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Small but Manifold – Hidden Diversity in "*Spumella*-like Flagellates"

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ABSTRACT

Colourless, nonscaled chrysophytes comprise morphologically similar or even indistinguishable flagellates which are important bacterivors in water and soil crucial for ecosystem functioning. However, phylogenetic analyses indicate a multiple origin of such colourless, nonscaled flagellate lineages. These flagellates are often referred to as "*Spumella*-like flagellates" in ecological and biogeographic studies. Although this denomination reflects an assumed polyphyly, it obscures the phylogenetic and taxonomic diversity of this important flagellate group and, thus, hinders progress in lineage- and taxon-specific ecological surveys. The smallest representatives of colourless chrysophytes have been addressed in very few taxonomic studies although they are among the dominant flagellates in field communities. To overcome the blurred picture and set the field for further investigation in biogeography and ecology of the organisms in question, we studied a set of strains of specifically small, colourless, nonscaled chrysomonad flagellates by means of electron microscopy and molecular analyses. They were isolated by a filtration-acclimatisation approach focusing on flagellates of around 5 μm. We present the phylogenetic position of eight different lineages on both the ordinal and the generic level. Accordingly, we describe the new genera *Apoikiospumella*, *Chromulinospumella*, *Segregatospumella*, *Cornospumella* and *Acrispumella* Boenigk et Grossmann n. g. and different species within them.

NONSCALED, coulourless chrysomonad flagellates (=nonscaled, colourlesschrysphytes) are major phagotrophs in freshwater and soil food webs (Boenigk and Arndt 2002; Del Campo and Massana 2011; Ekelund et al. 2001; Finlay and Esteban 1998; Richards and Bass 2005) and are among the dominant feeders on bacteria (Berninger et al. 1991; Šimek et al. 2013).

Scaled, colourless chrysomonad flagellates have recently been revised, specifically the genus *Paraphysomonas* and the newly erected genus *Clathromonas* (Scoble and Cavalier-Smith 2014). Colourless chrysomonad taxa lacking surface scales seem to be at least as diverse (Boenigk 2008b). Due to the lack of surface scales, these latter taxa are even harder to distinguish based on morphology. As one consequence, these flagellates have often been merged as *Spumella* spp. (Boenigk 2008a; Boenigk et al. 2005) irrespective of the molecular diversity and polyphyly of this group (Boenigk 2008a) calling for major revisions. As rDNA phylogenies suggest, colourless,

nonscaled chrysomonad flagellates are not monophyletic, but have lost photosynthesis at least five times independently in different lineages and, thus, became *Spumella*-like in morphology (Boenigk 2008a; Boenigk et al. 2005; Cavalier-Smith and Chao 2006; Stoeck et al. 2008). The terms "*Spumella*-like flagellates", "*Spumella*-like cells" or "*Spumella* sensu Cienkowsky" often used in the ecological literature hint at the current uncertainties and the potential polyphyly of the genus *Spumella* (Berglund et al. 2005; Boenigk et al. 2005; Charvet et al. 2012; Lepère et al. 2006). Eventually, this genus must be divided into several genera (Boenigk 2005; Findenig et al. 2010; Scoble and Cavalier-Smith 2014).

Generic affiliation of colourless, nonscaled chrysomonad taxa

Several colourless, nonscaled chrysophyte genera have been described including *Spumella* Cienkowsky 1870,

Monas Müller 1773, *Oikomonas* Kent 1880 (also *Eucomonas* or *Oicomonas*), *Synoikomonas* Skuja 1964 and *Paramonas* Kent 1880. Several further generic names have been proposed but are considered to be synonyms of one of the above names, specifically *Heterochromulina* Pascher 1912 and *Heterochromonas* Pascher 1912.

The genus *Monas* Müller 1773 is one of the first flagellate genera ever described [Müller 1773; "Vermis inconspicuus, simplicissimus, pellucidus, punctiformis" (inconspicuous, most simple, transparent and punctiform worm)]. Organisms affiliated with most major branches of eukaryotes and even bacteria have subsequently been lumped into this genus. Later, many of these organisms have been removed, however, still leaving the genus *Monas* as a polyphyletic collection of species and considerable doubts about the identity of its lectotype, *Monas mica* Müller 1773, selected by Diesing (1850).

Spumella Cienkowski 1870 has been synonymised with *Monas* Müller 1773, but Silva (1960) and later Preisig et al. (1991) recommended that the genus *Monas* should be abandoned because of the uncertainties pertaining to the identity of its lectotype. The strains of *Spumella* investigated so far were all characterised by a naked cell surface, heterokont flagella emerging from an apical depression with mastigonemes on the long flagellum, while the short flagellum is naked, and mitochondria with tubular cristae. A number of other structures differed between strains; for instance, they may or may not possess a leucoplast, a flagellar swelling, an eyespot or mucocysts (Bruchmüller 1998; Preisig and Hibberd 1983). Only a few studies have investigated the stomatocysts in colourless, nonscaled chrysomonad flagellates with a *Spumella*–like morphology (Belcher and Swale 1976; Cienkowsky 1870; Findenig et al. 2010; Yubuki et al. 2008). Cyst morphology, as well as scale morphology in scaled chrysophycean taxa, is believed to be species-specific and thus a suitable criterion for differentiating species (Findenig et al. 2010; Sandgren 1991). Based on the morphology of stomatocysts, Findenig et al. (2010) designated an epitype for *Spumella vulgaris* Cienkowsky 1870, i.e. for the type species of the genus.

Heterochromonas Pascher 1912 has been proposed as a generic name for biflagellated, colourless, and nonscaled chrysomonad flagellates based on the assumption that these flagellates are the colourless counterpart of *Ochromonas*, i.e. a phylogenetic sister group differing by the reduction in the plastids. Pascher (1912) and Bourrelly (1957) suggested to use the genus name *Heterochromonas* for those flagellates of the *Monas*/*Spumella* morphodeme with known stomatocysts. In contrast, Skuja (1939, 1948, 1956) rejected cyst formation as a criterion for the separation of genera. From a taxonomic perspective, the generic name *Heterochromonas* is a synonym of *Spumella,* and as *Spumella* has priority, this generic name can be rejected. From a phylogenetic point of view, this generic name should be used for a colourless sister taxon of the clade comprising the type species of *Ochromonas* Wysotzki 1887 (i.e. *Ochromonas triangulata* Wysotzki 1887). No sequence data exist for *O. triangulata* and the

type strain is unavailable and has never been found again. However, *Ochromonas moestrupii* Andersen 2011 is very similar to *O. triangulata* and has even been proposed as a promising candidate for an epitype (Andersen 2011). Based on the presumable monophyly of *O. triangulata* and *O. moestrupii,* the molecular affiliation of the type species of *Ochromonas* can, therefore, be deduced although molecular data for the type species itself are missing. *Heterochromonas* may be considered sister to the clade comprising *O. moestrupii,* which clusters close to *Dinobryon* in phylogenetic analyses, but not with one of the strains described herein.

Related to the above case of the genus *Heterochromonas* is the potential inclusion of some colourless, nonscaled chrysomonad flagellates within the genus *Ochromonas.* Wysotzki (1887) described two species from which *O. triangulata* Wysotzki was designated as lectotype for the genus by Bourrelly (1957). The polyphyly of *Ochromonas,* as shown in molecular studies, indicates that there are several genera that have a similar morphology (i.e. they are naked single cells with two heterodynamic flagella, but otherwise are distinctive genetically) (Andersen 2011). Nomenclaturally, the name *Ochromonas* will be applied to the clade containing the type species (Andersen 2011). Despite intensive investigations of the type locality (Lake Veisovo, a salt lake in the Ukraine) by Andersen 2011, the type species could not be found again. As there are neither sequence data nor type material to make such sequence data available, the molecular identity of the type species cannot be verified. It is, however, very likely that *O. moestrupii* is a very close relative as it resembles *O. triangulata* in several ways; furthermore, both species originate from salt water. Based on the close resemblance of both species, *O. moestrupii* has been proposed as a potential epitype and is, therefore, the currently best choice for rooting the genus *Ochromonas* in molecular trees (Andersen 2011). Species which are affiliated with *Ochromonas,* but are phylogenetically affiliated with other clades, therefore, probably do not belong into the genus *Ochromonas* and await taxonomic revision.

For heterotrophic, nonscaled chrysomonad flagellates with a largely reduced second flagellum the generic names *Oikomonas* Kent 1880 (also *Eucomonas* or *Oicomonas*) and *Heterochromulina* Pascher 1912 have been proposed: *Oikomonas* is regarded as the colourless counterpart of *Chromulina* Cienkowsky 1870 by some authors, however, the identity of the genus is not clear (Preisig et al. 1991; Silva 1960). For both genera, type material of the type species is not available for molecular analyses, however, strains affiliated with the type species of *Oikomonas* have been isolated later, and these latter strains have been sequenced. Based on these sequence data, *Oikomonas* spp. (with *Oikomonas mutabilis* Kent 1880 as the type species) cluster as a sister group to *Chromulina* spp. (with *Chromulina nebulosa* Cienkowsky 1870 as the type species) (Cavalier-Smith and Chao 2006). Pascher (1912) proposed the generic name *Heterochromulina* for colourless nonscaled chrysomonad flagellates with a very

short second flagellum which are sister to *Chromulina*. However, as *Oikomonas* Kent has priority over *Heterochromulina* Pascher the latter name is only a synonym and has been included in *Oikomonas* (Preisig et al. 1991). From a phylogenetic point of view, *Heterochromulina* is sister to the clade comprising *C. nebulosa*. However, as *Oikomonas* spp. cluster as a sister clade to *Chromulina* and *Oikomonas* has priority over *Heterochromulina*, the latter name must be regarded as a synonym to *Oikomonas* and should not be further used to avoid confusion.

The genus *Paramonas* Kent 1880 has been erected for uniflagellate taxa formerly affiliated with the genus *Monas* Müller, which have a distinct oral aperture. A type species has not been designated by Kent, but Cavalier-Smith and Chao (2006) designated *Paramonas globosa* as type for the genus providing further morphological and molecular data based on an ATCC isolate of this species. Based on the phylogenetic data, the genus *Paramonas* belongs to the Pseudodendromonadales (Bicoecia) (Cavalier-Smith and Chao 2006).

The genus *Physomonas* Kent 1880 and its type species *Physomonas socialis* (Ehrenberg) Kent 1880 differ considerably from the flagellates described herein (Kent 1880; as *Bodo socialis* in Ehrenberg 1832). *Physomonas* is usually attached to the substratum by a thread-like pedicle, and food uptake takes place at all parts of the periphery. In contrast, food uptake in small, colourless chrysomonad flagellates occurs only at the anterior part of the organisms near the flagellar roots, and thread-like pedicles do not occur in these flagellates. More importantly, the mode of spore formation is very different. A subdivision of the body into many spores, as described for *Physomonas,* has never been observed for the flagellates described herein and the genus *Physomonas* can be excluded.

The genus *Pedospumella* Boenigk et Findenig has recently been erected for small, colourless, nonscaled chrysomonad flagellates based on molecular data and cyst morphology. The vegetative cell of the type species *Pedospumella encystans* Findenig et Boenigk 2010 is similar to that of *S. vulgaris* (Cienkowsky) Findenig et Boenigk 2010, however, molecular data clearly show that *Pedospumella* forms a separate clade not related to the clade containing *Spumella*.

Similarly, the genus *Poteriospumella* Boenigk et Findenig 2010 has been erected for phylogenetically divergent lineages based on sequence information. Phylogenetically, this genus is the sister taxon to *Poterioochromonas* Scherffel 1901, but differs in lacking the characteristic stalk of *Poterioochromonas* as well as in lacking photosynthesis. Furthermore, considerable differences in gene sequence data support *Poteriospumella* as a separate genus.

The genus *Apoikia* Kim et al. 2010 has been erected by Kim et al. (2010) with *A. lindahlii* Skuja 1956 as the type species using *Monas lindahlii* Skuja 1956 as basionym. Molecular data show that this taxon and related strains form a separate clade (i.e. the Apoikiida) within Chrysophyceae (Scoble and Cavalier-Smith 2014). The genus is described as colonial with the cells growing in colourless mucilage. As none of our isolates grow in a colony or are surrounded by mucilage, this generic name can also be rejected for any of our isolates.

Species identity of colourless, nonscaled chrysomonad flagellates

As outlined above, most of the strains described in this study are not affiliated with one of the described genera. However, we need to consider whether one of our strains may belong to a previously described species, which (based on the above considerations) would then need to be transferred to another genus. More than 100 of such colourless, nonscaled chrysomonad taxa have been formally described. The described species affiliated with the above genera deviate from the strains described in this study (see Table S2 for *Monas* spp. and *Spumella* spp.). Due to the high degree of morphological similarities of colourless, nonscaled chrysomonad taxa, molecular data seem inevitable for differentiating species and genera. However, for most described species no sequence data or cultures are available. Exceptions are the type species of the genera *Pedospumella*, *Poteriospumella*, *Spumella* sensu Boenigk et Findenig, and *Apoikia* Kim et al. 2010 as well as few other strains all of which differ in SSU rRNA gene sequence from the taxa described herein.

