The enormous diversity of small free-living heterotrophic flagellates (Patterson and Larsen 1991) has in recent years become a focus of attention of ecologists and taxonomists. The importance of heterotrophic nanoflagellates in marine pelagic food webs as major consumers of bacteria and picophytoplankton has been widely acknowledged. Additionally, host specific parasitoid nano-flagellates, such as Cryothecomonas and Pirsonia, can cause great mortality among phytoplankton populations (Drebes et al. 1996; Kühn et al. 1996; Tillmann et al. 1999). The ecological importance of flagellates has made particular demands on systematists. Some groups are relatively well-defined and are easily identified by diagnostic features. Electron microscopy has proved a powerful tool to reveal the ultrastructure of characteristic organellar appearances. Although flagellates can be distinguished by their structural properties, their taxonomic position often remains uncertain (e.g. Patterson and Zöllf 1991). Ribosomal RNA sequence comparisons have been proven to be a useful tool in molecular eukaryote and prokaryote evolution because this technique offers a measure of evolutionary relatedness that is independent of morphology (Bhattacharya et al. 1995; Sogin et al. 1989; Van De Peer and De Wachter 1997; Woese 1987). Although the number of available ribosomal RNA sequences for molecular evolutionary analysis has grown continuously during the last two decades, only a tiny proportion of the great
diversity of protists has been subjected to molecular genetic analysis (Cavalier-Smith 1998a; Schlegel 1991). Ribosomal RNA sequencing has led to fundamental revisions of protist taxonomy. It has been shown, for example, that lobose amoebae, such as Hartmannella and Acanthamoeba are not closely related to euglypid filose amoebae (Bhattacharya et al. 1995). These discoveries have led to the classification of the Cercozoa (syn. Rhizopoda Von Siebold 1845). Unfortunately, supporting morphological reasons (Cavalier-Smith 1996/97). He pointed out that the theca of Cryothecomonas was transferred from the Thecomonadidae to the Sarcomonadidae without segmentation (Phytomyxa (Cavalier-Smith 1996/97; 1998a; 2000).

Six species of the heterotrophic nanoflagellate Cryothecomonas have been described so far. Cryothecomonas appears to be a ubiquitous flagellate that occurs in polar and temperate waters tolerating salinity from 0.0 to 34 psu and with cell densities up to $2.6 \times 10^6$ cells l$^{-1}$ (Ikävalko and Thomsen 1997). Cryothecomonas was first described by Thomsen et al. (1991) based on three species from Danish waters (C. inermis, C. scybalophora, C. vesiculata) and C. armigera from the Weddell Sea, Antarctica. Recently two new species, Cryothecomonas longipes and Cryothecomonas aestivialis (strain 1 in this study) were described from the North Sea (Drebess et al. 1996; Schnepf and Kühn 2000). In this study we report the occurrence of another C. aestivialis strain (strain 2). Whereas some species (C. armigera and C. inermis) ingest nanoplanktonic algae (2–4.5 µm), others feed on diatoms much larger than themselves by gradually phagocytizing host protoplasm, either by attaching to the outside of the frustule of the host diatom (e.g. C. longipes) or by invading the frustule (e.g. C. aestivialis strains 1 and 2).

Since its first description, the taxonomic position of Cryothecomonas has been a matter of discussion. Patterson and Zöllfel (1991) hypothesized Cryothecomonas to be related to cercomonad amoeboflagellates. Cavalier-Smith (1993) erected the new order Cryomonadida with the sole family Cryothecomonadidae and placed it within the phylum Neomonada, Class Thecomonadidae (Cavalier-Smith 1996/97). He pointed out that the theca of Cryothecomonas is probably not homologous to that of Apusomonas (Cavalier-Smith 1998b). Recently Cryothecomonas was transferred from the Thecomonadidae into the Sarcomonadidae without supporting morphological reasons (Cavalier-Smith 2000). Unfortunately, Cryothecomonas had not been analysed with molecular evolutionary methods. We have now sequenced the small subunit ribosomal RNA gene (SSU rRNA) from Cryothecomonas longipes and two strains of Cryothecomonas aestivialis and compared these sequences to SSU rRNAs from diverse eukaryotes. Our analyses confirm the current systematic position of Cryothecomonas as a sarcomonad flagellate.

