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High diversity of the '*Spumella*-like' flagellates: an investigation based on the SSU rRNA gene sequences of isolates from habitats located in six different geographic regions

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Summary

We isolated 28 strains of '*Spumella*-like' flagellates from different freshwater and soil habitats in Austria, People's Republic of China, Nepal, New Zealand, Uganda, Kenya, Tanzania and Hawaii by use of a modified filtration–acclimatization method. '*Spumella*-like' flagellates were found in all of the samples and were often among the dominant bacterivorous flagellates in the respective environments. The small subunit ribosomal RNA (SSU rRNA) gene sequence of the isolates was determined and aligned with previously published sequences of members belonging to the Chrysophyceae *sensu stricto*. Phylogenetic analysis of the 28 new sequences confirmed their position within the Chrysophyceae *sensu stricto* and positioned them within different clades. Most of the sequences grouped within clade C and formed several subclusters separated from each other by green taxa including flagellates belonging to *Ochromonas*, *Dinobryon*, *Poterioochromonas* and others. All soil isolates clustered together (subcluster C1) with the soil strain *Spumella elongata* and the undescribed soil strain 'Spumella danica'. Aquatic isolates were affiliated with at least two branches (C2 and C3). Sequence similarity to the closest related member of the Chrysophyceae ranged between 92% and 99.6%, sequence divergence among the '*Spumella*-like' flagellates was as high as 10%. We conclude that (i) the '*Spumella*-like' flagellates are a diverse group both in terms of sequence dissimilarity between isolates and in terms of the number of genotypes, (ii) *Spumella* and *Ochromonas* are polyphyletic, and (iii) based on the SSU rRNA gene no biogeographical restriction of certain branches could be observed even though different ecotypes may be represented by the same genotype.

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Introduction

Since 1983 Azam and colleagues (Azam *et al*., 1983) introduced the concept of the microbial loop, the significance of heterotrophic single-cell eukaryotes for carbon transfer through aquatic food webs has become generally accepted (Wylie and Currie, 1991; Sanders *et al*., 1994; Sherr and Sherr, 1994; Arndt *et al*., 2000; Boenigk and Arndt, 2002). The nanoflagellate genera *Spumella*/*Monas* are among the most important heterotrophic eukaryotes in many different ecosystems: On annual average 20–50% of the pelagic heterotrophic nanoflagellate (HNF) biomass in freshwaters is formed by small heterokont taxa, mainly colourless chrysophytes (= chrysomonads) and bicosoecids (Salbrechter and Arndt, 1994; Arndt *et al*., 2000). *Spumella*, which represents a typical colourless chrysophyte has been reported to be generally common in freshwaters (Carrick and Fahnenstiel, 1989; Sanders *et al*., 1989; Bennet *et al*., 1990; Carrias *et al*., 1998). Even in benthic sites, colourless chrysophytes make up to 30%, but usually much less (Arndt *et al*., 2000). In addition, the chrysophyte genera *Ochromonas* and *Poterioochromonas* are assumed to be among the dominant mixotrophs (Bennet *et al*., 1990). The primary mode of nutrition of these mixotrophs often is bacterivory (Andersson *et al*., 1989). These bacterivorous chrysophytes (family Chromulinaceae *sensu*; Preisig, 1995) therefore are responsible for a significant part of the bacterivory in freshwater systems and are an important link between bacterial production and higher trophic levels. Thus far, field investigations and food web models have been primarily focused on the so-called 'functional groups', i.e. bacteria, heterotrophic nanoflagellates, ciliates, etc. It became evident, however, that such models cannot sufficiently describe the specific interactions and pathways within the microbial food web. For this reason,

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attention is increasingly drawn towards species- or taxon-specific investigations and assessing the diversity of the free-living flagellates is becoming increasingly important (e.g. Kinner *et al*., 1998; Cleven and Weisse, 2001).

The attempt for specific investigations is often hampered by the sparseness of diagnostic characteristics for taxonomic identification. This is valid in particular for the small heterotrophic chrysophytes which are often summed up as '*Spumella*-like flagellates' or *Spumella* spp. in many field studies (Weisse, 1997; Auer and Arndt, 2001; Cleven and Weisse, 2001; Weitere and Arndt, 2003). The sparseness of diagnostic features and molecular data does not provide any clarification whether these organisms form either a mono- or a polyphyletic group: *Spumella* is considered to be the colourless counterpart of *Ochromonas* (Preisig *et al*., 1991), but *Ochromonas* ssp. have already been reported to cluster in different branches, i.e. together with *Poterioochromonas*, *Chrysoxys* and *Chromulina,* and have therefore been suggested to be polyphyletic (Andersen *et al*., 1999). In addition, occasional loss of colour has been independently described for several species of *Ochromonas* (Bourrelly, 1957) and for these reasons the separation of *Ochromonas* and *Spumella* is doubtful (Fenchel, 1982a,b; Preisig *et al*., 1991). Similarly, in ecophysiological laboratory investigations, members of the heterotrophic chrysophytes have been widely used as model organisms, but these investigations are based on very few strains (cf. Cowling, 1991) that often lack precise taxonomic identification (Holen and Boraas, 1991; Zwart and Darbyshire, 1992; Rothhaupt, 1997; Boenigk, 2002). In laboratory and field studies, these organisms are usually treated as a black box assuming, basically, similar ecological characteristics. Recent studies revealed, however, that even closely related protist taxa differ in their basic response to environmental factors (Weisse, 2002; Boenigk *et al*., 2004). It is therefore of urgent interest to survey the diversity and the taxonomic and ecological integrity of the so-called '*Spumella*-like' flagellates. To minimize confusion resulting from the different botanical (Chrysophyceae) and zoological (Chrysomonadida) nomenclatures we will generally follow the concept of Preisig (1995) throughout our manuscript and use the term Chrysophyceae.

We isolated 28 strains of '*Spumella*-like' flagellates from soil and freshwater habitats in Austria, People's Republic of China, Nepal, Uganda, Tanzania, Kenya, New Zealand and Hawaii, and conducted phylogenetic analysis using the small subunit ribosomal RNA (SSU rRNA) gene sequences. We were specifically interested in assessing the diversity of this taxonomically vague group. We hypothesized that (i) the diversity of the '*Spumella*-like' flagellates is dramatically underestimated by means of conventional light microscopical investigations; (ii) the '*Spumella*-like' flagellates are not a monophyletic group; and (iii) the '*Spumella*-like' flagellates do not represent a consistent ecophysiological group.

