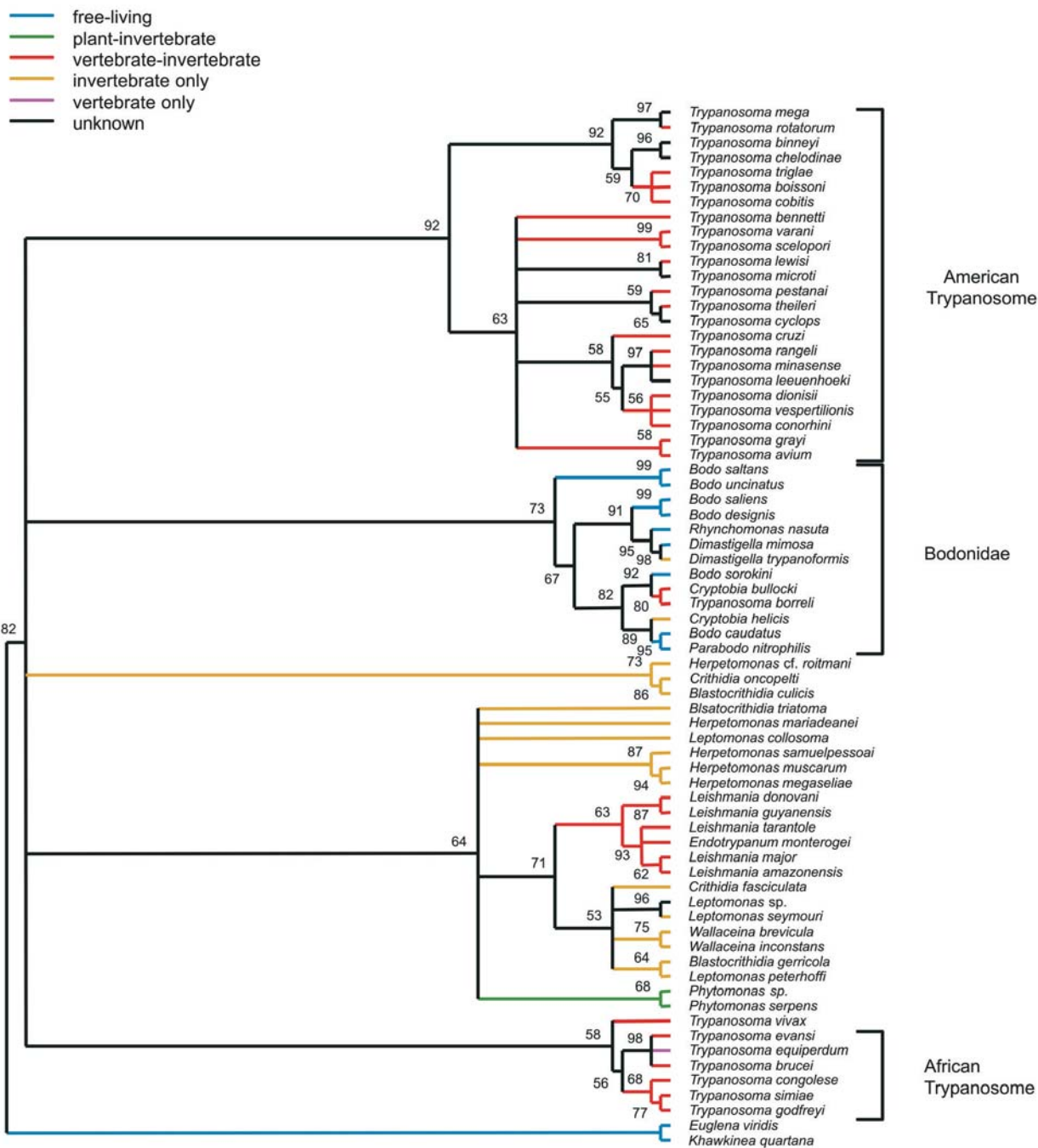


Figure 2
 Quartet maximum likelihood (QML) tree of 18S rRNA sequences from Trypanosomatidae and Bodonidae, constructed using the Tamura-Nei model. Numbers on the branches represent the percentage of puzzling steps supporting the branch.