**Results**

**Phylogenetic Analysis**

SSU rRNA sequences were determined for Cryothecomonas longipes and two strains of Cryothecomonas aestivialis and deposited in Genbank. The SSU rRNA sequences of Cryothecomonas longipes, Cryothecomonas aestivialis strain 1 and strain 2 are 1819, 1815 and 1817 nucleotides in length respectively. In contrast to morphological similarity between Cryothecomonas aestivialis strains 1 and 2, both strains differ from each other by 8 nucleotide substitutions in their SSU rRNA sequences. The absolute number of nucleotide differences separating these strains is comparable to species differences between the bipolar haptophytes Phaeocystis antarctica and Phaeocystis pouchetii (6–10) (Medlin et al. 1994), within species of the diatom Skeletonema (11) (Medlin et al. 1991) and of the protozoan Tetrahymena (0–33) (Sogin et al. 1986). Cryothecomonas longipes is separated by 68 and 74 base substitutions from Cryothecomonas aestivialis strain 1 and Cryothecomonas aestivialis strain 2 respectively.

We have used two SSU rRNA data sets for the phylogenetic analyses. The large data set with representatives from all major eukaryotic lineages was used to infer the phylogenetic position of Cryothecomonas. The small data set contained all available SSU rRNA sequences from taxa belonging to the phylum Cercozoa. This data set was used to infer the position of Cryothecomonas within the Cercozoa.

The phylogenetic position of Cryothecomonas as determined from a maximum likelihood analysis is shown in Figure 1 for the large data set and in Figure 2 for the small one. Cryothecomonas forms a monophyletic group within the phylum Cercozoa with 100% bootstrap support in both maximum parsimony and neighbor joining distance analysis (Figure 1). The branch leading to the Cercozoa is supported by high bootstrap values in the maximum parsimony and neighbor joining distance analysis, but Plasmodiophora brassicae (Phytomyxea) recently classified as belonging to the Cercozoa (Cavalier-Smith 1996/1997) is not included in this clade (Fig. 1). The sarcomonad flagellate Heteromita globosa appears as a sister taxon to Cryothecomonas in both maxi-
Figure 1. SSU-rRNA phylogeny of 47 eukaryotes based on sequence comparisons inferred with the maximum likelihood method using 1,546 unambiguously aligned nucleotides. The bootstrap values (500 replications) above the internal nodes are inferred from a maximum parsimony analysis, whereas the bootstrap values (500 replications) below the internal nodes are inferred using the neighbor-joining method. Only bootstrap values greater than 50% are reported. The scale below the tree indicates the branch length corresponding to ten changes per 100 nucleotide positions.
mum likelihood trees; however, this relationship is not supported by bootstrap values (Figs. 1 and 2). Differences within the branching order between the maximum likelihood trees inferred from the large and small data set were observed (Figs. 1 and 2). In both trees Cercomonas appeared not as a monophyletic genus and Cercomonas sp. 1 was placed as the first divergence of the clade that contains the remaining Cercomonas species. However, in the maximum likelihood tree calculated from the small data set, Cercomonas sp. 1 was placed as a sister taxon to the Sarcomonadea/Testaceafilosia clade (Fig. 2), whereas Cercomonas sp. 1 was placed between this clade in the maximum likelihood tree calculated from the large data set (Fig. 1). The Testaceafilosia appeared as a sister clade to the Sarcomonadea (Fig. 1), whereas the Testaceafilosia clade was placed as a sister to Cercomonas sp. 1–3 within the Sarcomonadea (Fig. 2). The tree inferred from the small data set utilised more unambiguously aligned nucleotide positions in the phylogenetic analyses and more likely reflects the correct evolutionary position of taxa in the Cercozoa (Fig. 2).

