An updated view of kinetoplastid phylogeny using environmental sequences and a closer outgroup: proposal for a new classification of the class Kinetoplastea

David Moreira,¹ Purificación López-García¹ and Keith Vickerman²

Correspondence

David Moreira

david.moreira@ese.u-psud.fr

¹UMR CNRS 8079, Ecologie, Systématique et Evolution, Université Paris-Sud, bâtiment 360, 91405 Orsay Cedex, France

²Division of Environmental & Evolutionary Biology, University of Glasgow, Glasgow G12 8QQ, UK

Given their ecological and medical importance, the classification of the kinetoplastid protists (class Kinetoplastea) has attracted much scientific attention for a long time. Morphology-based taxonomic schemes distinguished two major kinetoplastid groups: the strictly parasitic, uniflagellate trypanosomatids and the biflagellate bodonids. Molecular phylogenetic analyses based on 18S rRNA sequence comparison suggested that the trypanosomatids emerged from within the bodonids. However, these analyses revealed a huge evolutionary distance between the kinetoplastids and their closest relatives (euglenids and diplomonads) that makes very difficult the correct inference of the phylogenetic relationships between the different kinetoplastid groups. Using direct PCR amplification of 18S rRNA genes from hydrothermal vent samples, several new kinetoplastid-like sequences have been reported recently. Three of them emerge robustly at the base of the kinetoplastids, breaking the long branch leading to the euglenids and diplomonads. One of these sequences belongs to a close relative of *Ichthyobodo necator* (a fish parasite) and of the '*Perkinsiella amoebae*'-like endosymbiont of *Neoparamoeba* spp. amoebae. The authors have studied the reliability of their basal position and used all these slow-evolving basal-emerging sequences as a close outgroup to analyse the phylogeny of the apical kinetoplastids. They thus find a much more stable and resolved kinetoplastid phylogeny, which supports the monophyly of groups that very often emerged as polyphyletic in the trees rooted using the traditional, distant outgroup sequences. A new classification of the class Kinetoplastea is proposed based on the results of the phylogenetic analysis presented. This class is now subdivided into two new subclasses, Prokinetoplastina (accommodating the basal species *I. necator* and '*Perkinsiella amoebae*') and Metakinetoplastina (containing the Trypanosomatida together with three additional new orders: Eubodonida, Parabodonida and Neobodonida). The classification of the species formerly included in the genus *Bodo* is also revised, with the amendment of this genus and the genus *Parabodo* and the creation of a new genus, *Neobodo*.

INTRODUCTION

Kinetoplastid protists belong, together with euglenids and diplomonads, to the phylum Euglenozoa (Cavalier-Smith, 1981). They are grouped in the class Kinetoplastea

(Cavalier-Smith, 1981), which is equivalent in composition to the order Kinetoplastida of previous classifications (Honigberg, 1963). The name of this group derives from the presence of a characteristic structure, the kinetoplast, which occurs as a mass of concentrated extranuclear DNA within the single mitochondrion (Vickerman, 1976). This kinetoplast DNA (kDNA) can display different distribution patterns, being massed close to the flagellar base (eukinetoplast) or scattered through the mitochondrion as several identical bodies (polykinetoplast) or unequally disseminated as diffuse masses (pankinetoplast). Not only is the peculiar arrangement of mitochondrial DNA unique among eukaryotes, but also some other features have attracted the attention of researchers upon this group for some time

Published online ahead of print on 12 March 2004 as DOI 10.1099/ijss.0.63081-0.

Abbreviations: GTR, general time reversible; kDNA, kinetoplast DNA; LBA, long branch attraction; ML, maximum-likelihood; MP, maximum-parsimony; NJ, neighbour-joining; PP, posterior probability.

The GenBank/EMBL/DDBJ accession numbers for the 18S rRNA gene sequences obtained from uncultured kinetoplastid-like species discussed in this study are AF530516 to AF530522.

(Vickerman, 1990, 2000). For instance, significant disease-causing parasites (such as *Trypanosoma* spp. and *Leishmania* spp.) belong to this class. On the other hand, free-living kinetoplastids are abundant and active microbial predators in terrestrial and aquatic ecosystems (Arndt *et al.*, 2000; Foissner, 1991; Vickerman, 2000). A consequence of this interest has been a long quest towards the reconstruction of a robust phylogeny that provides a framework to study the origin and evolution of genetic, biochemical and structural traits in this group.

According to morphological criteria, kinetoplastids have been classified within two main suborders, the uniflagellate Trypanosomatina and the biflagellate Bodonina (Vickerman, 1976). Also on the grounds of morphology and ultrastructure, the suborder Bodonina has been subdivided in different families according to a number of concurrent classification schemes. Vickerman first proposed two families, Bodonidae and Cryptobiidae (Vickerman, 1976), although later on he re-unified all bodonids within a single family, Bodonidae (Vickerman, 1978, 1990, 2000). In contrast, the classification scheme proposed by Zhukov (1991) divides the Bodonina into two families: Bodonidae and Cephalothamniidae (Vickerman included *Cephalothamnium* in the Cryptobiidae). This confusing situation has not been clarified by the advent of molecular phylogenetic analyses of kinetoplastids one decade ago. The first molecular phylogenies, constructed using the 18S rRNA as marker, included a poor bodonid sampling (Landweber & Gilbert, 1994; Maslov *et al.*, 1994). More recent 18S rRNA phylogenies used a broader sampling but yielded contradictory results. For example, the common bodonid species *Bodo saltans* emerges (with a weak statistical support) as the closest relative to trypanosomatids in many rooted phylogenetic trees reconstructed using 18S rRNA (Callahan *et al.*, 2002; Lukeš *et al.*, 2002; Simpson *et al.*, 2000, 2002), CoxII (Blom *et al.*, 1998) and Hsp90 (Simpson *et al.*, 2002) sequences, but it is included within a large group of bodonids and cryptobiids in alternative 18S rRNA trees (Doležal *et al.*, 2000). Moreover, the species emerging first in these rooted trees differ between the different analyses. A basal emergence in 18S rRNA trees has been thus suggested either for *Cruzella marina* and *Bodo designis* (Doležal *et al.*, 2000), *Cryptobia helici* and *Bodo caudatus* (Lukeš *et al.*, 2002) or for *Proccryptobia sorokini* (Callahan *et al.*, 2002; Dyková *et al.*, 2003). This uncertainty has precluded a reliable rooting of the kinetoplastid tree, making very difficult any inference about the evolutionary history of important traits in this group, such as the kDNA distribution or parasitism. Therefore, several authors have had recourse to unrooted trees for reconstructing more stable and better supported phylogenies to provide the framework required for the analysis of some of these characters (Doležal *et al.*, 2000; Simpson *et al.*, 2002). Using this strategy, the most comprehensive phylogenetic analysis of kinetoplastid 18S rRNA sequences to date has recently lent support to the subdivision of bodonids within three strongly supported groups (Simpson *et al.*, 2002). One of these

groups, clade 3, containing the species *B. saltans* and '*Bodo* cf. *uncinatus*', appears to be the closest relative to the trypanosomatids.

How do we explain these problems afflicting kinetoplastid phylogenies? Two concurrent factors are, at least partially, most likely responsible. The first is the use of very distant outgroup sequences to root the kinetoplastid tree. Most often, these are sequences from other euglenozoa (euglenids and/or diplomonids), although sequences from some slow-evolving eukaryotic groups have also been used. All 18S rRNA phylogenetic trees show a very long unbroken branch connecting the kinetoplastid sequences to the outgroup. It is usually the longest internal branch retrieved in these trees, with up to ~20 times the length of the longest internal branch within kinetoplastids (Simpson *et al.*, 2002). This distant outgroup is an obvious source of phylogenetic instability, as demonstrated by the fact that removal of the outgroup helps to obtain more-robust trees (Doležal *et al.*, 2000; Simpson *et al.*, 2002). It has been consequently stated that '*it is clear that a resolution of this problem will require finding an outgroup more closely related to kinetoplastids or analysing another dataset*' (Doležal *et al.*, 2000). The second identified factor disrupting the kinetoplastid phylogeny is the massive accumulation of multiple overlapping mutations (mutational saturation) in the kinetoplastid sequences. The entire euglenozoan 18S rRNA dataset exhibits a severe degree of mutational saturation, which has considerably hindered the reconstruction of the evolutionary relationships between euglenids, diplomonids and kinetoplastids (Moreira *et al.*, 2001). As a result, removing the outgroup significantly increases the number of unambiguously aligned sites while simultaneously reducing the number of sites with multiple mutations (Doležal *et al.*, 2000; Simpson *et al.*, 2002). The confluence of a distant outgroup and mutational saturation can give rise to major phylogenetic reconstruction artefacts (Philippe & Adoutte, 1998; Philippe & Laurent, 1998). In fact, under these circumstances, the sequences tend to emerge according to their evolutionary rates rather than to their true phylogenetic affinities, i.e. the fastest evolving sequences are attracted towards the base of the tree by a long branch attraction (LBA) artefact (Philippe & Adoutte, 1998; Philippe & Germot, 2000; Philippe *et al.*, 2000). In agreement with this, several rooted kinetoplastid trees show a most likely artefactual basal branching of the species *Proccryptobia sorokini*, which corresponds to one of the longest branches in unrooted kinetoplastid trees (Callahan *et al.*, 2002; Simpson *et al.*, 2002).

