J. Eukaryot. Microbiol., 51(4), 2004 pp. 402–416

Ribosomal RNA Phylogeny of Bodonid and Diplonemid Flagellates and the Evolution of Euglenozoa

SOPHIE VON DER HEYDEN,[a] EMA E. CHAO,[a] KEITH VICKERMAN[b] and THOMAS CAVALIER-SMITH[a]

[a]*Department of Zoology, University of Oxford, South Parks Road, Oxford, OX1 3PS, UK*
[b]*Division of Environmental and Evolutionary Biology, University of Glasgow, Glasgow, G12 8QQ, UK*

ABSTRACT. Euglenozoa is a major phylum of excavate protozoa (comprising euglenoids, kinetoplastids, and diplonemids) with highly unusual nuclear, mitochondrial, and chloroplast genomes. To improve understanding of euglenozoan evolution, we sequenced nuclear small-subunit rRNA genes from 34 bodonids (*Bodo*, *Neobodo*, *Parabodo*, *Dimastigella*-like, *Rhynchobodo*, *Rhynchomonas*, and unidentified strains), nine diplonemids (*Diplonema*, *Rhynchopus*), and a euglenoid (*Entosiphon*). Phylogenetic analysis reveals that diplonemids and bodonids are more diverse than previously recognised, but does not clearly establish the branching order of kinetoplastids, euglenoids, and diplonemids. *Rhynchopus* is holophyletic; parasitic species arose from within free-living species. Kinetoplastea (bodonids and trypanosomatids) are robustly holophyletic and comprise a major clade including all trypanosomatids and most bodonids ('core bodonids') and a very divergent minor one including *Ichthyobodo*. The root of the major kinetoplastid clade is probably between trypanosomatids and core bodonids. Core bodonids have three distinct subclades. Clade 1 has two distinct *Rhynchobodo*-like lineages; a lineage comprising *Dimastigella* and *Rhynchomonas*; and another including *Cruzella* and *Neobodo*. Clade 2 comprises *Cryptobia/Trypanoplasma*, *Procryptobia*, and *Parabodo*. Clade 3 is an extensive *Bodo saltans* species complex. *Neobodo designis* is a vast genetically divergent species complex with mutually exclusive marine and freshwater subclades. Our analysis supports three phagotrophic euglenoid orders: Petalomonadida (holophyletic), Ploeotiida (probably holophyletic), Peranemida (paraphyletic).

Key Words. *Bodo saltans*, Euglenoidea, *Dimastigella*, *Diplonema*, kinetoplastids, *Neobodo*, *Rhynchobodo*, *Rhynchopus*, 18S rRNA.

PHYLUM Euglenozoa (Protozoa) (Cavalier-Smith 1981) was established to embrace two traditionally separate protozoan classes: kinetoplastids, characterised by kinetoplast DNA and bizarre RNA editing in their mitochondria (Landweber and Gilbert 1994; Maslov et al. 1994), and the typically larger euglenoid flagellates, which lack kinetoplast DNA but often have green chloroplasts bounded by three membranes. The relationship between kinetoplastids and euglenoids was initially suggested by cytochrome c phylogeny (Schwartz and Dayhoff 1978) and four shared morphological characteristics: discoid mitochondrial cristae; a flagellar pocket; flagellar paraxonemal rods (= paraxial or paraflagellar rods); and a pellicle with a corset of microtubules underlying most of the cell surface (Vickerman and Preston 1976). Kinetoplastids comprise the typically free-living bodonid flagellates and the disease-causing trypanosomatid and leishmanial parasites (Vickerman 1976, 1990). It was later found that some, but not all, Euglenozoa had unique tubular extrusomes (Brugerolle 1985) and that rRNA analysis supported their specific relationship and marked divergence from other eukaryotes (Cavalier-Smith 1993a; Montegut-Felkner and Triemer 1997; Sogin, Elwood, and Gunderson 1986). Both groups consistently have much larger nuclear small subunit (SSU) rRNA genes than most other phyla, and the rRNA of *Euglena* at least is fragmented (Gray et al. 2001; Schnare, Cook, and Gray 1990).

Kinetoplastids are unique among eukaryotes in having glycosomes, modified peroxisomes bearing the glycolytic enzymes, most of which are absent from the cytosol (Michels and Hannaert 1994). Euglenoids are unusual in having permanently condensed chromosomes and a reversed anaphase A/B sequence (Triemer and Farmer 1991a, b), whereas kinetoplastids have closed mitosis with an endonuclear spindle and chromosomes that do not condense during mitosis, being partially condensed during interphase (Frolov and Skarlato 1998). Some trypanosomatids at least have numerous minichromosomes in addition to regular ones with visible kinetochores (Ersfeld and Gull 1997). Sexual processes have rarely been demonstrated in Euglenozoa. Syngamy has been observed only in the euglenoid *Scytomonas pusilla* (= *Copromonas subtilis*) (Dobell 1908); there is genetic evidence for syngamy and meiosis in African trypanosomes (Gibson 1995) and the South American *Trypanosoma cruzi* (Gaunt et al. 2003); the bodonid *Dimastigella* is the only free-living kinetoplastid with evidence for sexuality, in the form of synaptonemal complexes (suggesting meiosis) observed in one isolate of *D. trypaniformis* (Vickerman 1991) though not in another (Breunig et al. 1993).

The third euglenozoan group is the diplonemids: *Diplonema* (Griessmann 1914; Larsen and Patterson 1990; Montegut-Felkner and Triemer 1994, 1996; Triemer and Ott 1990) and *Rhynchopus* (Schnepf 1994; Skuja 1948). As they do not possess discoid mitochondrial cristae and the flagella generally lack paraxonemal rods (Triemer and Farmer 1991b), it was uncertain whether they really belonged to Euglenozoa. Phylogenetic analyses of nuclear 18S rRNA and mitochondrial partial cytochrome oxidase subunit I (CO1) protein genes showed that *Diplonema* falls within Euglenozoa (Maslov, Yasuhira, and Simpson 1999). Nuclear 18S rRNA analysis also showed the same for *Rhynchopus* (Cavalier-Smith 2000) and the close affinity of *Rhynchopus* with *Diplonema* (Busse and Preisfeld 2002a) confirming their inclusion in the phylum by Cavalier-Smith (1993a), supported by Simpson (1997).

Euglenozoan nuclear mRNAs are also remarkable in that most are formed by trans-splicing of a miniexon to the actual coding region in euglenoids (Frantz et al. 2000; Muchal and Schwartzbach 1992) and apparently all are in kinetoplastids (Sutton and Boothroyd 1986). At least in kinetoplastids, this trans-splicing is achieved by a ribonucleoprotein machinery related to the spliceosomes that remove spliceosomal introns (Palfi et al. 2000; Palfi, Lane, and Bindereif 2002), even though they have almost no cis-spliced introns, unlike euglenoids and other mitochondrial eukaryotes. As expected (Cavalier-Smith 1993b) diplonemids also make mRNA by trans-splicing (Sturm et al. 2001). Euglenozoa are the sister group to the phylum Percolozoa which probably lacks trans-splicing; both are grouped together as superphylum Discicristata in the protozoan infrakingdom Excavata (Cavalier-Smith 1993d; 2003a, c).

The phylogenetic branching order of the three main euglenozoan classes is uncertain: some 18S rRNA analyses associate diplonemids and kinetoplastids (Busse and Preisfeld 2002a; Maslov, Yasuhira, and Simpson 1999; Simpson, Lukeš, and Roger 2002) with low to moderately strong bootstrap support, whereas others show diplonemids as sisters of or even branching within euglenoids (Cavalier-Smith 2003a) in accord with the grouping of Diplonemea and Euglenoidea as subphylum

Corresponding Author: T. Cavalier-Smith—Telephone number: +44 01865 281065; FAX number: +44 1865 281310; Email: tom.cavalier-smith@zoo.ox.ac.uk

Plicostoma (Cavalier-Smith 1998). Additionally, analyses are hampered by divergent evolutionary rates of the 18S rRNA genes of these groups (Busse and Preisfeld 2002b; Busse, Patterson, and Preisfeld 2003). Resolving the earliest relationships within Euglenozoa is also impeded by mutational near-saturation of euglenoid 18S rRNA (Moreira, López-García, and Rodríguez-Valera 2001). A recent analysis of cytosolic forms of heat shock protein 70 (Hsp70) and 90 (Hsp90) supports a sister relationship between diplonemids and kinetoplastids, to the exclusion of *Euglena gracilis* (Simpson and Roger 2004). Further, in diplonemids and kinetoplastids, tryptophan is encoded by TGA codons, whereas euglenoid mitochondria use the universal genetic code (Inagaki et al. 1997; Maslov, Yasuhira, and Simpson 1999; Yasuhira and Simpson 1997). Relationships among the free-living/endo-ecto commensal members of the order Bodonida are unclear, as is the question whether trypanosomatids arose from within bodonids or are their sisters. Recent phylogenetic analyses of new kinetoplastid sequences of the fish parasite *Ichthyobodo* and a *Perkinsiella*-like symbiont of paramoebid amoebae place them as a divergent sister group to other bodonids and trypanosomatids (Callahan, Litaker, and Noga 2002; Dyková et al. 2003; López-García, Philippe, and Moreira 2003; Moreira, López-García, and Vickerman 2004). This closer outgroup should help in deciding the position of the root of the remaining bodonids, which we here refer to as core bodonids.

To clarify further relationships within Euglenozoa, we sequenced the 18S rRNA genes of four *Diplonema* and five *Rhynchopus* isolates (both free-living and parasitic), as well as 34 free-living bodonids and two isolates of uncertain affinities: a *Cryptaulaxoides*-like organism and a *Phanerobia*-like strain. Six of these sequences were included in a preliminary illustrative tree (Cavalier-Smith 2000). Our new data and analyses provide many insights into the internal phylogeny and diversity of bodonids and, to a lesser extent, within diplonemids and euglenoids. In particular, we isolated strains from a novel, highly divergent *Rhynchobodo*-like lineage within Clade 1 of Simpson, Lukeš, and Roger (2002). Our analyses confirm the polyphyly of the former genus *Bodo* and support its replacement by three genera: *Bodo sensu stricto* (solely *Bodo saltans*); *Parabodo* (including the former *Bodo caudatus*); and *Neobodo* (including the former *Bodo designis*), as suggested by Moreira, López-García, and Vickerman (2004). We adopt this revised nomenclature here. The basal branching order of Euglenozoa remains unresolved by 18S rRNA, as parsimony trees weakly support a sister relationship between diplonemids and kinetoplastids, whereas distance analyses weakly support one between diplonemids and euglenoids.

MATERIALS AND METHODS

Cultures. Table 1 shows the strains used, their sources, growth media and 18S rRNA gene lengths. *Procryptobia sorokini* and *Parabodo nitrophilus* were kindly donated by Dr. A. P. Mylnikov, Borok, Russia. All other strains were obtained from culture collections as stated or isolated from environmental samples. For free-living strains cells were isolated by repeated serial dilutions of 50 μl of environmental samples into sterile microtitre wells (Nunclon, Scientific Laboratory Supplies, Nottingham, UK) containing 230 μl of growth medium; all were in uni-bodonid culture, but some contained other eukaryotes (see Table 1). *Rhynchopus* spp. I, II and IV were isolated from *Nephrops norvegicus* trawled from the Firth of Clyde, UK. *Rhynchopus* spp. I and IV were obtained from the blood of *N. norvegicus* infected with the parasitic dinoflagellate *Hematodinium* sp. Blood was drawn aseptically from a walking leg sinus of the animal by syringe, distributed into microtitre well plates containing FCSG medium (Appleton and Vickerman 1998) and separated from *Hematodinium* by serial dilution. *Rhynchopus* sp. II was isolated from the gills of a freshly killed animal, washed in sterile balanced *Nephrops* saline (BNS) before explanting to a six-well plate containing FCSG medium. *Rhynchopus* sp. II multiplied and was cloned by serial dilution in the same medium.

PCR amplification and sequencing. DNA was extracted and amplified from strains marked with an asterisk (*) in Table 1 as described previously (Cavalier-Smith and Chao 1995). Cells of other strains were harvested under low-pressure filtration onto glass fibre filters (Whatman) and DNA was extracted using an UltraClean DNA soil extraction kit (MoBio Laboratories, Cambio Ltd., Cambridge, UK). Their 18S rRNA genes were PCR-amplified using either general eukaryote primers G and F (Cavalier-Smith and Chao 1995) or for cultures with other eukaryotes present with the kinetoplastid-specific primers (kineto14F: 5′CTGCCAGTAGTCATATGCTTGTTTCAAGG A 3′ and kineto2026R: 5′GATCCTTCTGCAGGTTCACCTA CAGCT 3′). PCR with these specific primers used 50–200 ng of gDNA, 2.5 mM primer, 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 1× PCR Buffer and 1 unit Taq-Polymerase (Invitrogen Ltd., Paisley, UK). Amplifications used an automated thermocycler (Touchgene Gradient, Techne, Scientific Laboratory Supplies, Nottingham, UK) with initial denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 63 °C for 36 s, extension at 72 °C for 1.5 min, and a final extension at 72 °C for 5 min. All fragments were gel purified using a GFX kit (Amersham Biosciences UK Ltd., Chalfont St. Giles, UK). Strains marked with # in Table 1 were directly bidirectionally sequenced. All others were cloned into pCR 2.1-TOPO vectors using a TOPO TA cloning kit (Invitrogen); between three and eight clones were picked and partially sequenced using M13F primer. There was low heterogeneity among all clones (< 1%); one for each strain was chosen for complete bidirectional sequencing. Sequencing used Big Dye terminators and an automated ABI-377 sequencer. GenBank accession numbers for strains isolated in this study: AY425008-AY425025 and AY490209-AY490235. GenBank accession numbers for all additional taxa used in the analyses can be found in Appendix 1.

