International Journal of Systematic and Evolutionary Microbiology (2000), **50**, 1943–1951 Printed in Great Britain

Phylogeny of the bodonid flagellates (Kinetoplastida) based on small-subunit rRNA gene sequences

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The phylogeny of kinetoplastid flagellates was investigated by determining the sequences of the small-subunit (18S) rRNA from *Bodo designis, Bodo saltans* K, *Bodo saltans* P, *Bodo sorokini*, *Bodo* sp. (cf. *uncinatus*), *Cruzella marina*, *Cryptobia helicis*, *Dimastigella mimosa* and *Parabodo nitrophilus* and analysing these data together with several previously obtained sequences. The root of the kinetoplastid tree was tentatively determined to be attached to the branch of *B. designis* and/or *Cruzella marina*. Within this topology, the suborder Trypanosomatina appears as a late-emerging monophyletic group, while the suborder Bodonina is paraphyletic. Within the bodonid subtree, the branches of parasitic organisms were intermingled with free-living ones, implying multiple transitions to parasitism. The tree indicates that the genera *Cryptobia* and *Bodo* are artificial taxa. In addition, the separation of the fish cryptobias and *Trypanoplasma borreli* as different genera was not supported.

Keywords: Kinetoplastida, Bodonina, *Bodo*, *Cryptobia*, phylogeny

INTRODUCTION

Studies of protozoa from the order Kinetoplastida (phylum Euglenozoa) has yielded a large number of unexpected findings, probably more than for any other comparable group of protists (Vickerman, 1994; Donelson *et al.*, 1999). The order has been defined by the presence of a unique organelle, the kinetoplast, which represents a DNA-containing compartment of the single mitochondrion of a cell.

These studies have revealed organization of the mitochondrial (kinetoplast) DNA in the form of giant networks, an unusual uridylate insertion/deletion type of RNA editing, intriguingly small kinetoplast rRNAs, importation of all mitochondrial tRNAs from the cytosol, *trans*-splicing of cytosolic mRNAs, trypanosome surface antigen variation and a novel nucleotide in DNA referred to as base J (reviewed by Donelson *et al.*, 1999) with this list likely to be continued in the future. Due to the early separation of Euglenozoa (Sogin *et al.*, 1986), some of these features could have been directly inherited from the early eukaryotes, while others can represent more recent adaptations, having evolved, for example, as an adaptation to facultative anaerobiosis (Cavalier-Smith, 1997) or even at later stages, as adaptations to parasitism.

Based on morphology, Kinetoplastida is traditionally divided into two suborders – Bodonina and Trypanosomatina (Vickerman, 1976, 1978; Lom, 1979; Kivic & Walne, 1984). Members of Trypanosomatina are characterized as obligatory parasitic species with a single flagellum and a relatively small kinetoplast, while species of Bodonina include free-living, commensal and ecto- and endoparasitic species with two flagella and a larger kinetoplast.

Because of their importance to human and animal health, trypanosomatids have been studied more intensively than bodonids. Investigation of trypanosomatid evolution at the molecular level included the

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Abbreviations: kDNA, kinetoplastid DNA; LSU large-subunit; SSU, small-subunit; T-PTP, topology-dependent cladistic permutation tail probability.

The GenBank accession numbers of the determined SSU rRNA sequences are: AF209856 (*B. designis*), AF208878 (*C. marina*), AF208880 (*C. helicis*), AF208882 (*D. mimosa*), AF208884 [*Bodo* sp. (cf. *uncinatus*)], AF208886 (*P. nitrophilus*), AF208887 (*B. saltans* P), AF208888 (*B. sorokini*), AF208889 (*B. saltans* K); and of the determined partial LSU rRNA sequences are: AF208879 (*C. marina*), AF208881 (*C. helicis*), AF208883 (*D. mimosa*), AF208885 (*P. nitrophilus*), AF208890 (*B. saltans* K).

analysis of rRNA gene sequences from more than fifty trypanosome species (reviewed by Stevens *et al.*, 1999). Early work addressed general aspects of the evolution of parasitism (Fernandes *et al.*, 1993; Maslov & Simpson, 1995; Maslov *et al.*, 1996) and investigated evolution of RNA editing (Landweber & Gilbert, 1994; Maslov *et al.*, 1994). Special attention has been paid to the evolution of the genus *Trypanosoma* with its more advanced characteristics and diverse parasitic adaptations (Lukeš *et al.*, 1997; Haag *et al.*, 1998; Stevens *et al.*, 1999). These works also shed light on the evolution of bacterial endosymbiosis in this group (Du *et al.*, 1994). Several studies refined the trypanosomatid taxonomy by showing that some genera (such as *Crithidia* and *Herpetomonas*) are polyphyletic and represent artificial taxa (Hollar *et al.*, 1998), while others (*Trypanosoma* and *Phytomonas*) are monophyletic and represent natural taxa (Hollar & Maslov, 1997; Lukeš *et al.*, 1997).