Most of the taxa described to date have a cell size of 12 μm or more. This is in contrast to the dominance of colourless chrysomonad flagellates of around 5 μm or less in ecological studies over recent decades. Ecological as well as phylogenetic research has increasingly focused on these small representatives. The disproportionate number of descriptions of large species is likely due to isolation protocols. A recent example is the isolation of predominantly scaled strains by J.M. Scoble (personal communications) in contrast to the isolation of predominantly nonscaled strains by J. Boenigk (own unpublished data). Similar biases are to be expected between different past and recent isolation approaches, specifically considering enrichment cultures which made up for the majority of previous studies vs. direct isolation approach and dilution techniques such as filtration–acclimatisation approaches. The latter approaches exclude large taxa by a filtration step and allow for successful isolation and cultivation of otherwise sensitive strains by gradual acclimatisation (Boenigk et al. 2005; Hahn et al. 2004).

Boenigk et al. (2005) isolated several strains of colourless, nonscaled chrysomonad flagellates with cell sizes around 5 μm (between 1.2 and 8.6 μm) based on such filtration–acclimatisation protocols. To address the diversity of these small colourless nonscaled chrysomonad flagellates, we studied 16 clonal cultures which are, based on molecular data, placed within eight different genera. We describe six new species and introduce three new taxonomic combinations.

Although our isolates differ from all taxa described to date and, therefore, must be considered as new species, some of the formerly described species may be related to the taxa described herein and may eventually need to be

transferred to one of the new genera. This holds true specifically for taxa for which electron microscopic data are available but molecular data are missing. Future molecular studies on these strains – as far as available – may shed light on their phylogenetic position.

Taking into account the present level of research, we hypothesise that colourless, nonscaled chrysophytes are far more diverse than currently documented. We further hypothesise that microscopic methods (LM, light microscopy; SEM, scanning electron microscopy; TEM, transmission electron microscopy) are unable to resolve this diversity and, thus, molecular methods must be integrated in taxonomic and systematic studies of this group. We hypothesise that the phylogeny of "*Spumella*-like flagellates" is supported by analyses of different genetic loci (SSU, LSU, ITS, COX1) and suggest to routinely integrate molecular analyses in ecological and biogeographical studies of this group and of nanoflagellates in general.

MATERIALS AND METHODS

Cultivation of strains

The investigated strains were obtained from the culture collection at the University of Duisburg-Essen and originate from geographically and ecologically distinct sampling sites worldwide (for details see Table 1). Clonal cultures have been established by serial dilution and enrichment culturing (Boenigk et al. 2005, 2006). The strains are permanently cultivated in IB-medium (Hahn et al. 2003) supplemented with a sterilised wheat grain at 15 °C and in a light-dark cycle of 14:10 h with 65 µE. When grown solely in inorganic medium and better light conditions, all strains die. To achieve high abundances (up to 200,000 individuals per ml) for EM fixation and DNA isolation, strains were fed with bacteria (gammaproteobacterium *Limnohabitans pelagia*, strain CB5, GenBank synonym *Vibrio pelagius*, from Lake Constance, see Hahn and Höfle 1998). Three axenic strains (JBC07, JBM10, JBNZ41) were cultivated in NSY-medium (Hahn et al. 2003) achieving equally high abundances without bacterial prey.

Light microscopy

Light microscopic analyses were carried out with a Nikon Eclipse Ti inverse microscope (Nikon Corporation, Tokyo, Japan). Cultures were observed in specially prepared thin bottom petri dishes (Boenigk and Arndt 2000) and documented using NIS-Elements Basic Software.

Electron microscopy

To detect ultrastructural features on whole cells not visible by LM, both SEM and TEM (applied on positive-stained specimens) were applied. Furthermore, ultrathin sections were produced for six of the chosen strains (i.e. for strains 199hm [*S. vulgaris*], JBM10 [*Poteriospumella lacustris*], JBNZ41, JBC07, AR4D6 and JBMS11 [*P. encystans*]). Electron microscopy was carried out with a Zeiss EM10 at 60 kV for ultrathin sections and positive-stained specimens and with a Zeiss 940A for SEM. Strains were cooled down before fixation and fixed with 2.5% glutaraldehyde (final concentration) in 0.1 M Hepes buffer (pH = 7.2) for 1 h on ice. Positive contrast was carried out on an EM copper grid with 1% aqueous uranyl acetate for 30–60 s. For ultrathin sections, fixed cells were pelleted by centrifugation at 2,000 g, stained with 1% osmium tetroxide in 0.1 M Hepes buffer (pH = 7.2) on ice, dehydrated in an acetone concentration series (30%, 50%, 75%, 90%, 100%, 100%) for 15 min at room temperature respectively and embedded in Spurr Epoxy Resin (Spurr 1969). Ultrathin sections were obtained with a Leica UCT ultramicrotome and poststained on grids with 1% aqueous uranyl acetate for 6 min and lead citrate (after Venable and Coggeshall 1965) for 4 min at room temperature. For SEM, strains were filtered on polycarbonate filters (0.2 µm), critical point dried with CO_2 in a Balzers CPC 030 and sputtered with gold in an Emitech K 550 sputter coater.

DNA isolation, PCR and sequencing

Genetic analyses were carried out with both conservative (SSU, 5.8S, and LSU) and more variable (ITS) nuclear gene sequences, as well as with COX1. We isolated DNA from the cultures using BioBudget DNA mini-kit (BioBudget Technologies GmbH, Krefeld, Germany) and carried out PCR in a 33-cycle-programme (5-1-2-3 min; see Table 2) (Bock et al. 2011) with a BioRad MyCycler (Bio-Rad Laboratories, Hercules, CA). Sequencing was provided by Beckman Counter Genomics (Takeley, UK). For both PCR and sequencing, different forward and reverse primer combinations per locus were tested until sufficient and comparable results were achieved (see Table 2).

Phylogenetic analysis

Sequences were edited with DNADragon 1.5.2 (Hepperle 2012) and aligned in BioEdit Sequence Alignment Editor 7.1.3.0 (Hall 2011) using the ClustalW algorithm (default settings) and manual editing by eye. The SSU alignment (1,491 bp) follows a compilation of sequences (provided by J.M.Scoble) covering all known lineages of Chrysophyceae. Sequences of *Spumella* strains were added using *Sellaphora blackfordensis* and *Nannochloropsis granulata* as outgroups. For ITS1+5.8S+ITS2 (1,009 bp), LSU (1,034 bp) and COX1 (399 bp), mainly sequences of "*Spumella*-like flagellates" were aligned (see Table 1 for accession numbers) and an unrooted phylogeny was inferred. As a proof of principle for polyphyly, two photosynthetic chrysophycean species were added. The ITS sequences (ITS1+5.8S+ITS2) of the different *Spumella* strains showed such great diversity that proper alignment and phylogenetic inference was only possible for the 5.8S (156 bp). Phylogenetic trees and corresponding robustness measures (bootstrap analyses with 1,000 replicates, and posterior probabilities) were inferred for all loci with MEGA 5.0.1.102 (Tamura et al. 2011) for neighbour-joining and maximum-parsimony, with Treefinder (Jobb et al. 2004)

Table 1. Strains of *Spumella* morphology used in this study + *Spumella* 'species' and strains for which sequences were available (bolt print for herein investigated strains and newly designated species)

Strain designation	Origin	Accession no. SSU	Accession no. ITS	Accession no. LSU	Accession no. COX1	BGBM no.	SAG no.	species designation (former designation)
JBM08	Austria, Lake Mondsee[a,b]	AY651098	KF697325	KF697336	KF697353	B 40 004 1261	SAG 2428	***Apoikiospumella mondseeiensis* n.sp. (*Spumella* spec.)**
JBM18	Austria, Lake Krottensee[a]	AY651092	EF577169	x	KF697355	x	x	x
JBC27	People's Republic of China, Small pond in Huqiu[b]	AY651093	KF697326	KF697335	KF697351	B 40 004 1262	SAG 2429	***Chromulinospumella sphaerica* nov.comb. (*Spumella* spec.)**
AR3A3	Austria, River Fuschler Ache[c]	GU073467	KF697322	KF697337	KF697345	B 40 004 1263	SAG 2432	***Segregatospumella dracosaxi* n.sp. (*Spumella* spec.)**
N1846	Japan, Tsukuba, freshwater pond	x	x	x	x	x	x	x
JBNA45	USA, Hawaii, freshwater[b]	DQ388541	EF577173	KF697332	KF697356	x	x	*Spumella* spec. (*Spumella* spec.)
JBNZ39	New Zealand, Shallow tarn near Karangarua[a,b]	AY651088	KF697324	KF697331	KF697357	B 40 004 1264	SAG 2434	***Spumella lacusvadosi* n.sp.**
AR4A6	Austria, River Fuschler Ache[c]	GU073468	KF697328	KF697344	x	B 40 004 0670	SAG 2321	*Spumella rivalis*
194f	Antarctica, Alexander Island, freshwater[b]	DQ388551	EF577179	KF607330	x	x	x	*Spumella* spec.
JBL14	Austria, puddle in Lunz[a,b]	AY651086	EF577172	KF697329	x	B 40 004 1265	SAG 2433	***Spumella bureschii* nov.comb (*Spumella* spec.)**
199hm	Antarctica, Davis Valley, freshwater[b,c]	DQ388552	EF577180	KF697342	x	B 40 004 0672	SAG 2322	*Spumella vulgaris*
JBM10	Austria, Small artificial pond in Mondsee, Karlsgarten[a,b,c]	AY651074	EF577166	KF697339	KF697354	B 40 004 0673	SAG 2323	*Poteriospumella lacustris*
JBNZ41	New Zealand, Lake Aviemore[a,b]	AY651075	EF577167	KF697340	KF697358	x	x	***Poteriospumella lacustris* (*Spumella* spec.)**
JBC07	People's Republic of China, Lake Tai Hu[a,b]	AY651097	EF577165	KF697341	KF697350	x	x	***Poteriospumella lacustris* (*Spumella* spec.)**
AR4D6	Austria, River Fuschler Ache[c]	GU073469	KF697327	KF697343	KF697361	B 40 004 1266	SAG 2430	***Cornospumella fuschlensis* n.sp. (*Spumella* spec.)**
JBAF33	Tanzania, Msimbazi River[a,b]	AY651077	x	KF697338	KF697359	B 40 004 1267	SAG 2427	***Acrispumella msimbaziensis* n.sp. (*Spumella* spec.)**
1006	Antarctica, Signy Island, soil[b]	DQ388558	EF577176	KF697333	x	x	x	***Pedospumella encystans* (*Spumella* spec.)**
JBMS11	Austria, soil, Mondsee near, Rauchhaus[a,b,c]	AY651083	KF697323	KF697334	KF697352	B 40 004 0671	SAG 2324	*Pedospumella encystans*
JBAS36	Nepal, Nag Pokhari, Kathmandu[a,b]	AY6510	x	x	x	x	x	*Pedospumella* spec. (*Spumella* spec.)
JBCS23	People's Republic of China, soil, near Badaling[b]	AY651081	EF577170	x	KF697349	B 40 004 1268	SAG 2431	***Pedospumella sinomuralis* n.sp. (*Spumella* spec.)**
JBAF35	Kenya, River Sagana[b]	AY651071	x	x	x	x	x	*Oicomonas* spec. (*Spumella* spec.)
"*Spumella obliqua*"	Germany, Lake Constance	AJ236860	x	x	x	x	x	x
"*Spumella danica*"	Denmark, Jutland, soil	AJ236861	x	x	x	x	x	x
CCAP 955/1	UK, Girton, soil	AJ236859	x	EF681931	x	x	x	***Pedospumella elongata* nov.comb. (*Spumella elongata*)**

[a]For further details, see Boenigk et al. (2005).
[b]For further details, see Boenigk et al. (2006).
[c]For further details, see Findenig et al. (2010).

Table 2. Primers used in this study

Primer	Sequence 5'-3' (melting temperature)	Target	Reference
18SF1 (forward)	AATCTGGTTGATCCTGCCAG (58.7 °C)	SSU	Katana et al. (2001)
18SR1 (reverse)	TGATCCTTCTGCAGGTTCACCTA (61.6 °C)	SSU	mod. a. Katana et al. (2001)
1420F (forward)	CAGGTCTGTGATGCCC (57.3 °C)	ITS	Rogers et al. (2006)
ITSF (forward)	CGTAACAAGGTTTCCGTAGG (57 °C)	ITS	Barth et al. (2006)
ITSR (reverse)	TCCTCCGCTTACTGATATGC (56.9 °C)	ITS	Barth et al. (2006)
ITS055R (reverse)	CTCCTTGGTCCGTGTTTCAAGACGGG (68.6 °C)	ITS	Marin et al. (1998)
ITS2R (reverse)	CCTCACGGTACTTGTTC (53.7 °C)	ITS	An et al. (1999)
25F (forward)	ACCCGCTGAATTTAAGCATATA (53.5 °C)	LSU	Jo et al. (2011)
1440R (reverse)	TGCTGTTCACATGGAACCTTTC (59.1 °C)	LSU	Jo et al. (2011)
2160R (reverse)	CCGCGCTTGGTTGAATTC (58.2 °C)	LSU	Jo et al. (2011)
CoChryF (forward)	TCTACTAAyCATAAAGATATCGG (~50 °C)	COX1	Jost unpublished
Cox1BR (reverse)	ACGGTAAACATATGATGAGCCCAAAC (59.9 °C)	COX1	Jost et al. (2010)

for maximum-likelihood (ML) and with MrBayes 3.2.1 (Ronquist et al. 2012) for Bayesian analyses. Models for Bayesian analyses were calculated by MrModeltest (SSU and LSU: GTR+I+gamma, 5.8S: SYM+gamma, COX1: F81+I+gamma) in PAUP* 4.0b10 (Swofford 2002). The most similar models available in Treefinder were used for the ML analysis. Bayesian analyses were performed until passing a threshold of 0.01 for the average standard deviations of split frequencies between two runs (sample frequency = 0.01).