Sequence analysis and phylogenetic methods. Four data sets were created. Data set 1 with 145 taxa and 1233 sites included all available diplonemid sequences, many eukaryotrophic, osmotrophic and photosynthetic euglenoids, a diverse range of kinetoplastids (*Ichthyobodo*, trypanosomatids and parasitic and free-living bodonids), and a selection of Percolozoa (Cavalier-Smith 1993d) as the closest sister group to Euglenozoa (Cavalier-Smith 1999) and other short-branch excavates (Metamonada—*Trimastix* and oxymonads—and Loukozoa: see Cavalier-Smith 2003a) as outgroups; inclusion of several close excavate outgroups should increase the reliability of basal euglenozoan branching orders. Data set 2 includes 80 taxa and 1233 positions and was restricted to a broad sample of 68 euglenoids, plus diplonemids as outgroup, in order to clarify basal euglenoid phylogeny. Data set 3 includes nearly all available core bodonids and a selection of trypanosomatids (69 taxa), but prokinetoplastids were deliberately omitted to allow inclusion of more nucleotides (1613 positions aligned with confidence) and therefore greater resolution within core bodonids. To allow maximum likelihood bootstrap analysis for kinetoplastids, data set 4 comprised many fewer taxa, including a representative selection of core bodonid and trypanosomatid sequences plus the *Ichthyobodo* clade (total 48 taxa), but included only 1342 positions because alignment of other parts of the *Ichthyobodo* sequence was uncertain. Data sets 1 and 2 were based on a

Table 1. Sources of isolates, culture media and lengths of new 18S rDNA sequences.

Strain and accession numbers	Source	Medium	Sequence length
Entosiphon sp. SA *# **AY425008**	Soil, Kommetjie, South Africa (SA)	SES	2155
Diplonema ambulator *# **AY425009**	Washington, DC, USA (ATCC 50223)	PE	1988
Diplonema sp. 2* # **AY425010**	Gaithersburg, MD, USA (ATCC 50224)	PE	1971
Diplonema sp. 3* # **AY425011**	Gaithersburg, MD, USA (ATCC 50225)	PE	1992
Diplonema sp. 4* # **AY425012**	Solomon's Island, MD, USA (ATCC 50232)	PE	2073
Rhynchopus sp. 1* # **AY425013**	New Bedford, MA, USA (ATCC 50226)	PE	1966
Rhynchopus sp. 2* # **AY425014**	Cliff Pool, Bermuda (ATCC 50229)	PE	1946
Rhynchopus sp. I # ^ **AY490209**	Blood of *Nephrops norvegicus* (Clyde Estuary, UK)	FCSG	1954
Rhynchopus sp. II # ^ **AY490210**	Gills of *Nephrops norvegicus* (Clyde Estuary, UK)	FCSG	1962
Rhynchopus sp. IV # ^ **AY490211**	Blood of *Nephrops norvegicus* (Clyde Estuary, UK)	FCSG	1954
Neobodo designis GLASGOW ^ **AY490235**	Soil, Glasgow Botanic Gardens, UK	TSB	2039
Neobodo designis LONGSTOCK ^ **AY490221**	Soil, Longstock, Hampshire, UK	BE	2043
Neobodo designis SA * **AY425016**	Mouille Point, Cape Town, South Africa (SA)	PE	2027
Neobodo sp. CPT *# **AY425017**	Garden Soil, Cape Town, SA	SES	2046
Bodo saltans JC02 ^ **AY490227**	Sediment, Priest Pot, Cumbria, UK	SES	2091
Bodo saltans JC03 **AY490228**	Sediment, Priest Pot, Cumbria, UK	SES	2079
Bodo saltans JC18 **AY490229**	Sediment, Priest Pot, Cumbria, UK	SES	2072
Bodo saltans PP5 ^ **AY490223**	Sediment, Priest Pot, Cumbria, UK	SES	2087
Bodo saltans NZ 1 ^ M **AY490232**	Soil, University of Waikato, Hamilton, New Zealand	SES	2076
Bodo saltans NZ 2 ^ **AY490230**	Sediment, Rotawiti Lake, NZ	SES	2080
Bodo saltans NZ 3 ^ M **AY490231**	Sediment, Karapiro Lake, NZ	SES	2078
Bodo saltans NZ 4 ^ **AY490234**	Soil near Karapiro Lake, NZ	SES	2069
Bodo saltans NZ 5 ^ M **AY490233**	Rotting seaweeds, Raglan, NZ	PE	2089
Bodo saltans BOTS ^ M **AY490224**	Algae and sediment, Okavango Delta, Botswana	SES	2075
Bodo saltans ML **AY490226**	Soil, Gudung Gading N.P., Sarawak, Malaysia	SES	2066
Bodo saltans SA ^ M **AY490222**	FW sediment, Klein Pakhuis, Western Cape, SA	SES	1946
Cryptaulaxoides-like *# **AY425021**	Soil, Punta Morales, Costa Rica	SES	2087
Dimastigella sp. (*Phanerobia*-like) *# **AY425020**	Soil, La Selva, Costa Rica	SES	2061
Novel bodonid NZ 1 ^ M **AY490220**	Marine sediment, Auckland, New Zealand	PE	2071
Novel bodonid NZ 2 **AY490219**	'Jockey Pool', near Mata Mata, NZ	PASA	2066
Novel bodonid UK ^ **AY490225**	Sediment, River Thames, Oxford, UK	SES	2083
Parabodo(=*Bodo*) *nitrophilus* *# **AY425019**	Russia (Dr. A. P. Mylnikov)	SES	1972
Parabodo (=*Bodo*)-like ML **AY490215**	Soil, Gudung Gading N.P., Sarawak, Malaysia	PE	1975
Parabodo (=*Bodo*) *caudatus*-like RDF * **AY425015**	Red Deer faeces, Glasgow, UK	SES	1978
Parabodo (=*Bodo*) sp. ^ **AY490218**	Lawn grass, Glasgow, UK	TSB	1964
Procryptobia sorokini *# **AY425018**	Russia (A. P. Mylnikov)	SES	2020
Procryptobia sp. SA **AY490216**	Sediment, Camps Bay, South Africa	PE	1987
Procryptobia sp. NZ ^ M **AY490217**	Marine Sediment, Raglan, NZ	SES	1992
Rhynchomonas nasuta ATCC 50105 * **AY425023**	Sargasso Sea, ATCC 50105	SES	2104
Rhynchomonas nasuta DB8 *# **AY425024**	Upper Chesapeake Bay, WHOI, DB8	PE	2103
Rhynchomonas nasuta OV1 *# **AY425025**	Sediment, Burnham Overy Staithe, Norfolk, UK	PE	2103
Rhynchomonas nasuta PP ^ **AY490214**	Algal scrape, Priest Pot, Cumbria, UK	SES	2043
Rhynchobodo-like NZ ^ **AY490213**	Sediment, Hamilton Lake, NZ	SES	2139
Rhynchobodo sp. UK ^ **AY490212**	Soil, Sourhope, UK	TSB	2088

The length of sequences is given in base pairs (bp) from the end of primer kineto14F to the beginning of kineto2026R, because sequences generated with this primer pair are slightly shorter than those generated with general eukaryotic primers (Cavalier-Smith and Chao, 1995). Media used: SES = Soil extract with added salt (UKNCC catalogue), PE = Plymouth Erdschreiber (UKNCC), TSB = 0.1% Tryptone Soya Broth (Difco), BE = 0.1% Beef Extract (Oxoid), FCSG = 10% Fetal Calf Serum in balanced *Nephrops* saline, PASA = Page's Amoeba Saline Agar. Asterisks indicate that the DNA isolation amplification and sequencing methods were as in Cavalier-Smith and Chao (1995), # indicates strains that were directly bi-directionally sequenced; all others were cloned before sequencing. Strains marked with ^ are still alive in our own culture collection. Those marked with an M are mixed with other eukaryotes, but are in uni-bodonid culture.

large 18S rRNA alignment with an even more comprehensive selection of Euglenozoa than used in the present analyses. The 18S rRNA sequences of data set 3 and 4 were aligned with ClustalX (Thompson, Higgins, and Gibson 1994) and then manually adjusted using Genetic Data Environment software (Smith et al. 1994).

Phylogenetic analyses used PAUP*4.0b10 (Swofford 1993). Distance matrices were calculated using the general time reversible (GTR) model and allowed for rate variation across sites, assuming a gamma distribution and a proportion of invariable sites, estimated by ModelTest (Posada and Crandall 1998). Data set 1 was analysed by Maximum Parsimony (MP) and distance methods using Neighbor Joining (NJ) and BioNJ; bootstrap analyses used 1000 BioNJ replicates and 500 for MP. Data set 2 was analysed using NJ, BioNJ and Minimum Evolution (ME: heuristic search with 100 random stepwise addition replicates) and 250 (each with 5 addition sequence replicates) heuristic bootstrap replicates. Data set 3 was analysed using all distance methods (NJ, BioNJ, ME and weighted least squares). Weighted least squares and ME trees were constructed using a heuristic search of 100 random stepwise addition sequence replicates; bootstrap analyses used 500 re-samplings (each with 5 addition sequence replicates). A subset of kinetoplastid sequences (data set 4) was analysed using maximum likelihood

ML+Γ+I; with 4 discrete rate categories); heuristic bootstrap analyses used 75 re-samplings. We carried out four separate ML analyses always using the same Γ+I, but with various time constraints and best tree searches and obtained the same tree 65 out of 167 times.

RESULTS

As in previous studies, all euglenozoan sequences were longer than the approximately 1850 nucleotides typical for most 18S rRNA genes (Table 1). The longest was *Entosiphon* (2155 nt) and the shortest *Rhynchopus* sp. 2 and *Bodo saltans* SA 1946 nt). The phylum Euglenozoa is robustly supported in Fig. 1 (84%); diplonemids and kinetoplastids are both holophyletic with 100% bootstrap support, but holophyly of euglenoids is weakly supported. Branching orders among the classes vary with different phylogenetic methods; parsimony analysis grouped diplonemids with kinetoplastids, whereas distance analyses group diplonemids with euglenoids, the latter clade corresponding to subphylum Plicostoma (Cavalier-Smith 1998), but support is very weak for both. The diplonemid clade is markedly shorter overall than for kinetoplastids and euglenoids, indicating slower evolution of their rRNA.

Phylogeny of Euglenoidea. Euglenoids exhibit the longest branches of any class within Euglenozoa. The BioNJ distance tree (Fig. 1) shows four strongly supported major euglenoid clades: the predominantly and ancestrally phototrophic superorder Euglenophycidae (= class Euglenophyceae sensu Marin et al. (2003), but of lower rank, so Euglenoidea can remain as a class, as in Cavalier-Smith 1998) (95% support); the ancestrally osmotrophic and heterotrophic Rhabdomonadida (87% support); the third clade comprises *Notoselenus* and *Petalomonas cantuscygni* (99% support) as shown by Busse, Patterson, and Preisfeld (2003) using Baysian analysis, supporting their grouping in the order Petalomonadida (Cavalier-Smith 1993); *Peranema* sp., *Anisonema*, and *Dinema* (all order Peranemida: Cavalier-Smith 1993a) form a fourth robust clade (99% support). Because this part of the tree might have been perturbed by the distant long-branch out groups these are removed in the analysis shown in Fig. 2, which shows the same four major euglenoid clades (92/93%, 85/91%, 99/96%, 99/96% support respectively). In this minimum evolution tree their branching order is different: rhabdomonads are now sisters to euglenophycids (this clade corresponding to subclass Aphagia Cavalier-Smith 1993a; 1998—the term Aphagea was incorrectly used by Busse, Patterson, and Preisfeld 2004 as a synonym for Rhabdomonadida alone) instead of the *Anisonema* clade. On both trees the grouping of these three clades has weak to moderate support (52–60%) and other phagotrophic euglenoids are more distant, suggesting that phagotrophic euglenoids are probably paraphyletic. Figure 2 also has a weakly supported clade comprising the morphologically very similar bacteriotrophic *Entosiphon sulcatum* and *Ploeotia costata*, providing the first molecular support for the order Ploeotiida (Cavalier-Smith 1993a). Our *Entosiphon* sequence is distinct from but quite closely related to the previously published one. *Peranema trichophorum* groups weakly as a sister to *Entosiphon* (Fig. 1) or to Ploeotiida. In Fig. 2 largely bacteriotrophic Petalomonadida are the earliest diverging euglenoids. Except for the robust sister relationship between Euglenida and Eutreptiida (previously found by Brosnan et al. 2003; Marin et al. 2003; and Busse, Patterson, and Preisfeld 2003), the branching order of the six euglenoid orders is poorly resolved, suggesting rapid early radiation among the phagotrophs and/or near mutational saturation.

