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Phylogenetic position of *Coronastrum ellipsoideum* and description of *Parachlorella hussii* sp. nov.

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Abstract: Following traditional morphological concepts, the genus *Coronastrum* is considered to be a rare member of the Scenedesmaceae (Chorophyceae). This classification may be called into question when molecular data are taken into account as well. Recent molecular phylogenetic studies revealed the polyphyletic origin of the family Scenedesmaceae within the Chlorophyceae and Trebouxiophyceae. In a combined approach of morphological analyses, SSU/ITS rRNA gene phylogeny and comparison of the ITS secondary structure, we analysed the systematics of *Coronastrum* strains available in public strain collections. Our molecular analyses revealed a new subclade within the *Chlorella* clade of the Chlorellaceae consisting of *Coronastrum ellipsoideum*, two strains with *Dictyosphaerium*-like morphology and one strain which fits the description of the genus *Parachlorella*. Four additional strains formed together a new lineage within the genus *Parachlorella* in the *Parachlorella* clade of the Chlorellaceae. These strains differ from the already known *Parachlorella* species in complementary base changes within the ITS2 and are here described for the first time as *Parachlorella hussii* sp. nov.

Key words: *Chlorella*; *Coronastrum*; *Dictyosphaerium*; ITS; *Parachlorella*; phylogeny; taxonomy

Introduction

In recent years, several attempts were made to gain a better understanding of the phylogenetic relationship of green algae by using molecular tools (e.g. Chapman et al. 1998; Friedl 1997; Lewis & McCourt 2004; Melkonian & Surek 1995; Pröschold & Leliaert 2007). The SSU of the rRNA gene turned out to be a useful marker for higher ranks such as classes and orders due to its highly conserved domains (Krienitz et al. 2003; Marin et al. 2003; Nakada et al. 2008). Whereas the SSU was often proved to be too conserved for genera and species delineation, the more variable regions of the ITS1 and ITS2 rRNA gene achieved more and more acceptance for phylogenetic reconstruction of lower ranks (Coleman 2003, 2007; Keller et al. 2010; Schultz et al. 2005). It was shown that the combination of the primary sequence with structural information such as compensatory base changes (CBCs) in the secondary structure provided sufficient information to separate species (Coleman 2007, 2009; Müller et al. 2007).

Under this context, the polyphyletic origin of several families and genera was revealed (Huss et al. 1999; Wolf et al. 2003). One example is the genus *Dictyosphaerium* Nägeli which was traditionally designated to the family Botryococcaceae (Komárek & Fott 1983). Recent molecular studies revealed a relationship of the type species *D. ehrenbergianum* Nägeli to the *Parachlorella* clade of the Chlorellaceae (Krienitz et al. 2010). It was further shown that morpho-species of *Dictyosphaerium* form independent lineages within the *Chlorella* clade of the Chlorellaceae, representing new genera and even intermixing with members of the genus *Chlorella* Beijerinck (Bock et al. 2010; Krienitz et al. 2004, 2010; Luo et al. 2010). Similar studies detected that former members of the genus *Chlorella* evolved independently within the Trebouxiophyceae and Chlorophyceae, demonstrating the paraphyletic origin of *Chlorella* (Darienko et al. 2010; Huss et al. 1999; Krienitz et al. 2004).

A similar pattern could be observed for the Scenedesmaceae (Chorophyceae). This family was morphologically characterized by spindle, curved or three-dimensional coenobia and syncoenobia (Komárek & Fott 1983). As consistent morphological criteria considered for all Scenedesmaceae were the occurrence of the cells in plane coenobia and the characteristic reproduction by 4 autospores (Komárek & Fott 1983). Molecular analyses reshaped the understanding of the Scenedesmaceae and its type genus *Scenedesmus* Meyen in different ways. Morphological observations divided the genus *Scenedesmus* Meyen in several different subgroups (Komárek & Fott 1983) which is only partly supported by molecular analyses. The genera *Scenedesmus* s. str. and *Desmodesmus* (Chodat) An, Friedl & E. Hegewald were proposed, corresponding to the subgroups *Scenedesmus/Acutodesmus* and *Desmodesmus* (An et al. 1999). Other genera of Scenedesmaceae were transferred after molecular revisions to the Trebouxiophyceae, e.g. members of *Crucigeniella* Lemmermann clustered within the Oocystaceae (Krienitz et al. 2003). Surprisingly, some genera of the Scenedesmaceae were found to cluster within the Chlorellaceae, e.g. *Dicloster acuatus* C.C.Jao, Y.S.Wei & H.C.Hu was found within the *Parachlorella* clade and *Didymogenes* Schmidle within the *Chlorella* clade (Hegewald & Hanagata 2000; Krienitz et al. 2004; Luo et al. 2010). The release of autospores by disrupture of the mother cell wall as observed in *Dicloster*, as well as in *Didymogenes* (the remnants of the cell wall remain attached to the autospores for a long time) is also a typical feature for members of the genus *Coronastrum* Thompson (Komárek & Fott 1983; Schnepf & Hegewald 1993).

The genus *Coronastrum* was considered to be a rare member of the Scenedesmaceae (Pfiester 1977). It was erected in 1938 based on *Coronastrum aestivale* Thompson (Thompson 1938). The genus is morphologically characterized by four-celled compounded coenobia, reproduction via four autospores which are released through disrupture of the mother cell wall in four parts and connected by hyaline strands of cell wall substances. The remnants of the mother cell wall are attached to the daughter cells, forming colourless appendages. These criteria gave the genus an easy recognizable appearance. The genus includes at the moment three additional species: *C. chodatii* Komárek, *C. ellipsoideum* Fott, *C. lunatum* Thompson (Fott 1946; Komárek 1974; Thompson 1950).

