Phylogenomic Analysis of Kinetoplastids Supports That Trypanosomatids Arose from within Bodonids

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Abstract

Kinetoplastids are a large group of free-living and parasitic eukaryotic flagellates, including the medically important trypanosomatids (e.g., Trypanosoma and Leishmania) and the widespread free-living and parasitic bodonids. Small subunit rRNA- and conserved protein-based phylogenies support the division of kinetoplastids into five orders (Prokinetoplastida, Neobodonida, Parabodonida, Eubodonida, and Trypanosomatida), but they produce incongruent results regarding their relative branching order, in particular for the position of the Trypanosomatida. In general, small subunit rRNA tends to support their early emergence, whereas protein phylogenies most often support a more recent origin from within bodonids. In order to resolve this question through a phylogenomic approach, we carried out massive parallel sequencing of cDNA from representatives of three bodonid orders (Bodo saltsan -Eubodonida-, Procryptobia sorokini -Parabodonida-, and Rhynchomonas nasuta -Neobodonida-). We identified 64 well-conserved proteins shared by these species, four trypanosomatids, and two closely related outgroup species (Euglena gracilis and Diplonema papillatum). Phylogenetic analysis of a concatenated data set yielded a strongly supported tree showing the late emergence of trypanosomatids as a sister group of the Eubodonida. In addition, we identified homologues of proteins involved in trypanosomatid mitochondrial mRNA editing in the three bodonid species, suggesting that editing may be widespread in kinetoplastids. Comparison of expressed sequences from mitochondrial genes showed variability at U positions, in agreement with the existence of editing activity in the three bodonid orders most closely related to trypanosomatids (Neobodonida, Parabodonida, and Eubodonida). Mitochondrial mRNA editing appears to be an ancient phenomenon in kinetoplastids.

Key words: kinetoplastids, phylogenomics, eukaryotic phylogeny, editing.

Kinetoplastid flagellates form a species-rich clade (the Kinetoplastea) within the phylum Euglenozoa (Cavalier-Smith 1981; Adl et al. 2005). Their name reflects the presence of a characteristic structure, the kinetoplast, which consists of a dense mass of extranuclear DNA contained within their single mitochondrion (Vickerman 1976). Kinetoplastea was traditionally considered to be composed of two subgroups: the uniflagellate trypanosomatids, which included significant parasitic genera, such as Trypanosoma and Leishmania, and the biflagellate bodonids, with a variety of free-living and parasitic genera (Vickerman 1976). As for many other protist groups, the phylogenetic relationships within the Kinetoplastea have been studied mostly using the small subunit ribosomal RNA gene (SSU rDNA) as marker. The acquisition of SSU rDNA sequences from divergent bodonids such as Ichthyobodo (Callahan et al. 2002) and Perkinsiella (Dyкова et al. 2003) has recently led to the withdrawal of the traditional division into Trypanosomatina and Bodonina and the establishment of a new classification (Moreira et al. 2004). According to taxon-rich SSU rDNA phylogenies, the class Kinetoplastea can be divided into two subclasses, the Prokinetoplastina (with the single order Prokinetoplastida) and the Metakinetoplastina, the latter containing the largest known diversity of kinetoplastids, which are classified into three orders for the bodonid species (Eubodonida, Parabodonida, and Neobodonida) and one for the trypanosomatids (Trypanosomatida) (Moreira et al. 2004; Simpson et al. 2006). With the exception of the relatively unstable position of the genus Rhynchobodo, each one of these orders was supported both by SSU rDNA and conserved protein (heat-shock proteins HSP70 and HSP90) phylogenies (Simpson et al. 2002, 2004; Moreira et al. 2004). In contrast, the relative order of emergence of the four orders within the Metakinetoplastina, in particular the position of the Trypanosomatida, remained more problematic. Whereas in SSU rDNA phylogenies, they often emerged as a sister group of a clade comprising the rest of metakinetoplastids (i.e., Eubodonida, Parabodonida, and Neobodonida) (Moreira et al. 2004; von der Heyden et al. 2004), in protein phylogenies Trypanosomatida tended to branch well nested within the Metakinetoplastina as sisters of the Eubodonida (Simpson et al. 2002, 2004). However, in both cases, the statistical support for their phylogenetic position...
was very variable, depending on the taxon sampling and analysis methods, and in general remained moderate. Thus, efforts to improve the sampling of protein data sets for these organisms have been qualified as highly desirable (Simpson et al. 2004).