Phylogeny of Kinetoplastea. There are two established lineages within kinetoplastids (Fig. 1), one comprising only *Ichthyobodo*, the *Perkinsiella*-like organism and some environmental DNA samples (Dyková et al. 2003; López-García, Philippe, and Moreira 2003), the other composed of a longer-branch clade containing all other bodonids (core bodonids) and trypanosomatids. A third lineage of solely environmental samples is a sister-group to both kinetoplastid clades, breaking the otherwise long stem of kinetoplastids (López-García, Philippe, and Moreira 2003). Different analyses place the root of the trypanosomatid/core bodonid clade (i.e. all except the divergent *Ichthyobodo* clade) in contradictory places. Parsimony analyses place the root between *Rhynchomonas* and *Dimastigella* and the rest of the clade, whereas distance and maximum likelihood place it between trypanosomatids and core bodonids (Fig. 1, 4), which ought to be more reliable as the use of gamma corrections should reduce long-branch artefacts to which parsimony is particularly prone. Bootstrap support values for the holophyly of core bodonids and trypanosomatids (Fig. 1) are 63% and 58% respectively.

Trees confined to core kinetoplastids (Subclass Metakinetoplastina of Moreira, López-García, and Vickerman (2004)), thus excluding the highly divergent *Ichthyobodo* clade (Prokinetoplastina of Moreira, López-García, and Vickerman (2004)), and rooted as on Fig. 1 and 4 show the three major robust clades indicated by Simpson, Lukeš, and Roger (2002) (bootstrap values of 76–100%: Fig. 3). On trees rooted between trypanosomatid and core bodonids, clades 2 and 3 group together, with significant support in distance (77–79%: Fig. 3) but weakly in ML analyses (48%: Fig. 4). With maximum likelihood core bodonids are also holophyletic but support is weak (32%).

Clade 1 (Neobodonida of Moreira, López-García, and Vickerman (2004)) is considerably more complex than noted by Simpson, Lukeš, and Roger (2002). It has five robust subclades (A-E: Fig. 3) . Clade 1A (two *Rhynchobodo* strains, *Klosteria* and *Rhynchobodo*-like) is sister (with good (78%) support only with weighted least squares) to clades 1B (*Dimastigella* and *Rhynchomonas*, with the longest branches), 1C (the *Neobodo designis* complex plus *Cruzella*) and clade 1D (*Cryptaulaxoides*-like, plus an environmental sequence from a marine hydrothermal vent (AT5–25) of unknown morphology). Clades 1C and 1D group together strongly on distance trees (78–84%: Fig. 3), but with only moderate support by ML (57%: Fig. 4). The novel bodonid Clade 1E is most divergent of all and includes both marine and freshwater strains morphologically very similar to *Rhynchobodo*. The great divergence of clades 1A and 1E imply either that their *Rhynchobodo*-like appearance is convergent or that it was ancestral for clade 1. The *Cryptaulaxoides*-like strain was originally designated *Cryptaulax* because of its spirally twisted groove and two heterodynamic flagella (Cavalier-Smith 2000) without realising that Skuja's (1948) name was preoccupied by a mollusc and hymenopteran under the zoological code that we consider appropriate for all Euglenozoa, or that Novarino (1996) had proposed *Cryptaulaxoides* as replacement. Assignment of *Cryptaulax* Skuja to the kinetoplastids (Bernard, Simpson, and Patterson 2000) is fully supported by our present trees, as well as our electron microscopy on this strain that revealed a paraxonemal rod, cortical microtubules and discoid cristae (T.C-S and B. Oates, unpubl. data). However, the assignment of all described *Cryptaulax* spp., except *C. marina* Throndsen 1969 (now deemed to be a junior synonym of *Hemistasia phaeocysticola* (Scherffel) Elbrächter, Schnepf, and Balzer (1996) by Bernard, Simpson, and Patterson (2000), specifically to *Rhynchobodo* (Vørs 1992; Bernard, Simpson, and Patterson 2000) may be premature, given that we find two very divergent *Rhynchobodo*-like lineages in clade 1A and neither groups specifically with the sequence from our twisted flagellate. We therefore refer to it simply as *Cryptau-*

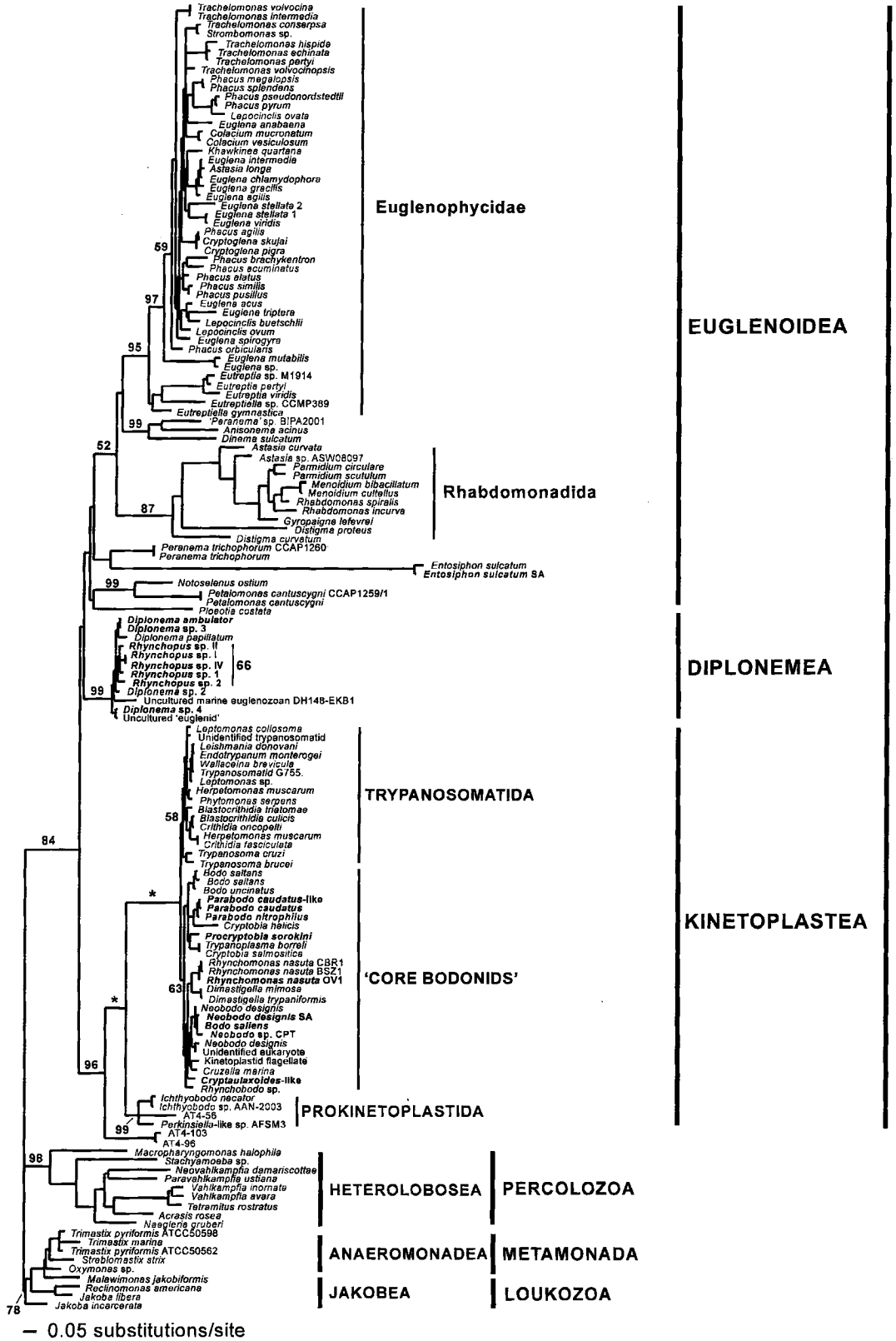

Fig. 1. Distance tree of 145 18S rRNA sequences, comprising Euglenozoa, Percolozoa, and as outgroups, Metamonada (= *Trimastix* and oxymonads) and Loukozoa, based on 1233 positions (BioNJ: GTR+Γ+I model: α = 0.760826, I = 0.140619). New sequences generated in this study are in bold. Numbers at major nodes only are bootstrap support percentages from 1000 BioNJ replicates. Values less than 50% are omitted. Asterisks denote 100% bootstrap support.

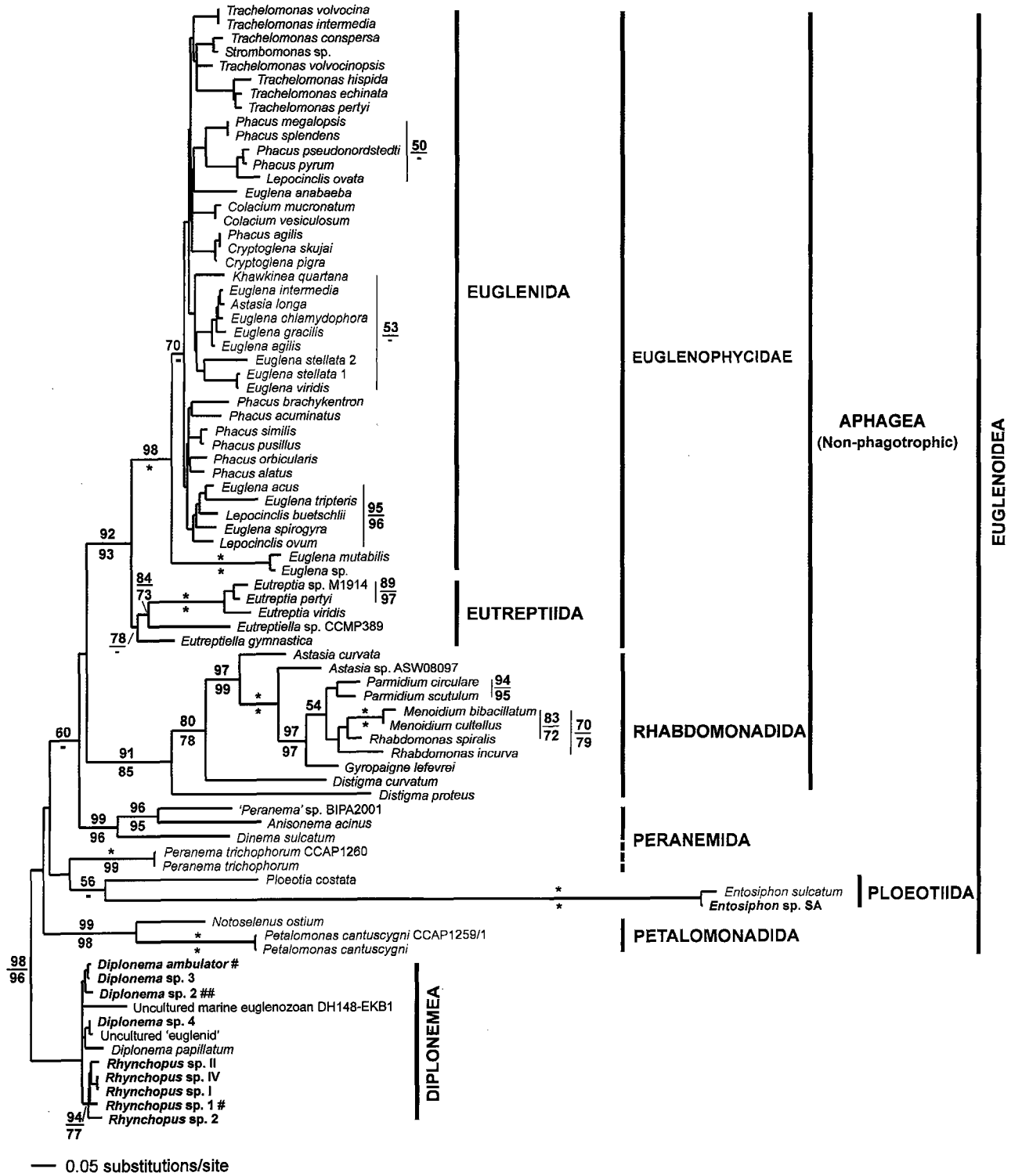

Fig. 2. Distance tree of 80 18S rRNA sequences, comprising euglenoids and diplonemids as an outgroup using 1233 positions (Minimum evolution: GTR+Γ+I model: α = 0.7678, I = 0.1642). This tree with the highest score (7.73163) was found 88 out of 100 times. Numbers at major nodes are bootstrap percentages (ME upper, BioNJ lower) from 250 replicates; values less than 50%, are omitted or marked by -. Asterisks denote 100% bootstrap support. Euglenoid orders are as in Cavalier-Smith (1993a). Newly sequenced taxa are in bold. Diplonemid strains marked with a # were also sequenced independently by Busse and Preisfeld (2002), those with ## by Maslov et al. (1999) after we had done so; as their sequences are almost identical to ours, apart from a few probable sequencing errors, they were not included in this analysis.