RESULTS

All investigated strains are small chrysomonad flagellates with a mean diam. of 1.2–8.6 μm that lack pigmentation and scales (Fig. 1, S1). The cells have a smooth surface. They have one long apical anteriorly oriented flagellum that is, approximately, two to three times the length of the cell body and a second shorter flagellum. The long apically anterior-oriented flagellum is always covered by tripartite mastigonemes as revealed by positive contrast (TEM). For some strains, smaller hairs on these mastigonemes are visible (strains 1006, JBM/S11, AR4D6, JBM10). Strain JBC27 shows smaller hairs between the mastigonemes on the long flagellum. However, the short flagellum is always naked (Fig. 2). Absolute as well as relative measurements of cell bodies (> 30 individuals from actively growing cultures), flagella length and mastigoneme length in SEM, positive contrast, and LM reveal differences in cell size and shape. However, differences in size and shape within strains are comparable in magnitude to those among the different strains and are, thus, not conclusive for delimitation of the investigated strains. All investigated strains are considerably smaller than most of the described species (compare Table S1). A presumptive plastid was observed close to the nucleus in all investigated strains (Fig. 3 circles). The presence of thylakoids was observed only in strain AR4D6 (Fig. 3), although all strains lacked visible pigmentation and failed to grow at low food concentrations, independent of light regime. All investigated strains are very similar to each other based on morphology (Fig. 1–3) whereas molecular

data clearly show that they cluster in different branches within Chrysophyceae and are unrelated to each other (Fig. 4). Despite the high morphological similarity between these small nonscaled chrysomonad taxa, they differ considerably from the species described to date (Table S2) except for the cases outlined below. Although the morphological diversity of large colourless, nonscaled chrysomonad flagellates seems considerable, it is much less conclusive regarding the smallest fraction of these flagellates.

Distinct strains

Based on SSU rRNA sequence data, all investigated strains cluster within the Chrysophyceae (Fig. 4). The strain JBC27 clusters within the order Chromulinales, the strains JBM08 and JBM18 cluster within a clade comprising the genus *Apoikia*. The strain AR3A3 is not closely related to any of the known orders within Chrysophyceae, but clusters separately. Together with an *Oikomonas* species, it forms a neighbouring lineage to the Synurales. All other investigated strains cluster within the order Ochromonadales. Phylogenetic trees calculated based on 5.8S rRNA gene sequences, LSU rRNA gene sequences and COX1 gene sequences are much less conclusive due to the sparseness of sequence data available in public databases. Nevertheless, the tree topologies largely correspond to those of the SSU rRNA tree. The three clusters within Ochromonadales can be identified accordingly in the 5.8S, the LSU and the COX1 phylogeny though their relative position changes (Fig. 5–7).

Within Ochromonadales, the strains JBNA45, JBNZ39, 194f and JBL14 cluster within clade C2, which is currently regarded as genus *Spumella* (Cienkowsky) Boenigk et Findenig. Based on the molecular distance to previously described species (*S. vulgaris*, *Spumella rivalis*, and *Spumella obliqua*), all but strain JBNA45 must be considered as new species. Although strain JBNA45 also differs in the SSU rRNA sequence from its closest relative (i.e. *S. obliqua*), these differences are small (0.1% difference). It is therefore uncertain whether this strain must be considered a different species or as a variant of *S. obliqua*.
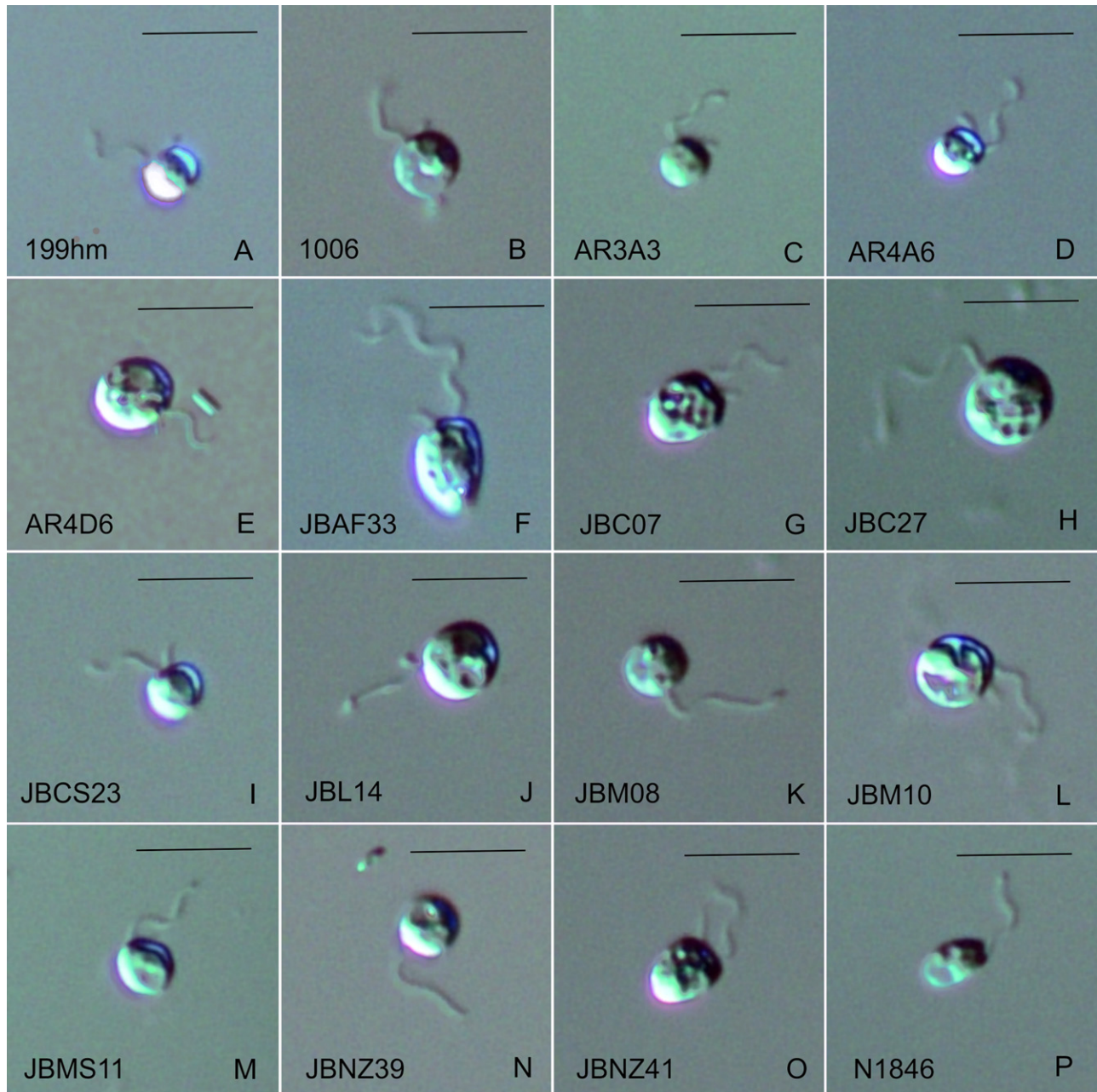
Figure 1 Light microscopic images (**A–P**) showing vegetative cells of strains of colourless nonscaled chrysophytes. **A.** 199hm = *Spumella vulgaris*. **B.** 1006 = *Pedospumella encystans*. **C.** AR3A3 = *Segregatospumella dracosaxi* n. gen. n. sp. **D.** AR4A6 = *Spumella rivalis*. **E.** AR4D6 = *Cornospumella fuschlensis* n. gen. n. sp. **F.** JBAF33 = *Acrispumella msimbaziensis* n. gen. n. sp. **G.** JBC07 = *Poteriospumella lacustris*. **H.** JBC27 = *Chromulinospumella sphaerica* n. gen. nov. comb. **I.** JBCS23 = *Pedospumella sinomuralis* n.sp. **J.** JBL14 = *Spumella bureschii* nov. comb. **K.** JBM08 = *Apoikiospumella mondseeiensis* n. gen. n. sp. **L.** JBM10 = *Poteriospumella lacustris*. **M.** JBMS11 = *Pedospumella encystans*. **N.** JBNZ39 = *Spumella lacusvadosi* n. sp. **O.** JBNZ41 = *Poteriospumella lacustris*. **P.** N1846. Scale bars = 10 μm for (A–P).

The strains JBNZ41, JBC07, JBAF33 and AR4D6 also cluster within Ochromonadales, but within clade C3. Hereof, the strains JBNZ41 and JBC07 are nearly identical in all investigated gene sequences to *Poteriospumella lacustris* (0.000% in SSU, LSU, 5.8S and Cox). We, therefore, assign these strains to this species. The next known relatives are species affiliated with the genus *Poterio-*

ochromonas with an SSU rRNA sequence difference of 4.4–4.7%. Strain JBAF33 strongly deviates from its next relative (*Uroglena* sp.) with 3.1% sequence difference in SSU rRNA. Based on this strong sequence difference, and the clear morphological and physiological (autotrophic vs. heterotrophic) differences, we designate this strain as type for the genus *Acrispumella* n. gen. Strain AR4D6
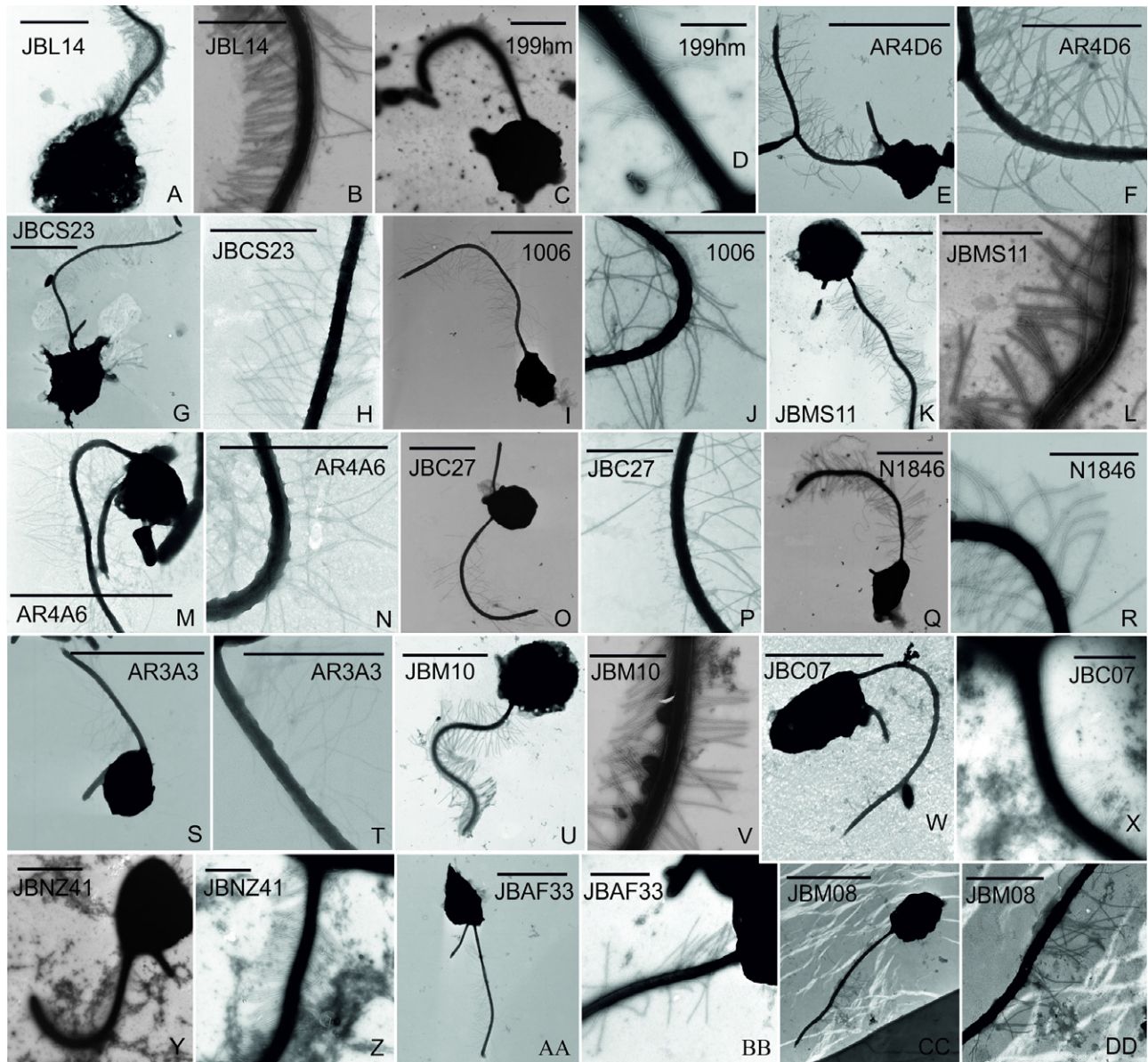
Figure 2 TEM images (positive contrast) (**A–DD**) showing vegetative cells of strains of colourless nonscaled chrysophytes. Two images per strain are given respectively: 1. whole cell, 2. zoom on mastigonemes of large flagellum. **A+B.** JBL14 = *Spumella bureschii* nov. comb. **C+D.** 199hm = *Spumella vulgaris*. **E+F.** AR4D6 = *Cornospumella fuschlensis* n. gen. n. sp. **G+H.** JBCS23 = *Pedospumella sinomuralis* n. sp. **I+J.** 1006 = *Pedospumella encystans*. **K+L.** JBMS11 = *Pedospumella encystans*. **M+N.** AR4A6 = *Spumella rivalis*. **O+P.** JBC27 = *Chromulinospumella sphaerica* n. gen. nov. comb. **Q+R.** N1846. **S+T.** AR3A3 = *Segregatospumella dracosaxi* n. gen. n. sp. **U+V.** JBM10 = *Poteriospumella lacustris*. **W+X.** JBC07 = *Poteriospumella lacustris*. **Y+Z.** JBNZ41 = *Poteriospumella lacustris*. **AA+BB.** JBAF33 = *Acrispumella msimbaziensis* n. gen. n. sp. **CC+DD.** JBM08 = *Apoikiospumella mondseeiensis* n. gen. n. sp. Scale bars = 3 μm for (A, C, E, G, I, K, M, O, Q, S, U, W, Y, AA, CC). Scale bars = 1 μm for (B, D, F, H, J, L, N, P, R, T, V, X, Z, BB, DD).