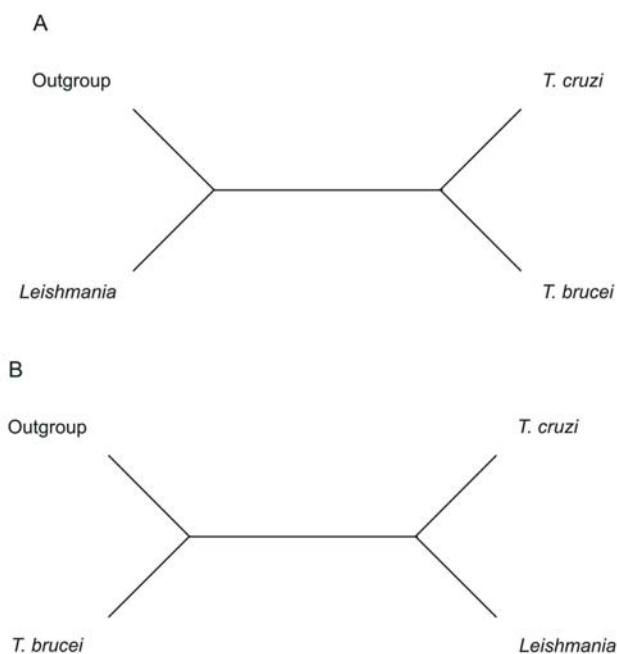


Figure 3
 Alternative topologies of trees including an American trypanosome (*T. cruzi*), and African trypanosome (*T. brucei* or a closely related species), one or more species of the genus *Leishmania*, and an outgroup used to root the tree. In (A) monophyly of *Trypanosoma* is supported, whereas in (B) it is not supported.

Protein Phylogenies

Phylogenetic studies of Trypanosomatidae using the sequences of protein-coding genes or their predicted amino acid sequences have been comparatively few. Alvarez and colleagues [13] published phylogenies of four protein-coding genes: ATPase subunit 6, α tubulin, glyceraldehyde-3-phosphate dehydrogenase, and trypanothione reductase. Three of these phylogenies could not address the question of monophyly of *Trypanosoma* because no outgroup outside the Trypanosomatidae was used to root the tree. The α tubulin phylogeny was rooted with a sequence from *Euglena gracilis* [13]. This phylogeny supported monophyly of *Trypanosoma*, in that *T. cruzi* clustered with *T. brucei* and apart from one sequence from the genus *Leishmania* [13]. Phylogenetic analyses of heat shock protein 90 (HSP90) by Simpson and colleagues [14] likewise supported monophyly of *Trypanosoma*, in that sequences from *T. brucei* and *T. cruzi* clustered together and apart from sequences of two *Leishmania* species. Interestingly, these analyses did not support monophyly of Bodonidae [14].

Because relatively few amino acid sequences for Trypanosomatidae are available at the present time, use of these sequences to address the question of monophyly of *Trypanosoma* reduces in many cases to a choice between the two topologies illustrated in Figure 3. As in previous studies [13,14], monophyly of *Trypanosoma* is supported when *T. cruzi* and *T. brucei* cluster together (Figure 3A). The most frequently observed alternative topology is one where *T. cruzi* clusters with *Leishmania* (Figure 3B). The latter topology corresponds to that seen in the ME tree of 18S rRNA genes (Figure 1).

In Table 1, we summarize the results of phylogenetic analyses of 42 protein families using three different methods. Further details of these analyses, including accession numbers and alignments, are provided in supplemental text [see additional file 1 "supplement.txt"]. Contrary to the results of 18S rRNA analyses [7], the majority of these analyses supported monophyly of *Trypanosoma* (Table 1). In 29 families (69%), all three methods supported monophyly of *Trypanosoma*; i.e., a topology like that of Figure 3A (Table 1). Furthermore, in 16 of these families, support for this topology was statistically significant (at the 95% level) by all three methods (Table 1). An example (the DNA-directed RNA polymerase II, large subunit family) of a topology of this form that received highly significant support is shown in Figure 4a.