Results

Isolation of strains and morphology

'*Spumella*-like' flagellates were present in all of the samples. Successful isolation was, however, hampered in some cases by fast growing bodonids, which in some samples overgrew the colourless chrysophytes. As the '*Spumella*-like' flagellates could hardly be differentiated by morphological features during the isolation, only one or two, i.e. a small and a large forms, were isolated per sample to avoid multiple isolation of the same clone. All strains possess a spherical to ovate cell body. The strains tended to either attach to the substratum when well fed or to actively swim when starved. Swimming cells tended to spin in small circles even though swimming behaviour differed between the strains. Attached cells did not detach even during cell division, and flagellates were therefore often found in small colonies.

All isolated strains were between 3.2 and 8.3 μm long, corresponding to a cell volume of 13–292 μm^3 (Table 1). Two unequal flagella inserted close together at the anterior end of the cell, the long flagellum being about 2–4 times longer than the cell body, the short flagellum around 1/2 to 3/4 of the cell diameter and usually of <4 μm length. Only in the strain JBAF35 we found just one flagellum that was visible in the light microscope. The isolates originating from soil (i.e. JBM/S11, JBM/S12, JBC/S23 and JBC/S24) and the freshwater isolate JBC07 had the ability to build cysts, but this ability became weaker in culture and the strains JBC07 and JBC/S23 seemed to have lost the ability to build cysts altogether. Changes in temperature and food conditions did not induce cyst formation in these strains. All strains were bacterivorous and no autofluorescence could be detected.

Isolation efficiency

For determining the efficiency of the isolation method, i.e. the fraction of flagellate cells which could be successfully isolated in percent of the field abundance of flagellates, filtrated lake water containing the original background bacterial community at *in situ* temperatures was used. The test on isolation efficiency showed that only 1–2% of the nanoflagellates could be successfully isolated by way of direct dilution. In contrast, after acclimatization of the filtrates, isolation efficiency increased to >80% and was mostly near 100%. It is not clear as to which extent individual cells adapted to the laboratory conditions during this treatment and to which extent more resistant forms replaced the original community. The short acclimatization period of 16–24 h would allow for not more than three to

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four subsequent cell divisions only. This implies that a significant fraction of at least 10–20% of the original flagellates were able to individually adapt to the laboratory conditions. Successfully isolated strains could be transferred to a permanent culture with two exceptions: Two isolates could be subcultured at least four times but did finally die back. One of these isolates proved during the isolation process to be a very fast growing flagellate with a generation time of less than 4 h (J. Boenigk, unpublished data). Even different treatments during cultivation such as varying culture media, amounts of wheat grains and bacterial food sources did not result in any permanent culture (J. Boenigk, unpublished data).

SSU rRNA gene sequence analysis and phylogenetic affiliation of strains

The SSU rRNA gene sequence was determined for 28 '*Spumella*-like' isolates originating from freshwater and soil habitats in Austria, People's Republic of China, New Zealand, Nepal, Uganda, Tanzania and Kenya (Table 1). The results of the phylogenetic analysis of the SSU rRNA gene sequences showed that the isolates represent a wide diversity within the chrysophyceae, even though morphological distinction by way of light microscopy was in most cases quite difficult or impossible. Sequence differences among the SSU rRNA gene sequences from our '*Spumella*-like' flagellates ranged from 0% to 10% (excluding the strains JBAS37 and JBNZ43, which had large A/T-rich insertions). Sequence similarity to the closest-related cultured chrysophyceae was between 92.0% and 99.6% (Table 1). The highest sequence divergence to a database rRNA gene sequence was observed for the strains JBC27, JBM18 and JBM43 (92.0%, 93.9% and 94.3%). In contrast to that, SSU rRNA gene sequences of 50% of the isolates were closely related (i.e. >97% sequence identity) to the known chrysophyceae cultures (Table 1). No correlation could be found between the different regions from which the flagellates were isolated and sequence similarity. Soil as well as aquatic organisms with the same or very similar 18S rRNA gene sequence were isolated from widely different geographic regions. For instance, the soil isolates JBM/S11, JBC/S24 and *Spumella elongata* showed a sequence similarity of 99.6%, but were isolated from Austria, the People's Republic of China and the UK (Tables 1 and 2). Similarly, the strains JBM10, JBC07, JBNZ41, JBC30, JBC31 and JBAF32 (sequence identity 100%) originate from locations in Austria, People's Republic of China, New Zealand and Uganda.

The neighbour-joining phylogenetic analysis positioned the isolates in different clusters (Fig. 1); however, bootstrap support (bootstrap value, BV >60%) was observed only for clades A, B1, B2 and E. In general, the clades introduced by Andersen and colleagues (1999) were confirmed by our analysis. The parsimony analysis produced a tree, which recovered all clades, except clade D (Fig. 2). However, the placement of the clades was different and bootstrap support was lower for all of the clades.

The majority, i.e. 19 of our isolates affiliated in both analyses with clade C, which however, had no bootstrap support. None of our isolates affiliated with the clade A, which contains the Synurophycean taxa and with clade B1, containing taxa belonging to *Hibberdia* and related genera (Figs 1 and 2). Sixteen of our clade C isolates grouped within three subclusters of this clade, i.e. the '*Spumella*-like' soil cluster C1, and the two '*Spumella*-like' aquatic clusters C2 and C3. Subcluster C2 had different bootstrap values in the distance and the parsimony tree, whereas subcluster C1 showed bootstrap support only in the distance tree. The '*Spumella*-like' clusters contain exclusively non-green '*Spumella*-like' isolates, and no green chrysophyceaen strains were affiliated with any of these clusters. All published sequences from isolates originating from soils (Table 2), i.e. the strains *Spumella elongata* (Belcher and Swale, 1976) and 'Spumella danica' (I. Bruchmüller, A. Mylnikov, K. Juergens and T. Weisse, unpublished), were affiliated with cluster C1. Three aquatic isolates (JBAS36, JBM19 and JBC13) were also affiliated with this cluster C1. The aquatic cluster C2 contained both strains from larger lakes, for instance, from Lake Constance (*S. obliqua*) and Lake Plußsee in Germany (*Spumella* spp. SpiG, 15G and 37G) (Table 2), and strains from small puddles in Mondsee (JBM09) and Lunz (JBL14) in Austria. All of the new sequences affiliated with the '*Spumella*-like' cluster C3 had identical 18S rRNA gene sequences and seem to be a sister group to *Poterioochromonas* spp. A lorica as described for *Poterioochromonas* spp. has, however, never been observed for new isolates of the cluster C3. In contrast to the distance tree, parsimony analysis indicated that *Ochromonas danica* and *Ochromonas sphaerocystis* represent a sister group to C3.