Different, but not necessarily exclusive, hypotheses can be advanced to explain the unbroken long basal kinetoplastid branch. These include the extinction of intermediate-branching taxa, an abrupt acceleration of evolutionary rate at the origin of this group, or just an incomplete examination of the extant kinetoplastid diversity leading to an imbalanced taxonomic sampling in phylogenetic analyses. Under the first hypothesis, use of markers

alternative to the rRNA will raise similar problems since for all of them the outgroup sequences will be very distantly related while, under the second hypothesis, it would be possible that alternative genes could have not suffered that hypothetical acceleration, so that they would provide closer and less-saturated outgroup sequences. A recent report seems to have discerned among these possibilities. The determination and phylogenetic analysis of the 18S rRNA sequence of the bodonid *Ichthyobodo necator*, formerly known as *Costia necatrix*, supported the view that this ectoparasite of fishes branches very deeply at the base of the kinetoplastid tree (Callahan *et al.*, 2002). More recently, additional support has been published by Dyková *et al.* (2003), who found that '*Perkinsiella amoebae*'-like organisms, found as endosymbionts of amoebae of the genus *Neoparamoeba*, are actually kinetoplastids related to *I. necator*, as revealed by the 18S rRNA phylogenetic analysis. Therefore, intermediate kinetoplastid species breaking the long basal branch of the group appear to exist. We have recently confirmed this conclusion with the determination of three additional sequences branching at basal positions in the kinetoplastid tree. We retrieved these early-emerging sequences, together with those of several late-emerging bodonids, by PCR amplification and sequencing of 18S rRNA genes directly from samples collected in the proximity of deep-sea hydrothermal vents in the Mid-Atlantic Ridge (López-García *et al.*, 2003). In the present work, we have studied the impact of these environmental sequences on the kinetoplastid 18S rRNA phylogeny. Their incorporation in the analyses, together with those from the genera *Ichthyobodo* and *Perkinsiella*, provides a closer outgroup that stabilizes the kinetoplastid tree. This allows retrieval of the monophyly of clades that otherwise emerge as paraphyletic or polyphyletic in most phylogenetic trees constructed using the traditional, distant outgroup sequences. Our species-rich 18S rRNA phylogenetic tree has provided us with the basis to propose a revised classification of the Kinetoplastea within two subclasses (Prokinetoplastina and Metakinetoplastina) and five orders (Prokinetoplastida, Eubodonida, Parabodonida, Neobodonida and Trypanosomatida), which accommodate virtually all described kinetoplastids.

METHODS

Seven 18S rRNA gene sequences from uncultured kinetoplastid-like species were obtained by PCR amplification using eukaryotic-specific primers and DNA extracted from several environmental samples collected in deep-sea hydrothermal areas of the Mid-Atlantic Ridge at depths between 1695 and 2264 m (for details, see López-García *et al.*, 2003). These sequences were automatically incorporated and aligned within a large alignment containing 2481 18S rRNA gene sequences retrieved from GenBank (<http://www.ncbi.nlm.nih.gov/>). The multiple alignment was then manually edited using the program ED from the MUST package (Philippe, 1993). Gaps and ambiguously aligned positions were excluded from our analyses. A preliminary phylogenetic analysis of all these eukaryotic sequences was done by neighbour-joining (NJ) using the MUST package (Philippe, 1993). This analysis allows the unambiguous ascription of the new seven sequences to the Kinetoplastea (López-García *et al.*, 2003).

From the large eukaryotic alignment, different kinetoplastid datasets (with or without sequences from other eukaryotic clades as outgroup) were constructed. For all these datasets, the corresponding maximum-parsimony (MP) trees were used to estimate the parameter values for a general time reversible (GTR) model of nucleotide substitution, with an eighth-category discrete approximation of a Γ distribution plus invariable sites (GTR + Γ + *I* model), using the program PAUP* 4b10 (Swofford, 2000). In fact, in all cases the program MODELTEST (Posada & Crandall, 1998) selected the GTR + Γ + *I* model as the best of 56 testable substitution models. NJ and maximum-likelihood (ML) analyses using this model were carried out with PAUP* 4b10 (Swofford, 2000). The ML trees were constructed using 100 random additions of species and the Tree Bisection-Reconnection (TBR) branch swapping option. Bootstrap replicates ($n=1000$) were performed for all datasets. MP trees were constructed by 100 random taxon additions and TBR branch swapping, and Mulpars and steepest descent option activated. Bootstrap replicates ($n=1000$) were performed for all datasets. Bayesian analyses (with a GTR + Γ + *I* model) were carried out using the program MrBayes 3 (Huelsenbeck & Ronquist, 2003). The Markov chain Monte Carlo search was run with four chains for 1 000 000 generations, with trees being sampled every 100 generations (the first 1500 trees were discarded as 'burnin'). Statistical comparisons of alternative tree topologies were done using the program CONSEL (Shimodaira & Hasegawa, 2001). Mutational saturation diagrams were constructed using the program COMP_MAT from the MUST package (Philippe, 1993). Alignments are available upon request.

RESULTS AND DISCUSSION

Identification of basal kinetoplastid-like sequences

Callahan *et al.* (2002) recently reported the phylogenetic analysis of the 18S rRNA gene sequence of the bodonid *I. necator*, which is an important ectoparasite of fish. Using a ML phylogenetic analysis, they retrieved a strong bootstrap support (100%) for the basal emergence of this species before the rest of kinetoplastid taxa. However, this basal position appeared to be unstable, since analyses using diplomemids as the only outgroup and analyses carried out with other tree reconstruction methods supported other branching locations for this bodonid (Callahan *et al.*, 2002). Moreover, Simpson *et al.* (2002) noticed that the 18S rRNA gene sequence from *I. necator* appears to be very divergent and that its phylogenetic position in unrooted analyses is unstable. As a consequence, its basal emergence in the rooted analyses could be due to an artefactual attraction to the outgroup sequences, i.e. an LBA artefact. One way to examine this possibility is by the incorporation of additional sequences related to *I. necator*, since it is well known that increasing the taxonomic sampling can help to alleviate LBA problems (Hendy & Penny, 1989).

Therefore, in addition to the sequence of the '*Perkinsiella amoebae*'-like endosymbiont (hereafter referred to as '*Perkinsiella cf. amoebae*') determined by Dyková *et al.* (2003), we have incorporated into the phylogenetic analyses three additional basal-branching putative kinetoplastid sequences. These were recently retrieved from an 18S rRNA-based survey of protist diversity from different substrates around deep-sea hydrothermal vents at the

Mid-Atlantic Ridge (López-García *et al.*, 2003). All our phylogenetic analyses, carried out using distance (NJ), MP, ML and Bayesian tree reconstruction methods, and a variety of sequence evolution models and of ingroup and outgroup sequences, recovered again the basal emergence of *I. necator* and '*Perkinsiella cf. amoebae*' with a strong statistical support (see Fig. 1 for a Bayesian tree). In addition, our environmental sequence AT4-54 branches as a sister group of *I. necator* and '*Perkinsiella cf. amoebae*', while the two other environmental sequences, AT4-96 and AT4-103, branch still deeper at the base of the kinetoplastid subtree. This result is in agreement with our previous analysis using a narrower euglenozoan sampling but a much wider representation of different eukaryotic lineages (López-García *et al.*, 2003). The congruence between the different analyses with an increased taxonomic sampling suggests that the basal position of *I. necator*, '*Perkinsiella cf. amoebae*' and the three environmental sequences is correct and not the result of an artefactual attraction towards the outgroup sequences.

Nevertheless, it is still possible that these congruent results arose by an LBA artefact, especially if the sequences emerging at the base are indeed very divergent, which can induce their branching at a midpoint position. We have addressed this question by studying the degree of mutational saturation for the complete set of euglenozoan sequences presented in Fig. 1. For each possible pair of sequences, we plotted the number of observed differences against the number of substitutions calculated upon an ML tree (Philippe *et al.*, 1994). The diagram obtained shows that the number of substitutions calculated between the diplomemid + euglenid outgroup sequences and the five basal sequences is in general smaller than the number of substitutions between the outgroup sequences and the other (hereafter referred as to 'apical') kinetoplastid sequences (Fig. 1b). This would not be expected if the basal sequences were just very divergent ones. On the contrary, our results suggest that their basal position is correctly inferred. Additional support for this was obtained by comparing the preferred Bayesian tree topology (i.e. with *I. necator*, '*Perkinsiella cf. amoebae*', AT4-54, AT4-96 and AT4-103 branching at the base of the kinetoplastid subtree) against different topologies where the basal sequences were constrained to branch at different positions within the apical kinetoplastid groups. The apical branching for these basal

sequences was significantly rejected by the Shimodaira's approximately unbiased (Shimodaira, 2002) statistical test (data not shown).

A final source of evidence was lent by the close inspection of the sequence alignment. The 18S rRNA gene sequence of *I. necator*, '*Perkinsiella cf. amoebae*' and the three environmental sequences may appear to be very divergent when they are compared exclusively with the apical kinetoplastid sequences. However, many of the differences correspond to positions that are conserved in the outgroup sequences, including not only diplomemids and euglenids, but also other eukaryotes (Fig. 2). This suggests that these sequences are indeed less divergent than the apical kinetoplastid sequences, in agreement with their emergence at the base of the kinetoplastid tree.

We, therefore, have a set of five slow-evolving 18S rRNA gene sequences that help to break the long basal branch leading to the apical kinetoplastid sequences. These sequences provide a closer outgroup to study the phylogenetic relationships among the apical kinetoplastids. They can also be used as slow-evolving representatives of the Kinetoplastea to study the position of this class within the Euglenozoa.