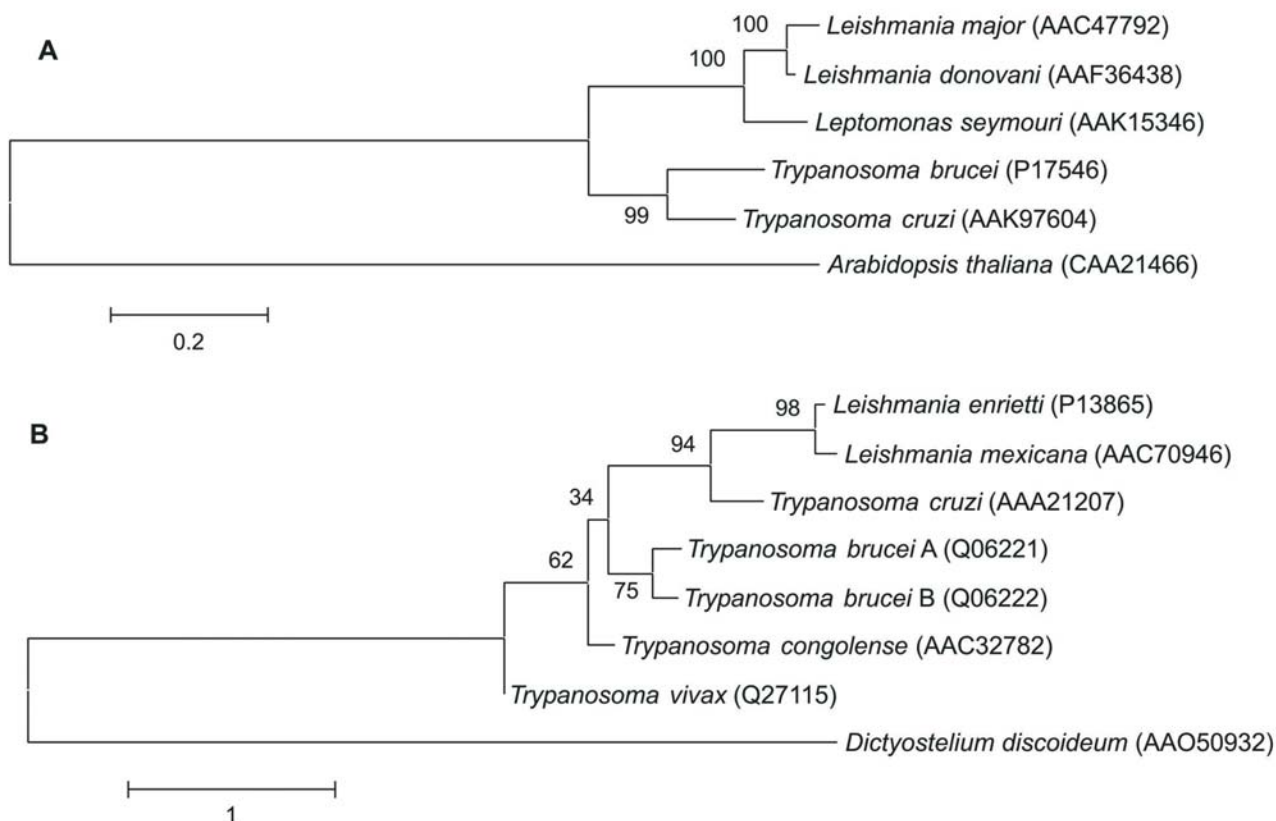
In only four families, monophyly of *Trypanosoma* was not supported by at least one of the three methods (Table 1). An example (the THT family) is shown in Figure 4b. In the phylogenetic trees of the THT family, *T. cruzi* clustered with *Leishmania* rather than with *T. brucei* (Figure 5b). Furthermore, *T. vivax* clustered outside all other sequences from *Trypanosoma* and *Leishmania*. This topology was thus reminiscent of the 18S rRNA ME tree (Figure 1). Interestingly, a *T. vivax* sequence was available for two of the four families for which monophyly of *Trypanosoma* was not supported by any method (Table 1).

Some of the protein families analyzed are encoded by multi-gene families in at least some of the species analyzed. In these cases, it was still possible to use these families to address the issue of monophyly of *Trypanosoma* if the branch order in the phylogeny made clear when the gene duplications occurred relative to speciation events. For example, in the case of S-adenosyl methionine decarboxylase, the phylogeny suggested that multiple gene duplication events occurred after the divergence of the three species of Trypanosomatidae for which sequences were available (Figure 5a). In the case of multi-drug resistance proteins, on the other hand, the phylogeny suggested that there were two separate subfamilies (MDR-A and MDR-E), which arose by a gene duplication prior to speciation within the Trypanosomatidae (Figure 5b). In this

Table 1: Support for monophyly of the genus *Trypanosoma* in protein phylogenies constructed by three different methods.