Five sequences were related to members of the genus *Paraphysomonas*. Electron microscopical investigations of these isolates provided evidence that, in contrast to isolates affiliated with the C cluster, all these isolates possessed scales, i.e. morphologically they belong to the genus *Paraphysomonas* (G. Novarino, pers. comm.). In our analysis bootstrap support did not provide significant measures of confidence that this group is monophyletic. Both analyses resulted in three well-supported lineages within the genus *Paraphysomonas*, with *P. butcheri* diverging earlier to the other *Paraphysomonas* species. The two strains affiliated to the lineage including *P. vestita* PV10 (Caron *et al*., 1999) and SOTON1 (Rice *et al*., 1997), and *P. foraminifera* HT3, were similar to the two *P. vestita*

Table 1. Origin and characteristics of isolates.

Isolate	Next known sequence	Sequence similarity	Origin	Latitude longitude	Altitude (m)	Date of isolation	Cell size (μm³)	Cysts	Autofluorescence
JBM06	*P. foraminifera* TPC2	99.3%	Austria, Lake Mondsee	47°52′0N 13°20′60E	500	17/09/2002	195 ± 63		No
JBC07	*P. malhamensis*	94.9%	Peoples Republic of China, Lake Tai Hu	31°30′0N 120°20′0E	3	17/11/2002	101 ± 27	x	No
JBM08	*O. tuberculata*	95.4%	Austria, Lake Mondsee	47°52′0N 13°20′60E	500	14/10/2002	55 ± 13		No
JBM09	*Spumella* sp. 15G	99.3%	Austria, Puddle in Mondsee	47°52′0N 13°20′60E	500	14/10/2002	85 ± 37		No
JBM10	*P. malhamensis*	94.9%	Austria, Small artificial pond in Mondsee, Karlsgarten	47°52′0N 13°20′60E	500	14/10/2002	34 ± 8		No
JBM/S11	*S. elongata*	99.6%	Austria, Soil, Mondsee near 'Rauchhaus'	47°52′0N 13°20′60E	500	14/10/2002	36 ± 30	x	No
JBM/S12	*S. elongata*	98.8%	Austria, Soil, Mondsee near 'Rauchhaus'	47°52′0N 13°20′60E	500	14/10/2002	41 ± 18	x	No
JBC13	'S. danica'	98.9%	Peoples Republic of China, Pond in Beijing, Prince Gong's Mansion	39°53′60N 116°24′46E	56	14/11/2002	36 ± 11		No
JBL14	*Spumella* sp. 15G	99.1%	Austria, Puddle in Lunz	47°51′0N 15°03′0E	884	24/10/2002	135 ± 21		No
JBM18	*O. tuberculata*	93.9%	Austria, Lake Krottensee	47°47′0 N 13°23′20E	580	13/11/2002	35 ± 13		No
JBM19	*S. elongata*	99.1%	Austria, Lake Hallstatt	47°32′60N 13°39′0E	556	13/11/2002	16 ± 4		No
JBC22	*O. sphaerocystis*	97.8%	People's Republic of China, Pond 1 in Sushou, The Humble Administrator's Garden	31°18′28N 120°37′10E	3	19/11/2002	61 ± 21		No
JBC/S23	'S. danica'	98.4%	People's Republic of China, Soil near Badaling	40°20′15N 115°58′10E	795	14/11/2002	13 ± 3	x	No
JBC/S24	*S. elongata*	99.6%	People's Republic of China, Soil from Shanghai	31°06′21N 121°22′31E	5	21/11/2002	15 ± 6	x	No
JBC27	*C. annularis*	92.0%	Peoples Republic of China, Small pond in Huqiu	31°20′05N 120°34′27E	4	20/11/2002	205 ± 22		No
JBM28	*C. dendrolepidota*	95.1%	Austria, Lake Schwarzensee	47°45′0N 13°29′50E	716	13/11/2002	31 ± 12		No
JBC29	*P. formamifera* SOTON A	95.2%	People's Republic of China, Lake Tai Hu	31°30′0N 120°20′0E	3	17/11/2002	58 ± 18		No
JBC30	*P. malhamensis*	94.9%	People's Republic of China, Lake Tai Hu	31°30′0N 120°20′0E	3	19/11/2002	28 ± 10		No
JBC31	*P. malhamensis*	94.9%	Peoples Republic of China, Pond 2 in Sushou, The Humble Administrator's Garden	31°18′28N 120°37′10E	3	19/11/2002	27 ± 9		No
JBAF32	*P. malhamensis*	94.9%	Uganda, Lake Nkuruba	0°37′0N 30°16′0E	1400	22/03/2003	66 ± 23		No
JBAF33	*P. malhamensis*	94.9%	Tanzania, Msimbazi River	5°15′0S 38°49′60E	151	20/03/2003	68 ± 20		No
JBAF35	*Oikomonas*	98.9%	Kenya, River Sagana	0°40′0S 37°12′0E	1207	28/03/2003	58 ± 13		No
JBAS36	'S. danica'	98.7%	Nepal, Nag Pokhari, Kathmandu	27°43′0N 85°19′0E	1298	27/03/2003	35 ± 17		No
JBAS37	*P. vestita* PV10	99.7%	Nepal, Ranipokhari, Kathmandu	27°43′0N 85°19′0E	1298	30/03/2003	202 ± 52		No
JBNZ39	*S. obliqua*	97.9%	New Zealand, Shallow tarn near Karangarua	43°37′0S 169°46′0E	1118	01/02/2003	25 ± 8		No
JBNZ40	*P. formamifera* SOTON A	95.2%	New Zealand, Small lake near Mandeville	46°0′0S 168°49′0E	104	02/02/2003	119 ± 26		No
JBNZ41	*P. malhamensis*	94.9%	New Zealand, Lake Aviemore	44°40′60S 170°22′0E	212	03/02/2003	62 ± 11		No
JBNZ43	*P. vestita* SOTON 1	94.3%	New Zealand, Small stream near Ashburton	43°58′0S 171°46′0E	60	05/02/2003	292 ± 129		No

Table 2. Known sequences of clade C of the Chrysophyceae (18S rRNA gene) following Andersen and colleagues (1999). The origin of the strains and the GenBank entry number are shown.