strongly deviates from its closest relatives (i.e. *Ochromonas sphaerocystis* and *Ochromonas danica*). As the latter two species do not cluster with *O. moestrupii*, i.e. with the clade presumably comprising the type species of *Ochromonas*, strain AR4D6 cannot be affiliated with the genus *Ochromonas* and we, therefore, designate this strain as type for the genus *Cornospumella* n. gen.

A third clade within Ochromonadales (C1-clade) comprises taxa affiliated with the genus *Pedospumella*. Strain 1006 is nearly identical in sequence data to the type strain and is, therefore, considered as affiliated with the type species *P. encystans*. *Spumella elongata* differs considerably in cell size and in the ratio of cell body and long flagellum from *P. encystans* and is, therefore, regarded a
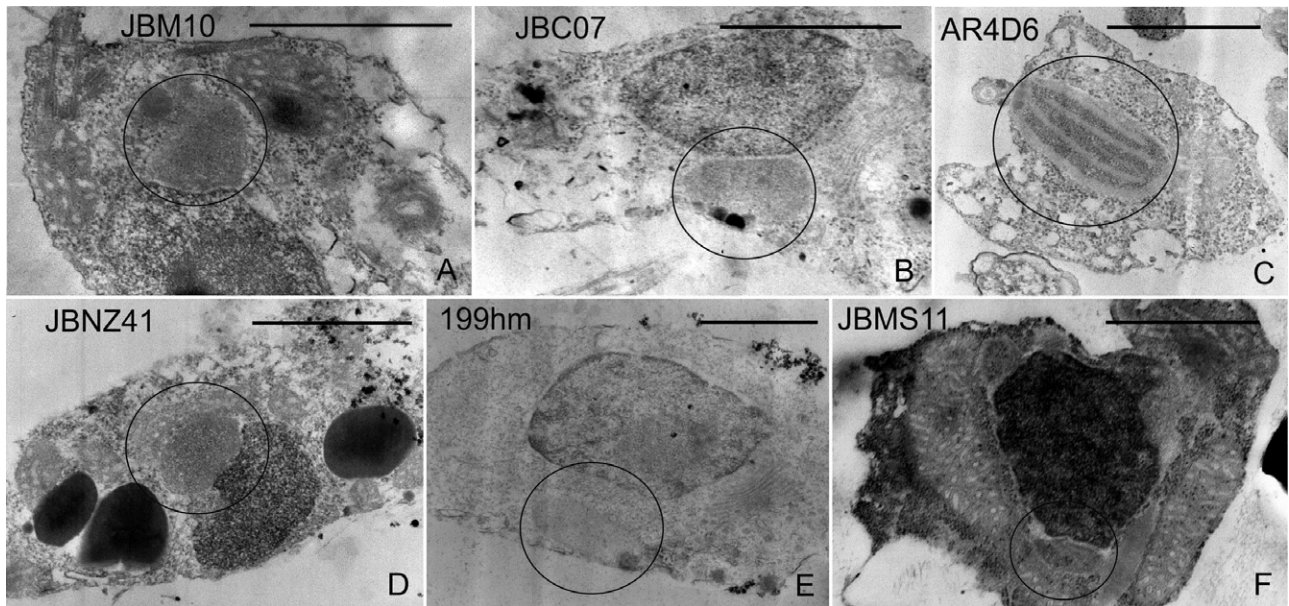
Figure 3 TEM images (ultrathin sections) (**A–F**) showing cell interior of strains of colourless nonscaled chrysophytes. Core-associated plastidal organells are highlighted. **A.** JBM10 = *Poteriospumella lacustris*. **B.** JBC07 = *Poteriospumella lacustris*. **C.** AR4D6 = *Cornospumella fuschlensis* n. gen. n. sp. **D.** JBNZ41 = *Poteriospumella lacustris*. **E.** 199hm = *Spumella vulgaris*. **F.** JBMS11 = *Pedospumella encystans*. Scale bars = 1 µm for (A–F).

different species, which must, however, be recombined to *Pedospumella elongata* nov. comb. The strains JBC23 and JBAS36 cluster in a sister clade to these former two species with a sequence difference of 0.7–2.2%. They cluster together with a sequence labelled *Spumella danica* which is, however, a nomen nudum, i.e. the organism has not formally been described (cf. Bruchmüller 1998; see Table S2).

The strain AR3A3 clusters within Chrysophyceae together with a strain designated as *Oikomonas* sp. (as this strain does not cluster with *Chromulina* this designation must be regarded as incorrect), but without statistical support to reveal the next related lineages. We designate this strain as type for the genus *Segregatospumella* n. gen. within family Segrataceae n. fam. and order Segregatales n. ord.

Within the order Chromulinales, strain JBC27 clusters separately from the only colourless genus within Chromulinales [i.e. *Oikomonas* (=*Heterochromulina*)]. In contrast to the genus *Oikomonas*, in which only one flagellum is visible in the light microscope, strain JBC27 has a second flagellum clearly visible by LM. *Cyclonexis annularis* Stokes 1886, a colonial photosynthetic chrysophyte, is the next relative to strain JBC27 based on available sequence data. Based on the high sequence difference of 7% from its next relative and the morphological differences, we designate this strain as type for the genus *Chromulinospumella* nov. gen.

The strains JBM08 and JBM18 cluster within the clade comprising *Apoikia* with strain JBM08 being the next relative to *Apoikia* with a molecular difference of 2.5–2.7% in

the SSU rRNA. However, *Apoikia* is a genus of colonial flagellates embedded in mucilage, and the second flagellum is clearly visible. In contrast, strain JBM08 is unicellular and the second flagellum is hardly visible in the light microscope. As its morphology strongly deviates from that of the genus *Apoikia* and its sequence also shows considerable differences, we designate strain JBM08 as type of the genus *Apoikiospumella* nov. gen.

DISCUSSION

Taxonomy and phylogeny

Colourless, nonscaled chrysomonad flagellates have been described and named since Müller's description of *Monas* species in 1773 (see Boenigk 2008b and references therein). However, most of the described flagellates which can be considered as colourless, nonscaled chrysomonad flagellates differ from the strains described in this study (see Table S2). Carefully considering the taxonomic literature, we thus come to the conclusion that only two of the observed strains in this study (i.e. strains JBC27 and JBL14) can be identified with one of the existing descriptions (for details see Table S2). Many taxa, originally described as *Monas* spp., have already been reclassified to other protistan groups or cannot be assigned with Chrysophyceae (Boenigk 2008b; Table S2). The described species which are affiliated with Chrysophyceae (including those for which such an affiliation is unclear, but cannot be rejected) differ from the strains investigated in this study. Quite a number of described species do not match
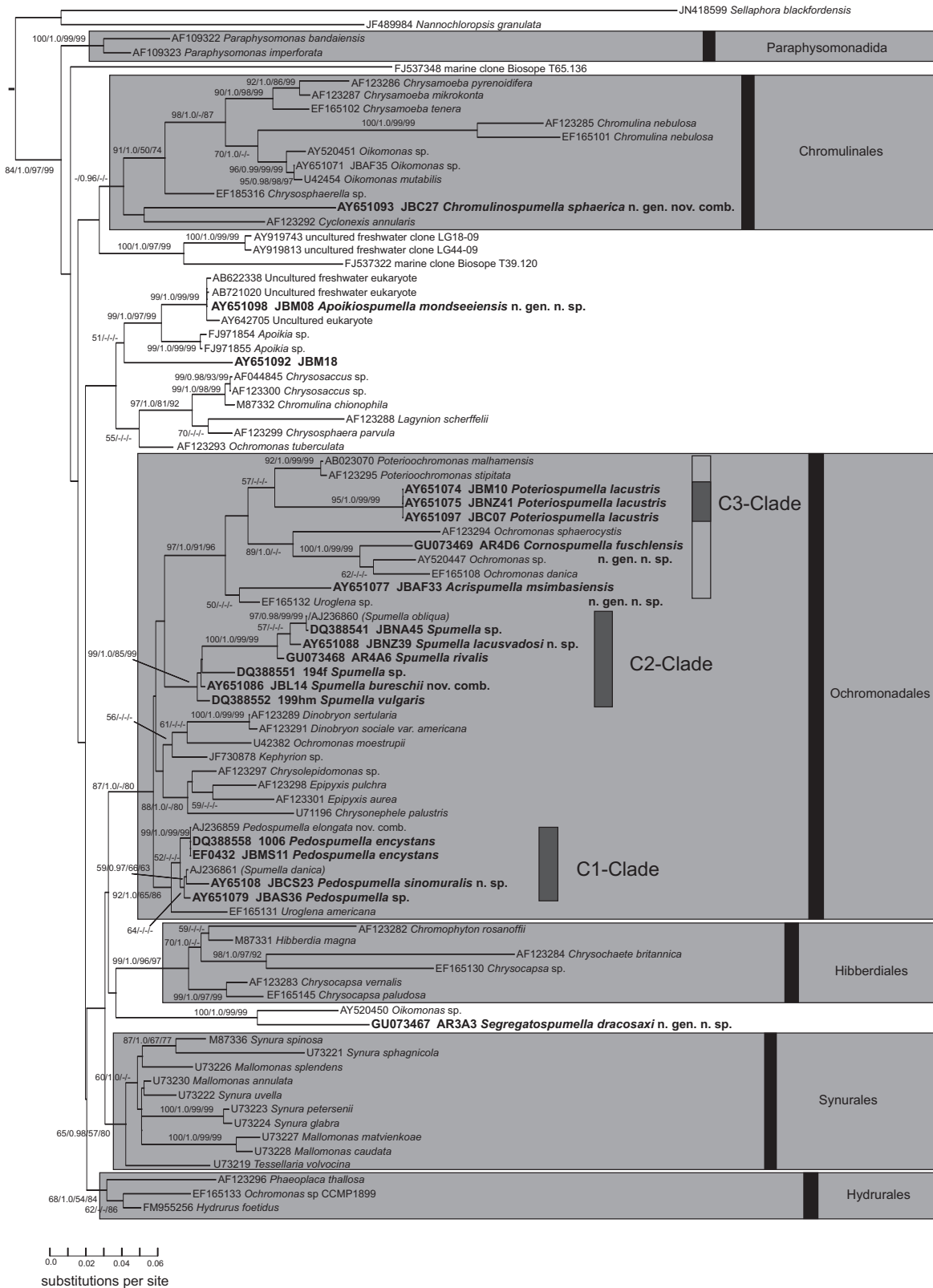
JN418599 *Sellaphora blackfordensis*
JF489984 *Nannochloropsis granulata*
100/1.0/99/99
AF109322 *Paraphysomonas bandaiensis*
AF109323 *Paraphysomonas imperforata*
Paraphysomonadida
FJ537348 marine clone Biosope T65.136
92/1.0/86/99 AF123286 *Chrysamoeba pyrenoidifera*
90/1.0/98/99 AF123287 *Chrysamoeba mikrokonta*
EF165102 *Chrysamoeba tenera*
98/1.0/-/87
100/1.0/99/99 AF123285 *Chromulina nebulosa*
EF165101 *Chromulina nebulosa*
91/1.0/50/74
70/1.0/-/- AY520451 *Oikomonas* sp.
96/0.99/99/99 AY651071 JBAF35 *Oikomonas* sp.
95/0.98/98/97 U42454 *Oikomonas mutabilis*
EF185316 *Chrysosphaerella* sp.
84/1.0/97/99
-/0.96/-/- **AY651093 JBC27 *Chromulinospumella sphaerica* n. gen. nov. comb.**
AF123292 *Cyclonexis annularis*
Chromulinales
100/1.0/97/99
100/1.0/99/99 AY919743 uncultured freshwater clone LG18-09
AY919813 uncultured freshwater clone LG44-09
FJ537322 marine clone Biosope T39.120
AB622338 Uncultured freshwater eukaryote
99/1.0/99/99 AB721020 Uncultured freshwater eukaryote
AY651098 JBM08 *Apoikiospumella mondseeiensis* n. gen. n. sp.
AY642705 Uncultured eukaryote
99/1.0/97/99
51/-/-/- FJ971854 *Apoikia* sp.
99/1.0/99/99 FJ971855 *Apoikia* sp.
AY651092 JBM18
99/0.98/93/99 AF044845 *Chrysosaccus* sp.
99/1.0/98/99 AF123300 *Chrysosaccus* sp.
97/1.0/81/92 M87332 *Chromulina chionophila*
AF123288 *Lagynion scherffelii*
55/-/-/-
70/-/-/- AF123299 *Chrysosphaera parvula*
AF123293 *Ochromonas tuberculata*