Family	Aligned sites	<i>Trypanosoma</i> species ¹	ME ²	MP	QML
Actin	375	Tb, Tcr	yes (A,71) ³	yes (A,71)	yes (A,100)
Adenylate cyclase	1142	Tb,Tcr	yes (A, 63)	yes (A, 94)	no
ATPase, subunit 6	225	Tb,Tcr	no (B,37)	yes (A, 92)	no
α tubulin	451	Tb,Tcr	yes (A,99)	yes (A,99)	yes (A,100)
β tubulin	442	Tb,Tcr	yes (A,98)	yes (A,96)	yes (A,100)
Calreticulin	393	Tco,Tcr	yes (A,95)	yes (A,97)	yes (A,100)
Cdc2-related kinase 3	290	Tb,Tcr	yes (A,97)	yes (A,68)	yes (A,99)
CDKRS	73	Tb,Tcr	yes (A,96)	yes (A,72)	yes (A,100)
CRK I	289	Tb,Tco,Tcr	yes (A,100)	yes (A,99)	yes (A,98)
Cyclophilin A	164	Tb,Tco,Tcr, Tvi	no (B,34)	no (B,49)	no
Cysteine proteinase	223	Tb,Tco,Tcr,Tr	yes (A,98)	yes (A,98)	yes (A,98)
Cytochrome b	171	Tb,Tcr	no	no (B,60)	no
Cytochrome-c oxidase II	198	Tb,Tcr	no (B,66)	no (B,35)	no (B,87)
DHFR-TS	455	Tb,Tcr,Tve	no (B,49)	no (B,65)	yes (A,100)
DNA-directed RNA polymerase II, large subunit	1599	Tb,Tcr	yes (A,99)	yes (A,100)	yes (A,100)
DNA topoisomerase II	1171	Tb,Tcr	yes (A,100)	yes (A,100)	yes (A,100)
EFH5	147	Tb,Tcr	yes (A,78)	yes (A,67)	no
EF-1a	444	Tb,Tcr	yes (A,99)	yes (A,93)	yes (A,100)
GAPDH	337	Tb,Tcr,Tev, Tr,Tvi	yes (A,98)	yes (A,94)	yes (A,100)
Glucose-6-phosphate isomerase	550	Tb,Tcr	yes (A,97)	yes (A,70)	yes (A,100)
GRP78	624	Tb,Tco,Tcr	yes (A,98)	yes (A,97)	yes (A,100)
HEXBP	161	Teq,Tcr	yes (A,100)	yes (A,100)	yes (A,100)
HGPRT	177	Tb,Tcr	yes (A,96)	yes (A,60)	yes (A,94)
HSP60	557	Tb,Tcr	yes (A,85)	yes (A,96)	yes (A,100)
HSP70	511	Tb,Tcr	yes (A,79)	yes (A,50)	yes (A,100)
HSP90	613	Tb,Tcr	yes (A,99)	yes (A,100)	yes (A,100)
Malate dehydrogenase	310	Tb,Tcr	yes (A,64)	yes (A,57)	no
MDR-A	877	Tb,Tcr	yes (A,99)	yes (A,100)	yes (A,100)
MDR-E	877	Tb,Tcr	yes (A,100)	yes (A,100)	yes (A,100)
Oligopeptidase B	667	Tb,Tcr	yes (A,99)	yes (A,100)	yes (A,100)
PAR-2	328	Tb,Tcr	yes (A,81)	yes (A,81)	no (B,87)
PAR-3	328	Tb,Tcr	yes (A,58)	yes (A,66)	yes (A,51)
Periredoxin	188	Tb,Tcr	yes (A,90)	yes (A,51)	³ yes (A,99)
Proteasome subunit α -5	237	Tb,Tcr	yes (A100)	yes (A,99)	yes (A,100)
Protein kinase A reg. subunit	372	Tb,Tcr	yes (A,97)	yes (A,99)	yes (A,100)
Pteridine reductase	238	Tb,Tcr	yes (A,94)	yes (A,55)	yes (A,100)
P-type H ⁺ -ATPase	832	Tb,Tcr	yes (A,76)	no (B,53)	no (B,91)
Ribosomal protein P0	312	Tb,Tcr	yes (A,97)	yes (A,97)	yes (A,100)
S-adenosyl methionine decarboxylase	324	Tb,Tcr	yes (A,63)	no (B,68)	yes (A,72)
THT	432	Tb,Tco,Tcr, Tvi	no (B,94)	no (B,86)	no (B,96)
TPIS	247	Tb,Tcr	yes (A,98)	yes (A,95)	yes (A,92)
Trypanothione reductase	407	Tb, Tco, Tcr, Tve	yes (A,100)	no (B,100)	yes (A,100)