Strain name	Origin of strain and identification number	GenBank entry	Reference
Spumella danica nov. sp.	Jutland, Denmark: soil	AJ236861	I. Bruchmüller, A. Mylnikov, K. Juergens and T. Weisse, unpublished
Spumella elongata	**Type strain** Girton, Cambridgeshire, UK: soil. CCAP strain no. 955/1	AJ236859	Bruchmüller (1998)
Spumella obliqua	Baden-Württemberg, Germany: Lake Constance – freshwater	AJ236860	Bruchmüller (1998)
Spumella sp. 15G	Schleswig-Holstein, Germany: Lake Behler See – freshwater	AJ236857	Bruchmüller (1998)
Spumella sp. 37G	Schleswig-Holstein, Germany: Pond near Plön – freshwater	AJ236858	Bruchmüller (1998)
Spumella sp. SpiG	Schleswig-Holstein, Germany: Lake Plußsee – freshwater	AJ236862	Synonym to *Spumella* sp. SpG Bruchmüller (1998)
Poterioochromonas malhamensis	Freshwater MCC-NIES strain MBI HT2 (no longer available)	AB02307	Andersen *et al.* (1999)
Poterioochromonas stipitata	Michigan, USA: roadside ditch – freshwater CCMP strain 1862	AF123295	Andersen *et al.* (1999)
Ochromonas CCMP1278	Port Phillip Bay, Melbourne, Australia: marine CCMP strain 1278	U42382	Andersen *et al.* (1999)
Ochromonas CCMP 584	Sargasso Sea: marine CCMP strain 584	U42381	Andersen *et al.* (1999)
Ochromonas danica	**Type strain** Everdrup, Denmark: Bog-pool – freshwater. UTEX strain 1298	M32704	Gunderson *et al.* (1987)
Ochromonas sphaerocystis	Arkansas, USA: small stream – freshwater CCMP strain 586	AF123294	Andersen *et al.* (1999)
Ochromonas tuberculata	Illinois, USA: Volo Bog – freshwater CCMP strain 1861	AF123293	Andersen *et al.* (1999)
Uroglena americana	Alberta, Canada: Glenmore reservoir – freshwater CCMP strain 1863	AF123290	Andersen *et al.* (1999)
Chrysoxis sp.	Washington, USA: North Atlantic – marine CCMP strain 591	AF123302	Andersen *et al.* (1999)
Dinobryon sociale var. *americana*	Maine, USA: west Boothbay Harbour – marine CCMP strain 1860	AF123291	Andersen *et al.* (1999)
Dinobryon sertularia	Alberta, Canada: Glenmore reservoir – freshwater CCMP strain 1859	AF123302	Andersen *et al.* (1999)
Chrysonephele palustris	Tasmania, Australia: Golden Cloud Swamp – freshwater	U71196	Saunders *et al.* (1997)
Chrysolepidomonas dendrolepidota	Michigan, USA: Lake Medora – freshwater CCMP strain 293	AF123297	Andersen *et al.* (1999)
Epipyxis aurea	Minnesota, USA: Darling Pond – freshwater CCMP strain 385	AF123298	Andersen *et al.* (1999)
Epipyxis pulchra	Minnesota, USA: Darling Pond – freshwater CCMP strain 382	AF123301	Andersen *et al.* (1999)

CCAP: Culture Collection of Algae and Protozoa UK; CCMP: Provasoli – Guillard National Centre for Culture of Marine Phytoplankton; MCC-NIES: Microbial Culture Collection at the national institute for environmental studies; UTEX: University of Texas, Culture Collection of Algae.

sequences characterized by the A/T-rich insertion sequences. The largest A/T-rich insertions as found in *P. vestita* PV10 were identically present in JBAS37.

Discussion

Older systems of classification have been based on vegetative morphological features and characteristics of the motile cell, especially the flagellar number and position (Preisig, 1995). Even though the flagellar number has been disregarded as a major taxonomic criterion (Kristiansen, 1986, 1990) it is still used for separating genera. Members of the chrysophycean order Chromulinales (following Preisig, 1995) possess two flagella where the second flagellum is short in *Chromulina* spp. and *Oikomonas* spp. and long in *Ochromonas* spp. and *Spumella* spp. Except for this difference *Ochromonas* and *Chromulina* on the one hand, and *Spumella* and *Oikomonas* on the other are similar (Preisig, 1995).

All of our isolates possessed a spherical to ellipsoidal cell body and attached to the substrate when satiated. Most strains possessed two flagella, a long and a short one, emerging from the anterior end. The short flagellum was several micrometres in length and following the light microscopical and electron microscopical investigations the isolates belong to the *Spumella*/*Monas* group. The strains specifically affiliated with cluster F possess scales and thus belong to the genus *Paraphysomonas* (G. Novarino, pers. comm.).

Only in the strain JBAF35 was the short flagellum not visible in the light microscope, or extremely short, and the strains may therefore be affiliated with *Oikomonas*. This