Light Microscopy

Because of the relatively high number of base substitutions found between Cryothecomonas aestivalis strain 1 and 2, we investigated both strains for morphological differences using light microscopy. Morphologically, Cryothecomonas aestivalis strain 2 is very similar to C. aestivalis strain 1 Drebes, Kühn et Schneplf (Drebes et al. 1996). C. aestivalis strain 2 flagellates are oblong to oval, 5–6 µm wide (strain 1: 4–5 µm) and 8.5–10 µm long (strain 1: 9–12 µm), and have two heterodynamic flagella that are inserted apically. The anteriorly directed flagellum is 10–14 µm long (strain 1: up to 15 µm), and the posteriorly directed flagellum measures 16–23 µm (strain 1: up to 25 µm). Also, the life cycles of C. aestivalis strain 1 and strain 2 are identical: infective flagellates squeeze inside the host diatom frustule between the girdle bands and feed on the diatom protoplast by means of a pseudopodium. After the trophic phase flagellates divide several times and, depending on the size of the host cell, form up to 40 offspring. There are, however, slight differences in the host

Figure 2. Eukaryotic phylogeny based on SSU rRNA sequence comparisons inferred with the maximum likelihood method using 1,559 unambiguously aligned nucleotides. The bootstrap values (500 replications) above the internal nodes are inferred from a maximum parsimony analysis, whereas the bootstrap values (500 replications) below the internal nodes are inferred using the neighbor-joining method. Only bootstrap values greater than 50% are reported. The scale below the tree indicates the branch length corresponding to five changes per 100 nucleotide positions.
ranges: in contrast to *C. aestivalis* strain 1, *C. aestivalis* strain 2 infects not only the chain-forming diatom *Guinardia delicatula* but also *G. flaccida*. In both parasitoid species, offspring develop after approximately 24 hours (at 15–20 °C) when feeding on *G. delicatula*. Whereas *C. aestivalis* strain 1 offspring leave the empty frustule immediately when digestion is finished, *C. aestivalis* strain 2 offspring remain inside the frustule of *G. delicatula* for up to several days but leave the frustule of *G. flaccida* immediately. Apparently, *G. delicatula* is not the ideal host for *C. aestivalis* strain 2. Whereas *G. delicatula* cells are soon separated from sibling cells within the chain when infected with *C. aestivalis* strain 2, chains remain intact when infected with *C. aestivalis* strain 1.

**Discussion**

The genus *Cryothecomonas* is related to the phylum Cercozoa and not to the phylum Neomonada (e.g. *Apusomonas* proboscidea, *Thecomonadae*, see *Cavalier-Smith* and *Chao* 1995; 1996/97) as proposed earlier by *Cavalier-Smith* (1993, 1996/97, 1998b). Recently, *Cryothecomonas* was removed from the *Thecomonadae* and placed in the Sarcomonadae but the reasons for this transfer were not discussed (*Cavalier-Smith* 2000). Our SSU rRNA phylogenies confirm that *Cryothecomonas* is a sarcomonad flagellate. The morphological/ ultrastructural data corroborate this result because *Cryothecomonas* shares several characteristics with the sarcomonad flagellates *Heteromita globosa*, *Thaumatomonas* sp. and *Cercomonas* sp., but shows significant differences to *Apusomonas* proboscidea. Most characteristically, *Apusomonas* has an anterior proboscis with two emerging flagella that terminate in an acronema and a posteriorly located nucleus (*Karpov* and *Zhukov* 1986). In sarcomonads the nucleus is located anteriorly and an acronema is absent.

Sarcomonad flagellates, including *Cryothecomonas*, are colourless amoeboid flagellates that feed by means of pseudopodia because they lack a discrete mouth. Pseudopodia emerge ventrally from a groove in *Thaumatomonas* and *Cryothecomonas*, whereas in *Cercomonas* and *Heteromita*, pseudopodia formation is not restricted to a particular area of the body. Two flagella are inserted apically or subapically at the anterior cell pole. They are retained but remain immobile during pseudopodial engulfment of food. The anterior basal bodies are directed forward, the posterior basal bodies at an angle of 90° (*Thaumatomonas*: 30°). The glycosalyx is generally thick and distinct. Extrusomes are present in some species. The nucleus with a central dense nucleolus is located anteriorly. Mitochondrial cristae are tubular or vesicular. Food vacuoles are located in posterior region of the cell (*Drebes* et al. 1996; *Karpov* 1997; *MacDonald* et al. 1977; *Mignot* and *Brugerolle* 1975; *Mylnikov* 1986; *Robertson* 1928; *Schnepf* and *Kühn* 2000; *Schuster* and *Pollak* 1978; *Shirkina* 1987).