18S rRNA phylogeny of the trypanosomatids and bodonids rooted on the basal kinetoplastid-like sequences

As shown before, the basal sequences can provide an outgroup for the analysis of the apical kinetoplastid phylogeny that is closer than the sequences traditionally used to root the kinetoplastid tree (i.e. diplomemids and euglenids). A mutational saturation analysis for the dataset used to construct the tree in Fig. 1 shows that the pairwise sequence comparisons involving 'basal' versus 'apical' kinetoplastids are much less saturated by multiple substitutions than the sequence comparisons involving euglenids or diplomemids versus apical kinetoplastids (Fig. 1c). Therefore, the basal sequences, although still quite distant, constitute a much more appropriate outgroup to examine the phylogeny of the apical kinetoplastid groups.

Fig. 3 shows a Bayesian tree constructed using a GTR model with a Γ law with eight discrete rate classes plus invariant sites (GTR + Γ + I) and rooted on the basal kinetoplastid

Fig. 1. (a) Bayesian phylogenetic tree of euglenozoan 18S rRNA sequences showing the position of several basal kinetoplastid species. Environmental sequences from deep-sea hydrothermal settings are in bold. Numbers at nodes are posterior probabilities except for the nodes concerning the basal kinetoplastids, for which the PP and ML, MP and distance-(NJ) bootstrap proportions are shown. The apical bodonid clades 1, 2 and 3 are named according to Simpson *et al.* (2002), T coding for the Trypanosomatida. The tree was constructed using 1150 aligned sites. The scale bar represents the number of substitutions per position. (b) Mutational saturation analysis for the euglenozoan 18S rRNA sequences. The dashed line represents the ideal case where the number of inferred substitutions is equal to the number of observed differences between pairs of sequences. Small points correspond to intragroup comparisons, open circles to comparisons of apical kinetoplastid versus outgroup (diplomemid and euglenid) sequences, and grey circles to comparisons of basal kinetoplastid versus outgroup sequences. (c) The same as in (b) but with small points representing intragroup comparisons, open circles comparisons of apical kinetoplastid versus outgroup sequences, and grey circles comparisons of apical versus basal kinetoplastid sequences.

<i>Gymnodinium beii</i>	1018	ACCATAAACTAT	1029	..	1120	GC-AAGGCTGAA	1130	..	1533	ATCGTGATG-GGGATAGATCA	1552	EK
<i>Mallomonas striata</i>	1013	ACCATAAACTAT	1024	..	1113	GC-AAGGCTGAA	1123	..	1523	GTCGTGATA-GGGATAGATTA	1542	
<i>Chlorella vulgaris</i>	1001	ACCATAAACGAT	1012	..	1103	GC-AAGGCTGAA	1113	..	1511	ATCGTGATG-GGGATAGATTA	1530	
<i>Mucor mucedo</i>	995	ACCACAAACTAT	1006	..	1094	GC-AAGGCTGAA	1104	..	1520	ATCGTGCTG-GGGATAGAGCA	1539	
<i>Monosiga brevicollis</i>	1004	ACCATAAACTAT	1015	..	1105	GC-AAGGCTGAA	1115	..	1513	GTCGTGCTG-GGGATAGATCT	1532	D
<i>Homo sapiens</i>	1077	ACCATAAACGAT	1088	..	1179	GC-AAAGCTGAA	1189	..	1595	TTCGTGATG-GGGATCGGGGA	1614	
<i>Diplonema papillatum</i>	1189	GCTCTAAACGAT	1200	..	1292	GC-AAGGCTGAA	1302	..	1760	GTCGTGATG-AGGATCGATGA	1779	
<i>Diplonema ambulator</i>	1204	ACTCTAAACGAT	1215	..	1314	GC-AAGGCTGAA	1324	..	1780	GTCGTGATG-AGGATCGATGA	1799	
<i>Rhynchopus sp.</i>	1199	ACTCTAAACGAT	1210	..	1307	GC-AAGGCTGAA	1317	..	1765	GTCGTGATG-AGGATCGATGA	1784	E
<i>Petalomonas cantuscygni</i>	1189	TCTCTAAATTCT	1200	..	1300	GC-AAGGCTGAA	1310	..	1770	GTCGTGATG-GGGATGATGC	1789	
<i>Peranema trichophorum</i>	1317	ACTCTAAACTAT	1328	..	1397	GC-AAGGCTGAA	1407	..	1889	GCCGTGTCA-GGGATAGATGT	1908	
<i>Euglena gracilis</i>	1418	ACTCTAAACGAT	1429	..	1531	GC-AAGGCTGAA	1541	..	2033	GACGTACTG-GGGATAGATGG	2052	
<i>Lepocinclis ovata</i>	1469	GCCGTAAACCAT	1480	..	1587	GC-AAGGCTGAA	1597	..	2157	GTTGTCCTG-GGGATAGATGA	2176	B
AT4-103	1132	GCTCTAAATGCT	1143	..	1239	GC-AAGGCTGAA	1249	..	1698	GTCGTGATG-GGGATCGCAGA	1717	
AT4-96	1128	GCTCTAAATGCT	1139	..	1236	GC-AAGGCTGAA	1246	..	1694	GTCGTGATG-GGGATCGCAGA	1713	
<i>Ichthyobodo necator</i>	1158	ACTGCAAACGAT	1169	..	1282	GC-AAGGCTGAA	1292	..	1752	GTCGTGCTA-GGGATCGAAGC	1771	
<i>Perkinsiella amoebae</i>	1168	ACTGCAAACGAT	1179	..	1282	GC-AAGGCTGAA	1292	..	1728	GTCGTGCTT-GGGATCGAAGC	1747	T
AT4-56	1136	ACTGCAAACGAT	1147	..	1252	GC-AAGGCTGAA	1262	..	1719	GTCGTGCTT-GGGATCGAAGC	1738	
<i>Crithidia oncopelti</i>	1182	ACTGCAAACGAT	1193	..	1359	GC-AAGAGTGAA	1369	..	1834	GACCCACTT-GGGACCGAGGA	1853	
<i>Leptomonas sp.</i>	1167	ACTGCAAACGAT	1178	..	1384	GC-AAGAGTGAA	1394	..	1860	GACCCACTT-GGGACCGAGGG	1879	
<i>Leishmania major</i>	1247	ACTGCAAACGAT	1258	..	1454	GC-AAGAGTGAA	1464	..	1930	GACCCACTT-GGGACCGAGGA	1949	2
<i>Trypanosoma brucei</i>	1269	ACTGCAAACCAT	1280	..	1499	GC-AAGAGTGAA	1509	..	1997	GACCCACTT-GGGACCGAGTA	2016	
<i>Trypanosoma pestanaei</i>	1281	ACTGCAAACGAT	1292	..	1539	GC-AAGAGTGAA	1549	..	2016	GACCCACTT-GGGACCGAGTA	2035	
<i>Trypanosoma cruzi</i>	1352	ACTGCAAACGAT	1363	..	1592	GC-AAGAGTGAA	1602	..	2064	GACCCACTT-GGGACCGAGTA	2083	
<i>Cryptobia helici</i>	1124	ACCACAAACGGT	1135	..	1337	GC-AAGTGTGAA	1347	..	1804	GACCCGATT-GGGACCGAGGA	1823	1
<i>Bodo caudatus</i>	1139	ACCACAAACGGT	1150	..	1334	GC-AAGAGTGAA	1344	..	1806	GACCCGATT-GGGACCGAGGA	1825	
<i>Parabodo nitrophilus</i>	1105	ACCACAAACGGT	1116	..	1296	GC-AAGAGTGAA	1306	..	1759	GACCCATTG-GGACCGAGGA	1778	
<i>Proccryptobia sorokini</i>	1125	ACCACAAACGAT	1136	..	1331	GC-AAGAGTGAA	1341	..	1800	GACCCGATT-GGGACCGAGGA	1819	
<i>Cryptobia bullocki</i>	1148	ACCACAAACGAT	1159	..	1346	GC-AAGAGTGAA	1356	..	1815	GACCCGATT-GGGACCGAGGA	1834	3
<i>Trypanoplasma borreli</i>	1146	ACCACAAACGAT	1157	..	1343	GC-AAGAGTGAA	1353	..	1812	GACCCGATT-GGGACCGAGGA	1831	
<i>Rhynchomonas nasuta</i>	1189	ACCACAAACGAT	1200	..	1401	GC-AAGAGTGAA	1411	..	1879	GACCCGATT-GGGACCGAGTA	1898	
<i>Dimastigella trypaniformis</i>	1190	ACCACAAACGGT	1201	..	1406	GCAAGAGTGAA	1417	..	1883	GACCCGATT-GGGACCGAGGA	1902	
<i>Cruzella marina</i>	1217	ACCACAAACGAT	1228	..	1423	GC-AAGAGTGAA	1433	..	1902	GACTCACTTGGGGCCCCAGGA	1922	1
<i>Bodonid clone LFS2</i>	1183	ACCACAAACGGT	1194	..	1385	GC-AAGAGTGAA	1395	..	1860	GACCCGCTT-GGGACCGAGGA	1879	
<i>Bodonid clone LKM101</i>	1184	ACCACAAACGGT	1195	..	1380	GC-AAGAGTGAA	1390	..	1851	GACCCGCTT-GGGACCGAGTA	1870	
<i>Bodo designis</i>	1166	ACCACAAACGAT	1177	..	1362	GC-AAGAGTGAA	1372	..	1835	GACCCGCTT-GGGACCGAGTA	1854	
<i>Bodo saliens</i>	1151	ACCACAAACGAT	1162	..	1352	GC-AAGAGTGAA	1362	..	1826	GACCCGCTT-GGGACCGAGTA	1845	3
<i>Rhynchobodo sp.</i>	1181	ACCACAAACGGG	1192	..	1390	GC-AAGAGTGAA	1400	..	1867	GACCCGATT-GGGACCGAGGA	1886	
<i>'Bodo c.f. uncinatus'</i>	1213	ACCACAAACGGT	1224	..	1404	GC-AAGAGTGAA	1414	..	1870	GACCCGATT-GGGACCGAG-A	1888	
<i>Bodo edax</i>	1223	ACCACAAACGGT	1234	..	1414	GC-AAGAGTGAA	1424	..	1890	GACCCGATT-GGGACCGAGTA	1909	
<i>Bodo sp.</i>	1227	ACCACAAACGGT	1238	..	1423	GC-AAGAGTGAA	1433	..	1898	GACCCGATT-GGGACCGAGTA	1917	3
<i>Bodo saltans</i>	1220	ACCACAAACGGT	1231	..	1414	GC-AAGAGTGAA	1424	..	1889	GACCCGATT-GGGACCGAGTA	1908	