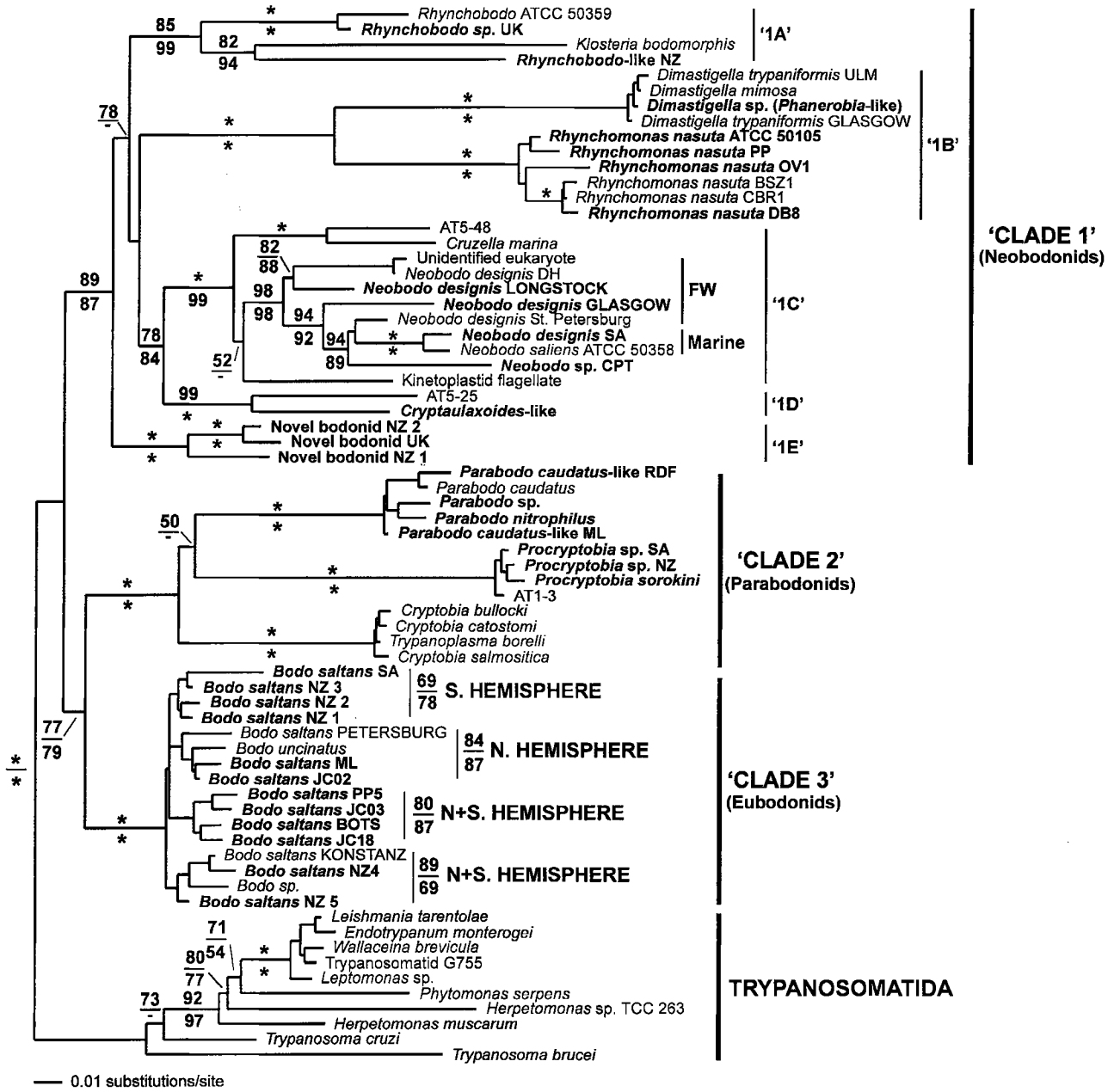

Fig. 3. Distance tree of 69 bodonid and trypanosomatid 18S rRNAs using 1613 positions (Weighted least squares: GTR+Γ+I model: α = 0.57747, i = 0.465950). The tree with the best score (7.65542) was found 100 out of 100 times. The 34 newly sequenced taxa are in bold. Numbers at major nodes only are bootstrap support percentages from 500 replicates using the weighted least squares method (upper) and 500 replicates using the ME method (lower). Where bootstrap support was less than 50%, no values are shown. Asterisks denote 100% support. Bodonid clades are labelled 1–3, after (Simpson, Lukeš, and Roger 2002). Freshwater and marine strains within certain clades are labelled and geographical locations of new isolates are shown.

laxoides-like. The strain now designated *Dimastigella*-like was identified on isolation as *Phanerobia pelophila* Skuja (1948) based on its apparent identity with the illustration in Patterson and Zölffel (1991) and therefore labelled *P. pelophila* in the tree of Cavalier-Smith (2000). It is so closely related to *Dimastigella*, branching among other *Dimastigella* species (all of which have very similar sequences) that it seems likely that it was a very elongated, more flexible variant of *Dimastigella*. Electron microscopy confirmed polykinetoplast mitochondria like those of *Dimastigella*. Unfortunately our strain died before we had access to Skuja's original description and could make a critical comparison.

Within clade 1C there is a very robust subclade of eight lineages, five identified as *Neobodo* (formerly *Bodo*) *designis* even though they are highly divergent from each other in sequence—more divergent than many trypanosomatid genera. One of our *N. designis* strains (SA) is quite closely related to an ATCC strain named as *B. saliens*. As we have examined hundreds of pure and mixed cultures of bodonids and never convinced ourselves that we had a *Bodo saliens*, which was

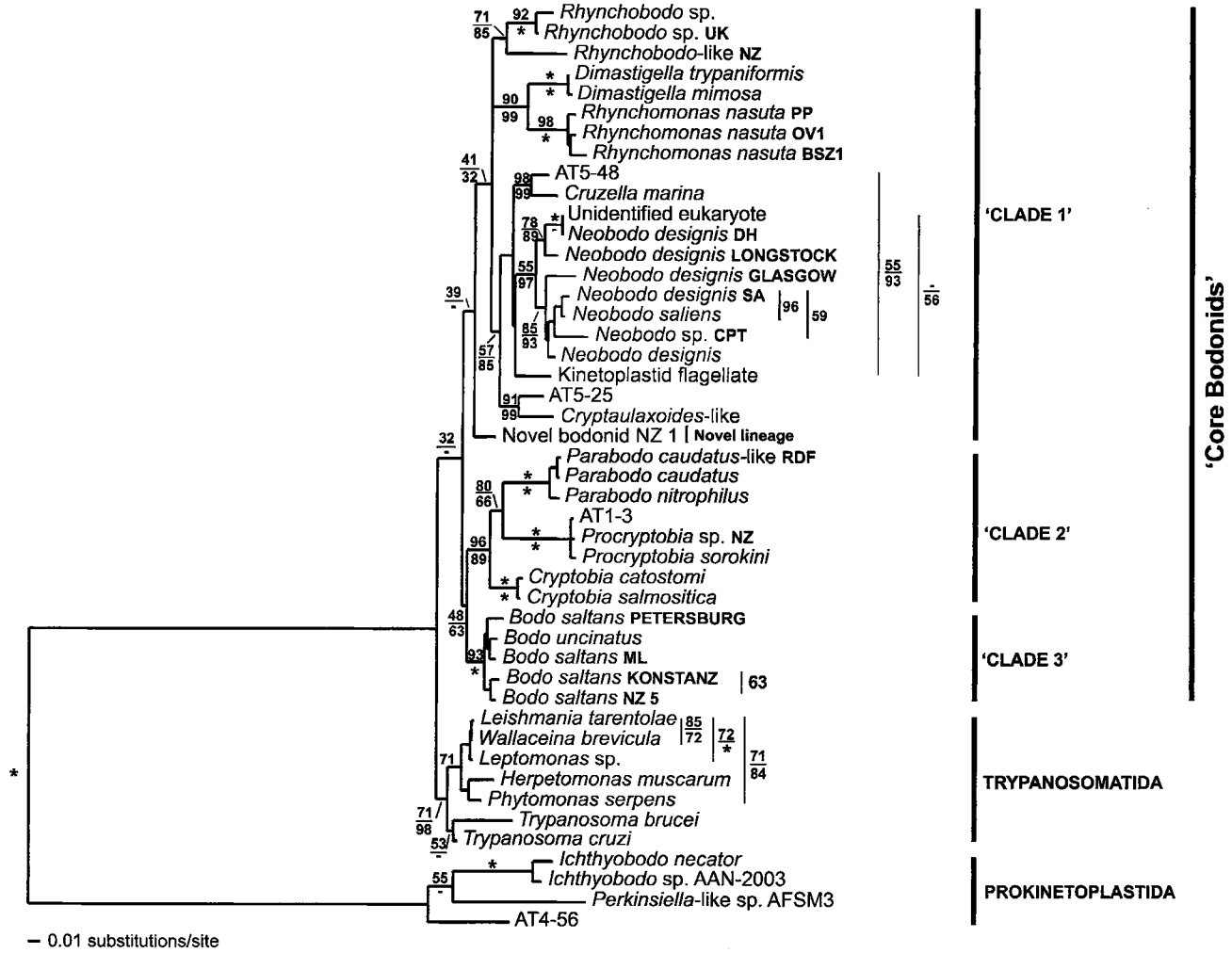

Fig. 4. Maximum likelihood tree of 46 kinetoplastid 18S rRNA sequences, using 1342 positions (a = 0.51466, i = 0.44012, – *ln*L = 8305.48578). Numbers at nodes are bootstrap support percentages using 75 replicates (above) and 500 bootstrap replicates using weighted least squares (below). Where bootstrap support was less than 50%, no values are shown (or -), except for the four basal nodes of the core bodonids. Asterisks denote 100% bootstrap support. Bodonid clades are labelled 1–3, after (Simpson, Lukeš, and Roger 2002).

differentiated from *designis* mainly in mode of swimming, length of posterior flagellum and posterior cell shape (Larsen and Patterson 1990; Tong 1997), we suspect that '*B. saliens*' may not be reliably distinguishable from *N. designis*. Our *Bodo rostratus*-like strain clearly differed from *N. designis*, but was identified as *B. rostratus* simply from the illustration in Lemmerman (1914); however, as our strain (now dead) lacked the complicated life cycle originally described for this species (as *Heteromita rostrata*: Kent (1880–82): Klebs (1892) transferred it to *Bodo*) the name *B. rostratus* used in a preliminary tree for this sequence (Cavalier-Smith 2000) was inapplicable, so we now refer to it only as *Neobodo* sp. CPT (Weitere and Arndt (2003) recently recorded a *B. rostratus*-like cell from the River Rhine). As over half the lineages in this subclade are *N. designis*, and there are no others with a really clear identity we refer to it as the *N. designis* species complex. It contains both marine and freshwater or soil isolates.

Clade 2 (Parabodonida of Moreira, López-García, and Vickerman (2004)) has three major branches (*Parabodo*; *Procryptobia*; *Cryptobia/Trypanoplasma*); their branching order is unresolved on distance trees, but ML analysis strongly suggests that *Parabodo* and *Procryptobia* clades are sisters to the exclusion of the parasitic cryptobias (80% support: Fig. 4); this topology is also seen on weighted least squares trees (Fig. 3). The sequence designated *Parabodo* sp. was from a strain that closely resembled *Parabodo caudatus* in both movement and morphology, but had an attached recurrent flagellum like *Procryptobia*, suggesting that the long stem at the base of the *Procryptobia* branch may exaggerate the evolutionary separation of the two genera.

Clade 3 (Eubodonida of Moreira, López-García, and Vickerman (2004)) contains exclusively 14 diverse *Bodo saltans* and two ATCC strains that, though labelled as *Bodo edax* and *Bodo uncinatus*, have never been critically examined and are likely actually to be *Bodo saltans* also. We isolated a marine strain of *B. saltans* (NZ 5), a species less frequently reported from marine environments and more often from freshwater and soil. This isolate from marine seaweeds lies within the rest of the clade.

The trees highlight the considerable genetic diversity within some clades comprising one 'morphospecies', especially within *Neobodo designis*, *Parabodo caudatus*, *Rhynchomonas nasuta* and above all *Bodo saltans*.

Phylogeny of Diplonemea. Diplonemids form a robust

clade, which has the shortest branch lengths within Euglenozoa (Fig. 1). In distance analyses *Rhynchopus* is holophyletic (66–94%), and arises from within *Diplonema*, whereas parsimony analyses support holophyly of both taxa. Within *Rhynchopus*, ecto-commensal/parasitic species arise from free-living ones.

DISCUSSION

Bodonid diversity and ancestry of trypanosomatids. Kinetoplastids are a diverse group, encompassing both free-living and parasitic species. Their overall phylogeny has been problematic because of poor taxon sampling, the lack of an agreed root to their tree, the paraphyly or polyphyly of taxa such as *Bodo* (now largely rectified by Moreira, López-García, and Vickerman 2004), and the uncertain position of trypanosomatids in relation to bodonids. We confirm the relationship of the core bodonid/trypanosomatid clade and the *Ichthyobodo/Perkinsiella*-like symbiont clade demonstrated previously (Callahan et al. 2002; Dyková et al. 2003; Moreira, López-García, and Vickerman 2004) and that the third lineage containing two environmental sequences is sister to recognised kinetoplastids (López-García, Philippe, and Moreira 2003). Establishing the phenotype of this lineage would be invaluable in answering questions about kinetoplastid ancestry—is it a third kinetoplastid lineage or a different type of euglenozoan?