92/1.0/99/99 AB023070 *Poterioochromonas malhamensis*
AF123295 *Poterioochromonas stipitata*
57/-/-/- **AY651074 JBM10 *Poteriospumella lacustris***
95/1.0/99/99 **AY651075 JBNZ41 *Poteriospumella lacustris***
AY651097 JBC07 *Poteriospumella lacustris*
C3-Clade
AF123294 *Ochromonas sphaerocystis*
97/1.0/91/96
89/1.0/-/- 100/1.0/99/99 **GU073469 AR4D6 *Cornospumella fuschlensis***
AY520447 *Ochromonas* sp.　**n. gen. n. sp.**
62/-/-/- EF165108 *Ochromonas danica*
50/-/-/- **AY651077 JBAF33 *Acrispumella msimbaziensis***
EF165132 *Uroglena* sp.　**n. gen. n. sp.**
97/0.98/99/99 /AJ236860 (*Spumella obliqua*)
57/-/-/- **DQ388541 JBNA45 *Spumella* sp.**
100/1.0/99/99 **AY651088 JBNZ39 *Spumella lacusvadosi* n. sp.**
GU073468 AR4A6 *Spumella rivalis*
DQ388551 194f *Spumella* sp.
C2-Clade
99/1.0/85/99 **AY651086 JBL14 *Spumella bureschii* nov. comb.**
DQ388552 199hm *Spumella vulgaris*
56/-/-/- 100/1.0/99/99 AF123289 *Dinobryon sertularia*
61/-/-/- AF123291 *Dinobryon sociale var. americana*
U42382 *Ochromonas moestrupii*
JF730878 *Kephyrion* sp.
AF123297 *Chrysolepidomonas* sp.
87/1.0/-/80 AF123298 *Epipyxis pulchra*
88/1.0/-/80 AF123301 *Epipyxis aurea*
59/-/-/- U71196 *Chrysonephele palustris*
99/1.0/99/99 AJ236859 *Pedospumella elongata* nov. comb.
DQ388558 1006 *Pedospumella encystans*
59/0.97/66/63 52/-/-/- **EF0432 JBMS11 *Pedospumella encystans***
AJ236861 (*Spumella danica*)
AY65108 JBCS23 *Pedospumella sinomuralis* n. sp.
C1-Clade
AY651079 JBAS36 *Pedospumella* sp.
92/1.0/65/86 EF165131 *Uroglena americana*
Ochromonadales
64/-/-/- AF123282 *Chromophyton rosanoffii*
59/-/-/- M87331 *Hibberdia magna*
70/1.0/-/- AF123284 *Chrysochaete britannica*
98/1.0/97/92 EF165130 *Chrysocapsa* sp.
99/1.0/96/97 AF123283 *Chrysocapsa vernalis*
99/1.0/97/99 EF165145 *Chrysocapsa paludosa*
Hibberdiales
AY520450 *Oikomonas* sp.
100/1.0/99/99 **GU073467 AR3A3 *Segregatospumella dracosaxi* n. gen. n. sp.**
87/1.0/67/77 M87336 *Synura spinosa*
U73221 *Synura sphagnicola*
60/1.0/-/- U73226 *Mallomonas splendens*
U73230 *Mallomonas annulata*
U73222 *Synura uvella*
100/1.0/99/99 U73223 *Synura petersenii*
U73224 *Synura glabra*
100/1.0/99/99 U73227 *Mallomonas matvienkoae*
U73228 *Mallomonas caudata*
65/0.98/57/80 U73219 *Tessellaria volvocina*
Synurales
AF123296 *Phaeoplaca thallosa*
68/1.0/54/84 EF165133 *Ochromonas* sp CCMP1899
62/-/-/86 FM955256 *Hydrurus foetidus*
Hydrurales

0.0　0.02　0.04　0.06
substitutions per site

Figure 4 Maximum-likelihood phylogeny based on SSU sequences showing the investigated strains of colourless nonscaled chrysophytes (bolt print) within Chrysophyceae. Numbers at nodes give bootstrap values and posterior probabilities in following order: maximum-likelihood/Bayesian/maximum-parsimony/neighbour-joining (values > 50 are shown; posterior probabilities > 0.95).
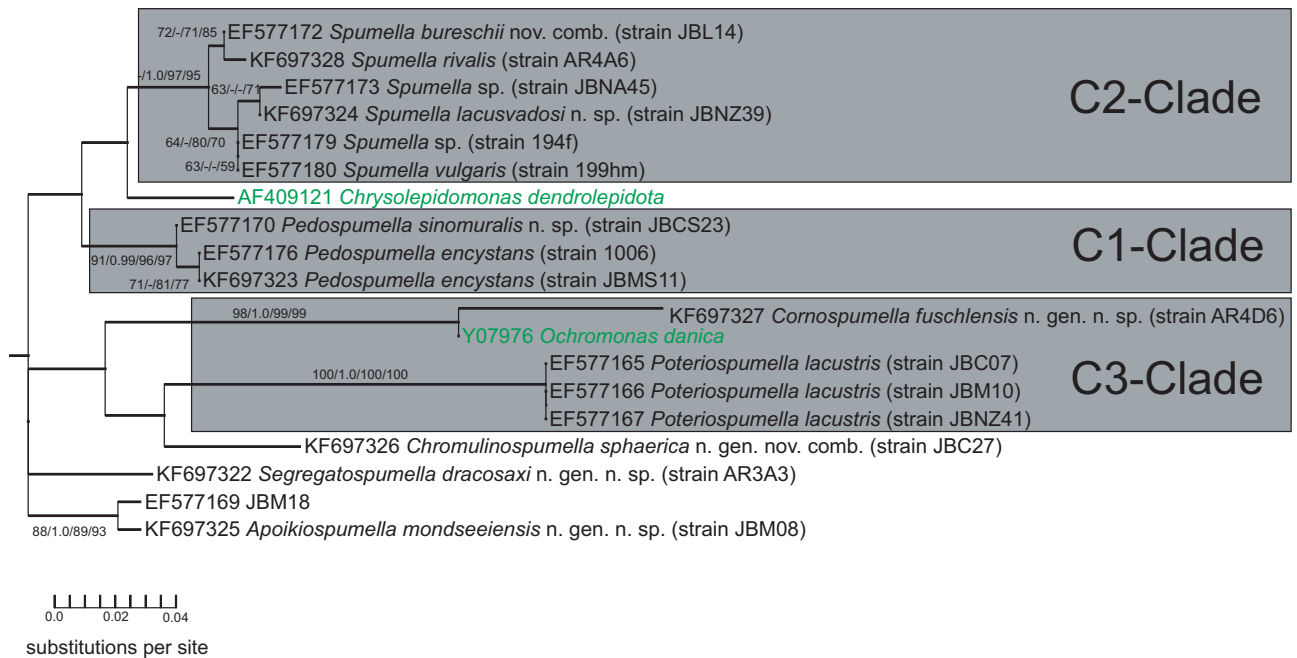
Figure 5 Maximum-likelihood phylogeny based on 5.8S sequences of strains of colourless nonscaled chrysophytes. Numbers at nodes give bootstrap values and posterior probabilities in following order: maximum-likelihood/Bayesian/maximum-parsimony/neighbour-joining (values > 50 are shown; posterior probabilities > 0.95). Two additional photosynthetic chrysophycean species (in green) show polyphyly of colourless nonscaled chrysophytes.
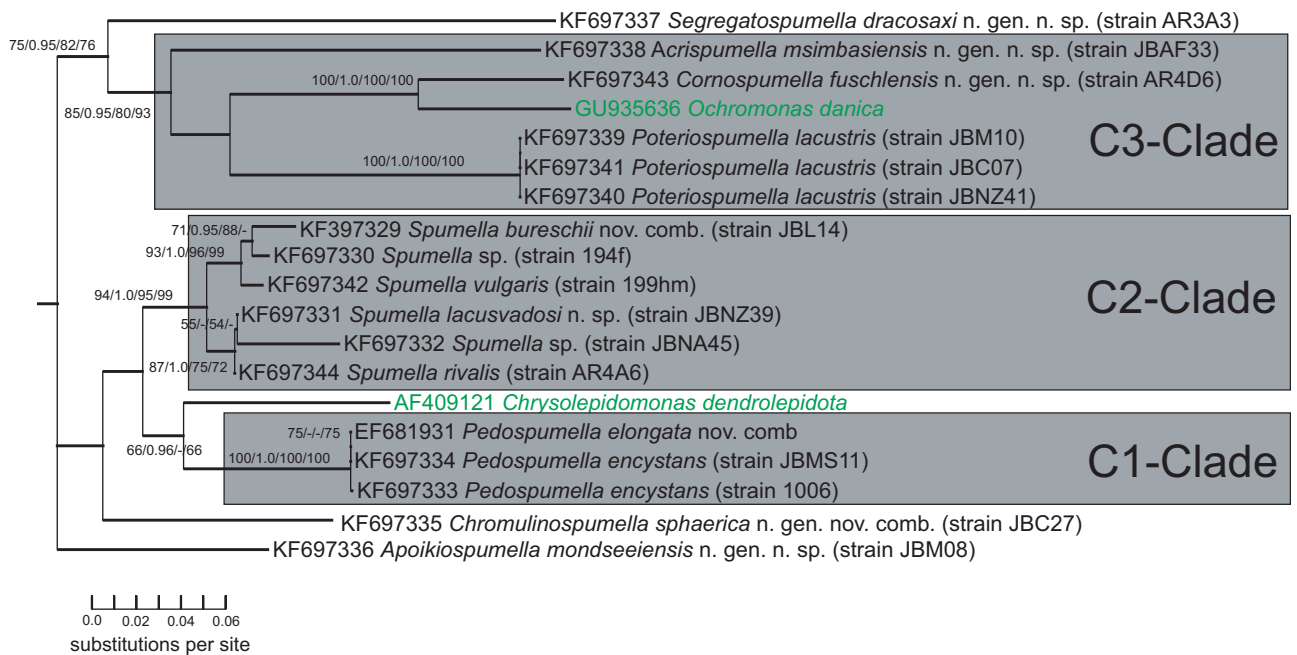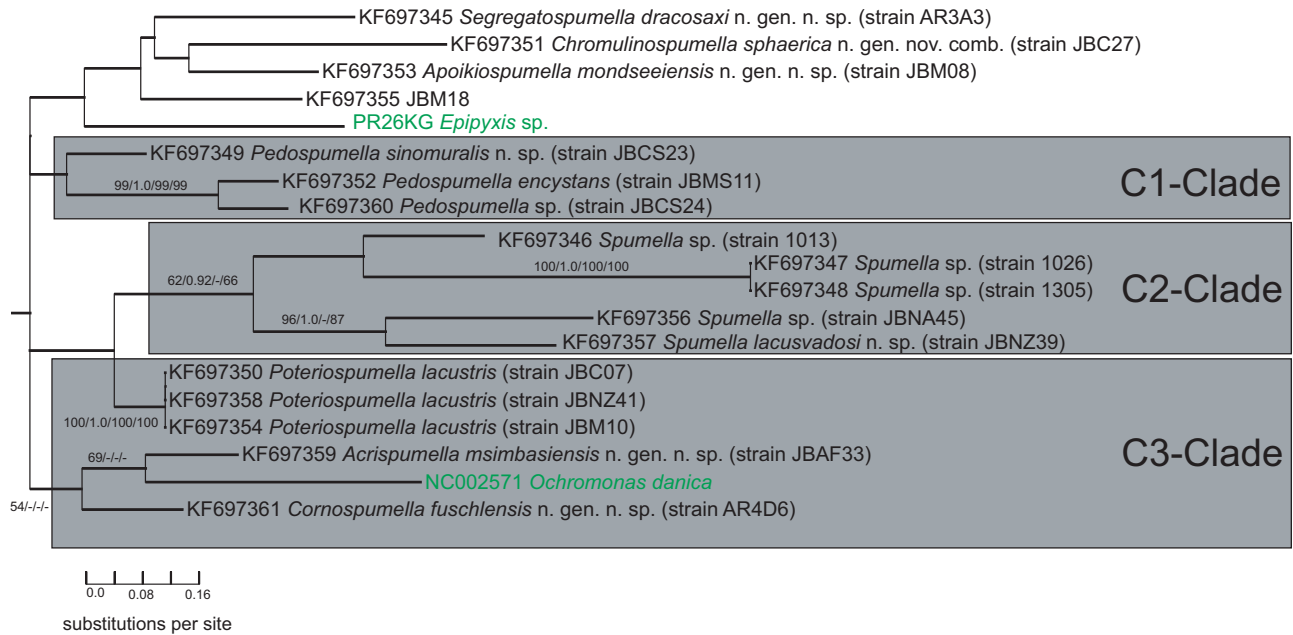

Figure 6 Maximum-likelihood phylogeny based on LSU sequences of strains of colourless nonscaled chrysophytes. Numbers at nodes give bootstrap values and posterior probabilities in following order: maximum-likelihood/Bayesian/maximum-parsimony/neighbour-joining (values > 50 are shown; posterior probabilities > 0.95). Two additional photosynthetic chrysophycean species (in green) show polyphyly of colourless nonscaled chrysophytes.

the strains investigated herein for the details provided, such as being pigmented as for *Monas bicolor* (Ehrenberg 1832) or having a gelatinous sheath as for *Monas coronifera* (Skuja 1948).