*Heteromita*, *Thaumatomonas* and *Cryothecomonas* have a spiral fiber or transitional helix in the transition zone of each flagellum (*Drebes* et al. 1996; *Karpov* 1997; *Karpov* and *Fogin* 1995; *Schnepf* and *Kühn* 2000; *Thomsen* et al. 1991), which is absent in *Cercomonas* (e.g. *Karpov* 1997).

From available morphological and molecular genetic data, we can confirm the current taxonomic placement of *Cryothecomonas* in the phylum Cercozoa, class Sarcomonadae (*Cavalier-Smith* 2000). Our phylogenetic analysis also shows that *Cryothecomonas*, euglyphid testate amoebae and sarcomonad flagellates shared a common evolutionary ancestor, whereas the Chlorarachniophyta (e.g. Chlorarachnion reptans) and the Phytomyxa (*e.g.* Plasmodiophora brassicae) diverged earlier. Euglypha rotunda and Paulinella chromatophora (*Euglypha*) form a separate cluster within the Cercozoa (*Figs. 1 and 2*). This result is in agreement with the hypothesis by *Cavalier-Smith* (1996/97) that euglyphid testate amoebae evolved from sarcomonad flagellates by the loss of flagella. The genus *Cryothecomonas* is polyphyletic (*Figs. 1 and 2*) as demonstrated in previous studies (*Cavalier-Smith* 1996/97; *Cavalier-Smith* and *Chao* 1996/1997; *Cavalier-Smith* 2000).

Our phylogenetic analysis indicates that *C. longipes* diverged prior to the separation of both *C. aestivalis* strains. *C. longipes* has a broad host range and infects at least 14 diatom species (*Schnepf* and *Kühn* 2000). *C. aestivalis* strain 1 infects only *Guinardia delicatula* (*Drebes* et al. 1996), whereas *C. aestivalis* strain 2 infects two diatom species of the same genus (*Guinardia delicatula* and *G. flaccida*). Thus host-specific species represents the derived condition and evolution perfects the parasitoids so that they are host-specific.

In our analysis Plasmodiophora brassicae appears not to be closely related to the Cercozoa or to any other eukaryote sequence included in our alignment. This organism contains three large insertion sequences in its SSU rRNA gene consisting of 388, 383 and 442 nucleotides (*Castlebury* and *Domier* 1998), which have to be excluded from the phylogenetic analysis. In earlier SSU rRNA phylogenetic studies, Plasmodiophora brassicae formed the
The unexpected high number of nucleotide differences (8) between two morphologically indistinguishable strains of Cryothecomonas aestivalis isolated near Sylt and Heligoland suggest the possibility of cryptic species within Cryothecomonas aestivalis. Variation within SSU or LSU rRNA sequences of strains of a morphologically well-defined species has been reported for many species in the marine environment (Knowlton 1993; Medlin et al. 1995). Sequence data from the D1/D2 region of the LSU rRNA, for example, revealed the existence of 46 sibling species within the dinoflagellate Cystrophecocidium cohnii and 25 sibling species within the protozoan Tetrahymena pyriformis (Beam et al. 1993, Preparata et al. 1992). Intraspecific sequence variation (up to 5 nucleotides) within the SSU rRNA of strains of Phaeocystis globosa (Lange 1997; Medlin et al. 1994) has been used together with differences in genome sizes within Phaeocystis globosa strains (Vaulot et al. 1994) to demonstrate the occurrence of cryptic species within the Phaeocystis globosa complex (Lange 1997; Medlin et al. 1995). The Cryothecomonas aestivalis strains could be separated by differences in their host specificity and by variation within their SSU rRNA sequences. The lack of any morphological differences between both strains can be explained by possible disparity between morphological and molecular rates of evolution. Similarities in body shape within marine phytoflagellate species is thought to reflect selection pressure and adaptation to the marine environment.