Our much larger bodonid data set has greatly increased resolution within core bodonids and clarifies the origin of trypanosomatids. ML analyses of data set 4 and all distance analyses place the root of the tightly clustered core bodonid/trypanosomatid clade between trypanosomatids and bodonids. Bootstrap support for the holophyly of core bodonids and trypanosomatids is 63% and 58% respectively (Fig. 1) in distance trees; with ML support for trypanosomatid holophyly is stronger but that for core bodonids is weaker (Fig. 4). Recent Hsp90 analyses (Stechmann and Cavalier-Smith 2003a) and 18S rRNA analyses by Marin et al. (2003) also support core bodonid holophyly, often with strong bootstrap support. However, Simpson, Lukeš, and Roger (2002) with a much smaller taxon sample for 18S rRNA, in particular lacking the *Ichthyobodo* clade, found the root to be variable. As we find clade 2 to be robustly holophyletic (100% support: Fig. 3; 96% support: Fig. 4) the apparent paraphyly of clade 2 on their trees may be a problem of too sparse taxon sampling and/or not including as many nucleotide positions as our much more extensive alignment allows. Hsp90 analyses also support holophyly of clade 2 (Simpson, Lukeš, and Roger 2002; Simpson and Roger 2004; Stechmann and Cavalier-Smith 2003a). However, Hsp90 analyses by Simpson, Lukeš, and Roger (2002) and Simpson and Roger (2004) suggested that *Bodo saltans* is sister to trypanosomatids (bootstrap support of 22–91%), that clade two is their sister (37–93% support) and that the root of the core bodonids may lie between clades 1 and clades 2, 3 and trypanosomatids. When Simpson and Roger (2004) increased the number of outgroup species to 19, *B. saltans* remained as sister to trypanosomatids, but with lower bootstrap support than in analyses with fewer outgroups. Analyses with three non-euglenozoan outgroup species (many fewer than in the analysis of Stechmann and Cavalier-Smith 2003a) usually gave the same topology with similar bootstrap support (their Fig. 3a; using only 1+2 codon positions), but sometimes grouped clades 2 and 3 together to form one superclade, as we consistently observe for 18S rRNA, albeit with only weak to moderate support. Since both 18S rRNA and Hsp90 trees with the broadest taxonomic representation agree in placing core bodonids as a holophyletic sister to trypanosomatids, we suspect that this topology is correct. The same topology had already been found in earlier analyses (Cavalier-Smith 1993a; Moreira, López-García, and Vickerman 2004). Although this topology needs testing from numerous proteins, it is possible that trypanosomatids did not evolve from core bodonids as recently argued (Simpson, Lukeš, and Roger 2002) but from a missing link between the ichthyobodonid clade and the common ancestor of core bodonids. Blom et al. (1998) also suggested that *Bodo saltans* was more closely related to trypanosomatids than to other bodonids based on mitochondrial DNA sequences. However, they compared only trypanosomatids, *B. saltans* and two parasitic cryptobiids; as no other free-living bodonid was analysed and they used only ~ 350 nucleotides, these results should be treated cautiously.

We are agreed on the merits of the recent separation of the ichthyobodonid clade from core bodonids as a new order Prokinetoplastida (Moreira, López-García, and Vickerman 2004), and that this new order should be in a separate subclass from core bodonids and Trypanosomatida (Moreira, López-García, and Vickerman 2004). We also agree that core bodonids deserve to be split into three suprafamilial taxa corresponding to clades 1–3, but it is an open question whether they should constitute separate orders; Neobodonida, Parabodonida and Eubodonida (Moreira, López-García, and Vickerman 2004) or remain as subordinal taxa within a single order Bodonida *sensu stricto* forming a sister to the Trypanosomatida. The vernacular terms neobodonids, parabodonids, and eubodonids are used here, being independent of rank.

Bodonid clade 1: neobodonids. We have discovered a novel lineage of free-living bodonids (clade 1E), which are morphologically close to *Rhynchobodo*, characterised by a free recurrent flagellum, very similar movement and flagellar activity; the rostrum is less well developed and smaller than in *Rhynchobodo*. Investigations with the electron microscope are needed to establish their kinetoplast structure. The clade has three free-living isolates, one marine and two freshwater (Table 1); one (novel bodonid NZ 2) was isolated from a thermal pool (around 37 °C) in New Zealand.

It was previously doubted whether ATCC strain 50359 really is a *Rhynchobodo* (Simpson, Lukeš, and Roger 2002). The close relationship to our independently isolated *Rhynchobodo* sp. suggests that its identification was correct, which one of us (K. Vickerman) has confirmed microscopically. We also isolated a free-living species (*Rhynchobodo*-like) that specifically groups with the recently described *Klosteria bodomorphis* (Nikolaev et al. 2003), albeit very distantly (Fig. 3), and resembles both *Rhynchobodo* species in its possession of a large preflagellar rostrum. As clade 1E is also very similar in morphology to *Rhynchobodo* and seems to be sister to clades 1A-D, the common ancestor of clade 1 was probably itself *Rhynchobodo*-like in morphology.

Strains identified as *Neobodo designis* are very widespread in clade 1C, and so mutually divergent genetically that this name probably signifies only a cluster of microscopically extremely similar morphotypes distinguished only by a distinctive mode of swimming, posterior flagella length and posterior cell shape (Eyden 1977; Larsen and Patterson 1990; Tong 1997) corresponding perhaps to a whole family in better characterised taxa. Considerable further work is needed to establish whether any lineages (e.g. *Neobodo* sp. CPT) within the *N. designis* species complex can be reliably distinguished morphologically. The striking genetic divergence within this complex can probably be related to physiological and ecological differences, as marine or freshwater isolates are clustered, not randomly arranged on the tree. We are now testing this further with more intensive sampling.

Bodonid clade 2: parabodonids. The taxa within Clade 2 are also morphologically diverse, and genetically so, as they have some of the longest branches within bodonids. They con-

tain free-living species such as *Parabodo* (= *Bodo*) *caudatus* and *Procryptobia sorokini* (= *Bodo sorokini* in the preliminary tree: Cavalier-Smith 2000) and the parasitic *Cryptobia/Trypanoplasma*. Maximum likelihood analysis strongly supports a sister relationship between the *Parabodo* clade and *Procryptobia*, to the exclusion of the fish cryptobias clade. This affinity is reflected in the difficulty of deciding from microscopy alone to which of the two genera *Parabodo* sp. belongs; this grouping was not satisfactorily resolved previously (Callahan, Litaker, and Noga 2002; Dyková et al. 2003; Simpson, Lukeš, and Roger 2002). *Cryptobia helicis* groups with *Parabodo caudatus* and *Procryptobia sorokini* (Fig. 1), never with other *Cryptobia* spp. (Callahan, Litaker, and Noga 2002; Dolezel et al. 2000; Simpson, Lukeš, and Roger 2002), suggesting multiple, independent transitions from free-living to parasitic lifestyles (Dolezel et al. 2000). Further information from protein analyses is required to confirm these relationships.

Clade 3: eubodonids—the *Bodo saltans* species complex. This clade of sixteen sequences is so genetically diverse that we agree with Callahan, Litaker, and Noga (2002) that it will eventually need to be divided into different species. There are four well-supported sub-clades (Fig. 3). Two contain strains exclusively from the northern and southern hemispheres, whereas the other two have a mixture of geographical origins. Might these strains of *B. saltans* be an exception to the theory that protists (and other microbes) are ubiquitously distributed around the globe, as there are no barriers to free dispersal of their astronomical populations (Finlay 2002)? More likely this geographical clustering is an artefact of undersampling; addition of further sequences may break it down. However, there has been no report of cyst formation for *B. saltans*, which may decrease the ability of this morphospecies to disperse over large distances and account for different genetic populations observed here. What clade 3 does show is extensive genetic diversity in what appears to be one morphospecies.

Ecological specialization within bodonid morphotypes. Some bodonids, notably *Neobodo designis*, are found in all types of habitats, and have been considered cosmopolitan and to have broad ecological tolerances (Atkins, Teske, and Anderson 2000; Patterson and Lee 2000). However, there appears to be a marked divergence between freshwater and marine species on our trees within the *N. designis* species complex (Fig. 2). We have demonstrated an even deeper separation between marine and freshwater strains for the cryptomonad *Goniomonas* (Herden, Chao, and Cavalier-Smith 2004). Culture studies of certain heterotrophic flagellates suggest that individual clones of some are physiologically adapted to certain environments only (Ekelund 2002). Thus among zooflagellates generally morphospecies may conceal great genetic diversity and ecological differentiation. Generalizations about the geographical distribution or cosmopolitanism of such flagellates cannot be made without detailed genetic studies, especially as light microscopy is often insufficiently discriminating to reveal their real biodiversity. Our finding of a strain from a thermal pool with a temperature of 37 °C indicates that bodonids are able to colonise and grow in a wide variety of temperatures, having also been observed in polar environments (Tong, Vørs, and Patterson 1997; Vørs 1992).

Diplonemid diversity. In earlier 18S rRNA analyses, *Rhynchopus* and *Diplonema* could not clearly be differentiated using molecular methods (Busse and Preisfeld 2002a). Morphologically, the two genera are often distinguished by their flagella: *Diplonema* has two short ones (3–4 μm), which vary in flexibility among species (Triemer and Ott 1990), whereas they barely emerge from the flagellar pocket in *Rhynchopus* species (Schnepf 1994; Vickerman 2002). Also *Diplonema* has extensive flat (non-discoid) cristae whereas *Rhynchopus* has abnormal mitochondria almost devoid of cristae (Schnepf 1994). Our distance analyses support the separation into two genera, and show that *Rhynchopus* is holophyletic and *Diplonema* paraphyletic, although in parsimony analyses both appear holophyletic. If the distance topology is correct, flagella were shortened and cristae were reduced during the origin of *Rhynchopus* from *Diplonema*. Within *Rhynchopus*, ecto-commensal/parasitic species group together and arise from within free-living species. The isolates from *Nephrops* assume a *Bodo*-like fully flagellated phase when the serum-containing culture medium is replaced with seawater (Vickerman 2002). Whether such a flagellated phase is peculiar to *Rhynchopus* and therefore serves to distinguish this genus from *Diplonema* remains to be seen. The more actively motile flagellate phase would obviously increase chances of epizoic/parasitic species finding a fresh host. Organisms with diplonemean ultrastructure but of uncertain genus have also described from gills of the crab *Cancer irroratus* (Bodammer and Sawyer 1981) and the haemocoel of larval clams in which they were pathogenic (Kent and Elston 1987), suggesting that diplonemeans may target a number of hosts. The two *Rhynchopus* spp. isolated from the blood of two different lobsters are identical in 18S rRNA sequence, so a single strain may have been present in that particular population of *N. norvegicus*. We postulate that this species invades the blood of the lobsters already stressed by infection with the syndinean dinoflagellate *Hematodinium*. Our findings show that *Nephrops norvegicus* harbours at least two species of parasitic *Rhynchopus*—one a gill epibiont, the other living in the same niche, but also capable of invading the host's blood.

Euglenoid diversity and early evolution. The separation of euglenoids into six orders as shown in Fig. 2 agrees completely with the ordinal classification of Cavalier-Smith (1993a) and, except for the probable paraphyly of Peranemida, with the cladistic interpretation of Cavalier-Smith (1995). Non-phagotrophic orders (Euglenida, Eutreptiida and Rhabdomonadida) are each robustly supported (78–100%) as is the holophyly of Euglenophycidae (Euglenida, Eutreptiida: Preisfeld et al. 2001; Müllner et al. 2001), but their grouping into a single osmotrophic subclass Aphagea is only sometimes found. The significantly deeper branching of the phagotrophic taxa is also poorly resolved. As Busse, Patterson, and Preisfeld (2003) pointed out, neither ultrastructure nor molecular evidence supports their traditional separation into two suborders, Sphenomonadina and Heteronematina (Leedale 1967). Instead our Fig. 2 gives molecular support to their recent subdivision into three orders based on the morphology of the feeding apparatus (Cavalier-Smith 1993a). The simplest feeding apparatus is that of the Petalomonadida (98–99% bootstrap support), which appear to be the earliest diverging euglenoids. This group has a simple MTR/pocket ingestion apparatus (Type I) like kinetoplastids and a non-plastic pellicle of few longitudinal strips (Leander, Triemer, and Farmer 2001). Although petalomonads are often said to be strictly bacterivorous because of these characters, Patterson and Simpson (1996) observed *Notoselenus ostium* to contain ingested eukaryotic algae.

Ploeotiida have a type II or IV feeding apparatus (vanes; two supporting rods with only peripheral microtubules) and an aplastic pellicle and are bacterivorous. Peranemida have a type III apparatus (curved vanes, 2 rods with internal microtubules). The position of *Dinema* was not resolved previously (Busse, Patterson, and Preisfeld 2003), but we have shown that it groups with *Anisonema* and *Peranema* sp. with 96–99% support. Possibly our trees are better resolved than previous ones because they include many more taxa or because they use closer

outgroups and were therefore able to include more of the sequence.

The fact that *Peranema trichophorum* groups with *Entosiphon* (Fig. 1) or both *Entosiphon* and *Ploeotia* (Fig. 1) with weak support, whereas the main peranemid clade is sister to Aphagia suggests that both *Peranema* and Peranemida may be paraphyletic. It implies that the curved vanes, plastic pellicle and eukaryotrophy of Peranemida are ancestral to the plicate vanes, aplastic pellicle and bacteriotrophy of Ploeotiida. The topology of Fig. 2 implies that both Aphagia and Ploeotiida evolved from peranemid ancestors and that vanes and rods were lost by the ancestral aphagean. *Entosiphon* and *Ploeotia* are distinguishable only with difficulty by light microscopy, the only difference being that *Entosiphon* has a protrusible ingestion apparatus (Type IV). In our analysis restricted to euglenoids and diplonemids *Entosiphon* and *Ploeotia* group together only weakly; however, this grouping is supported by shared character analysis (Leander, Triemer, and Farmer 2001) and likely to be correct. Ploeotiida and Peranemida were grouped together as a class Peranemea (Cavalier-Smith 1993a) (later subclass Peranemia: Cavalier-Smith 1998); almost certainly Peranemia is paraphyletic. The probable early divergence of Petalomonadida (Fig. 2) helps justify their treatment as a third euglenoid subclass (Petalomonadia Cavalier-Smith 1993a; 1998). However, as diplonemids have vanes and rods, it is likely that they were present in the common ancestor of euglenoids and diplonemids and lost independently in the ancestral petalomonads and aphagian. Thus petalomonads are probably secondarily simplified as argued by Cavalier-Smith (1995). The weak grouping of petalomonads with *Ploeotia* on Fig. 1 emphasises the poor resolution at the base of the tree and allows the possibility that petalomonads might not actually be the most divergent euglenoids but arose by simplification from ploeotiids and a common ancestral secondary rigidification of the pellicle.