In addition to their medical importance, trypanosomatids have extreme versions of several of the very unusual characters found in kinetoplastids, such as mitochondrial RNA editing, generalized mRNA trans-splicing, or a complex mitochondrial genome structure (Simpson et al. 2006). Therefore, the resolution of their phylogenetic position is important to better understand the evolution of these exotic traits (Simpson et al. 2006). Discrepancies between the SSU rDNA and protein-based phylogenetic analyses are most likely due to the limited amount of phylogenetic signal contained in these markers and probably also to the fast evolutionary rate affecting these organisms. This is particularly noticeable for the SSU rDNA, which yields phylogenies with a very long basal branch that reflects a strong rate acceleration early in the evolution of kinetoplastids (Dolezel et al. 2000; Simpson et al. 2002, 2004; Moreira et al. 2004; von der Heyden et al. 2004). To address this question, we tried to apply a multimarker approach, which is becoming the preferred choice to resolve difficult nodes in eukaryotic phylogeny (e.g., see Delsuc et al. 2005; Keeling and Gascuel 2008), and Bayesian inference (BI), with the mixture model CAT (Lartillot and Philippe 2004), phylogenetic analyses of this data set yielded the same, strongly supported tree (fig. 1). It showed the late emergence of trypanosomatids as sisters of the free-living bodonid *B. saltans*, as already found in several single-protein analyses (Simpson et al. 2002, 2004) but, in contrast to the moderate support found in most of them, this node received maximal statistical support (ML bootstrap value—BV—of 100% and BI posterior probability—PP—of 1). We obtained the same results from the analysis of a data set with all missing data removed (supplementary fig. 1, Supplementary Material online).

At present, complete genome sequences are available for trypanosomatids, including several *Trypanosoma* and *Leishmania* species (El-Sayed et al. 2005; Peacock et al. 2007) but almost no sequence data exist for free-living bodonids. To fill this gap, we carried out massive parallel sequencing of cDNA obtained from three free-living bodonid species using 454 pyrosequencing (see Materials and Methods in Supplementary Material online). The selected species were representative of the three bodonid orders in the Metakinetoplastina: *Bodo saltans* (Eubodonida), *Procrystalba sorokini* (Parabodonida), and *Rhynochomonas nasuta* (Neobodonida). To identify conserved markers, we carried out TBlastN searches upon the bodonid cDNA sequences using as queries a set of 143 protein sequences commonly employed in eukaryotic phylogenomic analyses (Rodríguez-Ezpeleta et al. 2005; Burki et al. 2007; Rodríguez-Ezpeleta, Brinkmann, Burger, et al. 2007; Hampl et al. 2009). We also looked for homologues of the 178 proteins (most of them hypothetical) identified in a pilot study of the genome of *B. saltans* (Jackson et al. 2008). Among all the homologues retrieved from the cDNA sequences, we kept only those proteins found in the three bodonid samples and also in trypanosomatids and in at least one closely related outgroup species (*Euglena gracilis* and/or *Diplonema papillatum*, Marande and Burger 2007; Rodríguez-Ezpeleta, Brinkmann, Burger, et al. 2007). In addition, we excluded those proteins that after multiple sequence alignment did not show any overlapping region for the three bodonids due to partial sequencing of the corresponding cDNAs. This yielded a final list of 64 proteins (supplementary table 1, Supplementary Material online).