The characters of *S. vulgaris* as described by Cienkowsky raised doubts about the purity of the type culture. Some characters such as noncontractile spines may even hint to species possibly affiliated with other chrysomonad

Figure 7 Maximum-likelihood phylogeny based on COX1 sequences of strains of colourless nonscaled chrysophytes. Numbers at nodes give bootstrap values and posterior probabilities in following order: maximum-likelihood/Bayesian/maximum-parsimoy/neighbour-joining (values > 50 are shown; posterior probabilities > 0.95). Two additional photosynthetic chrysophycean species (in green) show polyphyly of colourless nonscaled chrysophytes.

genera such as *Paraphysomonas* whereas other characters such as the flexible cell surface observed in some individuals presumably contradict such an affiliation. The stomatocyst as described by Cienkowsky (1870) corresponds well with cysts produced by flagellates, which are today predominantly considered to be *Spumella* sp. It needs to be noted that flagellates producing stomatocysts of the size as described by Cienkowsky can be much smaller than the vegetative cells described by Cienkowsky (Findenig et al. 2010). Again, these underpins doubts about the purity of the type culture of Cienkowsky. Based on the similarity of the lectotype with stomatocysts of an extant flagellate, Findenig et al. (2010) designated an epitype for *S. vulgaris*. Thus, the epitype of *S. vulgaris* corresponds to the original description of the stomatocyst by Cienkowsky (1870), and the vegetative cells correspond to what is today considered to be *Spumella*. It needs to be stated, however, that the vegetative cell of the epitype (Findenig et al. 2010) deviates in some characters to those provided by Cienkowsky (1870). A future discovery of a taxon with all the properties described by Cienkowsky may prove this basic assumption by Findenig et al. (2010) wrong and, thus, would require a transfer of the taxa affiliated with the genus *Spumella* Cienkowsky sensu Findenig and Boenigk into a new genus. Considering the doubts about the purity of the original culture of Cienkowsky, the choice of Findenig et al. (2010) seems justified, conserves the generic name for those flagellates which are today regarded as *Spumella*, and lays a sound basis for revising the colourless, nonscaled chrysomonad flagellates.

The flagellates investigated herein were morphologically very similar. Specifically, size is not a criterion allowing for the differentiation of the distinct investigated strains: all strains were small and intraspecific variation in size was high. The investigated strains were, however, considerably smaller than most described taxa. This difference in size between the investigated strains and most described taxa is presumably due to differences in the isolation protocol. While most previous studies applied enrichment techniques, we used a filtration–acclimatisation approach (i.e. selecting for flagellates of around 5 μm and smaller). We furthermore demonstrate plastidal remains in all strains that we investigated as TEM ultrathin sections (compare images in Fig. 3). We show plastidal remains in four different genera (independent heterotrophic lineages within Chrysophyceae) and expect the other described genera to also bear reduced plastids (TEM ultrathin sections were not carried out here). These remains support the assumption that heterotrophic chrysomonad flagellates derive from phototrophic ancestors.

The flagellates formerly lumped as being "*Spumella*-like" in morphology – despite being morphologically hardly or not at all distinguishable – show high polyphyly in phylogenetic analyses. Our analyses support the polyphyly of these flagellates (Boenigk et al. 2005; Scoble and Cavalier-Smith 2014) and the erection of the genera *Pedospumella* and *Poteriospumella* by Findenig et al. (2010). As well, we show that a further subdivision within the identified three clades of Ochromonadales is required to adequately differentiate between lineages of small colourless,

nonscaled chrysomonad flagellates that evolved independently. We furthermore identify lineages that are not part of the three described clades within Ochromonadales having evolved in the chrysophycean order Chromulinales, in a cluster together with the genus *Apoikia* and in one yet undescribed clade. We, therefore, describe flagellates with a *Spumella*-like morphology also outside Ochromonadales. This finding is in accordance with the observed huge diversity in the ITS1 and ITS2 sequences of the strains indicating only a distant relationship. On the basis of the above analyses, we agree with Findenig et al. (2010) in abandoning the generic name *Monas* and suggest the exclusive use of the genus names *Spumella* sensu Findenig, *Pedospumella, Poteriospumella* (see Findenig et al. 2010) and the generic names introduced herein, namely *Apoikiospumella, Chromulinospumella, Segregatospumella, Acrispumella,* and *Cornospumella* (see "Taxonomic Summary"). As none of our isolates cluster within the clade comprising *O. moestrupii* or as a sister to this clade, both from a taxonomic and a phylogenetic point of view, the generic names *Ochromonas* and *Heterochromonas* can be rejected for any strain described in this study. However, we consider it likely that the *Ochoromonas* clade comprising *O. moestrupii* also has a colourless sister clade – specifically because *O. moestrupii* has been reported to sometimes divide into two cells one of which lacks a plastid (Andersen 2011). The generic name *Heterochromonas* should, thus, be reserved for such a sister clade according to the original proposition by Pascher (1912). Several former *Spumella* spp. are, thus, reclassified within this paper. Other *Spumella* spp. have been described in detail by electron microscopic methods, but can be separated from the herein described taxa based on either molecular data (*S. obliqua* sensu Mylnikov et al. 2007), deviant morphology (*Spumella sociabilis* sensu Mylnikov and Mylnikova 2005; colony-forming), (*Spumella gregaria* Tanichev 1993; length of flagella), (*Spumella termo* sensu Tanichev 1993; forms exovacuoles), or size (*Spumella sphaerophora*: sensu Mylnikov and Mylnikova 2005). The description of *Spumella subterana* (Tanichev and Karpov 1995) is not valid and must, therefore, be considered as nomen nudum. Although these latter taxa differ from the strains investigated herein, they may be related to one of them. A re-examination of these latter strains with molecular methods may shed light on their phylogenetic position possibly requiring a transfer to another genus.

Such polyphyly in morphologically similar or indistinguishable groups has also been reported for several other protistan lineages, prominent examples of which are the Trebouxiophyceae of *Chlorella*-like morphology (Huss et al. 1999) and of *Dictyosphaerium*-like morphology (Krienitz et al. 2010), the choanoflagellates affiliated with Codonosigidae (Nitsche et al. 2011), the kinetoplastids of *Bodo*-like morphology (Moreira et al. 2004), and the pigmented chrysophytes of an *Ochromonas*-like morphology (Andersen et al. 1999). Thus, polyphyly of protistan morphodemes, rather than being unexpected and rare, is currently disclosed for more and more organisms.

Ecological and evolutionary scenarios behind the multiple evolution of small colourless, nonscaled chrysomonad flagellates

The high molecular diversity between the flagellate taxa investigated herein is not reflected by a respective morphological differentiation indicating multiple convergent evolution of this morphodeme. Despite morphololgical similarity, the investigated strains may differ considerably in ecology, ecophysiology and geographical distribution (Boenigk 2008a; Nolte et al. 2010).

The occurrence of multiple convergent evolution raises the question of reasons for the success of this morphology. The optimisation of nutrition and the trade-off between photosynthesis on the one hand and consumption of small bacteria, specifically ultramicrobacteria, may be the key behind this evolution: According to the predator–prey theory, a reduction in the plastid and with that a reduction in overall cell size increases the capture efficiency of small prey (De Castro et al. 2009). The loss of pigmentation and the evolution of small bacterivorous forms may, therefore, be driven by the availability of differential food sources (i.e. large vs. small bacteria) and the availability of nutrients and light for photosynthesis (De Castro et al. 2009).

Although being very similar in aspects such as general feeding behaviour (Boenigk et al. 2005) or tolerance to pH (generally 3.15 to ~11) (Pfandl et al. 2009), comparative research on different strains of small, colourless, nonscaled chrysomonad flagellates (named therein "*Spumella* spec." or "*Spumella*-like flagellates" and specified by strain names) reveals inequality in many respects including in temperature as well as salinity tolerance (Pfandl et al. 2009), in specific feeding behaviour and food selection (Boenigk et al. 2004; Šimek et al. 2013), in toxicity level (Boenigk and Stadler 2004), seasonal appearance (Nolte et al. 2010), required habitat character (Boenigk 2005) and in global distribution (Boenigk et al. 2006). These inequalities, however, have been observed on different taxonomic levels. Tolerance to salinity varies within "*Spumella*-like flagellates" from 1 to 6 g NaCl/L, but shows a comparable range of tolerances within cluster C3 of Ochromonadales itself from strain 1-8-A1 tolerating up to 2 g/L to strain JBC30 tolerating up to 6 g/L (Pfandl et al. 2009 – also compare species descriptions herein for specific data). Such high variance in phylogenetically closely related strains rather points to a quick evolutionary change concerning salinity. High intraclade variation has also been observed in comparable research of strains JBM10, JBC07 and JBNZ41, which show almost a 100% similarity in SSU sequences. JBC07 has proved to be more toxic to zooplankton than strains JBM10 and JBNZ41 (Boenigk and Stadler 2004). In feeding experiments, strain JBC07 in comparison grows the fastest on large bacteria, whereas strain JBNZ41grows faster than JBC07 and JBM10 on two ultramicrobacterial strains. Unlike strains JBC07 and JBNZ41, strain JBM10 grows equally well on all offered food bacteria (Boenigk et al. 2004). In contrast to these findings, temperature tolerance as well as habitat preference seem to be more stable within

clades indicating slower evolutionary change. This is most striking in clades C1, C2, and C3 of Ochromonadales. Clade C1 comprises mainly soil-associated strains, whereas clades C2 and C3 are mostly aquatic – C3 mostly from eutrophic and shallow waters, C2 from aquatic environments ranging from large lakes to puddles (Boenigk 2005). Differences in temperature tolerance of the three clades are most striking showing an overlapping, but unambiguous differentiation from cold- to warm-adapted. Clade C2, being the most cold-adapted of the three, grows well between 5 and 10 °C whereas C1 and C3 hardly grow or even die (Boenigk et al. 2006). Correspondingly and based on environmental data from samplings on a global scale as well as from seasonal succession studies (spring to autumn), clade C2 can be characterised as cold-temperate, C1 as temperate-moderately warm and C3 as temperate-warm (Nolte et al. 2010), including endemic adaptation as for strains of clade C2 originating from the Antarctica (Boenigk et al. 2006). On the level of genera of "*Spumella*-like flagellates" (described by Findenig et Boenigk 2010 and Grossmann et Boenigk herein), a preliminary ecological characterisation would thus be as follows:

Genus *Spumella*: predominantly cold-temperate, early-year (in temperate zone), freshwater

Genus *Poteriospumella*: predominantly temperate-warm, late-year, freshwater, rather eutrophic

Genus *Cornospumella*: predominantly temperate-warm, late-year, freshwater

Genus *Acrispumella*: predominantly temperate-warm, late-year, freshwater

Genus *Pedospumella*: predominantly temperate-moderately warm, mid-year, mostly soil

Genus *Apoikiospumella*: freshwater, slow-growing

Genus *Chromulinospumella*: freshwater

Genus *Segregatospumella*: freshwater

These genus-level characterisations are rough generalisations, and variation between species is to be expected. Published data so far, however, provide evidence that ecological and physiological differences between different genera and between different species of small, colourless, nonscaled chrysomonad flagellates do exist. The practice of lumping such organisms in ecological studies, such as occurs with "*Spumella*-like flagellates", is therefore questionable. It may be justified with respect to rough predator–prey interactions such as gross feeding rates on bacterioplankton (Boenigk 2005). However, different species feed preferentially on different bacteria (Šimek et al. 2013) and are adapted to different habitat characteristics. Future ecological investigations should, therefore, consider the different genera and preferentially also the different species separately.