¹ Species abbreviations: Tb = *Trypanosoma brucei*; Tco = *T. congolense*; Tcr = *T. cruzi*; Teq = *T. equiperdum*; Tev = *T. evansi*; Tr = *T. rangeli*; Tve = *T. vespertilionis*; Tvi = *T. vivax*. ² Phylogenetic methods: ME = minimum evolution [9]; MP = maximum parsimony [11]; QML = quartet maximum likelihood [10]. ME trees were constructed using the gamma-corrected amino acid distance [15], and the parameter of the gamma distribution was estimated by the TREEPUZZLE program [10]. QML trees were constructed assuming the JTT model of amino acid evolution [21] and that rates varied among sites following a gamma distribution. Amino acid sequences were aligned using the CLUSTALW program [22]. ³ Table entries indicate whether tree supported ("yes") or did not support ("no") monophyly of *Trypanosoma*. "A" and "B" refer to the alternative topologies illustrated in Figure 3. The numbers indicate the confidence level for the interior branch in the trees. For ME and MP, support is the percentage of 1000 bootstrap pseudo-samples supporting the interior branch. For QML, support is the percent of puzzling steps supporting the interior branch.

**Figure 4**

ME trees for two protein families: (A) DNA-directed RNA polymerase II, large subunit, which supports monophyly of *Trypanosoma*; and (B) THT, which does not support monophyly of *Trypanosoma*. Numbers on the branches represent the percentage of 1000 bootstrap pseudo-samples supporting the branch.

case, each subfamily provided separate evidence regarding the relationships among *T. cruzi*, *T. brucei*, and *Leishmania* (Figure 5b). Similarly, the paraflagellar rod components PAR-2 and PAR-3 represented separate subfamilies that arose before speciation of Trypanosomatidae (Table 1).

Discussion

Phylogenetic analyses of 42 protein families generally contradicted the results based on 18S rRNA sequences. Here we briefly discuss some of the considerations that may help lead to a resolution of this contradiction. There are a number of factors that might lead any tree based on a specific gene or protein to produce a phylogeny that is not identical to the phylogeny of the organisms sampled [15]. One such factor is stochastic error; since gene sequences are finite in length, a given gene may by chance yield results contrary to the species tree. In the case of gene families, it is possible that genes that are compared may not truly be orthologous (i.e., descended from an ances-

tral gene without gene duplication); if paralogous genes are mistaken for orthologous genes, the gene tree is likely to be very different from the species tree. Finally, there may be certain biases inherent in methods of phylogenetic reconstruction.

For example, it is well known that ME and MP methods can be prone to the problem known as "long-branch attraction" (or "short-branch attraction") [15]. This describes a tendency for long branches to cluster together, and likewise for short branches to cluster together. Maximum likelihood (ML) methods (including QML and Bayesian methods) are less prone to long-branch attraction. However, ML methods can be subject to a tendency that might be called "opposite-branch attraction." In opposite-branch attraction, short branches tend to cluster with long branches [15]. In a given data set, if ME and MP yield a topology consistent with long-branch attraction, while ML yields a topology consistent with opposite-