Fig. 2. Alignment of several regions of the 18S rRNA gene from several slow-evolving eukaryotes (EK), diplomonids (D), euglenids (E), basal bodonids (B), trypanosomatids (T) and apical bodonids of the clades 1 (1), 2 (2) and 3 (3). Numbers on the right and left of the sequence fragments are the distances (in nucleotides) to the ends of the sequences. Gaps are indicated by dashes. Positions with black background correspond to signatures supporting the separation of basal bodonids and trypanosomatids from the rest of kinetoplastids (first alignment segment) or the basal position of the early emerging kinetoplastid sequences (second and third alignment segments).

sequences. To further increase the kinetoplastid taxonomic sampling, we have included four additional apical-branching 18S rRNA environmental sequences also recovered from our analysis of deep-sea hydrothermal vent samples (López-García *et al.*, 2003). Notably, the groups that emerge as monophyletic in unrooted analyses (Simpson *et al.*, 2002) are also monophyletic in this rooted tree (although with a noticeable decrease of bootstrap support for clade 1, from 99% down to 77% for ML bootstrap values). The internal topologies for these groups are also in agreement with those found in unrooted analyses, with the exception of the preference in our trees for the grouping of *Proccryptobia sorokini* at the base of a group containing *B. caudatus*, *Cryptobia helici* and *Parabodo nitrophilus* within clade 2. Therefore, it appears that the use of these closer outgroup sequences to root the apical kinetoplastid tree has a positive effect. To test this hypothesis further, we have carried out

several additional Bayesian analyses with different sets of ingroup and outgroup sequences, but without varying the alignment and the alignment positions used (not shown). First, to test whether the monophyly of the apical groups was the result of the incorporation of the four apical-branching environmental sequences rather than of the use of a closer outgroup, we reconstructed a tree with similar settings but excluding these sequences. As for the complete dataset, the new tree also retrieved the monophyly of the apical groups, although with lower posterior probabilities (PPs) for the trypanosomatids and clade 1 bodonids (0.67 and 0.60, respectively). This result suggests that the inclusion of the apical bodonid environmental sequences is not the major factor in retrieving the monophyly of the apical groups. Second, we reconstructed trees with the apical sequences plus different combinations of outgroup sequences. A tree rooted on diplomonid sequences retrieved

Fig. 3. Bayesian phylogenetic tree of apical kinetoplastid 18S rRNA sequences rooted on several basal sequences. Environmental sequences from deep-sea hydrothermal settings are in bold. Numbers at nodes are posterior probabilities except for the node concerning the split between the trypanosomatids and the apical bodonids, for which the PP and ML, MP and distance- (NJ) bootstrap proportions are shown (numbers in parentheses indicate that the node was not recovered in the MP and NJ analyses). ML bootstrap proportions are also shown for the nodes supporting the monophyly of the four apical kinetoplastid groups. The numbers on a black background indicate the position of the root for the apical kinetoplastids found in alternative trees rooted with (1) diplomonids, (2) diplomonids plus the basal sequences, (3) the *Ichthyobodo*–*Perkinsiella* clade only, and (4) diplomonids plus the *Ichthyobodo*–*Perkinsiella* clade. The apical bodonid clades 1, 2 and 3 are named according to Simpson *et al.* (2002). The names of the five kinetoplastid orders proposed in our new classification are indicated on the right. The tree was constructed using 1333 aligned sites. The scale bar represents the number of substitutions per position.

a basal paraphyletic emergence of clade 1 bodonids, while the trees rooted on (i) diplomonids plus the basal sequences, (ii) the *Ichthyobodo*–*Perkinsiella* clade only and (iii) the *Ichthyobodo*–*Perkinsiella* clade plus diplomonids retrieved all of them a basal paraphyletic emergence of trypanosomatids,

i.e. a rooting of the apical kinetoplastids within the trypanosomatids (Fig. 3). Interestingly, a study supporting a similar rooting within the trypanosomatids has been published recently (Hughes & Piontkivska, 2003). Our results suggest that this rooting is probably due to the use of a very distant

outgroup. In fact, our analyses strongly suggest that the use of the closely related basal kinetoplastid and environmental sequences as outgroup allows retrieving the monophyly of all the apical clades.

Nevertheless, the most remarkable feature of our tree concerns the position of the root for the apical kinetoplastids, since the deepest node in this region of the tree separates the trypanosomatids from the rest of apical kinetoplastids. The best ML tree constructed using similar settings (GTR+ Γ +I) displayed the same topology (not shown). However, GTR+ Γ +I–NJ and MP trees showed a basal paraphyletic emergence of clade 2 bodonids, although with very low bootstrap support (<50%, not shown).

The statistical support for the trypanosomatids versus apical bodonids split in our Bayesian and ML trees is weak, as also occurs for the relationships among the bodonid clades 1, 2 and 3. Yet, this support is in the same range as, or even slightly higher than, those in other analyses favouring a late emergence of trypanosomatids nested within the bodonids (Callahan *et al.*, 2002; Doležal *et al.*, 2000; Simpson *et al.*, 2002). We did not find support for the early emergence of clade 1 bodonids previously proposed on the basis of Hsp90 phylogenetic analyses (Simpson *et al.*, 2002). The lack of strong support for the deep nodes of the tree is in agreement with the fact that alternative topologies showing a late emergence of trypanosomatids have similar likelihood values for our dataset (not shown). In addition, the majority-rule consensus tree constructed upon 1000 replicates in an ML bootstrapping analysis does not resolve the relationships between trypanosomatids and the bodonid clades 1, 2 and 3, since they all emerge within a multifurcation (not shown).

To look for additional support in favour or against this putative divergence between trypanosomatids and apical bodonids, we have analysed the conserved signature positions (i.e. those conserved in all sequences within monophyletic groups and polarizable using the outgroup sequences) in our extensive 18S rRNA dataset. Only one perfect signature position can be found (corresponding to nucleotide 1223 in the sequence of *B. saltans*), which indeed supports the basal separation of trypanosomatids from the apical bodonids (see Fig. 2). No perfect signature position can be found supporting the monophyly of trypanosomatids and clade 3 bodonids, despite this being a relationship favoured by several phylogenetic analyses (Callahan *et al.*, 2002; Simpson *et al.*, 2002). We have also looked for signatures in the Hsp90 dataset used by Simpson *et al.* (2002). One position (corresponding to amino acid 107 in the complete sequence of *Trypanosoma cruzi*, GenBank accession number A26125) supports the separation of trypanosomatids from the apical bodonids, but three positions (41, 218 and 490) support a sisterhood relationship between trypanosomatids and clade 3 bodonids. Other signatures in positions showing more variable amino acid content also yield controversial support: position 485 supports a basal branching of trypanosomatids, while positions 535 and 604

support the sisterhood of trypanosomatids with clade 2 and/or clade 3 bodonids. Interestingly, in the kinetoplastid 18S rRNA dataset used by these authors (Fig. 1a in their article), there is an additional signature (position 1226) supporting the basal emergence of trypanosomatids, but this signature disappears with the addition of our new sequences. This suggests that the larger number of sequence signatures observed in the Hsp90 dataset may be just due to the small number of Hsp90 sequences available, and that the future incorporation of new sequences may destroy some of these signatures. In summary, signatures appear to be too scarce and/or too equivocal to discern between the different alternative topologies.

Our results using the new slow-evolving basal kinetoplastid sequences reveal that the 18S rRNA does not give privileged support to the classical hypothesis of the late origin of trypanosomatids nested within the bodonids. Therefore, it appears that the resolution of the position of the trypanosomatids in the kinetoplastid tree is still an open question since radically different tree topologies cannot be excluded with the available data. The solution to this query will most likely come from the analysis of alternative protein markers for a wide variety of species, including *I. necator* and other early emerging ones.

Structural and genetic evolution in the Kinetoplastea

The enigmatic origin and evolution of the kDNA and RNA editing have been debated for a long time (e.g. Cavalier-Smith, 1997; Doležal *et al.*, 2000; Lukeš *et al.*, 2002; Maslov *et al.*, 1994; Simpson *et al.*, 2000, 2002; Vickerman, 1990, 2000). A robust phylogenetic tree for the kinetoplastids is a logical prerequisite to address this question. The most recent analyses prioritize the hypothesis of a late origin of trypanosomatids as sister-group of bodonid species such as *B. saltans* (Callahan *et al.*, 2002; Simpson *et al.*, 2002). This view has important implications regarding the evolution of kDNA. In this phylogenetic scheme, the characteristic catenation of the kDNA minicircles in the trypanosomatids would most likely be a derived trait. However, the relaxed open-conformation minicircles found in trypanosomatids and certain clade 1 and clade 3 bodonids do not have a clear origin, although it has been suggested that the most parsimonious scenario would be that this exceptional DNA conformation was ancestral in the kinetoplastids (Simpson *et al.*, 2002). The ancestral organization of the kDNA as a eukinetoplast, pankinetoplast or polykinetoplast is also uncertain.