Bodonids, on the other hand, have attracted substantially less attention. So far, sequences useful for phylogenetic analyses are available only for the fish parasites *Trypanoplasma borreli* (Maslov *et al.*, 1994) and *Cryptobia* spp. (Wright *et al.*, 1999), and the free-living species *Bodo caudatus* (Fernandes *et al.*, 1993), *Dimastigella trypaniformis* (Berchtold *et al.*, 1994) and '*Rhynchobodo*' sp. (Lukeš *et al.*, 1997). By putting into phylogenetic context the data obtained by the analysis of RNA editing (Maslov *et al.*, 1994; Lukeš *et al.*, 1994; Blom *et al.*, 1998, 2000), kDNA structure (Yasuhira & Simpson, 1996; Lukeš *et al.*, 1998), and glycosomes (Wiemer *et al.*, 1995) a better understanding of these processes and structures can be achieved. The aim of this work is to determine phylogenetic relationships within the suborder Bodonina, and to provide a framework for molecular and biochemical studies on bodonids.

METHODS

Strain origin and cultivation conditions. *Bodo saltans* strain St Petersburg (P), *Dimastigella mimosa* and *Parabodo nitrophilus* were isolated from the tanks of a sewage plant in Borok, Yaroslavl, Russia (Frolov *et al.*, 1997; Mylnikov, 1986). *Bodo designis* was isolated from the soil in St Petersburg, Russia, in 1992. All the aforementioned strains were provided by S. Karpov (Institute of Cytology, St Petersburg, Russia) and A. Frolov (Zoological Institute, St Petersburg, Russia). *Bodo* sp. (cf. *uncinatus*) represents the strain ATCC 30904 (Daggett & Nerad, 1982). It was originally isolated from a freshwater pool and provided to us by J. Kulda (Faculty of Sciences, Charles University, Prague). *D. mimosa* and *Bodo* sp. (cf. *uncinatus*) were cultivated in the ATCC medium 802 with the feeder bacteria *Alcaligenes xylosoxidans denitrificans* and *Aerobacter aerogenes* at 16 and 20 °C, respectively. The origin and cultivation of the *Bodo saltans* strain Konstanz (K) have been described previously (Blom *et al.*, 1998). '*Rhynchobodo*' sp. (ATCC strain 50359) and *Cruzella marina* (ATCC strain 50326) were obtained from the American Type Culture Collection. The ultrastructural examination of '*Rhynchobodo*' sp. demonstrated its similarity with other species of *Bodo*, especially with *B. designis* (A. Mylnikov, personal communication). Inclusion of *C. marina* in kinetoplastids is supported by analysis of the kinetoplast DNA (A. Žiková, M. Jirků & J. Lukeš, unpublished). The strain *Bodo sorokini* ATCC 50641 was isolated from marine sediments of the White Sea, Tshupa Bay, Russia, in 1986, and obtained from A. Mylnikov (Institute of Biology of Inland Waters, Borok, Russia). *B. sorokini* (Zhukov, 1975) is morphologically and ultrastructurally close to *B. caudatus* and *P. nitrophilus* (A. Mylnikov, personal communication). *C. marina*, *B. designis* and *B. sorokini* were cultivated with the feeder bacteria *A. x. denitrificans* in the ATCC medium 1525 at 16 or 22 °C (*B. sorokini*). The origin of *Cryptobia helicis* has been described elsewhere (Lukeš *et al.*, 1998).

DNA extraction, amplification and sequencing. Isolation of total cell DNA, PCR amplification of the small-subunit (SSU) rRNA genes and partial large-subunit (LSU) rRNA genes and sequencing with a set of conserved oligomer primers were performed as described previously (Maslov *et al.*, 1996, 1999; Lukeš *et al.*, 1997).