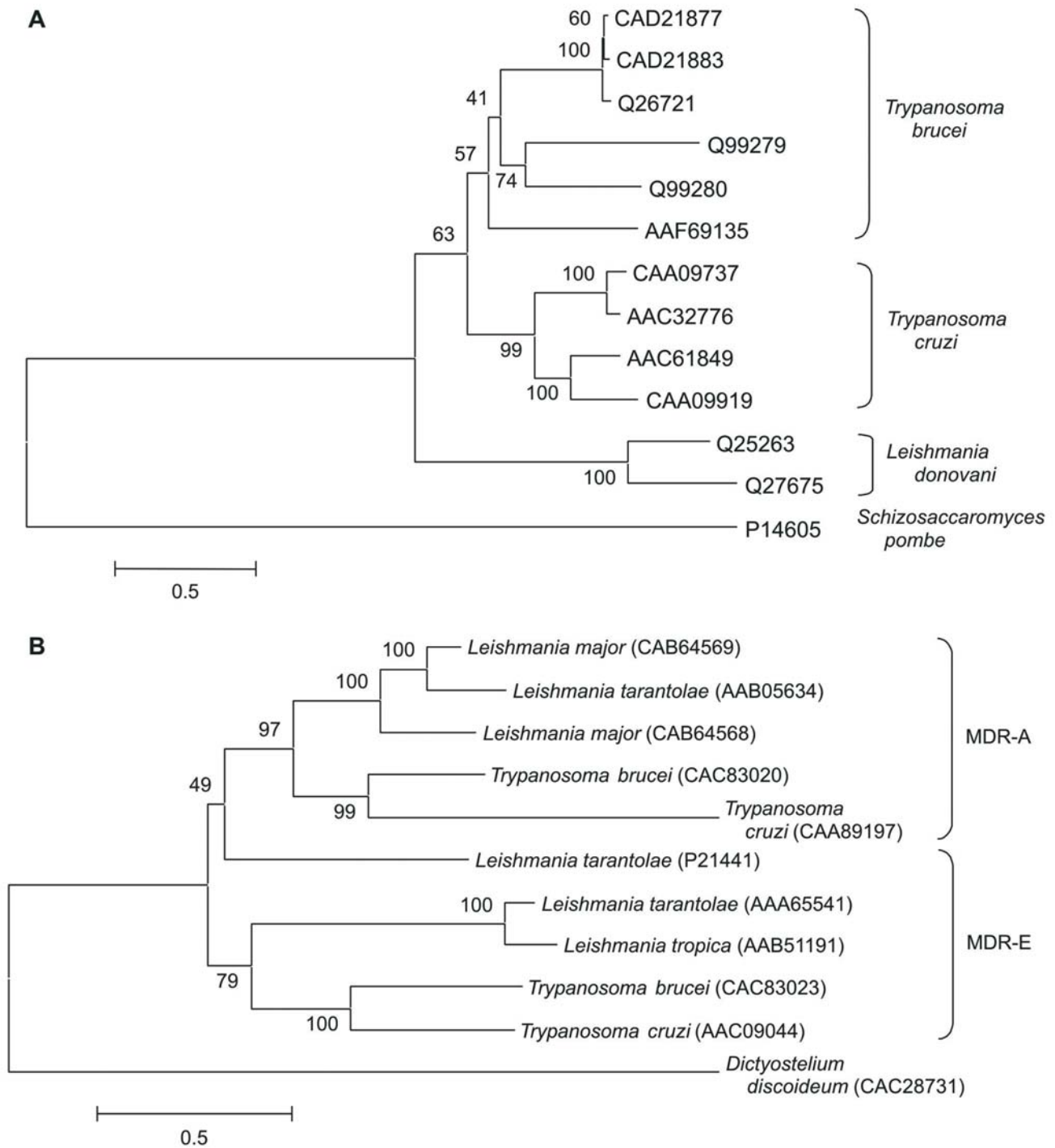


Figure 5
ME trees for (A) adenylate cyclase; and (B) multi-drug resistance proteins (MDR-A and MDR-E). . Numbers on the branches represent the percentage of 1000 bootstrap pseudo-samples supporting the branch.

branch attraction, it may be impossible to determine which topology is real and which is artifactual.

It might be argued that the phylogenies not supporting monophyly of *Trypanosoma* are explainable by stochastic error. In support of this interpretation, it might be noted that only a minority of protein families do not support monophyly (Table 1). Furthermore, those protein families that show strongest support for monophyly are often proteins with a large number of residues that are highly conserved because they play important cellular functions. Examples include DNA-directed RNA polymerase II, large subunit (Figure 4a); DNA topoisomerase II; and HSP90 (Table 1). By contrast, the proteins not supporting monophyly include a number that are quite short, such as cyclophilin A and cytochrome b (Table 1). Furthermore, in those families showing topologies inconsistent with monophyly, statistical support for that topology tends to be relatively weak.

On the other hand, it does not appear likely that biases of phylogenetic methods have played a major role in the outcome of either 18S rRNA or protein phylogenies. Different methods agreed in not supporting monophyly of *Trypanosoma* in the case of 18S rRNA [7]. In the case of protein phylogenies, all three methods used showed agreement in 35 of 42 (83.3%) of families. In the case of the 18S rRNA, comparisons of the pattern of nucleotide substitution between kinetoplast and outgroup sequences showed no striking rate differences among different members of the genus *Trypanosoma* [7]. This observation suggests that long-branch attraction of African trypanosomes toward the root was probably not a factor in the 18S rRNA phylogeny [7].