Distribution patterns of protists have been controversially discussed (Fenchel et al. 1997: "Everything-is-everywhere"; Foissner 2006, 2008: "moderate endemicity") taking into account the organisms' small size and potential dispersal as well as local and regional dynamics of habitat enclosure. For the investigated groups of organisms, taxa of presumable worldwide distribution (e.g. *Poteriospumella lacustris*) as well as presumable endemics (such as the cold-adapted Antarctic strains) are known. Furthermore, the finding of low-abundance taxa (Nolte et al. 2010) revisits the question of dispersal and distribution patterns. Due to the lack of mere numbers of investigations of this group of organisms, such issues can currently not be answered satisfactorily – despite the group being one of the most abundant flagellates in aquatic as well as soil environments, and with that being among the most abundant eukaryotes on earth. Given the high diversity specifically in freshwaters, large-scale sampling campaigns specifically focusing on freshwaters and soils would, therefore, be most desirable to further address this issue. Such studies should, however, be accompanied by analyses addressing the functional differentiation between these genera and species in the field as well as by further taxonomic analyses of such flagellates. As with respect to diversity, we are currently certainly just scraping the tip of an iceberg. The taxonomic amendment presented herein should provide a sound basis for addressing the yet enigmatic diversity of small, colourless, nonscaled chrysomonad flagellates.

Due to the high degree of morphological convergence between the different groups, we propose and call for using molecular phylogenetic information rather than morphology for the classification of such flagellates. Currently, the SSU as a conserved sequence for a gross classification and ITS sequences as variable sequences allowing for species discrimination seem to be the best choice with a good database reference. As shown, different molecular loci reveal a concordant phylogeny. Small, colourless, nonscaled chrysomonad flagellates are not limited to the order Ochromonadales. We describe the new genera *Apoikiospumella,* as a neighbour to the genus *Apoikia*, *Chromulinospumella* within the order Chromulinales and the genus *Segregatospumella* of uncertain connection within Chrysophyceae with their type species, respectively. For the genus *Segregatospumella*, we consequently describe the new family Segregataceae and the new order Segregatales. Within Ochromonadales, we describe two new genera with their respective type species. We describe and recombine additional species within the already known genera *Spumella* sensu Findenig and *Pedospumella* of Ochromonadales. Furthermore, three of the investigated strains cannot be regarded as independent species lineages and are, therefore, included in previously described species, namely strains JBNZ41 and JBC07 to *Poteriospumella lacustris* (Boenigk et Findenig 2010) and strain 1006 to *Pedospumella encystans* (Boenigk et Findenig 2010). According to their position in the SSU phylogeny, we include two further strains respectively in the genera *Spumella* and *Pedospumella*, namely strain JBNA45 ("*Spumella*-like flagellate") as *Spumella* spec. and strain JBAS36 ("*Spumella*-like flagellate") as *Pedospumella* spec. However, the species delimitation of these strains in not clear and we, therefore, do not formally describe them. Accordingly, strain JBAF35 changes its taxonomic affiliation from "*Spumella*-like flagellate" to *Oikomonas* spec.

The two species names *S. obliqua* and *S. danica* are only represented by sequence information in GeneBank (submitted in 1999). The publication containing their

descriptions and naming remains unpublished. Both names are, therefore, invalid. In the phylogenetic analysis undertaken herein, the sequence of "*Spumella obliqua*" clusters within the genus *Spumella* and the sequence of "*Spumella danica*" within the genus *Pedospumella*.

As Chrysophyceae are a mixed class of phototrophic, mixotrophic and heterotrophic organisms, we describe all species and genera under the ICN and ICZN both whenever possible. In cases where the two codes contradict each other, we follow the ICN. For the description of higher taxa, we likewise apply the ICN.

TAXONOMIC SUMMARY

Sar Adl et al. 2012
Stramenopiles Patterson 1989, emend. Adl et al. 2005
Class: Chrysophyceae Pascher 1914

Order: Apoikiida Boenigk et Grossmann n. ord.
Diagnosis. Nonphotosynthetic, nonscaled, bacterivorous chrysophycean bi-flagellates. Colonial as well as solitary living species. Order containing only the family Apoikiaceae. Distinct from other orders within Chrysophyceae by the gene sequences (SSU, ITS, LSU, COX1) of the described species within the order: *Apoikia lindahlii* Kim et al. 2010 (Basionym *Monas lindahlii* Skuja 1956) and *Apoikiospumella mondseeiensis* n. g. n. sp. (described below).
Type of the order. Apoikiaceae Boenigk et Grossmann n. fam.
Etymology. "Apoikiida" draws on the only known family of the new order: "Apoikiaceae".
Family: Apoikiaceae Boenigk et Grossmann n. fam.
Diagnosis. Nonphotosynthetic, nonscaled, bacterivorous chrysophycean bi-flagellates. Colonial as well as solitary living species. Family containing the genera *Apoikia* and *Apoikiospumella*. Distinct by the gene sequences (SSU, ITS, LSU, COX1) of the two known species within the family: *A. lindahlii* Kim et al. 2010 (Basionym *Monas lindahlii* Skuja 1956) and *Apoikiaspumella mondseeiensis* n. g. n. sp. (described below).
Type of the family. *Apoikia lindahlii* Kim et al. 2010 (Basionym *Monas lindahlii* Skuja 1956)
Etymology. "Apoikiaceae" draws on the genus "*Apoikia*".
Apoikiospumella Boenigk et Grossmann n. g.
Diagnosis. Nonphotosynthetic, noncolonial, nonscaled, bacterivorous chrysophycean bi-flagellate. Distinct phylogenetic lineage related to the genera *Apoikia* (SSU sequence difference: 2.5–2.7%), *Chrysosphaera* (4.3%) and *Chrysosaccus* (5%).
Typus generis. ***Apoikiospumella mondseeiensis*** Boenigk et Grossmann n. sp.
Etymology. Feminine. "*Apoikio-*" indicates the phylogenetic affiliation to the genus *Apoikia* and "*-spumella*" the taxonomic point of origin as so-called "*Spumella*-like flagellate".
Apoikiospumella mondseeiensis Boenigk et Grossmann n. sp.

Diagnosis. Nonphotosynthetic, noncolonial, nonscaled, bacterivorous chrysophycean bi-flagellate. Vegetative cells mostly spherical, sometimes elongated or posteriorly pointed, 2–7.1 µm in diam. Long flagellum up to 12 µm in length bearing tripartite mastigonemes (compare images Fig. 1(K), Fig. 2(CC+DD) in this paper). Second flagellum short and attached to the cell body so that scarcely visible in LM. Distinct from its closest relative *Apoikia* sp. (i.e. *A. lindahlii*) as noncolonial and not covered in mucilage, as well as in the SSU sequence by 2.5–2.7% sequence difference.

Distinct – on at least species level – from other "*Spumella*-like flagellates" of comparable morphology (as those described herein) by its gene sequences of SSU, ITS, LSU and COX1 (compare trees Fig. 4–7).
Holotype. Botanical Garden and Botanical Museum Berlin Dahlem, no. B 40 004 1261 (formaldehyde fixation of strain JBM08).
Type habitat. Mesotrophic freshwater lake.
Type locality. Austria, Lake Mondsee, 47°52′0 N, 13°20′0 E, 500 m asl.
Etymology. The species epithet "*mondseeiensis*" hints at the species' place of origin from Lake Mondsee, Austria.
Gene sequences. NCBI accession no. AY651098 (SSU), no. KF697325 (ITS), no. KF697336 (LSU), no. KF697353 (COX1); all from strain JBM08.
Further reference. SAG number 2428 (from strain JBM08); strain JBM08 in the culture collection of Jens Boenigk at University Duisburg-Essen.
Additional information. In experiments: temperature maximum of 30.7 °C, salinity maximum of 1 g NaCl/L, pH tolerance between 3.15 and 10.9 (Pfandl et al. 2009); all data from strain JBM08.

Order: Chromulinales Pascher 1910
Family: Chromulinaceae Engler 1897
Chromulinospumella Boenigk et Grossmann n. g.
Diagnosis. Nonphotosynthetic, noncolonial, nonscaled, bacterivorous chrysophycean bi-flagellate. Distinct phylogenetic lineage within the order Chromulinales of Chrysophyceae related to the genus *Cyclonexis* (SSU sequence difference: 7%).
Typus generis. ***Chromulinospumella sphaerica*** (Valkanov) Boenigk et Grossmann nov. comb.
Etymology. Feminine. "*Chromulino-*" indicates the phylogenetic affiliation to the order Chromulinales and "*-spumella*" the taxonomic point of origin as so-called "*Spumella*-like flagellate".
Chromulinospumella sphaerica (Valkanov) Boenigk et Grossmann nov. comb.
Basionym: *Monas sphaericus* Valkanov (1925).
Emended diagnosis. Nonphotosynthetic, noncolonial, nonscaled, bacterivorous chrysophycean bi-flagellate. Cells mostly spherical, sometimes elongated, 2–8.6 µm in diam. Long flagellum up to 15 µm in length bearing tripartite mastigonemes. In addition, smaller hairs in-between the mastigonemes, inserting at the plasma membrane of the long flagellum (compare images Fig. 1(H), Fig. S1(G), Fig. 2(O+P) in this paper). Distinct from the genus *Cyclo-*

nexis (closest relative) as neither photosynthetic nor building ring shaped colonies, as well as in the SSU sequence by 7% sequence difference.

Distinct – on at least species level – from other "*Spumella*-like flagellates" of comparable morphology (as those described herein) by its gene sequences of SSU, ITS, LSU and COX1 (compare trees Fig. 4–7).

Holotype. figure 7 in Valkanov (1925), designated by Valkanov.

Epitype (designated herein). Botanical Garden and Botanical Museum Berlin Dahlem, no. B 40 004 1262 (formaldehyde fixation of strain JBC27).

Type habitat. Small pond.

Type locality. People's Republic of China, Huqiu, 31°20′05 N, 120°34′27E, 4 m asl.

Gene sequences. NCBI accession no. AY651093 (SSU), no. KF697326 (ITS), no. KF697335 (LSU), no. KF697351 (COX1); all from strain JBC27.

Further reference. SAG number 2429 (from strain JBC27); strain JBC27 in the culture collection of Jens Boenigk at University Duisburg-Essen.

Additional information. In experiments: temperature maximum of 33.6 °C, salinity maximum of 3 g NaCl/L, pH tolerance between 3.15 and 10.9 (Pfandl et al. 2009); all data from strain JBC27.

Order: Segregatales Boenigk et Grossmann n. ord.

Diagnosis. Nonphotosynthetic, noncolonial, nonscaled, bacterivorous chrysophycean bi-flagellates. Order only containing the family Segregataceae. Distinct from other orders within Chrysophyceae by the gene sequences (SSU, ITS, LSU, COX1) of the known species *Segregatospumella dracosaxi* n.sp. (described below) and the SSU sequence AY520450 (NCBI accession number).

Type of the order. Segregataceae Boenigk et Grossmann n. fam.

Etymology. "Segregatales" draws on the only known family of the new order: "Segregataceae".

Family: Segregataceae Boenigk et Grossmann n. fam.

Diagnosis. Nonphotosynthetic, noncolonial, nonscaled, bacterivorous chrysophycean bi-flagellates. Family containing the genus *Segregatospumella*. Distinct by the gene sequences (SSU, ITS, LSU, COX1) of the known species within the family: *Segregatospumella dracosaxi* n. sp. (described below) and the SSU sequence AY520450 (NCBI accession number).

Type of the family. *Segregatospumella* Boenigk et Grossmann n. g.

Etymology. "Segregataceae" draws on the genus: "*Segregatospumella*".

Segregatospumella Boenigk et Grossmann n. g.

Diagnosis. Nonphotosynthetic, noncolonial, nonscaled, bacterivorous chrysophycean bi-flagellate. Distinct and independent phylogenetic lineage within Chrysophyceae.The only close relative is the NCBI sequence AY520450 with an SSU sequence difference of 4.7%.

Typus generis. ***Segregatospumella dracosaxi*** Boenigk et Grossmann n. sp.

Etymology. Feminine. "*Segregato-*" indicates the phylogenetic unrelatedness to all other known lineages of Chrysophyceae and "*-spumella*" the taxonomic point of origin as so-called "*Spumella*-like flagellate".

Segregatospumella dracosaxi Boenigk et Grossmann n. sp.

Diagnosis. Nonphotosynthetic, noncolonial, nonscaled, bacterivorous chrysophycean bi-flagellate. Cells mostly spherical, sometimes elongated or posteriorly pointed, 1.2–5.6 μm in diam. Long flagellum up to 8 μm in length bearing tripartite mastigonemes (compare images Fig. 1(C), Fig. S1(N), Fig. 2(S+T) in this paper). Stomatocysts 3.6–5.5 μm in diam. and with a smooth surface (compare Findenig et al. 2010). Distinct from its closest relative AY520450 (NCBI accession number) by 4.7% sequence difference in the SSU sequence.

Distinct – on at least species level – from other "*Spumella*-like flagellates" of comparable morphology (as those described herein) by its gene sequences of SSU, ITS, LSU and COX1 (compare trees Fig. 4–7).

Holotype. Botanical Garden and Botanical Museum Berlin Dahlem, no. B 40 004 1263 (formaldehyde fixation of strain AR3A3).

Type habitat. Small freshwater stream.