Phylogenetic analysis. Additional 18S rRNA sequences used in this work were retrieved from GenBank and include: the trypanosomatids – *Crithidia fasciculata* (X03450), *Herpetomonas muscarum* (L18872), *Phytomonas serpens* strain 1G (AF016323), *Herpetomonas roitmani* (AF038023) and *Trypanosoma cruzi* (M31432); the bodonids – *Dimastigella trypaniformis* (strain Ulm – X76494, strain Glasgow – X76495), '*Rhynchobodo*' sp. (U67183), *Trypanoplasma borreli* (L14840), *Cryptobia bullocki* (AF080224), *Cryptobia catostomi* (AF080226), *Cryptobia salmositica* (AF080225), *Bodo caudatus* (X53910); and the diplonemids – *Diplonema papillatum* (AF119811) and *Diplonema* sp. (AF119812).

Alignments were generated manually using an interactive multiple alignment editor SeqEdit, version 3.1 (Olsen, 1990). The alignments are available on request from D.A.M. or can be retrieved from the following URL: http://www.lifesci.ucla.edu/RNA/trypanosome/alignments.html/. Maximum-likelihood and parsimony analyses were performed using PAUP* 4.0 beta version (Swofford, 1998) with the outgroup represented as a monophyletic sister clade to the ingroup. A heuristic search was performed for likelihood trees, and branch-and-bound search for parsimony trees. The corresponding bootstrap analyses included 100 or 500 replicates, respectively. For the parsimony analysis, an additional evaluation of monophyletic groups was performed using T-PTP (topology-dependent cladistic permutation tail probability). This test included 500 permutations (Faith, 1991). The null hypothesis (non-monophyly) can be rejected at $P \leqslant 0{\cdot}05$. Statistical evaluation of the trees inferred under different topological constraints was performed using the Kishino–Hasegawa test (Kishino & Hasegawa, 1989). The null hypothesis (absence of significant differences between the trees in question) is also rejected at $P \leqslant 0{\cdot}05$. Both statistical tests are included in the PAUP package.

RESULTS

Alignments

Two alignments of the SSU rRNA sequences were constructed and used for analysis of the kinetoplastid phylogeny. The first alignment contained 1283 characters for the 22 kinetoplastid ingroup and two diplo-

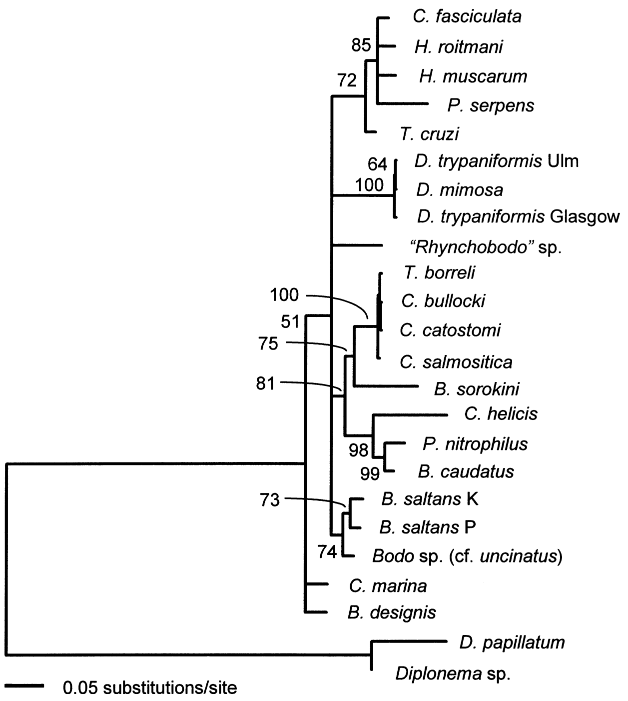

Fig. 1. Majority consensus (50 %) maximum-likelihood tree of Kinetoplastida constructed using the SSU rRNA alignment with 1283 characters and containing two diplonemid outgroup species. The bootstrap values determined for 100 replicates are given at each node. The distance scale is given under the tree. Other parameters were as described in Table 1 for unconstrained search with the full taxon set.

nemid outgroup taxa. The close relationship of the orders Kinetoplastida and Diplonemida within Euglenozoa is well supported by morphology and ultrastructure (Kivic & Walne, 1984; Simpson, 1997) and corroborated by the SSU rRNA and cytochrome oxidase I (COI) protein phylogenetic analysis (Maslov *et al.*, 1999). The ingroup contained nine new and eight known bodonid sequences. Although more than 50 trypanosomatid sequences are currently available, for practical reasons we have included only five sequences which were representative of the five major monophyletic clades of trypanosomatids: *T. cruzi* for trypanosomes; *H. roitmani* for the endosymbiont-bearing clade; *P. serpens* for phytomonads, *H. muscarum* for the endosymbiont-free herpetomonads and *C. fasciculata* for the clade of crithidias, leishmanias and related groups (Hollar *et al.*, 1998). The purpose of this alignment was to find the root of the kinetoplastid tree. The second alignment contained only 22 kinetoplastid taxa. With the absence of the outgroup it was possible to align a larger number of characters (1693), thus producing an alignment which was more informative for investigating relationships within the ingroup.