We have seen that, although not robustly rejected, this phylogenetic framework is not strongly favoured by the newly available 18S rRNA data. At any rate, the identification of *I. necator* and its allies as an outgroup for the apical kinetoplastids opens the way to solve these questions. Unfortunately, little is known about the characteristics of the kDNA and the existence of RNA editing in these basal bodonids. *I. necator* appears to possess polykinetoplast

kDNA (Joyon & Lom, 1969), while '*Perkinsiella* cf. *amoebae*' shows a particular kDNA structure that also resembles to a certain extent a polykinetoplast kDNA (Dyková *et al.*, 2003). This would suggest that this was the ancestral kDNA state in the kinetoplastids, which is at odds with the hypothesis that pankinetoplast kDNA is ancestral (Lukeš *et al.*, 2002). Nevertheless, the bodonid clade 1 shows a mixture of polykinetoplast and eukinetoplast species, suggesting that the conversion between these two conformations may occur. The presence of polykinetoplast kDNA in *I. necator* and several apical bodonids might be therefore not necessarily ancestral but the result of convergence. No data are available about the topological status (relaxed or supercoiled) of the kDNA in this species, so that the question about the ancestry of the open-conformation minicircles remains unsettled. Examination of this trait in *I. necator* and '*Perkinsiella* cf. *amoebae*' becomes an urgent task to solve this query.

An appealing deduction from the classical 18S rRNA-based phylogenies was that trypanosomatids, being nested among bodonids, derive from a biflagellate ancestor (Doležal *et al.*, 2000; Vickerman, 2000). Despite the lack of resolution for the apical region of the kinetoplastid tree and the possibility for other alternative topologies to be correct, the indisputable basal emergence of *I. necator* lends a robust support to this hypothesis.

Taxonomic implications

As noted in the Introduction, different taxonomic schemes have been proposed to classify the kinetoplastids. The most widespread one, namely the distinction of the two suborders Trypanosomatina and Bodonina (Vickerman, 1976), has been challenged recently by several authors who recommend abandoning the suborder Bodonina because the emergence of the trypanosomatids within it renders the group non-monophyletic (Callahan *et al.*, 2002; Simpson *et al.*, 2002). Our analysis of a larger 18S rRNA dataset keeps open the possibility that the species that we have called the 'apical' bodonids form a monophyletic group, though with moderate statistical support (Fig. 3). More data, in particular from protein sequences, are necessary to resolve the relationships among the apical kinetoplastid groups. At any rate, our analyses demonstrate the necessity of the erection of a new group to accommodate *I. necator*, as already suggested by Callahan *et al.* (2002), as well as '*Perkinsiella* cf. *amoebae*'. Actually, the phylogenetic position of these basal species and their wide evolutionary distance from the rest of bodonids strongly advocate for their own taxonomic status within the Kinetoplastea, which is not contemplated by any of the current taxonomic schemes. Consequently, we propose the division of the class Kinetoplastea into two new subclasses – Prokinetoplastina, including the genera *Ichthyobodo* and *Perkinsiella*, and Metakinetoplastina, including the apical kinetoplastid genera (see below). This major division implies the adoption of new orders to accommodate these apical kinetoplastids. Thus, we propose to classify the Metakinetoplastina within four orders, the

Trypanosomatida and three new orders: Eubodonida, Parabodonida and Neobodonida, which correspond to the three major bodonid clades retrieved in our analyses (Fig. 3). The future determination of the precise position of the root of the apical part of the kinetoplastid tree should not affect the stability of this classification, since this information can easily be accommodated simply by the erection of two superorders within the subclass Metakinetoplastina.

Neither the subdivision of the former suborder Bodonina into the two families Bodonidae and Cryptobiidae (Vickerman, 1976) nor the two subfamilies Bodoninae and Cryptobiinae (Zhukov, 1991) are compatible with the 18S rRNA- and Hsp90-based phylogenetic trees. These molecular phylogenies support several robust monophyletic groups, in particular the clades 2 and 3 (Fig. 1 and 3). Clade 2 corresponds to the new order Parabodonida and clade 3 to the new order Eubodonida. The latter groups several isolates of *B. saltans* and the dubiously distinct isolates labelled *Bodo edax* and '*B. c.f. uncinatus*' (see notes to the updated classification of the Kinetoplastea below). Given their different feeding habits, behaviour, and flagellar and oral structure (Brooker, 1971), it is not surprising that they branch far from the other *Bodo* species (*B. caudatus*, *Bodo saliens* and *B. designis*), belonging to the other two new bodonid orders Parabodonida and Neobodonida (see below). Therefore, if the generic name *Bodo* is retained for the type species *B. saltans*, the distant species *B. caudatus*, *B. saliens* and *B. designis* should be classified within at least two different genera (see the updated classification of the genus *Bodo* below). A similar situation is found for the genus *Cryptobia*, since the type species, *Cryptobia heliciis*, does not form a monophyletic group with the marine fish parasites (*C. bullocki*, *C. catostomi* and *C. salmositica*). *C. heliciis* possesses pankinetoplast kDNA, which is different from the unusual 'megakinetoplast' kDNA from *Trypanoplasma* and the marine cryptobias (Lukeš *et al.*, 1998). These marine cryptobias could be reclassified within the genus *Trypanoplasma*, which would be in agreement with the idea that most *Cryptobia* spp. and *Trypanoplasma* should be united into a single genus (Vickerman, 1976, 1990, 2000; Woo, 1987). More sequences are now needed from cryptobias of non-molluscan invertebrate hosts (e.g. *C. vaginalis* from leeches), however, to clarify the phylogenetic isolation of *Cryptobia heliciis*. Nevertheless, even with these amendments, the family Cryptobiidae (also including the genera *Procryptobia* and *Dimastigella*) would remain polyphyletic and should be abandoned. It appears that attachment of the recurrent flagellum (or loss of attachment) may have evolved more than once.

A genus that has been particularly problematic is *Rhynchobodo*. This genus almost uniquely among kinetoplastids possesses extrusomes (trichocysts) similar to those of euglenids and diplomonads (Brugerolle, 1985). The only molecular data available for this genus is the 18S rRNA sequence of the species *Rhynchobodo* sp. ATCC 50359

(Lukeš *et al.*, 1997). This sequence has shown an unstable position in previous phylogenetic analyses (e.g. Atkins *et al.*, 2000; Doležel *et al.*, 2000; Simpson *et al.*, 2002), being even possible that the root of the apical part of the kinetoplastid tree lies between this species and the rest of clade 1 bodonids (as in one of the ML trees shown by Simpson *et al.*, 2002). However, our trees rooted with closer outgroup sequences show a much more stable position of this species at the base of the clade 1 bodonids although with moderate statistical support (Fig. 3). We suggest therefore that it is a member of the new order Neobodonida. An additional problem concerning this species is that the generic assignment of *Rhynchobodo* sp. ATCC 50359 might be questionable, since no detailed morphological data are available for this strain (Simpson *et al.*, 2002). The examination of this ATCC isolate by one of us (K. Vickerman, unpublished data) authenticates its ascription to the genus *Rhynchobodo*, which therefore would be part of the order Neobodonida.

The position of the Kinetoplastea within the Euglenozoa

The new basal kinetoplastid sequences can be useful also to address the question of the deep phylogenetic relationships

between the three euglenozoan classes, Euglenoidea, Diplonemea and Kinetoplastea. Since the basal sequences display significantly shorter branches than the rest of kinetoplastids (Fig. 1a), we have used them as slow-evolving representatives for the entire class Kinetoplastea, following a strategy already applied by others to resolve the phylogenetic position of fast-evolving lineages (Aguinaldo *et al.*, 1997). The Bayesian analysis of a dataset incorporating four diplomids, four euglenids, four basal kinetoplastids and several slow-evolving representatives from other eukaryotic taxa retrieves a tree showing the sisterhood of kinetoplastids and diplomids with a weak to strong statistical support (Fig. 4). The high PP (0.95) assigned by the Bayesian analysis to this relationship is especially noticeable, in contrast with the weak support found by the other methods (bootstrap proportions between 51 and 61%). Disagreements between posterior probabilities calculated by Bayesian analysis and bootstrap support are frequent (Alfaro *et al.*, 2003; Douady *et al.*, 2003; Suzuki *et al.*, 2002), so we cannot draw conclusions about the robustness of this node. Other Bayesian trees constructed using diverse apical species as representatives of the Kinetoplastea also retrieve the sisterhood of kinetoplastids and diplomids, although

Fig. 4. Bayesian phylogenetic tree of euglenozoan 18S rRNA sequences rooted on several slow-evolving eukaryotic species. Environmental sequences from deep-sea hydrothermal settings are in bold. Numbers at nodes are posterior probabilities except for the node separating the diplomids (D) and kinetoplastids (K) from the euglenids (E). For this node the PP and ML, MP and distance- (NJ) bootstrap proportions are shown. The tree was constructed using 1150 aligned sites. The scale bar represents the number of substitutions per position.

with lower posterior probabilities (between 0.71 and 0.90, not shown).