Type locality. Austria, River Fuschler Ache near Mondsee, 47°50′N, 13°16′E, 500 m asl.

Etymology. The species epithet "*dracosaxi*" hints at the species' place of origin in Austria near the mountain "Drachenwand".

Gene sequences. NCBI accession no. GU073467 (SSU), no. KF697322 (ITS), no. KF697337 (LSU), no. KF697345 (COX1); all from strain AR3A3.

Further reference. SAG number 2432 (from strain AR3A3); strain AR3A3 in the culture collection of Jens Boenigk at University Duisburg-Essen.

Order: Ochromonadales Pascher 1910

Family: Ochromonadaceae Lemmermann 1899

Cornospumella Boenigk et Grossmann n. g.

Diagnosis. Nonphotosynthetic, noncolonial, nonscaled, bacterivorous chrysophycean bi-flagellate. Clusters within clade C3 in Ochromonadales and forms a distinct phylogenetic lineage with the closest relatives being *O. sphaerocystis* (AF123294) and *Ochromonas danica* (EF165108) with an SSU sequence difference of 6.1% (*O. sphaerocystis*) and 3.4% (*O. danica*).

Typus generis. ***Cornospumella fuschlensis*** Boenigk et Grossmann n. sp.

Etymology. Feminine. "*Corno-*" hints at the typical hook-like projections of the stomatocysts (as described in Findenig et al. 2010) and "*-spumella*" indicates the taxonomic point of origin as so-called "*Spumella*-like flagellate".

Cornospumella fuschlensis Boenigk et Grossmann n. sp.

Diagnosis. Nonphotosynthetic, noncolonial, nonscaled, bacterivorous chrysophycean bi-flagellate. Cells mostly spherical, sometimes elongated or posteriorly pointed, 1.3–6.9 μm in diam. Long flagellum up to 9.4 μm in length bearing tripartite mastigonemes. In addition, smaller hairs

on mastigonemes. Plastidal, nonpigmented compartment showing thylakoidal inner folding (compare images Fig. 1(E), Fig. S1(D), Fig. 2(E+F), Fig. 3(C) in this paper). Stomatocysts with distinctly deformed shape and hook-like projections, 4.35–5.7 μm in length and 3.96–5.45 μm in width (compare Findenig et al. 2010). Distinct from its closest relatives *O. sphaerocystis* and *Ochromonas danica* as not being photosynthetic. Sequence difference in the SSU sequence: 6.1% (*O. sphaerocystis*) and 3.4% (*O. danica*).

Distinct – on at least species level – from other "*Spumella*-like flagellates" of comparable morphology (as those described herein) by its gene sequences of SSU, ITS, LSU and COX1 (compare trees Fig. 4–7).

Holotype. Botanical Garden and Botanical Museum Berlin Dahlem, no. B 40 004 1266 (formaldehyde fixation of strain AR4D6).

Type habitat. Small freshwater stream.

Type locality. Austria, River Fuschler Ache near Mondsee, 47°50′N, 13°16′E, 500 m asl.

Etymology.: The species epithet "*fuschlensis*" hints at the species' place of origin from the river Fuschler Ache near Mondsee, Austria.

Gene sequences. NCBI accession no. GU073469 (SSU), no. KF697327 (ITS), no. KF697343 (LSU), no. KF697361 (COX1); all from strain AR4D6.

Further reference. SAG number 2430 (from strain AR4D6); strain AR4D6 in the culture collection of Jens Boenigk at University Duisburg-Essen.

Acrispumella Boenigk et Grossmann n. g.

Diagnosis. Nonphotosynthetic, noncolonial, nonscaled, bacterivorous chrysophycean bi-flagellate with a typical pointy elongation of the cell body at its posterior end. Clusters within clade C3 in Ochromonadales and forms a distinct phylogenetic lineage with the closest relative being a *Uroglena* species (EF165132) with an SSU sequence difference of 3.1%.

Typus generis. *Acrispumella msimbaziensis* Boenigk et Grossmann n. sp.

Etymology. Feminine. "*Acri-*" hints at the typical pointy shape of the cell body and "*-spumella*" indicates the taxonomic point of origin as so-called "*Spumella*-like flagellate".

Acrispumella msimbaziensis Boenigk et Grossmann n. sp.

Diagnosis. Nonphotosynthetic, noncolonial, nonscaled, bacterivorous chrysophycean bi-flagellate. Cells typically elongated with pointed posterior end, 2–7.7 μm in diam. (mean of length and width). Long flagellum up to 17.4 μm in length bearing tripartite mastigonemes (compare: images Fig. 1(F), Fig. S1(E), Fig. 2(AA+BB) in this paper). Distinct from its closest relative *Uroglena* sp. as not photosynthetic, with a sequence difference in the SSU sequence of 3.1%.

Distinct – on at least species level – from other "*Spumella*-like flagellates" of comparable morphology (as those described herein) by its gene sequences of SSU, LSU and COX1 (compare trees Fig. 4–7).

Holotype. Botanical Garden and Botanical Museum Berlin Dahlem, no. B 40 004 1267 (formaldehyde fixation of strain JBAF33).

Type habitat. Freshwater river.

Type locality. Tanzania, Msimbazi River, 5°15′0S, 38°49′60E, 151 m asl.

Etymology. The species epithet "*msimbaziensis*" refers to the species' place of origin from the river Msimbazi in Tanzania.

Gene sequences. NCBI accession no. AY651077 (SSU), no. KF697338 (LSU), no. KF697359 (COX1); all from strain JBAF33.

Further reference. SAG number 2427 (from strain JBAF33); strain JBAF33 in the culture collection of Jens Boenigk at University Duisburg-Essen.

Additional information. In experiments: temperature maximum of 34.6 °C, salinity maximum of 4 g NaCl/L, pH tolerance between 3.15 and 10.9 (Pfandl et al. 2009); all data from strain JBAF33.

Spumella (Cienkowsky) Findenig et Boenigk

Spumella bureschii (Valkanov) Boenigk et Grossmann nov.comb.

Basionym. *Monas bureschii* Valkanov (1925)

Emended diagnosis. Nonphotosynthetic, noncolonial, nonscaled, bacterivorous chrysophycean bi-flagellate. Cells mostly spherical, sometimes elongated or posteriorly pointed, 2.9–7.4 μm in diam. Long flagellum up to 14.2 μm in length bearing tripartite mastigonemes (compare images Fig. 1(J), Fig. S1(L), Fig. 2(A+B) in this paper). Stomatocysts 5.17–8.09 μm in diam., spherical to slightly oval with deep conical pore (compare Findenig et al. 2010). Distinct phylogenetic lineage within the genus *Spumella* with an SSU sequence difference of 0.7% to *S. vulgaris* and 1.2% to *Spumella* sp. (DQ388558).

Holotype. figure 18 in Valkanov (1925), designated by Valkanov.

Epitype (designated here). Botanical Garden and Botanical Museum Berlin Dahlem, no. B 40 004 1265 (formaldehyde fixation of strain JBL14).

Type habitat. Puddle water.

Type locality. Austria, Lunz, 47°51′0 N, 15°03′0E, 884 m asl.

Gene sequences. NCBI accession no. AY651086 (SSU), no. EF577172 (ITS), no. KF697329 (LSU); all from strain JBL14.

Further reference. SAG number 2433 (from strain JBL14); strain JBL14 in the culture collection of Jens Boenigk at University Duisburg-Essen.

Additional information. In experiments: temperature maximum of 29.1 °C (Boenigk 2006), salinity maximum of 6 g NaCl/L, pH tolerance between 3.15 and 11.2 (Pfandl et al. 2009); all data from strain JBL14.

Spumella lacusvadosi Boenigk et Grossmann n. sp.

Diagnosis. Nonphotosynthetic, noncolonial, nonscaled, bacterivorous chrysophycean bi-flagellate. Cells mostly spherical, sometimes elongated or posteriorly pointed, 1.5–7.1 μm in diam. Long flagellum up to 10.4 μm in

length bearing tripartite mastigonemes (compare image Fig. 1(N) in this paper). Distinct phylogenetic lineage within the genus *Spumella* with an SSU sequence difference of 1% to *Spumella* sp. (DQ388541) and 1% to *S. rivalis*.

Holotype. Botanical Garden and Botanical Museum Berlin Dahlem, no. B 40 004 1264 (formaldehyde fixation of strain JBNZ39).

Type habitat. Shallow tarn.

Type locality. New Zealand, near Karangarua, 43°37′0S, 169°46′0 E, 1118 m asl.

Etymology. The species epithet "*lacusvadosi*" hints at the species' origin from a shallow mountain lake.

Gene sequences. NCBI accession no. AY651088 (SSU), no. KF697324 (ITS), no. KF697331 (LSU), no. KF697357 (COX1); all from strain JBNZ39.

Further reference. SAG number 2434 (from strain JBNZ39); strain JBNZ39 in the culture collection of Jens Boenigk at University Duisburg-Essen.

Additional information. In experiments: temperature maximum of 36.7 °C (Boenigk 2006), salinity maximum of 4 g NaCl/L, pH tolerance between 3.15 and 10.9 (Pfandl et al. 2009); all data from strain JBNZ39.

Pedospumella Findenig et Boenigk
Pedospumella sinomuralis Boenigk et Grossmann n. sp.

Diagnosis. Nonphotosynthetic, noncolonial, nonscaled, bacterivorous chrysophycean bi-flagellate. Cells mostly spherical, sometimes elongated or posteriorly pointed, 1.9–5.6 μm in diam. Long flagellum up to 9.5 μm in length bearing tripartite mastigonemes (compare images Fig. 1(I), Fig. S1(H), Fig. 2(G+H) in this paper). Distinct phylogenetic lineage within the genus *Pedospumella* with an SSU sequence difference of 1% to *Pedospumella* sp. (AY651079) and 0.8% to *S. danica*.

Holotype. Botanical Garden and Botanical Museum Berlin Dahlem, no. B 40 004 1268 (formaldehyde fixation of strain JBCS23).

Type habitat. Soil.

Type locality. People's Republic of China, near Badaling, 40°20′15 N, 115°58′10E, 795 m asl.

Etymology. The species epithet "*sinomuralis*" refers to the species' place of origin in China close to the Great Wall.

Gene sequences. NCBI accession no. AY651081 (SSU), no. EF577170 (ITS), no. KF697349 (COX1); all from strain JBCS23.

Further reference. SAG number 2431 (from strain JBCS23); strain JBCS23 in the culture collection of Jens Boenigk at University Duisburg-Essen.

Additional information, In experiments: temperature maximum of 29.1 °C (Boenigk 2006), salinity maximum of 5 g NaCl/L, pH tolerance between 3.15 and 11.2 (Pfandl et al. 2009); all data from strain JBCS23.

Pedospumella elongata (Stokes) Boenigk et Grossmann nov.comb.
Basionym. *Physomonas elongata* Stokes (1886).

Synonym. *Spumella elongata* (Stokes) Belcher et Swale (1976), *Monas elongata* (Stokes) Lemmermann (1910).

Emended Diagnosis. (See descriptions as *Physomonas elongata* in Stokes 1886 and as *Spumella elongata* in Belcher and Swale 1976 and sequence information as *Spumella elongata* in Bruchmüller 1998 and Wylezich et al. 2010 – the latter two available in GenBank). Distinct from its closest relative *Pedospumella encyctans* in the larger cell body (8–11.2 μm) and the ratio of cell body and long flagellum (long flagellum as long as or shorter than the cell body).

Lectotype. figure 1 in Stokes (1886), designated here by Grossmann and Boenigk.

Epitype. Strain CCAP 955/1 (formally designated here following Belcher and Swale).

Type habitat. Soil.

Type locality. UK, Girton (Cambridgeshire), 1974 (by Belcher and Swale).

Gene sequences. NCBI accession no. AJ236859 (SSU) and NCBI accession no. EF681931 (LSU); all from strain CCAP 955/1.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. SEM images (**A**–**N**) showing vegetative cells of strains of colourless nonscaled chrysophytes. **A.** 199hm = *Spumella vulgaris*. **B.** 1006 = *Pedospumella encystans*. **C.** AR4A6 = *Spumella rivalis*. **D.**

AR4D6 = *Cornospumella fuschlensis* n.gen.n.sp. **E.** JBAF33 = *Acrispumella msimbaziensis* n.gen.n.sp. **F.** JBC07 = *Poteriospumella lacustris*. **G.** JBC27 = *Chromulinospumella sphaerica* n.gen.nov.comb. **H.** JBCS23 = *Pedospumella sinomuralis* n.sp. **I.** JBM10 = *Poteriospumella lacustris*. **J.** JBMS11 = *Pedospumella encystans*. **K.** JBNZ41 = *Poteriospumella lacustris*. **L.** JBL14 = *Spumella bureschii* nov.comb. **M.** N1846. **N.** AR3A3 = *Segregatospumella dracosaxi* n.gen. n. sp. Scale bars = 5 μm for (A–N).

Table S1. Measurements of investigated strains of colourless, nonscaled chrysophytes given as ranges (smallest to largest individual); > 30 individuals per strain were measured.

Table S2. Assessment of *Monas* and *Spumella* species with regard to the herein investigated strains of colourless, nonscaled chrysophytes.