Our preliminary analyses also included three species of Euglenida, as we did in the previous work (Maslov *et al.*, 1999). However, we found that the trees obtained (data not shown) were less robust with lower bootstrap values and a more frequent occurrence of polytomies, than trees rooted with diplonemids. At the same time the root of the maximum-likelihood tree and topology of the ingroup were identical as with the use of diplonemids only. Since the euglenoids formed the longest branches in the tree, it seemed likely that a decrease in resolution was due to a larger proportion of sites with mutational saturation in the kinetoplastid sequences versus the outgroup. We, therefore, omitted the euglenids from further analyses.

The root of the kinetoplastid tree

The maximum-likelihood bootstrap consensus tree for 22 ingroup and two outgroup taxa is shown in Fig. 1. In this case, the position of the root can be only marginally resolved with the ribosomal dataset. The basal trichotomy includes the branches of *C. marina* and *B. designis*, as well as the clade containing all the remaining kinetoplastids. However, this topology is seen only in 51 % of bootstrap replicates indicating a low level of statistical support. In addition, the position of the root was sensitive to the set of assumptions for the likelihood analysis: the root was obtained in this position only under the assumption of variable rates following γ-distribution with the shape parameter estimated via likelihood. Two other parameters, the proportion of invariable sites and the transition/transversion ratio, were also estimated by likelihood. Any simplification of assumptions resulted in a tree with a basal polytomy (data not shown) which included not only the branches of *C. marina* and *B. designis*, but also several individual branches and clades of the remaining kinetoplastids. Bootstrapping with maximum-parsimony produced an unresolved tree. These results suggest that the amount of phylogenetic information which determines a correct position of the root is very low, so that only a proven power of maximum-likelihood, most of all its reduced sensitivity to misleading multiple nucleotides changes, unequal rates and sampling variance (Swofford *et al.*, 1996), could be used to discern this phylogenetic signal.

The best maximum-likelihood tree (data not shown) revealed the same trichotomy, as seen in the consensus tree (*B. designis*, *C. marina* and the clade of remaining species). This result, and the results of the bootstrap analysis, indicated that either *B. designis* or *C. marina*, or the clade uniting both of them represents the earliest offshoot from the kinetoplastid tree. These topologies make the bodonids a paraphyletic assembly and the trypanosomatids a late diverging monophyletic clade. However, given the uncertainty regarding the root position, it was necessary to investigate alternative *a priori* hypotheses. If the root were attached at the internal branch between bodonids and trypanosomatids, this would make both groups monophyletic, and if the root were attached at the trypanosomatid clade, this group would be paraphyletic, while bodonids would constitute a monophyletic group. Testing these possibilities was done by imposing the corresponding

Table 1. Parameters of the phylogenetic trees produced with maximum likelihood and parsimony

Topological constraint	**Maximum likelihood**		**Parsimony**		
	ln likelihood (no. trees)	***P*-value in K–H test**	**No. steps (no. trees)**	***P*-value in**	
				T-PTP	**K–H test***
Full taxon set:					
No constraints	−5822·20292 (1)†	–	829 (2)	–	–
Constraints					
(O,(B,(T)))	−5822·20292 (2)‡	–	829 (1)	–	–
(O,(T,(B)))	−5822·51861 (1)	0·9172	833 (6)	0·190	0·4654–0·5373
(O,((B),(T)))	−5823·17311 (1)	0·7214	829 (1)	–	–
Ingroup set:					
No constraints	−8085·45042 (1)§	–	1151 (2)	–	–
Constraints for the monophyly of:					
(1) Trypanosomatids with *B. saltans*/*uncinatus*	−8090·40946 (1)	0·3852	1157 (2)	0·044	0·1088, 0·1574
(2) Trypanosomatids with the entire clade of *Trypanoplasma*/*Parabodo*	−8090·43298 (1)	0·3796	1159 (4)	0·052	0·1306, 0·1167, 0·0325, 0·0209
(3) All *Cryptobia* with *T. borreli*	−8107·19467 (1)	0·0298	1162 (2)	0·230	0·1086, 0·1011
(4) Trypanosomatids with *T. borreli* and fish *Cryptobia*	−8144·63156 (1)	0·0001	1176 (1)	0·616	0·0011
(5) All *Bodo*	−8385·32948 (1)	<0·0001	1256 (4)	1·000	<0·0001

* Multiple values appear in comparisons involving several suboptimal (constrained) trees.