The last two studies did not include representatives of the Cercozoa in their phylogenetic analyses. The deepest branch in the Cercozoa clade (Cavalier-Smith 1996/97; Cavalier-Smith and Chao 1996/1997; Cavalier-Smith 2000). Other phylogenetic studies with rDNA sequences indicate that Plasmodiophora brassicae is a distinct group and not closely related to any other eukaryotes (Castlebury and Domier 1998; Ward and Adams 1998). Unfortunately, the unexpected high number of nucleotide differences (8) between two morphologically indistinguishable strains of Cryothecomonas aestivalis isolated near Sylt and Heligoland suggest the possibility of cryptic species within Cryothecomonas aestivalis. Variation within SSU or LSU rRNA sequences of strains of a morphologically well-defined species has been reported for many species in the marine environment (Knowlton 1993; Medlin et al. 1995).

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**Methods**

**Cultures and DNA extraction:** C. aestivalis strain 1 was isolated in Autumn 1994 from plankton samples collected in the Wadden Sea off List/Sylt (North Sea, German Bight), when infecting Guinardia delicatula (Drebets et al. 1996). Cryothecomonas aestivalis strain 2 was isolated in July 1999 from plankton samples collected at Heligoland (North Sea, German Bight), feeding inside G. delicatula and G. flaccida (Castracane) H. Peragallo. Flagellates were maintained in F/2 medium (Guillard and Ryther 1962) with G. delicatula as host at 15 °C on a 16/8-h light/dark cycle and 25–30 µmol photons m⁻² s⁻¹. Twice a week a few µl of an infected culture was inoculated into a new host culture. Living cells were observed under the light microscope at room temperature. C. longipes was isolated at List/Sylt in September 1998 (Schnepf and Kühn 2000) and maintained with Thalassiosira rotula Meunier. Cultures were harvested when all diatoms were infected and effectively dead to prevent diatom DNA contamination and frozen immediately in liquid nitrogen until needed or used directly for DNA extraction. Total nucleic acids were obtained using a 3% CTAB (hexadecyltrimethylammonium bromide) procedure (Doyle and Doyle 1990). Cultures of the protist strains used in this study are available from the authors upon request.

**Amplification:** Total nucleic acid preparations were used as templates for the amplification of SSU rRNA genes using conserved primers (Medlin et al. 1988). Each 100 µL PCR reaction contained 100 ng total nucleic acids, 1 mM of each dATP, dGTP, dCTP and dTTP, 10 µM of each primer, 10 µL of 10× reaction buffer (100 mM Tris, (pH 8.4), 500 mM KCl, 20 mM MgCl₂, 0.1% gelatin) and 1.5 units AmpliTaq DNA polymerase (PERKIN-ELMER, Foster City, CA). PCR reactions were performed using a Perkin-Elmer-Cetus thermocycler with an initial denaturation step of 95 °C for 6 min after which the Taq polymerase was added as the heating block cooled to 60 °C, followed by 29 cycles of 72°C for 4 min, 94 °C for 2 min, 45 °C for 2 min, and a final extension step 72 °C for 9 min. Ten µL of the amplification products were checked for correct length, purity and yield on ethidium bromide-stained 1.5% agarose gels (Sambrook et al. 1989) visualized by UV and photographed with Polaroid 667 film.

**Sequence analysis:** PCR products were prepared for sequencing using the PCR purification kit from QIAGEN (Hilden, Germany) following the instructions of the manufacturer. Sequencing was performed using the Sequi-Therm-Cycle Sequencing kit from BIOZYM (Hess. Oldendorf, Germany) using up to 200 ng PCR product per reaction and following the instructions of the manufacturer. Sequencing reactions were run on an automatedLicor sequencer (MWG, Ebersberg, Germany). Sequence outputs were checked and manually corrected.