The sisterhood of kinetoplastids and diplomonads is in disagreement with our previous analysis of the euglenozoan internal phylogeny, which moderately supported an alternative diplomonads + euglenids relationship (Moreira *et al.*, 2001). However, in contrast with this previous analysis, the kinetoplastids + diplomonads clade retrieved here appears to be less sensitive to G + C bias since it is repeatedly found using a variety of outgroup sequences with diverse G + C composition (not shown). Such a clade joining kinetoplastids and diplomonads would be congruent with previous 18S rRNA-based euglenozoan phylogenies (Maslov *et al.*, 1999) and especially with recent phylogenies based on protein sequences that provide very strong support for this relationship (Simpson & Roger, 2004).

A remarkable feature of the euglenozoan tree is the weak bootstrap support for the monophyly of the euglenids. Moreover, a proportion of the best-scored ML trees supports the emergence of the kinetoplastids + diplomonads clade within the euglenids, although this result might be due to LBA artefacts instead of true phylogenetic signal since the 18S rRNA sequences available for most basal euglenids (Eutreptiales, Rhabdomonadales, Sphenomonadales and Heteronematales) are extremely divergent (Busse & Preisfeld, 2002a, b; Preisfeld *et al.*, 2001). Nevertheless, the idea that the clade composed of kinetoplastids plus diplomonads could have had its origin from within the euglenids can be appealing, in particular because of the recent discovery of genes of possible chloroplast origin in the genome of trypanosomatid species (Hannaert *et al.*, 2003), which suggests that kinetoplastids had a photosynthetic ancestor. To test this hypothesis, the identification of slow-evolving basal euglenids and/or the construction and analysis of sequence datasets of a variety of genes from a wide spectrum of members of the three euglenozoan subphyla are essential.

Concluding remarks

The culture-independent approach based on the direct amplification of 18S rRNA genes from environmental samples has allowed us to detect three new sequences that, as occurs with those from the genera *Ichthyobodo* and *Perkinsiella*, emerge deeply on the kinetoplastid branch. In addition to their influence in breaking the otherwise unbroken long basal kinetoplastid branch, these sequences indicate that our knowledge on the diversity of the kinetoplastids probably remains very limited. 18S rRNA-based surveys can be a rapid way to get insight into kinetoplastid diversity. It is interesting to note that, despite the publication of several of such surveys, most of them of marine environments (Amaral Zettler *et al.*, 2002; Dawson & Pace, 2002; Edgcomb *et al.*, 2002; López-García *et al.*, 2001; Moon-van der Staay *et al.*, 2001; Stoeck & Epstein, 2003), our study of deep-sea hydrothermal environments has been the first to yield kinetoplastid sequences (López-García

et al., 2003). Given the profusion of kinetoplastid species in nature (Arndt *et al.*, 2000; Foissner, 1991; Vickerman, 2000), this result might probably reflect a PCR bias against the amplification of kinetoplastid rRNA genes. Surveys using kinetoplastid-specific primers will overcome this possible limitation and will most likely continue to increase the kinetoplastid rRNA dataset.

Our limited knowledge on kinetoplastid diversity seems to be a major reason for the observation of a very long basal kinetoplastid branch, although alternative explanations involving a dramatic acceleration of evolutionary rate at the origin of the kinetoplastids may have also been partially responsible. However, we can discard the hypothesis of the extinction of all intermediate lineages. At least two early emerging lineages (the first defined by the environmental sequences AT4-96 and AT4-103, and the second by *I. necator*, '*Perkinsiella* cf. *amoebae*' and the environmental sequence AT4-56, i.e. the new subclass Prokinetoplastina) still exist. Isolation of the organisms for which we have just retrieved their 18S rRNA sequences, and their subsequent detailed characterization (which remains also necessary for *I. necator* and '*Perkinsiella* cf. *amoebae*') will be central to our understanding of the origin and early evolution of the kinetoplastids.

Updated classification of the class Kinetoplastea^a

Class Kinetoplastea Honigberg 1963 emend. Vickerman 1976

Subclass: Prokinetoplastina subclass nov. Vickerman^b. Diagnosis: An independent early emerging clade in 18S rRNA phylogenies. Most recent common ancestor of *Ichthyobodo* and *Perkinsiella*. Polykinetoplastic.

Order: Prokinetoplastida ord. nov. Vickerman. With characters of subclass. Two genera: *Ichthyobodo* and *Perkinsiella*.^c

Subclass: Metakinetoplastina subclass nov. Vickerman. Diagnosis: An independent late-emerging clade in 18S rRNA phylogenies. Most recent common ancestor of *Bodo*, *Cryptobia*, *Trypanoplasma*, *Procryptobia*, *Rhynchobodo*, *Dimastigella*, *Crithidia*, *Leishmania* and *Trypanosoma*. Eukinetoplastic, pankinetoplastic or polykinetoplastic. Free-living and parasitic species described.

Order 1. Eubodonida ord. nov. Vickerman^d. Diagnosis: Eukinetoplastic; kDNA circles not forming a network. Biflagellate. Anterior flagellum bears non-tubular hairs; posterior flagellum lacks hairs, free from body. Phagotrophic. Cytostome anterolateral, surrounded by lappets; marked preoral ridge absent; cytopharynx traverses body. Free-living species described. One genus: *Bodo*.

Order 2. Parabodonida ord. nov. Vickerman^e. Diagnosis: Pankinetoplastic/eukinetoplastic (have potential for pankinetoplastic distribution of kDNA); kDNA circles do not

form a network. Biflagellate; both flagella lack hairs; posterior flagellum attached to body or free from it. Phagotrophic or osmotrophic. Cytostome anterolateral, usually with prominent preoral ridge. Free-living and parasitic species described. Most recent common ancestor of *Parabodo* [includes *Parabodo caudatus* comb. nov., basionym *Bodo caudatus* (Stein, 1878), *Cryptobia*, *Procryptobia* and *Trypanoplasma*].

Order 3. Neobodonida ord. nov. Vickerman^f. Diagnosis: Polykinetoplastic or eukinetoplastic. Biflagellate; usually both flagella lack hairs. Posterior flagellum attached to body or free of it. Phagotrophic. Cytostome apical, associated with conspicuous development of preflagellar rostrum. Free-living species (some possibly endocommensal). Most recent common ancestor of *Neobodo* gen. nov. [includes *Neobodo designis* comb. nov., basionym *Bodo designis* (Skuja, 1948), *Rhynchobodo*, *Rhynchomonas*, *Dimastigella* and *Cruzella*].

Order 4. Trypanosomatida Kent 1880 stat. nov. Hollande 1952^g. Diagnosis: Eukinetoplastic; kDNA in form of a network of catenated circles. Uniflagellate, flagellum hairless and emerging from pocket at anterior end of body or emerging laterally and attached to body along much of its length. Osmotrophic. Cytostome, if present, close to flagellar pocket, lacks associated oral structures. All species parasitic. Most recent common ancestor of *Leptomonas*, *Herpetomonas*, *Crithidia*, *Blastocrithidia*, *Rhynchoidomonas*, *Phytomonas*, *Leishmania*, *Sauroleishmania*, *Endotrypanum*, *Wallaceina* and *Trypanosoma*.

^aHonigberg's order Kinetoplastida is treated here as subclass Kinetoplastea within the phylum Euglenozoa Cavalier-Smith.

^bIn this updated view, the class is divided into two subclasses, Prokinetoplastina ('Pro-' signifying early branching) and Metakinetoplastina ('Meta-' signifying later), on the basis of gene sequences. Environmental sequences AT4-103 and AT4-96 would presumably belong to a third subclass.

^c*Ichthyobodo necator* and '*Perkinsiella* cf. *amoebae*' are reasonably well characterized morphologically, but the molecular organization of their kinetoplasts is unknown.

^dEubodonidae: 'Eu-' signifying true, as the type species of Ehrenberg's genus *Bodo*, *B. saltans* figures prominently in this group. The '*Bodo* sp.' sequenced by Callahan *et al.* (2002) from the ATCC, and assigned to this clade (see Fig. 3), is undoubtedly also *B. saltans*. The nature of '*B. edax*' and '*B. c.f. uncinatus*' is more difficult to assess. Callahan *et al.* obtained these two isolates from the ATCC: both lack an associated published description. Klebs' (1892) *Bodo edax* description applies to a protoalveolate (*Colpodella* sp.). In the same publication, Klebs also changed Saviile-Kent's name *Heteromita uncinatus* to *Bodo*, having confused the two genera. Kent's species, however, had amoeboid activity, which does not occur in *Bodo* spp. but is characteristic

of the cercomonad genus *Heteromita*. It appears likely that the two ATCC isolates are also *B. saltans*.

^eParabodonida: 'Para-' signifying alongside. This clade contains the species *Bodo caudatus* Stein and *Parabodo nitrophilus* Skuja. As separation of the former from the type species of the genus *Bodo* is now necessary, and the inclusion of *P. nitrophilus* in the same genus as *Bodo caudatus* has long been called for (Vickerman, 1976), it seems expedient to transfer *B. caudatus* to the genus *Parabodo* Skuja, 1939, further justifying the name of the new order. Intriguingly, this order contains all the genera of the former family Cryptobiidae (characterized by an attached recurrent flagellum) except *Dimastigella*. An interesting feature of this group is the potential for pankinetoplastic distribution of kDNA in the mitochondrion. This is evident in the cryptobias/trypanoplasms, but on encystation redistribution of kDNA into several masses occurs in *Procryptobia glutinosa* (Vickerman, 1978) and in a flagellate otherwise indistinguishable from *Bodo caudatus* (Brugerolle & Mignot, 1979).