† The tree parameters were estimated via likelihood and included: transition/transversion ratio (1·602427), value of proportion of invariable sites (0·375400), value of γ-shaped parameter (0·524818). The same values of these parameters were set in the search of the trees with topological constraints.

‡ One of the trees is rooted at *B. designis*, and the other has the trichotomy which also includes *C. marina* and the monophyletic clade of all remaining kinetoplastids. In most other cases, the differences were restricted to the internal branching order of trypanosomatid clade or the clade of *Trypanoplasma*/fish *Cryptobia*.

§ The estimated tree parameters were: transition/transversion ratio (1·621341), value of proportion of invariable sites (0·456734), value of γ-shaped parameter (0·605804).

topological constraints on the tree, and the results are given in Table 1. The best unconstrained tree and the trees with a constraint for the paraphyly of Bodonina show the same highest value of ln likelihood. This value was slightly lower under the constraint for the paraphyly of trypanosomatids, and it was even more so when both groups were forced to be monophyletic. According to the Kishino–Hasegawa test, these differences were not significant. The parsimony analysis showed that the paraphyly of Bodonina and the monophyly of both groups represented two most parsimonious topologies, while the paraphyly of trypanosomatids did not result in a significantly different score (Table 1). The T-PTP test, however, did not support the monophyly of bodonids which is implied by the latter topology. So, we can tentatively conclude that the tree topology with the root attached to bodonids, and within bodonids to the *B. designis*/*C. marina* branch, is most supported. However, the differences between alternative topologies were subtle, indicating that the problem of the earliest kinetoplastid branches was only marginally resolved by this analysis. Finding a closer outgroup would be needed for a more reliable rooting of the rRNA tree.

Relationships within the Kinetoplastida

A well-resolved tree topology was obtained with maximum-likelihood (Fig. 2), and to a lesser extent with maximum-parsimony, when the outgroup taxa were excluded from the alignment, thus allowing for a substantial increase of the number of alignable characters. This fact also indicates that the polytomies obtained with the previous dataset reflect a shortage of informative characters in that alignment rather than a real burst in the kinetoplastid radiation.

The ingroup topology consisted of several highly supported monophyletic clades which included the clade of trypanosomatids, the clade of *T. borreli*, fish *Cryptobia*, *B. sorokini* and *P. nitrophilius* together with the sister clade of *B. saltans* and *Bodo* sp. (cf. *uncinatus*), and the clade of *B. designis* and *C. marina*. Only the positions of the *Dimastigella* species and ‘*Rhynchobodo*’ sp. remained incompletely resolved. These organisms grouped together in 47% of replicates, and in 50% of cases *Dimastigella* spp. were monophyletic with *B. designis* and *C. marina*.