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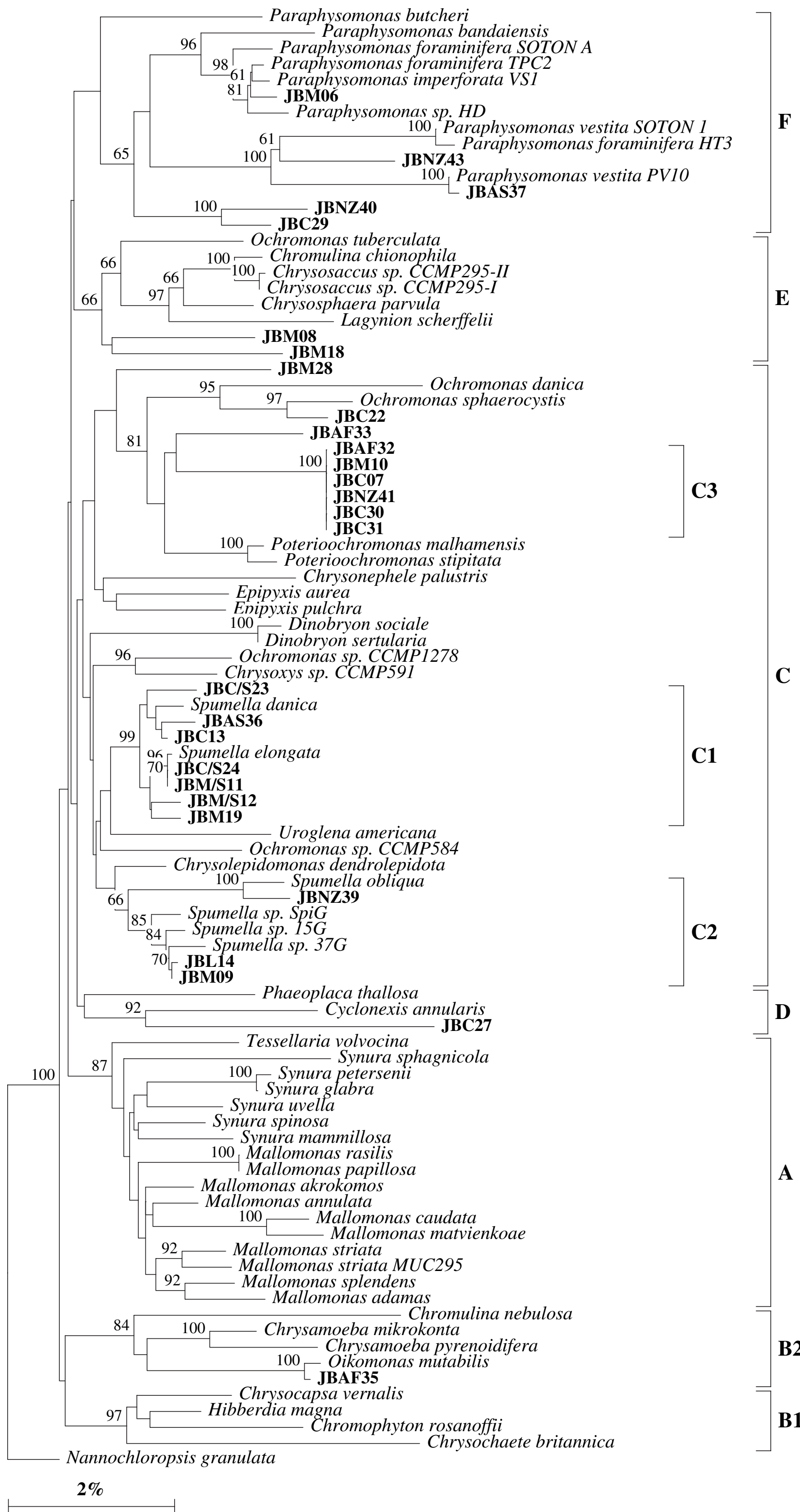

Fig. 1. Neighbour-joining tree showing the affiliation of 18S rRNA gene sequences from '*Spumella*-like' isolates to the Chrysophyceae *sensu stricto*. The numbers at the nodes of the tree indicate percentage of bootstrap values for each node out of 100 bootstrap resamplings (values above 60 are shown). The scale bar indicates 2% estimated sequence divergence.

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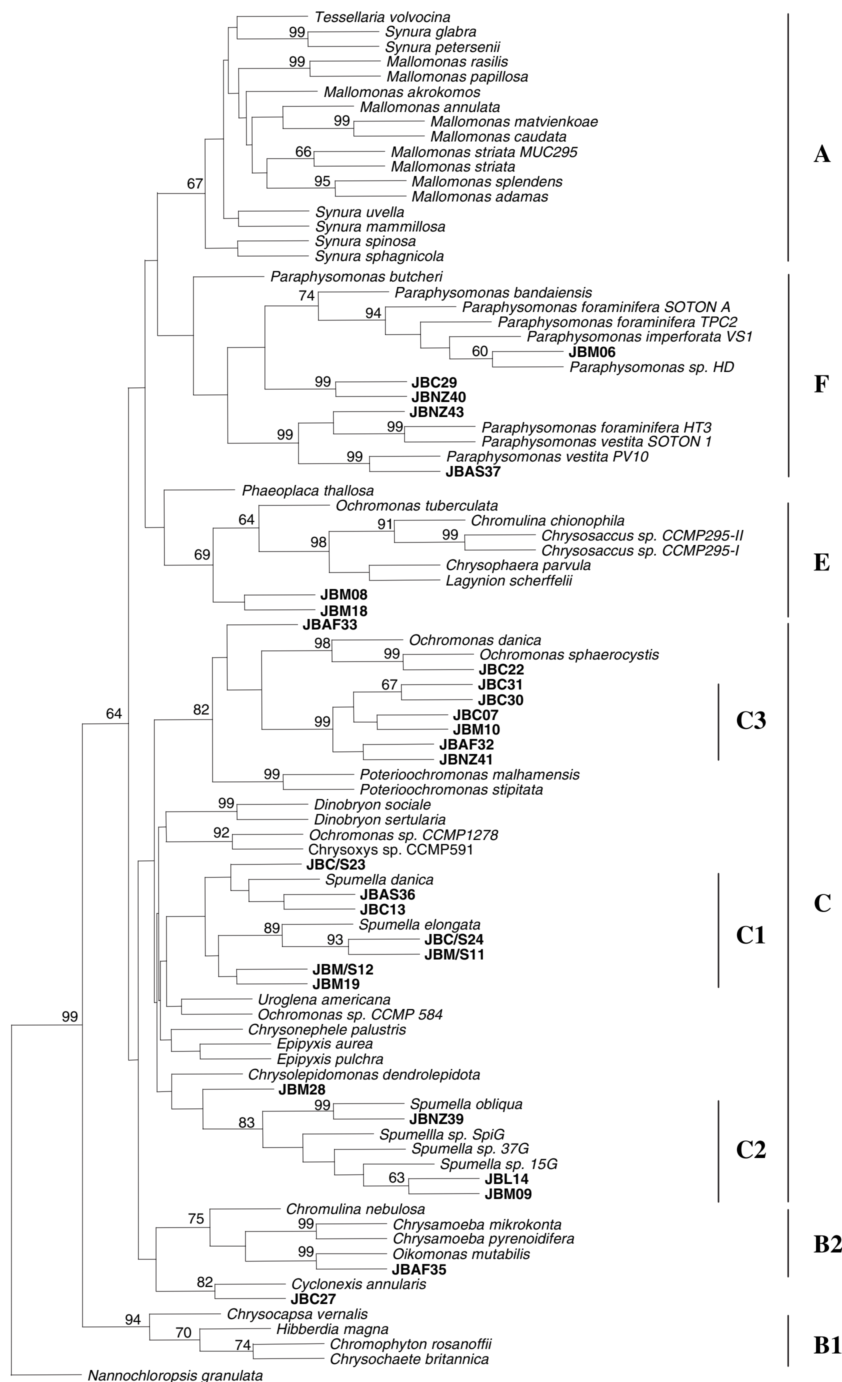