Composition and monophyly of Euglenozoa. The strong evidence from rRNA sequences for holophyly of Euglenozoa indicates that the ultrastructural characteristics chosen to define (Cavalier-Smith 1981) and refine (Cavalier-Smith 1993b; Simpson 1997) the phylum are reliable phylogenetic markers. In particular the latticed ciliary paraxonemal rods and tubular extrusomes which are both found in at least some members of all three groups (Simpson 1997) but in no other eukaryotes, are clear synapomorphies that must have been present in the common ancestor of all three groups. Clearly Euglenozoa comprise three very distinct groups, kinetoplastids, euglenoids and diplonemids, which are all appropriately treated as separate classes.

The position of *Hemistasia* and *Postgaardi*, other euglenozoan flagellates of uncertain affinities, is discussed by Simpson and Roger (2004) and the relationship of Euglenozoa to other excavates is treated by Cavalier-Smith (2003a, c) and Stechmann and Cavalier-Smith (2002, 2003b).

Evolutionary diversification of euglenozoan cells: multiple organelle losses. Our present analysis has not been able to resolve the basal branching order of the Euglenozoa, since distance analyses suggest that Plicostoma are holophyletic, whereas parsimony (equally weakly) suggests that they are paraphyletic. A recent study combining data from two proteins strongly supports this paraphyly (Simpson and Roger 2004); if this is correct, then the shared presence of vanes in the ingestion apparatus is ancestral for all Euglenozoa not simply for euglenoids and diplonemids (provided they are homologous between diplonemids and euglenoids, which has been questioned: Montegut-Felkner and Triemer 1996), and ancestral euglenoids would have been able to ingest eukaryotic algae, contrary to the arguments of Triemer and Farmer (1991b). However, according to the more recent cabozoan theory (Cavalier-Smith 1999; 2003a, b, c) of the origin of the euglenoid plastid, the enslavement of a green alga took place even earlier in a soft-surfaced common ancestor of excavates and Rhizaria long before the euglenozoan pellicle evolved. Recently it has been shown that some trypanosomes contain several 'plant-like' genes that appear to be homologues of proteins found in plants or algae (Hannaert et al. 2003), which supports the hypothesis of early chloroplast uptake and subsequent losses; other molecular evidence that the common ancestor of all excavates had plastids is reviewed by Cavalier-Smith (2003a).

rRNA phylogenies (Fig. 1) make it clear that there is at least one non-photosynthetic, osmotrophic euglenoid *Khawkinea quartana* (incorporated into *Euglena sensu stricto* as *Euglena quartana*: Marin et al. 2003) nested on trees within green, photosynthetic euglenoids and so has arisen from photosynthetic euglenoids by loss of plastids; although *Astasia longa* (incorporated into *Euglena sensu stricto* as *Euglena longa*: Marin et al. 2003), is a euglenophycid that recently lost photosynthesis but retained plastids (Gockel and Hachtel 2000); the unrelated *Astasia curvata* and *Astasia* sp. ASW 08097 are part of the far more ancient plastidless Rhabdomonadida. It is likely that all plastidless members of the non-phagotrophic subclass Aphagia (Fig. 2) (originally class Aphagea Cavalier-Smith 1993a, later subclass: Cavalier-Smith 1998) have arisen by plastid loss and are not ancestrally without plastids as sometimes thought (Leedale 1978); once the ancestral putatively photosynthetic aphagian had lost phagotrophy, those losing plastids could not regain it and thus became osmotrophs. The clear proof that some euglenoids have lost plastids adds to the growing evidence from chromophyte algae (both heterokont chromists (Cavalier-Smith 1997) and dinoflagellates (Saunders et al. 1997, Saldarriaga et al. 2001; 2004), for multiple evolutionary losses of chloroplasts in algae (Cavalier-Smith 2002)). Chloroplast loss seems to be more common in euglenoids, chromists and dinoflagellates, all of which obtained their chloroplasts by secondary symbiosis (Cavalier-Smith 1999; Fast et al. 2001; Roos et al. 1997) than in green plants, which acquired theirs by primary symbiosis (Cavalier-Smith 1993c). Loss of mitochondria has also been frequent during eukaryote evolution (Roger 1999). As organelle loss has been much commoner than the very rare symbiotic acquisition of organelles (Archibald and Keeling 2002; Cavalier-Smith 1992), it is not unreasonable that all heterotrophic Euglenozoa have arisen by such losses, and that the ancestral euglenozoan was photosynthetic (for detailed arguments see: Cavalier-Smith 1999, 2002, 2003a,b,c). If diplonemids and kinetoplastids are sisters (Simpson and Roger 2004), only one chloroplast loss would have occurred in their common ancestor, but vanes and rods were probably lost three times: independently in ancestral kinetoplastids, petalomonads and aphagians. If the euglenoid topology of Fig. 2 is correct, four more losses within euglenoids must be postulated by the cabozoan theory (or only three if petalomonads are sisters of or derived from ploeotiids). The posterior flagellum was lost independently by trypanosomatids and some petalomonads.

Euglenozoan genome evolution. The discovery of trans-splicing of mini-exons to make all nuclear-encoded mRNAs of trypanosomatids led to suggestions that this might be a primitive eukaryotic character (Simpson 1990). However, this was never phylogenetically plausible, and is even less so now that Euglenozoa are established as a highly derived group within excavates, which are themselves a derived group within the bikonts, rather than early diverging eukaryotes (Cavalier-Smith 2003a, c). Trans-splicing is unknown in their sister group, Percolozoa, which does have normal cis-splicing (Fulton et al. 1995), as do other excavate phyla (Simpson, MacQuarrie, and Roger 2002) and all properly studied eukaryote phyla. This

strongly favours the alternative idea that trans-splicing evolved secondarily from cis-splicing independently in Euglenozoa Cavalier-Smith 1993b), nematodes (Blaxter and Liu 1996) and a few genes in slime moulds. After trans-splicing was discovered in *Euglena* (Muchal and Scwartzbach 1992), Cavalier-Smith (1993b) postulated that trans-splicing of all protein-coding genes originated in the ancestral euglenozoan and would be found in all euglenozoan groups, proposing universal trans-splicing as a unique molecular synapomorphy for Euglenozoa (Cavalier-Smith 1993b, 1995). Subsequently trans-splicing was found in diplonemids (Sturm et al. 2001), bodonids (Santana et al. 2001) and prokinetoplastids (Dyková et al. 2003). It is now known in many euglenoids, but although all genes examined in most genera are trans-spliced, the three studied to date in *Distigma proteus* are not, though traces of mini-exons were found (Frantz et al. 2000). Ebel et al. (1999) first demonstrated typical spliceosomal introns in *Entosiphon* nuclear genes and Gray et al. (2001) did so in those of *Euglena*, which suggests that the ancestral euglenozoan had normal cis-splicing of nuclear introns as well as trans-splicing. Trans-splicing has now been found in all euglenoid orders except petalomonads and peranemids; it is important to investigate them too because of the possibility that they are particularly early diverging. Cis-splicing has also been reported in *Trypanosoma brucei* and *T. cruzi* (Mair et al. 2000).

Thus it is highly probable that trans-splicing of mini-exons did arise in the ancestral euglenozoan from cis-splicing. But whether it rapidly spread to all genes in that ancestor as formerly envisaged (Cavalier-Smith, 1993b) and was reversed in at least some genes of *Distigma proteus* is unclear. The contrast with nematodes where only about 15% of genes are trans-spliced in *Caenorhabditis elegans* (Blumenthal et al. 2002), using 10 different splice leader sequences (mini-exons), and kinetoplastids where all are spliced using only one is striking. In nematodes, which particular genes are grouped in operons and subject to trans-splicing varies somewhat among species (Lee and Sommer 2003). It will be interesting to see how the proportion of trans-spliced genes varies among euglenoids; is it universal in some? The fact that no unspliced genes have yet been found in several genera (Frantz et al. 2000) suggests that it may be. However, as grouping in operons may be in part an adaptation to small genome size, it could be that in euglenoids, among which cell size (and therefore genome size: Cavalier-Smith 2004) varies over a much greater range than in the typically tiny and small-genome kinetoplastids, fewer genes may be in operons and trans-spliced in larger-celled species than in small ones.

The absence of kinetoplasts in both diplonemids and euglenoids makes it likely that they also lack mitochondrial RNA U editing (Lukeš et al. 1994) and that this unusual feature is a derived property of kinetoplastids alone; it probably arose through mutation pressure and phenotypic repair not for adaptive reasons (Cavalier-Smith 1993a). A uniting feature of diplonemid and kinetoplastid mitochondria is that tryptophan is encoded by TGA codons, whereas euglenoid mitochondria utilize the universal code (Inagaki et al. 1997; Maslov, Yasuhira, and Simpson 1999; Yasuhira and Simpson 1997). However, this particular change has occurred more than once in evolution and might be convergent.

ACKNOWLEDGMENTS

S.H. thanks the UK Natural Environment Research Council (NERC: Marine and Freshwater Microbial Biodiversity Programme) for financial support and Dr. Ray Cursons (University of Waikato, Hamilton, New Zealand) for 'Jockey Pool' DNA sample. K.V. thanks NERC (Soil Biodiversity Programme) for funding and the Leverhulme Trust for an Emeritus Fellowship. T.C-S. thanks NERC for research grants, the Canadian Institute for Advanced Research and NERC for fellowship support and Dr. A. P. Mylnikov for bodonid strains.

LITERATURE CITED

Appleton, P. L. & Vickerman, K. 1998. In vitro cultivation and developmental cycle in culture of a parasitic dinoflagellate (*Hematodinium* sp.) associated with mortality of the Norway lobster (*Nephrops norvegicus*) in British waters. *Parasitology,* **116**:115–130.

Archibald, J. M. & Keeling, P. J. 2002. Recycled plastids: a 'green' movement in eukaryotic evolution. *Trends Genet.,* **18**:577–584.

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.

Bernard, C., Simpson, A. G. B. & Patterson, D. J. 2000. Some free-living flagellates (Protista) from anoxic habitats. *Ophelia,* **52**:113–142.

Blaxter, M. & Liu, L. 1996. Nematode spliced leaders: ubiquity, evolution and utility. *Int. J. Parasitol.,* **26**:1025–1033.

Blom, D., de Haan, A., van den Berg, M., Sloof, P., Jirku, M., Lukeš, J. & Benne, R. 1998. RNA editing in the free-living bodonid *Bodo saltans*. *Nucl. Acids. Res.,* **26**:1205–1213.

Blumenthal, T., Evans, D., Link, C. D., Guffanti, A. D, Lawson, D., Thierry-Mieg, J., Thierry-Mieg, D., Chiu, W. L., Duke, K., Kiraly, M. & Kim, S. K. 2002. A global analysis of *Caenorhabditis elegans* operons. *Nature,* **417**:851–854.

Bodammer, J. S. & Sawyer, T. K. 1981. Aufwuchs protozoa and bacteria on the gills of the rock crab, *Cancer irroratus*: a survey by light and electron microscopy. *J. Protozool.,* **28**:35–46.

Breunig, A., Konig, 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.

Brosnan, S., Shin, W., Kjer, K. M. & Triemer, R. E. 2003. Phylogeny of the photosynthetic euglenophytes inferred from the nuclear SSU and partial LSU rDNA. *Int. J. Syst. Evol. Microbiol.,* **53**:1175–1176.

Brugerolle, G. 1985. Des trichocystes chez les bodonides, un caractère phylogénétique supplémentaire entre Kinetoplastida et Euglenida. *Protistologica,* **21**:339–348.

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. *Gene,* **284**:83–91.

Busse, I., Patterson, D. J. and Preisfeld, A. 2003. Phylogeny of phagotrophic euglenids (Euglenozoa): a molecular approach based on culture material and environmental samples. *J. Phycol.,* **39**:828–836.

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. 1992. The number of symbiotic origins of organelles. *Biosystems,* **28**:91–106.

Cavalier-Smith, T. 1993a. Kingdom Protozoa and its 18 phyla. *Microbiol. Rev.,* **57**:953–994.

Cavalier-Smith, T. 1993b. Evolution of the eukaryotic genome. *In*: Sims, P. (ed.), The Eukaryotic Genome. Cambridge University Press, Cambridge. p. 333— 385.

Cavalier-Smith, T. 1993c. The origin, losses and gains of chloroplasts. *In*: Lewin R. A. (ed.), Origin of Plastids; Symbiogenesis, Prochlorophytes and the Origins of Chloroplasts. Chapman & Hall, New York. p. 291–348.

Cavalier-Smith, T. 1993d. Percolozoa and the symbiotic origin of the metakaryote cell. *In*: Ishikawa, H., Ishida, M. & Sato, S. (ed.), Endocytobiology V. Tübingen University Press, Tübingen. p. 399– 406.

Cavalier-Smith, T. 1995. Zooflagellate phylogeny and classification. *Cytology (St. Petersburg),* **37**:1010–1029.