**Phylogenetic analysis:** Sequences were added to an alignment, which contained over 300 small subunit rRNA genes from eukaryotic organisms (data not shown), using maximum primary and secondary structural similarity with the Olsen sequence editor (Larsen et al. 1993) on a VAX 6520 computer.
From this alignment a subset was selected consisting of 47 taxa, among which Trypanosoma theleri and Trypanosoma vespertilios were selected as outgroup taxa (Fig. 1). A second subset consisting of 14 sequences was selected from the large data set to investigate the phylogenetic position of Cryothecomonas within the phylum Cercozoa using Plasmodiophora brassicae as outgroup (Fig. 2). Maximum likelihood, distance and parsimony analyses were performed using 1546 and 1559 unambiguously aligned nucleotides in the large and small data set respectively.

**Maximum likelihood analysis:** Maximum likelihood analysis was performed with the fastDNAmI program (V1.0 Olsen et al. 1994). Global rearrangements of the full tree were performed and 44 branches (large data set) or 11 branches (small data set) were crossed. Taxon addition was performed randomly and a transition/transversion ratio = 2 was used. With this method a total of 21,511 trees (large data set) or 1,716 trees (small data set) were examined. From which the tree with the highest likelihood value was selected (large data set, Ln likelihood = –7048.84161), (small dataset, Ln likelihood = –23822.09759), (large data set respectively.

**Distance analysis:** A neighbor-joining bootstrap analysis (500 replicates) for both data sets was performed using the PHYLIP computer program (version 3.5, Felsenstein 1993). Dissimilarity values (Fitch and Margoliash 1967) based on pairwise comparisons of sequences, were transformed into distances using the Kimura-two-parameter model (Kimura 1980). Distance matrices were converted into trees using the neighbor-joining method (Felsenstein 1993) with jumbled taxon addition and the transition/transversion ratio = 2.

**Maximum parsimony analysis:** A maximum parsimony bootstrap analysis (500 replicates) for both data sets was performed using the PAUP computer program (V3.1.1 Swofford 1993). Introduced gaps were treated as missing data; informative characters were treated as multistate unordered. Maximum parsimony trees were analysed with a heuristic search method with a tree-bisection-reconnection (TBR) branch swapping option with random taxon addition. SSU rRNA sequences used in this study (with EMBL/Genbank numbers) are as follows: (Acanthamoeba castellanii (Douglas) Page, M13435), (Achlya bisexualis Coker, M32705), (Ane monia sulcata Pennant, X53498), (Apusomonas pro boscidea Alexieff, L37037), (Artemia salina Leach, X01723), (Aureobasidium pullulans (De Bary) Arnaud, M55639), (Ceratium furca (Kütz) Silva, Mattox et Blackwell, M95613), (Fucus distichus Linnaeus, M62995), (Fucus distichus Linnaeus, M97959), (Glaucohystis nostochinearum, Itzigsohn, X70803), (Glauco spherea vacuolata, Korschikov, X81903), (Hanusia phi Hill et Wetherbee = Cryptomonas phi, nuclear, X57162), (Hanusia phi Hill et Wetherbee = Cryptomonas phi, nucleomorph, X57008), (Hartmannella vermiformis Page, M95168), (Heteromita globosa Dujardin, U42447), (Klebsormidium flac cidium (Kütz) Silva, Mattox et Blackwell, M95613), (Lagenidium giganteum Couch, M54393), (Ochromonas danica Pringsheim, M32704), (Oxytricha nova D. Prescott, M14601), (Paulinella chromatophora Lauterborn, X81811), (Paramecium tetra relia, Sonneborn, X03772), (Phaeocystis globosa Scherffel, X77476), (Pythophthora megasperma, Drechsler, M54938), (Plasmodiophora brassicae Woronin, U18981), (Prorocentrum micans Ehren berg, M14649), (Rhodomonas salina (Wislouch) Hill et Wetherbee = Pyrenomonas salina (Wislouch) Santore, nuclear, X554276), (Rhodomonas salina (Wislouch) Hill et Wetherbee = Pyrenomonas salina (Wislouch) Santore, nucleomorph, X555032), (Skel etoneoma costatum (Greville) Cleve, X85395), (Synura spinosa Korschikov, M87336), (Thaumatomonas sp. De Saedeleer, M42446), (Trypanosoma theleri Laveran, AJ 009164), (Trypanosoma vespertilios Battaglia, AJ 009166), (Zea mays Linnaeus, M20017), (Zea mays Linnaeus, K02202).

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