The most remarkable feature of the tree obtained was

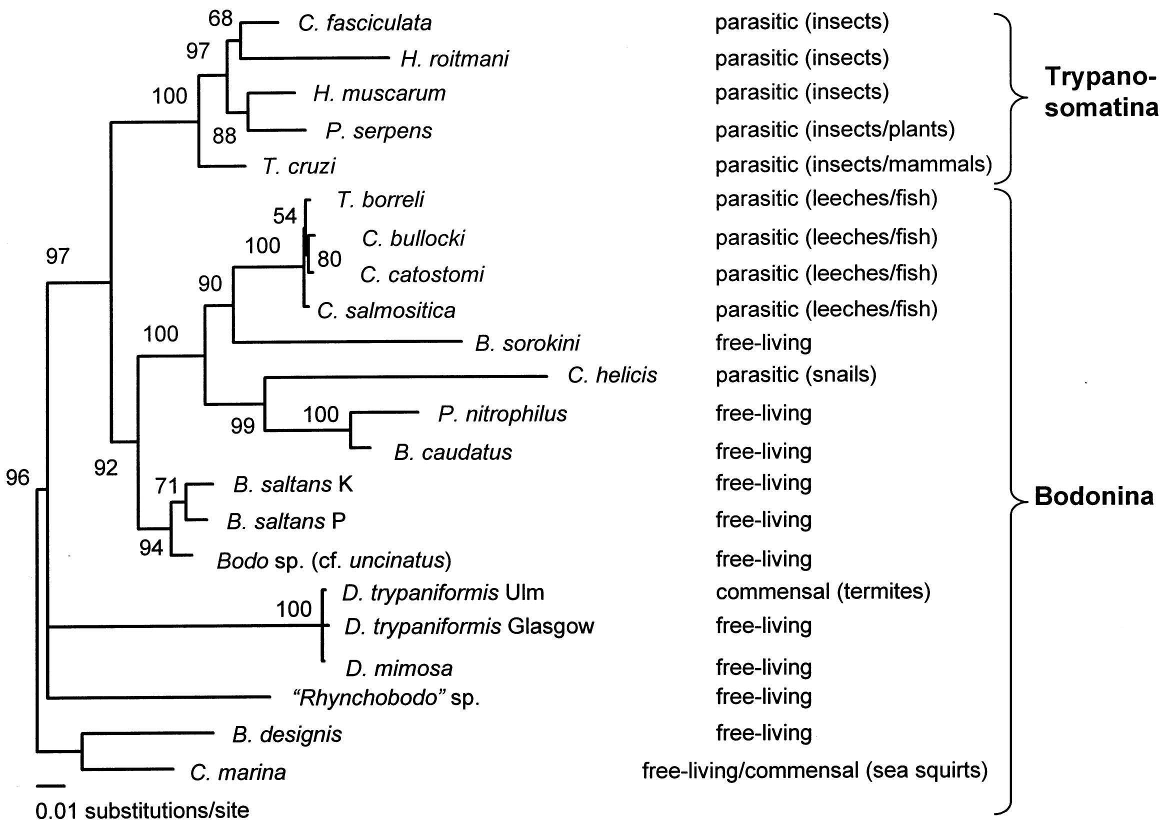

Fig. 2. Majority consensus maximum-likelihood SSU rRNA tree of Kinetoplastida constructed using the alignment with 1693 characters and only the ingroup species. Indication of the lifestyle and the hosts is shown on the right. The tree parameters were as described in Table 1 for unconstrained search with the ingroup set.

that the parasitic organisms did not form a separate cluster. The parasitic and the commensal bodonids were found interspersed among the free-living species within the large *Trypanoplasma–Cryptobia–Bodo–Parabodo* clade (Fig. 1). The topological constraint that enforced the monophyly of *C. helicis* from snails and the compact *Trypanoplasma*–fish *Cryptobia* group (Table 1, constraint 3) resulted in a tree which was significantly less likely using maximum-likelihood. Although the corresponding difference (11 steps) in parsimony was not significant, the monophyly still did not withstand the T-PTP test. Nor was there any support for a closer grouping of these fish parasites with the trypanosomatids (constraint 4). Alternative topologies, which could not be excluded *a priori*, were the monophyly of trypanosomatids with the clade of free-living *B. saltans*/*Bodo* sp. (cf. *uncinatus*) (constraint 1) and, to a lesser extent, the monophyly of trypanosomatids with the mixed clade of *Trypanoplasma–Cryptobia–Bodo–Parabodo* (constraint 2). From a taxonomic perspective, these results demonstrated the polyphyly of the genus *Cryptobia* (constraint 3).

Even more striking was the polyphyly of the genus *Bodo*, the members of which were found within four different monophyletic clades, all strongly supported; the trees constrained for the monophyly of *Bodo* (constraint 5) were significantly inferior to the unconstrained trees. At the same time, the close relatedness of the geographically distant isolates of *Dimastigella* spp. supported their taxonomic assignment into the same genus, although there was no consistency on the level of species. Similarly, the *B. saltans* strains originating from Germany and Russia were monophyletic, although the intraspecific sequence differences were larger than the interspecific differences among the fish cryptobias. An observed close relatedness of the latter group with *T. borreli* could be expected, since based on their morphology, these organisms are sometimes considered members of the same genus (see also Discussion). Results of a preliminary analysis with the limited set of the LSU sequences (data not shown) confirmed the phylogenetic relationships of *C. marina*, *C. helicis*, *D. mimosa*, *P. nitrophilus* and *B. saltans* inferred from the SSU sequences.

DISCUSSION

As members of all major aquatic and terrestrial ecosystems (Foissner, 1991), bodonids represent an ecologically and economically important group of organisms. They are also crucial components of sewage cleaning units and the causative agents of fish diseases in aquacultures (Woo, 1994). In an attempt to better understand the phylogenetic relationships within this group, we have investigated a variety of free-living, commensal, and parasitic species of bodonids from geographically distant localities. Our results show that bodonids are most likely paraphyletic, while trypanosomatids, in agreement with the current morphology-based taxonomy, represent a late-emerging monophyletic group. The tree is rooted at the free-living organisms *B. designis* and *C. marina* and also contains a number of free-living bodonids at its base.