Cavalier-Smith, T. 1997. Sagenista and Bigyra, two phyla of heterotrophic, heterokont chromists. *Arch. Protistenkd.,* **148**:253–267.

Cavalier-Smith, T. 1998. A revised six-kingdom system of life. *Biol. Rev.,* **73**:203–266.

Cavalier-Smith, T. 1999. Principles of protein and lipid targeting in secondary symbiogenesis: euglenoid, dinoflagellate, and sporozoan plastid origins and the eukaryote family tree. *J. Eukaryot. Microbiol.,* **46**:347–366.

Cavalier-Smith, T. 2000. Flagellate megaevolution, the basis for eukaryotic diversification *In*: Leadbeater, B. S. C & Green, J. C. (ed.), The Flagellates—Unity, Diversity and Evolution. Taylor & Francis, London, New York. p. 361–390.

Cavalier-Smith, T. 2002. Chloroplast evolution: secondary symbiogenesis and multiple losses. *Curr. Biol.,* **22**:12, R62–64.

Cavalier-Smith, T. 2003a. The excavate protozoan phyla: Grassé emend. (Anaeromonadea, Parabasalia, *Carpediemonas*, Eopharyngia) and Loukozoa emend. (Jakobea, *Malawimonas*): their evolutionary affinities and new higher taxa. *Int. J. Syst. Evol. Microbiol.,* **53**:1741–1758.

Cavalier-Smith T. 2003b. Genomic reduction and evolution of novel genetic membranes and protein-targeting machinery in eukaryote-eukaryote chimaeras (meta-algae). *Phil. Trans. Roy. Soc. B.,* **358**:109–134.

Cavalier-Smith, T. 2003c. Protist phylogeny and the high-level classification of Protozoa. *Eur. J. Protistol.,* **39**:338–348.

Cavalier-Smith, T. 2004. Economy, speed, and size matter: evolutionary forces driving nuclear genome miniaturisation and expansion. *Ann. Bot.*, (in press)

Cavalier-Smith, T. & Chao, E. E. 1995. The opalozoan *Apusomonas* is related to the common ancestor of animals, fungi and choanoflagellates. *Proc. R. Soc. Lond. Ser. B.,* **261**:1–6.

Dobell, C. C. 1908. Structure and life history of *Copromonas subtilis*. *Q. J. Microsc. Sci.,* **52**:75–120.

Dolezel, D., Jirku, 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.

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.

Ebel, C., Frantz, C., Paulus, F. & Imbault, P. 1999. *Trans*-splicing and *cis*-splicing in the colourless euglenoid *Entosiphon sulcatum*. *Curr. Genet.,* **35**:542–550.

Elbrächter, M., Schnepf, E. & Balzer, I. 1996. *Hemistasia phaeocysticola* (Scherffel) comb. nov., redescription of a free-living, marine, kinetoplastid flagellate. *Arch. Protistenkd.,* **147**:125–136.

Ekelund, F. 2002. Tolerance of soil flagellates to increased NaCl levels. *J. Eukaryot. Microbiol.,* **49**:324–328.

Ersfeld, K. & Gull, K. 1997. Partitioning of large and minichromosomes in *Trypanosoma brucei*. *Science,* **276**:611–614.

Eyden, B. P. 1977. Morphology and ultrastructure of *Bodo designis* Skuja 1948. *Protistologica,* **13**:169–179.

Fast, N. M., Kissinger, J. C., Roos, D. S. & Keeling, P. J. 2001. Nuclear-encoded, plastid-targeted genes suggest a single common origin for apicomplexan and dinoflagellate plastids. *Mol. Biol. Evol.,* **18**:418–426.

Finlay, B. J. 2002. Global dispersal of free-living microbial eukaryote species. *Science,* **296**:1061–1063.

Frantz, C., Ebel, C., Paulus, F. & Imbault, P. 2000. Characterization of *trans*-splicing in euglenoids. *Curr. Genet.,* **37**:349–355.

Frolov, A. O. & Skarlato, S. O. 1998. Unusual pattern of mitosis in the free-living flagellate *Dimastigella mimosa* (Kinetoplastida). *Protoplasma,* **201**:101–109.

Fulton, C., Remillard, S. P., Lai, E. Y. & Levy, Y. Y. 1995. A calcineurin-B-encoding gene expressed during differentiation of the amoeboflagellate *Naegleria gruberi* contains two introns. *Gene,* **154**:39–45.

Gaunt, M. W., Yeo, M., Frame, I. A., Stothard, J. R., Carrasco, H. J., Taylor, M. C., Mena, S. S., Veazey, P., Miles, G. A. J., Acosta, N., de Arias, A. R. & Miles, M.A. 2003. Mechanism of genetic exchange in American trypanosomes. *Nature,* **421**:936–393.

Gibson, W. C. 1995. The significance of genetic exchange in trypanosomes. *Parasitol. Today,* **11**:465–468.

Gockel, G. & Hachtel, W. 2000. Complete gene map of the plastid genome of the nonphotosynthetic euglenoid flagellate *Astasia longa*. *Protist,* **151**:347–351.

Gray, M. W., Greenwood, S. J., Schnare, M. N. & Cook, J. R. 2001. Analysis of intergenic spacer transcripts suggests 'read-around' transcription of the extrachromosomal circular rDNA in *Euglena gracilis*. *Nuc. Acids Res.,* **29**:2191–2198.

Griessmann, K. 1914. Uber marine Flagellaten. *Arch. Protistenkd.,* **32**: 1–78.

Hannaert, V., Saavedra, E., Duffieux, F., Szikora, J.-P., Rigden, D. J., Michels, P. A. M. & Opperdoes, F. R. 2003. Plant-like traits associated with metabolism of *Trypanosoma* parasites. *Proc. Natl. Acad. Sci. USA,* **100**:1067–1071.

Inagaki, Y., Hayashi-Ishimaru, Y., Ehara, M., Igarashi, I. & Ohama, T. 1997. Algae or protozoa: phylogenetic position of euglenophytes and dinoflagellates as inferred from mitochondrial sequences. *J. Mol. Evol.,* **45**:295–300.

Kent, M. L. & Elston, R. A. 1987. An *Isonema*-like flagellate (Protozoa Mastigophora) infection in larval geoduck clams, *Panope abrupta*. *J. Invert. Pathol.,* **50**:221–229.

Kent, W. S. 1880–1882. A manual of the Infusoria (3 vols.). Bogue, London.

Klebs, G. 1892. Flagellatenstudien. *Zeitschrift für Wissenschaftliche Zoologie,* **55**:265–445.

Landweber, L. F. & Gilbert, W. 1994. Phylogenetic analysis of RNA editing: a primitive genetic phenomenon. *Proc. Natl. Acad. Sci. USA,* **91**:918–921.

Larsen, J. & Patterson, D. J. 1990. Some flagellates from tropical marine sediments. *J. Nat. Hist.,* **24**:801–938.

Leander, B. S., Triemer, R. E. & Farmer, M. A. 2001. Character evolution in heterotrophic euglenids. *Europ. J. Protistol.,* **37**:337–356.

Lee, K. Z. & Sommer, R. J. 2003. Operon structure and *trans*-splicing in the nematode *Pristionchus pacificus*. *Mol. Biol. Evol.,* **20**:2097–2103.

Leedale, G. F. 1967. Euglenoid Flagellates. Prentice-Hall, Englewood Cliffs, New Jersey.

Leedale, G. F. 1978. Phylogenetic criteria in euglenoid flagellates. *Biosystems,* **10**:183–187.

Lemmermann, E. 1914. Protomastiginae. *In*: Pascher, A. (ed.), Die Süsswasser-Flora Deutschlands, Österreichs und der Schweiz, Heft 1: Flagellatae 1. Gustav Fischer Verlag, Jena.

López-García, P., Philippe, H. & Moreira, D. 2003. Autochthonous eukaryotic diversity in hydrothermal sediment and experimental microcolonizers at the Mid-Atlantic Ridge. *Proc. Nat. Acad. Sci. USA,* **100**: 697–702.

Lukeš, J., Arts, G. J., van den Burg, J., de Haan, A., Opperdoes, F., Sloof, P. & Benne, R. 1994. Novel pattern of editing regions in mitochondrial transcripts of the cryptobiid *Trypanoplasma borreli*. *EMBO. J.,* **13**:5086–5098.

Mair, G., Huafang, S., Hongjie, L., Appolinaire, A., Aviles, H. O., Bishop, J. R., Falcone, F. H., Gavrilescu, C., Montgomery, J. L., Santori, M. I., Stern, L. S., Wang, Z., Ullu, E. & Tschudi, C. 2000. A new twist in trypanosome RNA metabolism: *cis*-splicing of pre-mRNA. *RNA,* **6**:163–169.

Marin, B., Palm, A., Klingberg, M. & Melkonian, M. 2003. Phylogeny and taxonomic revision of plastid-containing euglenophytes based on SSU rDNA sequence comparisons and synapomorphic signatures in the SSU rRNA secondary structure. *Protist,* **154**:99–145.

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* with the Euglenozoa as inferred from the ssu rRNA gene and partial COI protein sequences. *Protist,* **150**:33–42.

Michels, P. A. M. & Hannaert, V. 1994. Structure, function, and biogenesis of glycosomes in Kinetoplastida. *J. Bioenerg. Biomembr.,* **26**: 205–212.

Montegut-Felkner, A. E. & Triemer, R. E. 1994. Phylogeny of *Diplonema ambulator* (Larsen and Patterson): 1. Homologies of the flagellar apparatus. *Eur. J. Protistol.,* **30**:227–237.

Montegut-Felkner, A. E. & Triemer, R. E. 1996. Phylogeny of *Diplo-*

nema ambulator (Larsen and Patterson): 2. Homologies of the feeding apparatus. *Eur. J. Protistol.*, **32**:64–76.

Montegut-Felkner, A. E. & Triemer, R. E. 1997. Phylogenetic relationships of selected euglenoid genera based on morphological and molecular data. *J. Phycol.*, **33**:512–519.

Moreira, D., López-García, P. & Rodríguez-Valera, F. 2001. New insights on the phylogenetic position of diplonemids: G+C content bias, differences in evolutionary rate and a new environmental sequence. *Int. J. Syst. Evol. Microbiol.*, **51**:2211–2219.

Moreira, D., López-García, P. & Vickerman, K. 2004. An updated view of kinetoplastid phylogeny using environmental sequences and a closer outgroup: proposal for a new classification of the class Kinetoplastea. *Int. J. Syst. Evol. Microbiol.*, (in press)

Muchal, U. S. & Schwartzbach, S. D. 1992. Characterization of a *Euglena* gene encoding a polyprotein precursor to the light-harvesting chlorophyll *a/b*-binding protein of photosystem II. *Plant Mol. Biol.*, **18**:287–299.

Müllner, A. N., Angeker, D. G., Samuel, R., Linton, E. W. & Triemer, R. E. 2001. Phylogenetic analysis of phagotrophic, phototrophic and osmotrophic euglenoids by using the nuclear 18S rDNA seqence. *Int. J. Syst. Evol. Microbiol.*, **51**:783–791.

Nikolaev, S. I., Mylnikov, A. P., Berney, C., Fahrni, J., Petrov, N. & Pawlowski, J. 2003. The taxonomic position of *Klosteria bodomorphis* gen. and sp. nov. (Kinetoplastida) based on ultrastructure and SSU rRNA gene sequence analysis. *Protistology*, **3**:126–135.

Novarino, G. 1996. Notes on flagellate nomenclature. 1. *Cryptaulaxoides* nom. n., a zoological substitute for *Cryptaulax* Skuja, 1948 (Protista incertae sedis) non *Cryptaulax* Tate, 1869 (Mollusca, Gastropoda) non *Cryptaulax* Cameron, 1906 (Insecta, Hymenoptera), with remarks on botanical nomenclature. *Acta Protozool.*, **35**:235–238.

Palfi, Z., Lücke, S., Lahm, H-W., Lane, W. S., Kruft, V., Bragado-Nilsson, E., Séraphin, B. & Bindereif, A. 2000. The spliceosomal snRNP core complex of *Trypanosoma brucei*: cloning and functional analysis reveals seven Sm protein constituents. *Proc. Nat. Acad. Sci. USA*, **97**:8967–8972.

Palfi, Z., Lane, W. S. & Bindereif, A. 2002. Biochemical and functional characterization of the *cis*-spliceosomal U1 small nuclear RNP from *Trypanosoma brucei*. *Mol. Biochem. Parasitol.*, **121**:233–243.

Patterson, D. J. & Lee, W. J. 2000. Geographic distribution and diversity of free-living heterotrophic flagellates. *In:* Leadbeater, B. S. C. & Green, J. C. (ed.), The Flagellates—Unity, diversity and evolution. Taylor & Francis, London. p. 269–287.

Patterson, D. J. & Simpson, A. G. B. 1996. Heterotrophic flagellates from coastal marine and hypersaline sediments in western Australia. *Europ. J. Protistol.*, **32**:423–448.

Patterson, D. J. & Zölffel, M. 1991. Heterotrophic flagellates of uncertain taxonomic position. *In:* Patterson, D. J. & Larsen, J. (ed.), The Biology of Free-living Heterotrophic Flagellates. Clarendon Press, Oxford. p. 427–476.

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 Rhabdomonales (Euglenozoa). *Int. J. Syst. Evol. Micobiol.*, **51**:751–758.

Roger, A. J. 1999. Reconstructing early events in eukaryotic evolution. *Am. Nat. (Suppl.)*, **154**:S146–163.