For each of the 42 protein families analyzed here, we computed the mean proportion of amino acid difference (p) between (1) *T. cruzi* and available *Leishmania* species; and (2) *T. brucei* and available *Leishmania* species. The mean p between *T. cruzi* and *Leishmania* (0.297 ± 0.026 S.E.) was slightly lower than that between *T. brucei* and *Leishmania* (0.311 ± 0.025 S.E.); and the difference was statistically significant (paired sample t-test; $P = 0.037$). However, this observation cannot be used to resolve the phylogenetic issue, since it can be interpreted differently depending on which phylogeny one accepts. If *Trypanosoma* is monophyletic (Figure 3A), then this result suggests that there is a slightly higher average rate of amino acid evolution in *T. brucei* than in *T. cruzi*. On the other hand, if *T. cruzi* is more closely related to *Leishmania* than it is to *T. brucei* (Figure 3B), it would not be unexpected that *T. brucei* proteins are more divergent from *Leishmania* proteins than are *T. cruzi* proteins.

A number of authors have suggested that taxon sampling – the choice of taxa to include in a phylogeny – may have a substantial impact on the results of phylogenetic analyses [16–18]. Some recent computer simulations have suggested that the effects of taxon sampling may not be as large as has been supposed [19], but the random sampling process used in these simulations may not correspond to the biased sampling of taxa that often occurs in actual data sets. Sampling of a diverse array of taxa is expected to improve the accuracy of phylogenetic reconstruction primarily because inclusion of numerous taxa is expected to break up long branches within the tree. Thus, inclusion of numerous can help to minimize the problems of long-branch attraction and of opposite-branch attraction.

In the case of Trypanosomatidae, it seems plausible that taxon sampling may have played a role in causing the different outcomes of 18S rRNA and protein analyses. Of the 29 data sets for which all methods supported monophyly of *Trypanosoma*, 25 included representatives of only a single American trypanosome species (*T. cruzi*) and a single African trypanosome species (usually *T. brucei*) (Table 1). It may be that the results would have been different in many of these families if more taxa had been available.

The role of *T. vivax* seems particularly important with regard to the issue of taxon sampling. Two of the three families for which *T. vivax* sequences were available did not support monophyly of *Trypanosoma* (Table 1). The THT family (Figure 4b) was particularly interesting in this regard. In this family, *T. cruzi* clustered with *Leishmania*; and this pattern received strong statistical support with all methods used (Table 1). Also, it is of interest that five of the families for which at least one method did not support monophyly of *Trypanosoma* included sequences either from Bodonidae (cytochrome b and cytochrome-c oxidase II) or from other genera of Trypanosomatidae besides *Trypanosoma* and *Leishmania* (ATPase, subunit 6, DHFR-TS, and trypanothione reductase).

Conclusion

Phylogenetic analyses of 18S rRNA genes from a large number of species and of much smaller data sets for 42 protein families have failed to provide a consistent answer regarding the question of whether or not the genus *Trypanosoma* is monophyletic. A majority of the protein data sets supported monophyly of *Trypanosoma* while 18S rRNA and a few proteins did not. One possible explanation for this discrepancy is the poor taxon sampling in most of the protein data sets. An accurate phylogeny of the Trypanosomatidae will require sequencing of protein-coding genes from more species of Trypanosomatidae and from the related family Bodonidae. It will be particularly important to sequence from more genes from *Trypano-*

soma vivax, which seems to be a highly divergent member of this group. Only when a substantial number of taxa have been sampled for a large number of genes will it be possible to resolve the evolutionary relationships of this important group of parasites.

Abbreviations Used

ME = minimum evolution; MP = maximum parsimony; p = proportion of amino acid difference; QML = quartet maximum likelihood; rRNA = ribosomal RNA.

Competing Interests

None declared.

Authors' Contributions

AH wrote the manuscript and conducted computational analyses. HP assisted with the writing and with computational analyses.

Supplemental text

The text file Additional file: 1 includes accession numbers, alignments, and quartet puzzling trees for the 42 families used in protein phylogenies and summarized in Table 1.

Additional material

Additional file 1

Supplement

Click here for file

[<http://www.biomedcentral.com/content/supplementary/1475-9292-2-15-S1.txt>]

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