Fig. 2. Parsimony tree showing the affiliation of 18S rRNA gene sequences from '*Spumella*-like' isolates to the Chrysophyceae *sensu stricto*. The numbers at the nodes of the tree indicate percentage of bootstrap values for each node out of 100 bootstrap resamplings (values above 60 are shown).

conclusion seems to be supported by the high 18S rRNA gene sequence similarity of JBAF35 and *Oikomonas mutabilis*. We observed, however, no consistent trend in length and visibility of the short flagellum, and a distinction between *Spumella* and *Oikomonas* based on only this character may be problematic. In general, the lack of distinctive characters in the '*Spumella*-like' flagellates, as discussed above, may not allow for the separation of taxa based solely on morphological characters.

Some prominent genera within the chrysophytes seem to be polyphyletic

In agreement with the study of Andersen and colleagues (1999) we found indications that *Ochromonas* is polyphyletic and occupied different branches within clade C and clade E. Polyphylie can also be expected for the colourless analogue of *Ochromonas*, i.e. *Spumella*. In fact, we found a high 18S rRNA gene diversity of the

'*Spumella*-like' flagellates, which grouped within different clades in the class Chrysophyceae *sensu stricto* (Andersen *et al.*, 1999). Isolates affiliated with the cluster F belong to the genus *Paraphysomonas* as they possess scales (G. Novarino pers. comm.). The genus *Spumella*, however, is polyphyletic and distributed throughout at least three subclusters which are characterized by isolates of different habitat types. All isolates from soil were affiliated with cluster C1. There are also three aquatic isolates grouping within this cluster, but we cannot exclude the possibility that these strains were introduced into the aquatic environment from the surrounding soils. Further studies are required to prove that SSU rRNA gene sequences are a useful marker to distinguish '*Spumella*-like' soil flagellates from aquatic strains. Similarly, it may be possible to attribute some rough ecological characteristics to the subcluster C3 strains. Most strains of the C3 cluster originated from shallow, often eutrophic habitats and our attempts to obtain axenic flagellate cultures succeeded only with isolates from this cluster (J. Boenigk, unpublished data). This suggests that some essential factors can be synthesized by these isolates in laboratory cultures. Specific nutritive requirements of chrysophytes are already indicated for the mixotrophic genera *Ochromonas* and *Poterioochromonas* (cf. Holen and Boraas, 1995).

*The '*Spumella*-like' flagellates are a diverse group both in terms of sequence dissimilarity between isolates and in terms of the number of genotypes*

The phylogenetic distance to known sequences was high for many of our isolates and thus the '*Spumella*-like' flagellates seem to be a very diverse group. Isolation attempts from samples obtained from 24 ecologically contrasting freshwater and soil habitats located in six climatic zones resulted in 28 isolates distributed over the entire SSU rRNA tree of the Chrysophyceae *sensu stricto*. Some of these isolates, e.g. JBM18 probably represent even novel lineages within this group, indicating that analysis of further isolates is necessary to support these lineages. Furthermore, we probably even underestimated the diversity of these flagellates, as the isolation approach we used in our study must be assumed to be selective: isolation efficiency using direct dilution of environmental samples was as low as 1–2% only, but could be increased to >10–20% by way of short-term acclimatization. The isolation procedure we used selected for small bacterivorous flagellates with medium to high growth rates, probably resulting in the isolation of only the fast-growing fraction of the '*Spumella*-like' organisms. Long-term controls confirmed that the positive growth of these flagellates could be definitely detected within the subculturing periods of 5–7 days and it can be assumed that at least these acclimatized flagellates in particular were the only fast-growing strains. Except the bias resulting from the initial treatment, i.e. cell losses because of sampling and shifts during the initial acclimatization period, a further bias resulting from the isolation protocol is therefore assumed to be low. The literature data on isolation efficiency are rare, but low isolation efficiency has also been observed by other researchers (G. Novarino, pers. comm.). Low efficiency of isolation is also supported by the contrasting high diversity of free-living protists and the relatively small group of protists successfully cultured in the laboratory (cf. Cowling, 1991). Recently, culture-independent molecular surveys on eukaryotic diversity in marine systems suggest that, there still is undescribed protistan diversity, particularly among small eukaryotic organisms (Moreira and Lopez-Garcia, 2002 and references therein). As isolation procedures often include an acclimatization period (Caron, 1993; Atkins *et al.*, 2000; Lim *et al.*, 2001), the dominance of the so-called 'laboratory weeds' do not, however, necessarily reflect a differential ability to grow in culture, but may indicate that these usually fast-growing organisms overgrow other protists during this acclimatization and consequently are isolated more often. The diversity of the '*Spumella*-like' flagellates must therefore be assumed to be even higher than is reported in this study. Given the fact that such a diverse group as the '*Spumella*-like' flagellates, which based on our sequence data probably comprises different independent lineages, is usually pooled in field investigations it is not surprising that correlations with habitat characteristics and environmental factors are often weak.