We first carried out individual phylogenetic analyses of these proteins with a large eukaryotic taxon sampling to check for potential hidden paralogy or horizontal gene transfer cases. All of them retrieved the monophyly of kinetoplastids and did not show any sign of these problems. Hence, we analyzed them simultaneously in a concatenated data set with only the kinetoplastid + close outgroup species (*E. gracilis* and *D. papillatum*) in order to maximize the number of conserved positions and to avoid potential artifacts due to the use of more distant outgroup taxa (Rodríguez-Ezepeleta, Brinkmann, Roure, et al. 2007). The resulting data set contained 11,425 amino acids and a maximum of 31% missing data for the kinetoplastid sequences (supplementary table 2, Supplementary Material online). Maximum likelihood (ML), with the LG + Γ model (Le and Gascuel 2008), and Bayesian inference (BI), with the mixture model CAT (Lartillot and Philippe 2004), phylogenetic analyses of this data set yielded the same, strongly supported tree (fig. 1). It showed the late emergence of trypanosomatids as sisters of the free-living bodonid *B. saltans*, as already found in several single-protein analyses (Simpson et al. 2002, 2004) but, in contrast to the moderate support found in most of them, this node received maximal statistical support (ML bootstrap value—BV—of 100% and BI posterior probability—PP—of 1). We obtained the same results from the analysis of a data set with all missing data removed (supplementary fig. 1, Supplementary Material online). The SSU rDNA phylogeny for the same set of species showed a poorly supported trypanosomatid-early topology (supplementary fig. 2, Supplementary Material online), as previously observed in taxon-rich SSU rDNA phylogenies (Moreira, Lópeza-García, and Vickerman 2004; von der Heyden et al. 2004). In order to evaluate the robustness of these different trees, we carried out approximately unbiased (AU) tests (Shimodaira 2002) using the concatenated protein and SSU rDNA data sets. In addition to the kinetoplastid-early and late topologies, we also evaluated all possible topologies for the kinetoplastid taxa, except those implying the nonmonophyly of the trypanosomatids (i.e., 12 topologies, see table 1). The
concatenated protein data set significantly rejected the SSU rDNA topology \((P = 0)\) and all other topologies, except one where \(P.\ sorokini\) and \(R.\ nasuta\) formed a clade at the base of the kinetoplastids \((P = 0.498)\). In fact, this relationship was retrieved in 14 of the trees reconstructed using the individual markers, although always with weak support \((BV < 70\%)\). Compared with the concatenated protein data set, the SSU rDNA data set was much less discriminant, as it only rejected 6 of the 12 topologies tested (table 1), which likely reflected the relatively weak phylogenetic signal provided by that single marker.

We evaluated the size of the sequence data set required to resolve the position of the trypanosomatids within the kinetoplastids by constructing cumulative concatenations of the 64 proteins, adding them progressively in random order and repeating this operation 50 times to calculate average bootstrap values (fig. 2). Although the monophyly of trypanosomatids rapidly received a BV of 100% from all concatenations \(>8\) proteins, their late emergence as sisters of \(B.\ saltans\) required the concatenation of at least 40 proteins to reach a BV \(> 95\%\). The deepest node in the kinetoplastid phylogeny, concerning the basal position of \(R.\ nasuta\), was supported by BV \(> 95\%\) by concatenations \(>50\) proteins, which suggested that this position was correct despite the fact that the alternative of the sister grouping of this species with \(P.\ sorokini\) was not rejected by an AU test upon the complete concatenated data set (see above). These results confirmed the need of a relatively large sequence data set to reconstruct the deep nodes of the kinetoplastid phylogeny.

As mentioned above, extensive mitochondrial mRNA editing is an astonishing process found in trypanosomatids. It is carried out through a complex series of insertions and deletions of uridylate residues (Us) with the help of guide RNAs to produce the mature and functional mRNAs (Blum et al. 1990; Alfonzo et al. 1997). Several proteins are involved, constituting complexes such as the 20S editosome (Worthey et al. 2003; Osato et al. 2009) and the MRB1 (Hashimi et al. 2008, 2009; Acestor et al. 2009) and KPAP1 (Etheridge et al. 2008) complexes. Among bodonids, editing has only been proved in \(Trypanoplasma\ borreli\) (Lukesˇet al. 1994; Maslov and Simpson 1994) and \(B.\ saltans\) mitochondria (Blom et al. 1998). To look for possible evidence of editing in other free-living bodonids, we searched for homologues of the 20S editosome and MRB1 and KPAP1 complexes characterized in \(Trypanosoma\) and \(Leishmania\).
species. Although we found very few hits against editing-related proteins in our cDNA sequences, likely due to the limited coverage of the cDNA sequencing, they were distributed in the three bodonid species (table 2). We found 7 occurrences in *P. sorokini*, 8 in *B. saltans*, and 5 in *R. nasuta*. In addition, homologues of the helicase Hel61 were found in the three bodonids but they were divergent and their orthology was unclear. Nevertheless, although a specific screening of editing-related genes will be necessary to verify the presence of functional editing complexes in the different bodonid groups, our results suggest that they may be present in species of the three bodonid orders of Metakinetoplastina. In addition to the presence of these genes, we looked for evidence of editing by examining the sequences of cDNAs of typical mitochondrial genes. We found homologues of *cox1*, *cox2*, and *nadhs* in our bodonid species showing a high proportion of U residues, which is characteristic of highly edited transcripts in kinetoplastids (Blum et al. 1990; Alfonzo et al. 1997). Moreover, we detected sequence variation at some positions corresponding to U residues, which has been used as evidence for editing in *T. borreli* (Lukesˇ et al. 1994; Maslov and Simpson 1994) and *B. saltans* (Blom et al. 1998). We observed a similar situation also in *P. sorokini* and *R. nasuta*, especially for the *cox2* gene, which paradoxically shows very limited editing in *B. saltans* (Blom et al. 1998) and no editing in *T. borreli* (Lukesˇ et al. 1994). Several *P. sorokini* and *R. nasuta* *cox2* transcripts showed several positions with a variable amount of missing U residues (fig. 3), probably corresponding to different stages in the editing process or to inefficient editing. This is particularly interesting for *R. nasuta* because it would be the first detection of editing in a member of the Neobodonida. These data and the presence of editing-related protein-coding genes support that editing, already demonstrated in *T. borreli* (Parabodonida) and *B. saltans* (Eubodonida) (Lukeš et al. 1994; Maslov and Simpson