^fNeobodonida: 'Neo-' signifying new. In many ways, this is the most puzzling clade of the three metakinetoplastid orders as it contains distinctly polykinetoplastic genera (*Rhynchobodo* and *Dimastigella*) and distinctly eukinetoplastic members (*Rhynchomonas nasuta* and *Bodo designis*). *Bodo designis* Skuja is well-studied ultrastructurally (Eyden, 1977). In its possession of a 'microtubular prism' supporting the cytostome-cytopharynx, it differs significantly in its feeding apparatus from both *B. saltans* and *P. caudatus* and its location in a different order from the former necessitates a new generic designation. It is proposed to make it the type species of the new genus *Neobodo* as indicated below (see updated classification of the genus *Bodo*). *Bodo curvifilis*, which also possesses a microtubular prism (Burzell, 1975), probably belongs in this genus. *Bodo saliens* has been described on the basis of size and behaviour only; the nature of its kinetoplast is undescribed as is its ultrastructure and, although it and other former *Bodo* spp. of this clade may tentatively be assigned to the genus *Neobodo*, creation of independent genera may eventually prove necessary. The genera *Rhynchobodo*, *Dimastigella* and *Rhynchomonas* are well characterized ultrastructurally (Brugerolle, 1985; Breunig *et al.*, 1993; Swale, 1973), the first two being polykinetoplastic, the last eukinetoplastic. Kinetoplast molecular organization has been studied only for *Dimastigella* (Štolba *et al.*, 2001) and the ATCC flagellate labelled *Cruzella marina* (Ziková *et al.*, 2003) also known to be polykinetoplastic (Frolov & Malysheva, 2002). In both these genera the minicircles are distributed in multiple discrete loci spread throughout the mitochondrion, but within each locus the circles are uncatenated.

^gOrder Trypanosomatida; corresponds exactly to the suborder Trypanosomatina and family Trypanosomatidae of previous classifications.

Updated classification of the genus *Bodo*

Diagnosis of the genera: The genus *Bodo* as presently constituted is clearly polyphyletic with species in three separate well-supported clades and demands revision. It is here proposed that the species in the three separate orders should be assigned to three different genera as follows.

Genus: *Bodo* Ehrenberg 1830 emend. Vickerman. Solitary phagotrophic flagellates with single discrete eukinetoplast. Recurrent flagellum free from the body and used in saltatory movements when not free-swimming. Lateral cytostome–cytopharynx lacks supporting prismatic rod. Type species: *Bodo saltans* Ehrenberg.

Genus: *Parabodo* Skuja 1939 emend. Vickerman. Solitary phagotrophic flagellates with kDNA arranged in eukinetoplast in trophic flagellate, but becoming dispersed as pankinetoplast in encysted stage. Body flattened. Recurrent flagellum free or mainly free from body and used as a skid when not free-swimming. Cytostome–cytopharynx lacks supporting prismatic rod. Type species: *Parabodo sacculifera* Skuja 1939.

Genus: *Neobodo* gen. nov. Vickerman. Solitary phagotrophic flagellates with a single discrete eukinetoplast. Recurrent flagellum free or mainly free from body and used as a skid when not free-swimming. Apical cytostome and cytopharynx supported by prismatic rod of microtubules. Type species: *Neobodo designis* (Skuja) Vickerman comb. nov.

ACKNOWLEDGEMENTS

This work was supported by GEOMEX (CNRS/INSUE) and ATIP (CNRS) grants to P.L.-G. K.V. is a Leverhulme Trust Emeritus Research Fellow. We thank A. G. B. Simpson and A. J. Roger for sharing preprints. J. Lukeš and an anonymous reviewer provided useful data and many valuable suggestions.

REFERENCES

- Aguinaldo, A. M., Turbeville, J. M., Linford, L. S., Rivera, M. C., Garey, J. R., Raff, R. A. & Lake, J. A. (1997). Evidence for a clade of nematodes, arthropods and other moulting animals. *Nature* **387**, 489–493.
- Alfaro, M. E., Zoller, S. & Lutzoni, F. (2003). Bayes or bootstrap? A simulation study comparing the performance of Bayesian Markov chain Monte Carlo sampling and bootstrapping in assessing phylogenetic confidence. *Mol Biol Evol* **20**, 255–266.
- Amaral Zettler, L. A., Gomez, F., Zettler, E., Keenan, B. G., Amils, R. & Sogin, M. L. (2002). Microbiology: eukaryotic diversity in Spain's River of Fire. *Nature* **417**, 137.
- Arndt, H., Dietrich, D., Auer, B., Cleven, E., Grafenham, T., Weitere, M. & Mylnikov, A. P. (2000). Functional diversity of heterotrophic flagellates in aquatic ecosystems. In *The Flagellates*, pp. 240–268. Edited by B. S. C. Leadbeater & J. C. Green. London: Taylor & Francis.
- Atkins, M. S., Teske, A. P. & Anderson, O. R. (2000). A survey of flagellate diversity at four deep-sea hydrothermal vents in the Eastern Pacific Ocean using structural and molecular approaches. *J Eukaryot Microbiol* **47**, 400–411.
- Blom, D., de Haan, A., van den Berg, M., Sloof, P., Jirků, M., Lukeš, J. & Benne, R. (1998). RNA editing in the free-living bodonid *Bodo saltans*. *Nucleic Acids Res* **26**, 1205–1213.
- Breunig, A., König, H., Brugerolle, G., Vickerman, K. & Hertel, H. (1993). Isolation and ultrastructural features of a new strain of *Dimastigella trypaniformis* Sandon 1928 (Bodonina, Kinetoplastida) and comparison with a previously isolated strain. *Eur J Protistol* **29**, 416–424.
- Brooker, B. E. (1971). Fine structure of *Bodo saltans* and *Bodo caudatus* (Zoomastigophora, Protozoa) and their affinities with the Trypanosomatidae. *Bull Brit Mus (Nat Hist)* **22**, 89–102.
- Brugerolle, G. (1985). Des trichocystes chez les bodonides, un caractère phylogénétique supplémentaire entre Kinetoplastida et Euglenida. *Protistologica* **21**, 339–348.
- Brugerolle, G. & Mignot, J.-P. (1979). Distribution et organisation de l'ADN dans le complexe kinétoplaste-mitochondrie chez un Bodonidé, protozoaire kinétoplastidé; variations au cours du cycle cellulaire. *Biol Cell* **35**, 111–114.
- Burzell, L. A. (1975). Fine structure of *Bodo curvifilus* Griessmann (Kinetoplastida: Bodonidae). *J Protozool* **22**, 35–39.
- Busse, I. & Preisfeld, A. (2002a). Phylogenetic position of *Rhynchopus* sp. and *Diplonema ambulator* as indicated by analyses of euglenozoan small subunit ribosomal DNA. *Gene* **284**, 83–91.
- Busse, I. & Preisfeld, A. (2002b). Unusually expanded SSU ribosomal DNA of primary osmotrophic euglenids: molecular evolution and phylogenetic inference. *J Mol Evol* **55**, 757–767.
- Callahan, H. A., Litaker, R. W. & Noga, E. J. (2002). Molecular taxonomy of the suborder Bodonina (Order Kinetoplastida), including the important fish parasite, *Ichthyobodo necator*. *J Eukaryot Microbiol* **49**, 119–128.
- Cavalier-Smith, T. (1981). Eukaryote kingdoms: seven or nine? *Biosystems* **14**, 461–481.
- Cavalier-Smith, T. (1997). Cell and genome coevolution: facultative anaerobiosis, glycosomes and kinetoplastan RNA editing. *Trends Genet* **13**, 6–9.
- Dawson, S. C. & Pace, N. R. (2002). Novel kingdom-level eukaryotic diversity in anoxic environments. *Proc Natl Acad Sci U S A* **99**, 8324–8329.
- Doležel, D., Jirků, M., Maslov, D. A. & Lukeš, J. (2000). Phylogeny of the bodonid flagellates (Kinetoplastida) based on small-subunit rRNA gene sequences. *Int J Syst Evol Microbiol* **50**, 1943–1951.
- Douady, C. J., Delsuc, F., Boucher, Y., Doolittle, W. F. & Douzery, E. J. (2003). Comparison of Bayesian and maximum likelihood bootstrap measures of phylogenetic reliability. *Mol Biol Evol* **20**, 248–254.
- Dyková, I., Fiala, I., Lom, J. & Lukeš, J. (2003). *Perkinsiella amoebae*-like endosymbionts of *Neoparamoeba* spp., relatives of the kinetoplastid *Ichthyobodo*. *Eur J Protistol* **39**, 37–52.
- Edgcomb, V. P., Kysela, D. T., Teske, A., de Vera Gomez, A. & Sogin, M. L. (2002). Benthic eukaryotic diversity in the Guaymas Basin hydrothermal vent environment. *Proc Natl Acad Sci U S A* **99**, 7658–7662.
- Eyden, B. P. (1977). Morphology and ultrastructure of *Bodo designis* Skuja 1948. *Protistologica* **13**, 169–179.
- Foissner, W. (1991). Diversity and ecology of soil flagellates. In *The Biology of Free-living Heterotrophic Flagellates*, pp. 93–112. Edited by D. J. Patterson & J. Larsen. New York: Clarendon Press.
- Frolov, A. O. & Malysheva, M. N. (2002). Ultrastructure of the flagellate *Cruzella marina* (Kinetoplastidea). *Tsitologiya* **44**, 477–484 (in Russian).