*Organisms affiliated with the '*Spumella*-like' clusters C1 through C3 seem to be distributed worldwide but ecophysiological characteristics seem to differ*

Diversity of protists and protist species richness are currently controversially discussed, mainly based on organisms possessing suitable morphological features, such as ciliates and the colourless chrysophyte genus *Paraphysomonas* (Finlay and Clarke, 1999a,b; Finlay and Fenchel, 1999; Foissner, 1999; Finlay, 2002). For instance, specific protistan morphotypes and even the same genotypes have been reported from different locations all over the world (Finlay, 2002). For the '*Spumella*-like' flagellates, because of the scarcity of both morphological features and molecular data, estimates of the diversity and their biogeographic distribution are even more complicated and it has even been proposed that most described species may belong to other taxa (Preisig *et al.*, 1991). Consequently, the '*Spumella*-like' flagellates are mostly pooled in ecological studies ('*Spumella*-like' flagellates or *Spumella* spp.) and this

group often comprises some *Paraphysomonas* spp. as well, because these two groups cannot sufficiently be separated by light microscopy (e.g. Weitere and Arndt, 2003). These '*Spumella*-like' flagellates are present in most ecosystems and are of general importance as one of the main eukaryotic heterotrophs in a variety of ecosystems often accounting for 20–50% of pelagic heterotrophic nanoflagellates (Carrick and Fahnenstiel, 1989; Sanders *et al*., 1989; Bennet *et al*., 1990; Carrias *et al*., 1998; Arndt *et al*., 2000; Weitere and Arndt, 2003). The '*Spumella*-like' flagellates therefore are regarded as a ubiquitous, worldwide-distributed group commonly found in freshwaters, soils and marine sites (Preisig *et al*., 1991). Accordingly, we detected '*Spumella*-like' organisms in all of the samples. In the 5 μm fraction these organisms were among the dominant organisms in most of the processed samples. Despite the lack of morphological distinctive characteristics we found evidence for a global distribution of closely related strains: For instance, the same genotype in terms of the 18S rRNA gene sequence similarity was successfully isolated from freshwater habitats located in Austria, China, New Zealand and East Africa, and very similar genotypes (>99.6% 18S rRNA gene sequence similarity) were obtained from soil habitats located in Austria, the UK and China. On the basis of the 18S rRNA gene sequence similarity, our findings may support the hypothesis of a worldwide distribution of microbial species (Finlay, 2002). Identical and very similar 18S rRNA sequences for eukaryotic microorganisms originating from geographically distant sites have been reported also for marine flagellates and marine clone sequences (Atkins *et al*., 2000; Massana *et al*., 2004). Although multiple rRNA gene copies with sequence variations seem to be present among different taxa of eukaryotic microorganisms (Ward *et al*., 1997; Rocio *et al*., 1998), we could not find any. However, eukaryotic microorganisms with very similar or identical rRNA gene sequence may show more differences in the more variable internal transcribed spacer (ITS) region (Tsuchiya *et al*., 2003). It remains to be evaluated if ITS-sequences may also aid in revealing cryptic speciation in heterotrophic nanoflagellates.

However, despite the high 18S rRNA gene sequence similarities of strains from geographically remote sampling sites, there are indications for ecological differences between these isolates. For instance, the isolates JBC07, JBM10 and JBNZ41 (18S rRNA gene sequence similarity 100%), respond differentially to ecological factors such as food concentration, food quality and suspended sediment concentrations (Boenigk *et al*., 2004; K. Pfandl, J. Boenigk and A. Wiedlroither, in prep.). These findings corroborate the idea that molecular data on the basis of the 18S rRNA gene may not suffice in separating ecologically different taxa and ecotypes. The above rough trends concerning ecological key parameters should therefore be interpreted carefully.

Conclusions

On the basis of these 18S rRNA gene sequence data, global distribution of the '*Spumella*-like' flagellates must be assumed, but there are indications that even closely related isolates show different ecophysiological abilities, which may restrict these organisms to a certain habitat or geographical region. Following our phylogenetic analysis we conclude that: (i) the '*Spumella*-like' flagellates are a very diverse group, and deduced from the relatively high phylogenetic distance of many isolates to the next known organisms, diversity seems to still be strongly underestimated; (ii) several chrysophycean genera including *Ochromonas* and *Spumella* are polyphyletic; and (iii) based on the SSU rRNA gene sequence there are thus far no indications for a geographic restriction, but there may be different ecophysiological abilities characterizing certain branches of '*Spumella*-like' flagellates.

Experimental procedures

Sampling sites

Samples from 24 ecologically contrasting freshwater and soil habitats located in six climatic zones were processed in order to isolate representative '*Spumella*-like' flagellates (Table 1). Samples taken from Lake Mondsee and sampling sites in Mondsee were immediately processed after sampling. Samples from the other lakes in the Salzkammergut area were processed within 6 h after sampling. All other samples were transported to the laboratory in sealed tubes and processed within a few days after sampling. All treatments after sampling were carried out under aseptic conditions.

Media for isolation and the maintenance of strains

Standard medium for the maintenance of strains and for the isolation process, except for the first step, was an artificial inorganic basal medium (NSY medium: Hahn *et al*., 2003). Sterile-filtrated lake water for the first isolation step was prepared using water sampled from a depth of 1 m in Lake Mondsee. Water was filtrated through 0.1 μm gauze and subsequently repeatedly heated in the microwave for 7.5 min at 700 W. This procedure killed all of the bacteria that might have been present in the filtrate. To obtain lake water containing a natural bacterial assemblage as a food source, the lake water was filtered through 1.2 μm filters. This filtrate contained $2–3 \times 10^6$ bacteria per millilitre.

Different bacterial strains, representatives of typical free-living aquatic bacteria and a natural bacterial community were used as food for isolating flagellates: The ultramicrobacterial actinobacterium strain (<0.1 μm^3) MWH-Mo1 [closest known relative *Clavibacter michiganensis* (Microbacteriaceae); Hahn *et al*., 2003], the bacterial strain *Listonella pelagia* CB5 (Hahn, 1997), and a natural bacterial

community from Lake Mondsee sampled from a depth of 1 m (for isolation of strains from Lake Mondsee only). The filtrate was subsequently checked for flagellate contamination. After successful isolation of the flagellate strains, they were transferred to permanent culture using NSY medium supplemented with wheat grain.