<table>
<thead>
<tr>
<th>Protein Complex</th>
<th>Bodo saltans</th>
<th>Procryptobia sorokini</th>
<th>Rhynchomonas nasuta</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tb11.01.0610 (Hel61)</td>
<td>20S editosome</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Tb11.03.0180 (MP44)</td>
<td>20S editosome</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Tb11.02.0490 (MP46)</td>
<td>20S editosome</td>
<td>–</td>
<td>?</td>
</tr>
<tr>
<td>Tb10.70.3850 (MP99)</td>
<td>20S editosome</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Tb09.160.2970 (REL1)</td>
<td>20S editosome</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Tb927.7.1550 (RET2)</td>
<td>20S editosome</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Tb10.406.0050</td>
<td>MRB1</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Tb927.2.3800 (GAP1)</td>
<td>MRB1</td>
<td>?</td>
<td>–</td>
</tr>
<tr>
<td>Tb927.5.3010</td>
<td>MRB1</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Tb927.6.2140</td>
<td>MRB1</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Tb927.7.2570 (GAP2)</td>
<td>MRB1</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Tb10.389.0070 (EF-Tu)</td>
<td>KAP1</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Tb11.01.7510</td>
<td>KAP1</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Tb11.02.5820 (KAP1)</td>
<td>KAP1</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Tb11.47.0024</td>
<td>KAP1</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Tb927.1.3010</td>
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<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Tb927.2.3180 (TBPR1)</td>
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<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Tb927.4.4150</td>
<td>KAP1</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

**Table 2.** Proteins Involved in Editing Found in the cDNA Sequences of Bodonid Species.

*Note.*—Reference sequences were from *T. cruzi*, (locus tag numbers are provided). +, a homologue was detected; −, no homologue was detected; ?, a homologue was detected, but sequence similarity was low. Only proteins with at least one homologue in the bodonid species are shown (for the complete list of proteins searched, see supplementary table 3, Supplementary Material online).

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**Fig. 3.** Alignment of *cox2* cDNA sequences from *Procryptobia sorokini* (A) and *Rhynchomonas nasuta* (B) showing variability at U sites (indicated by gray background) that suggests different degrees of editing of the corresponding mRNAs. For each species, the first sequence corresponds to the fully edited mRNA; the translated amino acid sequence is shown at the bottom of each alignment.
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1994; Blom et al. 1998), may be present also in Neobodo-
shima and, therefore, be general in the whole subclass Metaka-
kinetoplastina. Editing is likely an ancient phenomenon in
kinetoplastids.

In conclusion, our multiprotein phylogenetic analyses
provided strong support for the late emergence of the try-
panosomatids and the paraphyletic branching of the three
bodoniid groups within the Metakinetoplastina, overcom-
ing the contradictory results obtained up to now from the
analysis of small sequence data sets (SSU rDNA and a few
proteins). As already suggested (Simpson et al. 2004, 2006),
the sister relationship of Trypanosomatida with Eubodoni-
a, makes B. saltans a key species to study the origin of the
parasitic trypanosomatids, deserving high priority for com-
plete genome sequencing (Jackson et al. 2008).

Supplementary Material
Detailed Materials and Methods and supplementary tables
1–3 and figures 1–2 are available at Molecular Biology and
Evolution online (http://www.mbe.oxfordjournals.org/).

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