- Hannaert, V., Saavedra, E., Duffieux, F., Szikora, J. P., Rigden, D. J., Michels, P. A. & Opperdoes, F. R. (2003). Plant-like traits associated with metabolism of *Trypanosoma* parasites. *Proc Natl Acad Sci U S A* **100**, 1067–1071.
- Hendy, M. D. & Penny, D. (1989). A framework for the quantitative study of evolutionary trees. *Syst Zool* **38**, 297–309.
- Hollande, A. (1952). Ordre des Bodonides (Bodonidea ord. nov.). In *Traité de Zoologie*, pp. 669–693. Edited by P. P. Grassé. Paris: Masson & Cie.
- Honigberg, B. M. (1963). A contribution to systematics of the non-pigmented flagellates. In *Progress in Protozoology*, p. 68. Edited by J. Ludvik, J. Lom & J. Vavra. New York: Academic Press.
- Huelsenbeck, J. P. & Ronquist, F. (2003). MrBayes: a program for the Bayesian inference of phylogeny. Uppsala: Uppsala University.
- Hughes, A. L. & Piontkivska, H. (2003). Phylogeny of Trypanosomatidae and Bodonidae (Kinetoplastida) based on 18S rRNA: evidence for paraphyly of *Trypanosoma* and six other genera. *Mol Biol Evol* **20**, 644–652.
- Joyon, L. & Lom, J. (1969). Etude cytologique, systématique et pathologique d'*Ichthyobodo necator* (Henneguy 1883) Pinto 1928 (Zooflagellé). *J Protozool* **6**, 703–719.
- Klebs, G. (1892). Flagellatenstudien. *Z Wiss Zool* **55**, 265.
- Landweber, L. F. & Gilbert, W. (1994). Phylogenetic analysis of RNA editing: a primitive genetic phenomenon. *Proc Natl Acad Sci U S A* **91**, 918–921.
- López-García, P., Rodríguez-Valera, F., Pedrós-Alió, C. & Moreira, D. (2001). Unexpected diversity of small eukaryotes in deep-sea Antarctic plankton. *Nature* **409**, 603–607.
- López-García, P., Philippe, H., Gaill, F. & Moreira, D. (2003). Autochthonous eukaryotic diversity in hydrothermal sediment and experimental micro-colonizers at the Mid-Atlantic Ridge. *Proc Natl Acad Sci U S A* **100**, 697–702.
- Lukeš, J., Jirků, M., Doležal, D., Kral'ova, I., Hollar, L. & Maslov, D. A. (1997). Analysis of ribosomal RNA genes suggests that trypanosomes are monophyletic. *J Mol Evol* **44**, 521–527.
- Lukeš, J., Jirků, M., Avliyakov, N. & Benada, O. (1998). Pankinetoplast DNA structure in a primitive bodonid flagellate, *Cryptobia helcis*. *EMBO J* **17**, 838–846.
- Lukeš, J., Guilbride, D. L., Votypka, J., Zikova, A., Benne, R. & Englund, P. T. (2002). Kinetoplast DNA network: evolution of an improbable structure. *Eukaryot Cell* **1**, 495–502.
- Maslov, D. A., Avila, H. A., Lake, J. A. & Simpson, L. (1994). Evolution of RNA editing in kinetoplastid protozoa. *Nature* **368**, 345–348.
- Maslov, D. A., Yasuhira, S. & Simpson, L. (1999). Phylogenetic affinities of *Diplonema* within the Euglenozoa as inferred from the SSU rRNA gene and partial COI protein sequences. *Protist* **150**, 33–42.
- Moon-van der Staay, S. Y., De Wachter, R. & Vaulot, D. (2001). Oceanic 18S rDNA sequences from picoplankton reveal unsuspected eukaryotic diversity. *Nature* **409**, 607–610.
- Moreira, D., López-García, P. & Rodríguez-Valera, F. (2001). New insights into the phylogenetic position of diplomids: G+C content bias, differences of evolutionary rate, and a new environmental sequence. *Int J Syst Evol Microbiol* **51**, 2211–2219.
- Philippe, H. (1993). MUST, a computer package of Management Utilities for Sequences and Trees. *Nucleic Acids Res* **21**, 5264–5272.
- Philippe, H. & Adoutte, A. (1998). The molecular phylogeny of Eukaryota: solid facts and uncertainties. In *Evolutionary Relationships among Protozoa*, pp. 25–56. Edited by G. Coombs, K. Vickerman, M. Sleigh & A. Warren. London: Chapman & Hall.
- Philippe, H. & Germot, A. (2000). Phylogeny of eukaryotes based on ribosomal RNA: long-branch attraction and models of sequence evolution. *Mol Biol Evol* **17**, 830–834.
- Philippe, H. & Laurent, J. (1998). How good are deep phylogenetic trees? *Curr Opin Genet Dev* **8**, 616–623.
- Philippe, H., Sörhannus, U., Baroin, A., Perasso, R., Gasse, F. & Adoutte, A. (1994). Comparison of molecular and paleontological data in diatoms suggests a major gap in the fossil record. *J Evol Biol* **7**, 247–265.
- Philippe, H., Lopez, P., Brinkmann, H., Budin, K., Germot, A., Laurent, J., Moreira, D., Muller, M. & Le Guyader, H. (2000). Early-branching or fast-evolving eukaryotes? An answer based on slowly evolving positions. *Proc R Soc Lond B Biol Sci* **267**, 1213–1221.
- Posada, D. & Crandall, K. A. (1998). MODELTEST: testing the model of DNA substitution. *Bioinformatics* **14**, 817–818.
- Preisfeld, A., Busse, I., Klingberg, M., Talke, S. & Ruppel, H. G. (2001). Phylogenetic position and inter-relationships of the osmotrophic euglenids based on SSU rDNA data, with emphasis on the Rhabdomonadales (Euglenozoa). *Int J Syst Evol Microbiol* **51**, 751–758.
- Shimodaira, H. (2002). An approximately unbiased test of phylogenetic tree selection. *Syst Biol* **51**, 492–508.
- Shimodaira, H. & Hasegawa, M. (2001). CONSEL: for assessing the confidence of phylogenetic tree selection. *Bioinformatics* **17**, 1246–1247.
- Simpson, A. G. & Roger, A. J. (2004). Protein phylogenies robustly resolve the deep-level relationships within Euglenozoa. *Mol Phylogenet Evol* **30**, 201–212.
- Simpson, A. G., Lukeš, J. & Roger, A. J. (2002). The evolutionary history of kinetoplastids and their kinetoplasts. *Mol Biol Evol* **19**, 2071–2083.
- Simpson, L., Thiemann, O. H., Savill, N. J., Alfonzo, J. D. & Maslov, D. A. (2000). Evolution of RNA editing in trypanosome mitochondria. *Proc Natl Acad Sci U S A* **97**, 6986–6993.
- Skuja, H. (1939). Beitrag zur Algenflora Lettlands II. *Acta Horti Bot Univ Latv XI/XII*, 41–169.
- Skuja, H. (1948). Taxonomie des Phytoplanktons einiger Seen in Upplands, Schweden. *Symb Bot Ups* **9**, 1–399.
- Stein, F. R. (1878). Der Organismus der Infusionsthiere. III. Der Organismus der Flagellaten I. Leipzig: Engelmann.
- Stoeck, T. & Epstein, S. (2003). Novel eukaryotic lineages inferred from small-subunit rRNA analyses of oxygen-depleted marine environments. *Appl Environ Microbiol* **69**, 2657–2663.
- Štolba, P., Jirků, M. & Lukeš, J. (2001). Polykinetoplast DNA structure in *Dimastigella trypaniformis* and *Dimastigella mimosa* (Kinetoplastida). *Mol Biochem Parasitol* **113**, 323–326.
- Suzuki, Y., Glazko, G. V. & Nei, M. (2002). Overcredibility of molecular phylogenies obtained by Bayesian phylogenetics. *Proc Natl Acad Sci U S A* **99**, 16138–16143.
- Swale, E. M. F. (1973). A study of the colourless flagellate *Rhynchomonas nasuta* (Stokes) Klebs. *Biol J Linn Soc* **5**, 255–264.
- Swofford, D. L. (2000). *PAUP*, phylogenetic analysis using parsimony (*and other methods)*, version 4. Sunderland, MA: Sinauer Associates.
- Vickerman, K. (1976). The diversity of the kinetoplastid flagellates. In *Biology of the Kinetoplastida*, pp. 1–34. Edited by W. H. R. Lumsden & D. A. Evans. London: Academic Press.
- Vickerman, K. (1978). The free-living trypanoplasms: descriptions of three species of the genus *Procrystobia* n.g., and redescription of *Dimastigella trypaniformis* Sandon, with notes on their relevance

to the microscopical diagnosis of disease in man and animals. *Trans Am Microsc Soc* **97**, 485–502.

Vickerman, K. (1990). Phylum Zoomastigina, class Kinetoplastida. In *Handbook of Protoctista*, pp. 215–238. Edited by L. Margulis, J. O. Corliss, M. Melkonian & D. J. Chapman. Boston: Jones & Bartlett.

Vickerman, K. (2000). Order Kinetoplastea. In *The Illustrated Guide to the Protozoa*, pp. 1159–1185. Edited by J. J. Lee, G. F. Leedale & P. Bradbury. Lawrence, KS: Allen Press.

Woo, P. T. K. (1987). *Cryptobia* and cryptobiasis in fishes. *Adv Parasitol* **26**, 199–237.

Zhukov, B. F. (1991). The diversity of bodonids. In *The Biology of Free-living Heterotrophic Flagellates*, pp. 177–184. Edited by D. J. Patterson & J. Larsen. New York: Clarendon Press.

Ziková, A., Vancová, M., Jirků, M. & Lukeš, J. (2003). *Cruzella marina* (Bodonina, Kinetoplastida): non-catenated structure of poly-kinetoplast DNA. *Exp Parasitol* **104**, 159–161.