Roos, D. S., Koehler, S., Delwiche, C. F., Denny, P. W., Tilney, L. G., Webster, P., Wilson, R. J. M. & Palmer, J. D. 1997. A plastid of probable green algal origin in apicomplexan parasites. *Science*, **275**: 1485–1489.

Saldarriaga, J., Taylor, F. J. R., Keeling, P. J. & Cavalier-Smith, T. 2001. Dinoflagellate nuclear SSU rRNA phylogeny suggests multiple plastid losses and replacements. *J. Mol. Evol.*, **53**:204–213.

Saldarriaga, J. F., Taylor, F. J. R. "Max", Cavalier-Smith, T., Menden-Deuer, S. & Keeling P. J. 2004. Molecular data and the evolutionary history of dinoflagellates. *Eur. J. Protistol.*, **40**:80–111.

Santana, D. M., Lukeš, J., Sturm, N. R. & Campbell, D. A. 2001. Two sequence classes of kinetoplastid 5S ribosomal RNA gene revealed among bodonid spliced leader RNA gene arrays. *FEMS Microbiol. Lett.*, **204**:233–237.

Saunders, G. W., Hill, D. R. A., Sexton, J. P. & Andersen, R. A. 1997. Small subunit ribosomal RNA sequences from selected dinoflagellates: testing classical evolutionary hypotheses with molecular systematic methods. *Plant Syst. Evol. (Suppl.)*, **11**:237–259.

Schnare, M. N., Cook, J. R. & Gray, M. W. 1990. Fourteen internal transcribed spacers in the circular ribosomal DNA of *Euglena gracilis*. *J. Mol. Biol.*, **215**:85–92.

Schnepf, E. 1994. Light and electron microscopical observations in *Rhynchopus coscinodiscivorous* spec. nov., a colourless phagotrophic euglenozoan with concealed flagella. *Arch. Protistenkd.*, **144**:63–74.

Schwartz, R. M. & Dayhoff, M. O. 1978. Origins of prokaryotes, eukaryotes, mitochondria and chloroplasts. *Science*, **199**:395–403.

Simpson, A. G. B. 1997. The identity and composition of the Euglenozoa. *Arch. Protistenkd.*, **148**:318–328.

Simpson, A. G. B. & Roger, A. J. 2004. Protein phylogenies robustly resolve the deep-level relationships within Euglenozoa. *Mol. Phylogenet. Evol.*, **30**:201–212.

Simpson, A. G. B, MacQuarrie, E. K. & Roger, A. J. 2002. Eukaryotic evolution: early origin of canonical introns. *Nature*, **419**:270.

Simpson, A. G. B., Lukeš, J. & Roger, A. J. 2002. The evolutionary history of kinetoplastids and their kinetoplasts. *Mol. Biol. Evol.*, **19**: 2071–2083.

Simpson, L. 1990. RNA editing—a novel genetic phenomenon. *Science*, **250**:512–513.

Skuja, H. 1948. Taxonomie des Phyto-planktons einiger Seen in Uppland, Schweden. *Symb. Bot. Upsal.*, **9**:1–399.

Smith, S. W., Overbeek, R., Woese, C. R., Gilbert, W. & Gillevet, P. M. 1994. The genetic data environment and expandable GUI for multiple sequence analysis. *Comput. Appl. Biosci.*, **10**:671–675.

Sogin, M. L., Elwood, H. J. & Gunderson, J. H. 1986. Evolutionary diversity of eukaryotic small-subunit rRNA genes. *Proc. Natl. Acad. Sci., USA*, **83**:1383–1387.

Stechmann, A. & Cavalier-Smith, T. 2002. Rooting the eukaryote tree by using a derived gene fusion. *Science*, **297**:89–91.

Stechmann, A. & Cavalier-Smith, T. 2003a. Phylogenetic analysis of eukaryotes using heat-shock protein 90. *J. Mol. Evol.*, **57**:408–419.

Stechmann, A. & Cavalier-Smith, T. 2003b. The root of the eukaryote tree pinpointed. *Curr. Biol.*, **13**:R665–R666.

Sturm, N. R., Maslov, D. A., Grisard, E. C. & Campbell, D. A. 2001. *Diplonema* spp. possess spliced leader RNA genes similar to the Kinetoplastida. *J. Eukaryot. Microbiol.*, **48**:325–331.

Sutton, R. E. & Boothroyd, J. C. 1986. Evidence for *trans*-splicing in trypanosomes. *Cell*, **47**:527–535.

Swofford, D. L. 1993. Phylogenetic Analysis using Parsimony (PAUP) 3.1.1. Smithsonian Institute, Washington, D.C.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Res.*, **22**:4673–80.

Throndsen, J. 1969. Flagellates of Norwegian coastal waters. *Nytt. Mag. Bot.*, **16**:161–216.

Tong, S. 1997. Heterotrophic flagellates and other protists from Southampton water, UK. *Ophelia*, **47**:71–131.

Tong, S., Vørs, N. & Patterson, D. J. 1997. Heterotrophic flagellates, centrohelid heliozoa and filose amoebae from marine and freshwater sites in the Antarctic. *Polar Biol.*, **18**:91–106.

Triemer, R. E. & Farmer, M. A. 1991a. An ultrastructural comparison of the mitotic apparatus, feeding apparatus, flagellar apparatus and cytoskeleton in euglenoids and kinetoplastids. *Protoplasma*, **164**:91–104.

Triemer, R. E. & Farmer, M. A. 1991b. The ultrastructural organization of the heterotrophic euglenids and its evolutionary implications. *In*: Patterson D. J. & Larsen, J. (ed.), The Biology of Free-living Heterotrophic Flagellates. Clarendon Press, Oxford. p. 183–204.

Triemer, R. E. & Ott, D. W. 1990. Ultrastructure of *Diplonema ambulator*, Larsen & Patterson (Euglenozoa) and its relationship to *Isonema*. *Eur. J. Protistol.*, **25**:316–320.

UKNCC. 2001. Catalogue of the UK National Culture Collection—List of Algae and Protozoa.

Vickerman, K. 1976. The diversity of the kinetoplastid flagellates. *In*: Lumsden, W. H. R. & Evans, D. A. (ed.), Biology of the Kinetoplastida. Academic Press, London. p. 1–34.

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

Vickerman, K. 1991. Organization of the bodonid flagellates. *In*: Patterson, D. J. & Larsen, J. (ed.), The Biology of Free-living Heterotrophic Flagellates. Clarendon Press, Oxford. p. 159–176.

Vickerman, K. 2002. Diplonemids. *In*: Lee, J. J., Leedale, G. F. & Bradbury, P. (ed.), The Illustrated Guide to the Protozoa, 2nd ed. Allen Press Inc., Lawrence, Kansas, USA. p. 1157–1159.

Vickerman, K. & Preston, T. M. 1976. Comparative cell biology of kinetoplastid flagellates. *In*: Lumsden, W. H. R. & Evans, D. A. (ed.), Biology of the Kinetoplastida. Academic Press, London. p. 36–130.

Von der Heyden, S., Chao, E. E. & Cavalier-Smith, T. 2004. Genetic diversity of goniomonads: an ancient divergence between freshwater and marine species. *Eur. J. Phycol.*, (in press).

Vørs, N. 1992. Heterotrophic amoebae, flagellates and heliozoa from the Tvärminne area, Gulf of Finland, in 1988–1990. *Ophelia*, **36**:1–109.

Weitere, M. & Arndt, H. 2003. Structure of the heterotrophic flagellate community in the water column of the river Rhine (Germany). *Europ J. Protistol.*, **39**:287–300.

Yasuhira, S. & Simpson, L. 1997. Phylogenetic affinity of mitochondria of *Euglena gracilis* and kinetoplastids using cytochrome oxidase I and Hsp60. *J. Mol. Evol.*, **44**:341–347.

APPENDIX

The GenBank accession numbers for additional taxa used in analyses are as follows; *Acrasis rosea*: (AF011458), *Anisonema acinus* (AF403160), *Astasia curvata* (AJ532394), *A. longa* (AF283305), *Astasia* sp. ASW08097 (AF283307), AT1–3 (AF530519), AT4–56 (AF530520), AT4–96 (AF530521), AT4–103 (AF530522), AT5–25 (AF530518), AT5–48 (AF530517), *Blastocrithidia culicis* (L29265), *B. triatomae* (AF153040), *Bodo saltans* Konstanz (AF208889), *Bo. saltans* St. Petersburg (AF208887), *Colacium mucronatum* (AF326232), *C. vesiculosum* (AJ532439), *Crithidia fasciculata* (Y00055), *Cr. oncopelti* (L29264), *Cruzella marina* (AF208878), *Cryptobia bullocki* (AF080224), *Cry. catostomi* (AF080226), *Cry. salmositica* (AF080225), *Cryptoglena pigra* (AJ532437), *Cryp. skujai* (AY532438), *Dimastigella trypaniformis* ULM (X76494), *D. trypaniformis* GLASGOW (X76495), *D. mimosa* (AF208882), *Dinema sulcatum* (AY061998), *Diplonema ambulator* (AF380996), *Di. papillatum* (AF119811), *Distigma curvatum* (AF099081), *Dis. proteus* (AF386639), *Endotrypanum monterogei* (X-53911), *Entosiphon sulcatum* (AF220826), *Euglena acus* (AF152104), *E. agilis* (AF115279), *E. anabaena* (AF242548), *E. chlamydophora* (AY029407), *E. gracilis* (AF283309), *E. intermedia* (AY029408), *E. mutabilis* (AF096992), *Euglena* sp. (AY029278), *E. spirogyra* (AF150935), *E. stellata* (1:AF283310, 2:AF150936), *E. tripteris* (AF286210), *E. viridis* (AF445460), *Eutreptia pertyi* (AF081589), *Eutreptia* sp. M1914 (AJ532396), *Eu. viridis* (AF157312), *Eutreptiella gymnastica* (AJ532400), *Eutreptiella* sp. CCMP 389 (AF112875), *Gyropaigne lefevrei* (AF110419), *Herpetomonas muscarum* (AY180151), *Herpetomonas* sp. TCC 263 (AF038024), *Ichthyobodo necator* (AY224692), *Ichthyobodo* sp. (AY224692), *Jakoba incarcerata* (AY117419), *J. libera* (AF411288), *Khawkinea quartana* (U84732), kinetoplastid flagellate (AF174379), *Klosteria bodomorphis* (AY268046), *Leishmania donovani* (U42465), *L. tarentolae* (M84225), *Lepocinclis buetschlii* (AF096993), *Lep. ovata* (AF061338), *Lep. ovum* (AF110419), *Leptomonas collosoma* (AF153038), *Leptomonas* sp. (AF153042), *Macropharyngomonas halophila* AF011465), *Malawimonas jakobiformis* (AY117420), *Menoidium bibacillatum* (AF247598), *M. cultellus* (AF295019), *Naegleria gruberi* (M18732), *Neobodo designis* DH (AF464896), *N. designis* St. Petersburg (AF209856), *N. designis* ATCC 50358 (AF174379), *Neovahlkampfia damariscottae* (AJ224891), *Notoselenus ostium* (AF403159), *Oxymonas* sp. (AJ429101), *Paravahlkampfia ustiana* (AJ224890), '*Peranema*' sp. BIPA2001 (AY048919), *P. trichophorum* CCAP1260 (AF386636), *P. trichophorum* (AH005452), *Phytomonas serpens* (U39577), *Parabodo caudatus* (X53910), *Parmidium circulare* (AF309633), *Pa. scutulum* (AF295018), *Petalomonas cantuscygni* CCAP1259/1 (AF386635), *Pe. cantuscygni* (U84731), *Perkinsiella*-like sp. (AY163355), *Phacus acuminatus* (AY130820), *Ph. agilis* (AY014998), *Ph. alatus* (AY014999), *Ph. brachykentron* (AF286209), *Ph. megalopsis* (AF090870), *Ph. orbicularis* (AF283315), *Ph. pseudonordstedtii* (AJ532435), *Ph. pyrum* (AF112874), *Ph. similis* (AF119118), *Ph. pusillus* (AF190815), *Ph. splendens* (AF190814), *Ploeotia costata* (AF525486), *Reclinomonas americana* (AF053089), *Rhabdomonas incurva* (AF247601), *R. spiralis* (AF247600), *Rhynchobodo* ATCC 50359 (U67183), *Rhynchomonas nasuta* BSZ1 (AF174378), *Rh. nasuta* (AF174377), *Stachyamoeba* sp. (AF011461), *Streblomastix strix* (AY188885), *Strombomonas* sp. (AF445461), *Tetramitus rostratus* (M98051), *Trachelomonas conspersa* (AJ532450), *T. echinata* (AY015001), *T. hispida* (AY130817), *T. intermedia* (AY015002), *T. pertyi* (AJ532443), *T. volvocina* (AJ532451) *T. volvocinopsis* (AY015004), *Trimastix marina* (AF244905), *Tr. pyriformis* ATCC 50562 (AF244903), *Tr. pyriformis* ATCC 50598 (AF244904), *Trypanoplasma borreli* (L14840), *Trypanosoma brucei* (AJ009140), *T. cruzi* (AF228685), Trypanosomatidae G755 (U59491), Uncultured 'euglenid' (AY180037), Uncultured marine euglenozoan (AF290080), Unidentified eukaryote (AF130858), Unidentified trypanosomatid (AF071866), *Vahlkampfia inornata* (AJ224887), *V. avara* (AJ224886), *Wallaceina brevicula* (AF153045).

Received 02-06-04, 04-04-04; accepted 04-04-04