The problem of mutational saturation, which has hampered many rRNA-based phylogenetic analyses (Philippe & Adoutte, 1998), seems to be present in our analysis as well, and may be responsible for the difficulties in determining a position of the root. The topology of the tree (Fig. 1) showing multifurcation of lineages with similar lengths indicates mutational saturation in the ingroup with regard to the outgroup. It is remarkable that by removing the outgroup, thereby reducing the number of sites with multiple mutations, a better resolved tree (Fig. 2) can be obtained. Additional problems associated with reconstructions of trypanosomatid phylogeny using rRNA data are caused by 'long branch attraction' which occurs when substitution rates in some lineages far exceed those in others (Felsenstein, 1978; Maslov *et al.*, 1996; Lukeš *et al.*, 1997). It seems that this artefact is not directly responsible for the observed root of the maximum-likelihood trees, as the branches of *C. marina* and *B. designis* are relatively short. However, the long branch attraction may be responsible for an unstable position of the *Dimastigella* species and '*Rhynchobodo*' sp. (see Results) which group together in 47% of bootstrap replicates and the branches of which are among the longest in the tree (Fig. 2). In addition, this artefact is likely to produce the observed root in the corresponding parsimony trees (Table 1) in which it is attached to the branch of '*Rhynchobodo*' sp. (not shown).

Analysis of the topologically constrained trees shows that the root attached at the bodonids is only slightly preferred to alternative models that include the root attached between the clades of bodonids and trypanosomatids or even at trypanosomatids. Considering the last two models, a trypanosomatid root is less plausible, even though it has a higher support with likelihood (Table 1), since this scenario would contradict abundant morphological, ultrastructural, molecular and ecological data indicating the derived nature of trypanosomatids. On the other hand, the best tree topology confirms earlier hypotheses that the ancestral kinetoplastids were free-living bodonid-like organisms (Vickerman, 1994).

It is clear that a resolution of this problem will require finding an outgroup more closely related to kinetoplastids or analysing another dataset. It should be noted that the analysis of several mitochondrial genes (Lukeš *et al.*, 1994; Blom *et al.*, 1998) also supported the bodonid root. Unfortunately, the limited number of available protein sequences is insufficient for a more detailed analysis.

However, the unrooted topology of the kinetoplastid SSU rRNA tree is very robust and, assuming that it reflects a true phylogenetic history of the group, allows several important conclusions to be drawn concerning the origin and evolution of parasitism in Kinetoplastida, even in the absence of a well-defined root. Parasitic bodonids, such as *T. borreli* and three species of fish cryptobias on one hand, and *C. helicis* on another, belong to the separate monophyletic clades. Trypanosomatids, all of which are parasitic, also form a separate clade within Kinetoplastida. This topology is well supported by the data and it strongly suggests that several and apparently independent acquisitions of the parasitic lifestyle occurred during the evolution of kinetoplastid flagellates. Moreover, it seems that this process still continues and the different steps of it can be observed in the existing clades. The examples of *C. marina* and *D. trypaniformis*, for which both endocommensal and free-living strains have been described (De Faria *et al.*, 1922; Nerad, 1993; Vickerman, 1978; Breunig *et al.*, 1993), may reflect the very early stages of transition towards endoparasitism. A more advanced step in this direction is illustrated by the obligatory endocommensal *C. helicis* which is likely to have evolved from a bodonid with a free-living lifestyle still preserved in the related species *B. caudatus*, *P. nitrophilus* and *B. sorokini*. Another transition to parasitism has independently occurred in the lineage leading towards the *T. borreli*–fish *Cryptobia* group. In this case, the evolution reached the stage of transition from intestinal parasites to haemoparasites, as this compact group contains both types of organisms. All these events seem to correspond to the early hypothesis of Minchin, who postulated that the haemoparasites of vertebrates evolved from their endoparasites, which in turn were derived from the free-living species (Minchin, 1908). The fact that none of the groups illustrates the entire evolutionary process should not be surprising. First, the intermediate stages might have become extinct, and second, the present survey of extant kinetoplastids is far from being complete.