Isolation protocol

A modified filtration–acclimatization method (Hahn *et al*., 2004) adjusted for the isolation of flagellates was used. Briefly, 5–20 ml of the sample was filtered through 5 μm-pore-size filters (Minisart syringe filters; Sartorius, Göttingen, Germany) and collected in sterile Erlenmeyer flasks. After 16–24 h of acclimatization at 15–25°C depending on the origin of the samples, subsamples of 5 ml were stepwise diluted (three subsequent 1:1 dilutions) with sterile-filtrated lake water. After 2–4 h the flagellates were counted using a Sedgewick-Rafter chamber, and a subsample was diluted into a final flagellate abundance of 0.5–1 flagellates per millilitre and subsequently transferred to two to four 24-well cell culture plates. Wells were supplemented with food bacteria at a concentration of $3–5 \times 10^6$ bacteria per millilitre, i.e. either a 1.2 μm filtrate of freshly sampled lake water (only for samples from Lake Mondsee), or cultures of the bacterial strains MWH-Mo1 or *L. pelagia* CB5.

Wells were checked every second day for a period of at least 2 weeks for positive growth under the microscope using a total magnification of 200×. When flagellate growth was detected, the medium was transferred to a 50 ml Erlenmeyer flask containing inorganic NSY medium and fresh food bacteria. After 2–6 days the subsamples were further diluted to a final concentration of 0.05, 0.1, 0.2 and 0.4 flagellates per millilitre and supplemented with the bacterial strains MWH-Mo1 or CB5 at a concentration of $15–25 \times 10^6$ bacteria per millilitre. Each of these dilutions were transferred to wells of sterile 24-well cell culture plates (1 ml per well) and incubated at 15–22°C depending on the origin of the isolates. Screening of the wells for the growth of flagellates was again performed by direct microscopical investigation every second day. Finally, flagellates were transferred to an Erlenmeyer flask containing fresh medium and bacteria. This procedure was repeated until pure cultures were established, but at least four times. Pure cultures were acclimatized to 15°C and transferred to permanent culture.

Isolation efficiency

The cultivable fraction of HNF was estimated by way of the direct dilution of freshwater samples. This test was only performed exemplarily for four samples from Lake Mondsee. Flagellates in the samples were counted using a live counting technique in a Sedgewick-Rafter chamber. A subsample was then diluted to a concentration of 0.5–1 flagellate per millilitre using 1.2 μm filtrated water from the same sample. Flagellate abundance was again checked and 1 ml was transferred to each well of 24-well cell culture plates and incubated at an *in situ* temperature. Altogether 16 plates were used per sample. The wells were checked microscopically for flagellates every two to three days for a period of at least 30 days.

DNA extraction and PCR of 18S rRNA genes

DNA was isolated from cell pellets or 0.5 ml cell cultures. Cells were incubated with 1.5 ml extraction buffer (10 mM Tris-HCl, 0.4% SDS, 10 mM EDTA) at 65°C, followed by incubation with proteinase K at 60°C (0.2 mg ml^{-1}). DNA was pelleted with ethanol after phenol-chloroform extraction. Small subunit ribosomal RNA genes were amplified using one of the two forward primers CTGGTTGATCCTGCCAG (*Paraphysomonas foraminifera* AB022864 position 30) and GAAACTGCGAATGGCTC (*P. foraminifera* position 109), and one of the two reverse primers GTAGGTGAACCTGCAG (*P. foraminifera* position 1824) and GTGAACCTGCAGAAG GATCA (*P. foraminifera* position 1828). The polymerase chain reaction (PCR) mixture contained 0.2 μM of each primer, 200 μM of each deoxynucleoside triphosphate, 2 mM $MgCl_2$, 1.25 U of *Taq* DNA polymerase (Qiagen), and 1× PCR buffer. Reactions were carried out in a PTC 200 thermocycler (MJ Research) starting with a denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 58°C for 1 min, elongation at 72°C for 2 min, and a final extension step of 10 min at 72°C. PCR products were checked on an agarose gel.

Sequencing of 18S rRNA genes and phylogenetic analysis

Polymerase chain reaction products were either directly sequenced or used for subsequent cloning into the vector pGEM-T Easy (Promega) by following the manufacturer's recommendations. Putative positive colonies were picked and directly amplified, using the vector primers T7 and SP6. The products were checked on an agarose gel. Sequencing reactions were performed with an ABI Prism® Big Dye™ Terminator v 3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosciences) and an ABI PRISM model 3100 automated sequencer. Sequences were submitted to the BLAST search program of the National Center for Biotechnology Information (NCBI) to find closely related sequences. Sequences were aligned using the 'CLUSTAL W' option (Thompson *et al*., 1997) in the BioEdit 5.0.9 sequence analysis software (Hall, 1999). Where necessary alignments were subsequently manually processed and corrected. Positions of unclear homology and AT-rich insertions were excluded from further phylogenetic analysis. The result was a final alignment of 1630 positions (including gaps). Sequence identities between isolates and the next related sequences were based on aligned whole gene sequences of equal length, and were calculated using the 'sequence identity matrix' option in BioEdit. The position of the unclear homology and A/T-rich insertions were excluded from further phylogenetic analysis. The TREECON 1.3b software package was used to calculate distance matrices by the Kimura algorithm (Kimura, 1980) and to generate phylogenetic trees by the neighbour-joining method (van de Peer and de Wachter, 1994). Parsimony trees were calculated using the program package PHYLIP (version 3.5; J. Felsenstein, Department of Genetics, University of Washington, Seattle). One hundred bootstrapped replicate resampling data were generated with SEQBOOT (PHYLIP). The full-length 18S rRNA gene sequences determined in this study have a length of 1695–1803 bp and have

been deposited in the NCBI database under accession numbers AY651071–651098.

Cell size and autofluorescence

Live cells from early stationary growth phase were taken for measuring cell dimensions. Cells were transferred to observation vessels (Boenigk and Arndt, 2000) and recorded on video tape using 500× to 1000× magnification. The length and width of at least 30 cells was measured directly from the video screen and the cell volume was calculated assuming an ellipsoid or a spherical cell shape. Cells were also checked for the occurrence of chloroplasts.

In addition, cells were checked for autofluorescence: Cells were fixed with formaldehyde (final concentration 2%) and stained with DAPI (final concentration 10 μg ml^{-1}) for 30 min. The cells were then filtered onto a black nucleopore 0.2 μm filter backed by a 0.45 μm cellulose nitrate filter and examined under an epifluorescence microscope using UV and blue light excitation for DAPI and for chlorophyll autofluorescence respectively.

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