The tree supports an independent origin of parasitism in trypanosomatids. This group of obligatory parasites is well separated in the phylogenetic tree from any group of parasitic bodonids. The data almost exclude the origin of trypanosomatids from *Cryptobia*-like bodonids, a scenario derived from the ultrastructural analysis (Kivic & Walne, 1984). The only bodonid clade which may be more related to trypanosomatids than to other bodonids is the clade of free-living *B. saltans*/*Bodo* sp. (see below). If the evolution in this case had also followed Minchin's scenario, then organisms representing the intermediate stages, such as endoparasitic trypanosomatids of vertebrates, either remain unknown or have become extinct. Alternatively, the evolution of trypanosomatids might have followed the route outlined by Léger (1904), according to which the original parasites were infecting the gut of insects with the subsequent acquisition of haemoparasitism in vertebrates.

The most obvious taxonomic implication of this work is the lack of support for the division of bodonids into two families Bodonidae and Cryptobiidae (Vickerman, 1976, 1978), and the polyphyly of the genus *Bodo*. The ubiquitous and ecologically important members of this genus appear dispersed throughout the tree as four separate branches. This is quite unexpected, since based on their morphology, the *Bodo* species are rather uniform, although this may reflect the absence of any peculiar traits rather than the presence of specific unifying characters. However, any result which involves a single isolate from a species or a group of species should be considered as preliminary, since there is always a possibility of a strain misidentification. From this point of view, only the groups of *B. saltans*, fish cryptobias and *Dimastigella* spp., each of which represented by two or three isolates, do not cause any concern. From the previous analysis of mitochondrial *cox2* gene sequences, *B. saltans* and *Bodo* sp. (cf. *uncinatus*) were inferred to be more closely related to trypanosomatids than to *C. helicis* or *T. borreli* (Blom *et al.*, 1998). This conclusion was indirectly strengthened for *B. saltans* by the gene order, RNA editing patterns, and structure of the kDNA minicircles which resemble those of trypanosomatids (Blom *et al.*, 1998, 2000). As shown by the results of T-PTP and Kishino–Hasegawa tests (Table 1), the monophyly of *B. saltans* and *Bodo* sp. (cf. *uncinatus*) clade with trypanosomatids cannot be excluded, so the question remains open.

D. trypaniformis and *D. mimosa* differ in several morphological features ranging from the specific position of the cytostome as seen in light microscope to ultrastructural differences of the cytoskeleton and the number of mitochondria (Vickerman, 1978; Frolov *et al.*, 1997). Interestingly, in spite of such morphological variation, *D. mimosa* branch is invariably located between the Glasgow and Ulm strains of *D. trypaniformis* in the best maximum-likelihood and parsimony trees (data not shown). The Ulm strain was isolated from the hindgut of a termite while all other *Dimastigella* isolates are free-living. However, although differences in growth conditions and in ultrastructure of nucleus between the two strains have been described (Breunig *et al.*, 1993), the authors did not consider these features significant enough for description of a new species. This view was upheld even when 1·9% differences were found in the 18S rRNAs of both strains (Berchtold *et al.*, 1994). Our results indicate that the taxonomic status of the Ulm strain of *D. trypaniformis* needs to be reconsidered.

In agreement with the previous analysis (Wright *et al.*, 1999), *T. borreli* and three haematozoic *Cryptobia* spp. formed a strongly supported monophyletic group, while *C. helicis*, a type species of the genus *Cryptobia*, constituted a separate branch. The differences in morphology and life cycles between the genera *Cryptobia* and *Trypanoplasma* have been embraced as sufficient by several authors (e.g. Lom, 1976; Becker, 1977; Brugerolle *et al.*, 1979), while deemed insufficient by others (e.g. Bower & Margolis, 1983; Woo, 1994). Recently described differences in the kDNA structure between *T. borreli* (Maslov & Simpson, 1994; Yasuhira & Simpson, 1996) and *C. helicis* (Lukeš *et al.*, 1998), along with the 18S and 28S rRNA data are considered by us as substantial. We therefore suggest that the subgeneric status of *Trypanoplasma* within the genus *Cryptobia*, as proposed by Woo (1994), is inadequate, and that the genus *Trypanoplasma* created early in the last century for biflagellated haematozoic species with a two-host life cycle (Laveran & Mesnil, 1901) should be considered valid.

ACKNOWLEDGEMENTS

We thank Drs A. Frolov, S. Karpov, A. Mylnikov, J. Kulda, K. Šimek and T. Nerad, who provided strains of bodonids used in this study, and Dr M. Peris for comments on the manuscript. The work was supported by the Grant Agency of the Czech Academy of Sciences no. A6022903 to J. L. and also in part by a Burroughs Wellcome New Investigator Award in Molecular Parasitology and the grant AI40634 from the National Institutes of Health to D. A. M.

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