Due to the recent revisions of the Scenedesmaceae and Chlorellaceae, it became questionable, if the members of the genus *Coronastrum* belong to the Scenedemaceae.

The aim of this study was to reveal the phylogenetic relationship of *Coronastrum* and to discover its closest allies. We analysed and compared the SSU and ITS rRNA gene sequences and the secondary structure of all strains (only two) of *Coronastrum* available in public strain collections: UTEX LB1382 (designated as *C. ellipsoideum*) and ACOI 473 (designated as *C. aestivale*). We revealed the phylogenetic relationship of *C. ellipsoideum* within the *Chlorella* clade, however, the phylogeny of the type species *C. aestivale* remained unresolved, awaiting new collection material whose morphology fits better to the type description. We could further show a new phylogenetic lineage within the genus *Parachlorella*, establishing the new species *Parachlorella hussii* sp. nov.

Material and methods

Algal strains and culture conditions

The strains analysed in this study were either kindly provided by the Culture Collection of Algae and Protozoa (CCAP, UK), the Culture Collection of Algae at the University of Texas (UTEX, USA), Coimbra Collection of Algae (ACOI, Portugal) or isolated from field material using microcapilars (for details on origin and isolator *see* Table 1). All strains were grown at 15 °C under a 14/10h light/dark regime either in liquid modified Bourrelly medium (Krienitz & Wirth 2006) or solid on 1% agar of BBM medium (Bischoff & Bold 1963).

Light microscopic observations

Cell shape, size and arrangement of cells and connecting strands within the colonies were analysed under the Light microscope ZEISS Axio Imager Z1 (Carl-Zeiss, Jena, Germany) with the software Axio vision for recording. The algae were determined according to Komárek & Fott (1983) and Krienitz et al. (2004).

DNA isolation, amplification and sequencing

Genomic DNA of the strains ACOI 1508, ACOI 939, ACOI 938 was extracted, the SSU amplified and sequenced as previously described (Pažoutová et al. 2010). Cells of remaining strains were mechanically disrupted in the presence of glass beads using the Tissuelyser II (Qiagen, Hilden, Germany). Total Genomic DNA was isolated using the Lysozym/sodium phosphate method (Allgaier & Grossart 2006). The polymerase chain reactions (PCR) for the SSU and ITS rRNA gene were carried out with the Taq PCR Mastermix Kit (Qiagen GmbH, Hilden, Germany) using 0.2 µL of the enzyme with approx. 10 ng extracted DNA in 40 µL reaction volume. The SSU was amplified on a gradient thermo cycler (BIO-RAD, München, Germany) with the primer set 18SF and 18SR (Katana et al. 2001) with the following cycle: initial denaturation 95 °C, 5 min; following by 33 cycles of denaturation 94 °C, 1 min – annealing 55 °C, 2 min – elongation 72 °C, 3 min; and final elongation 72 °C, 10 min. The ITS for all strains was amplified as described above with the primer set NS7m and LR1850 (An et al. 1999), using an annealing temperature of 45 °C. PCR products were purified with the PEG-precipitation after Rosenthal et al. (1993). Genes were sequenced using the sequencing primers after Marin et al. (1998) with Applied-Biosystems 3130-Genetic-Analyzers (Applied Biosystems GmbH, Darmstadt). The overlapping SSU and ITS partial sequences were assembled to a complete consensus sequence consisting of SSU, ITS1, 5.8S, ITS2 regions using the software SeqAssem (Hepperle 2004a). Eight strains were newly sequenced during this study. Part of the genomic DNA was stored at the BGBM DNA Network (Gemeinholzer et al. 2010).

Phylogenetic analyses

The sequences were manually aligned with the SequentiX Alignment Editor (Hepperle 2004b) using the rRNA secondary structure of *Micractinium pusillum* Fresenius as reference (Figs S1 and S2 in Luo et al. 2006). A data set of 37 taxa with 2518 aligned base positions was used for the phylogenetic analyses, excluding introns. We calculated Modeltest 3.7 to obtain the substitution model (Akaike criteria) which best fits our data set (Posada & Crandall 1998; Posada & Buckley 2004). Phylogenetic trees were calculated using PAUP* (portable version 4.0b10) (Swofford 2002) with *Catena viridis* (KR 1991/4) as outgroup. The GenBank accession numbers for all included strains are given in Table 1. We calculated a maximum likelihood tree, using the Model GTR+G+I (freqA = 0.2034, freqC = 0.2914, freqG = 0.2732, freqT = 0.2320, R [AC] = 0.9298, R [AG] = 1.3392, R [AT] = 0.9925, R [CG] = 0.7134, R [CT] = 3.1892, R [GT] = 1.0000) with the proportion of invariable sites (I) set to 0.6755 and gamma distribution shape parameter (G) set to 0.484. To confine the tree topology, we calculated bootstrap analyses by distance (neighbor-joining; NJ; 1000 replicates), maximum parsimony (MP; 1000 replicates) and maximum likelihood (ML; using the GTR+G+I;

Table 1. List of strains used in this study.

Strain number	Species	Origin$^{a)}$	Accession Number	Reference
SAG 2015	*Actinastrum hantzschii*	KR 1996/4, River Elbe near Aken, Sachsen-Anhalt, Germany	FM205841	Luo et al. 2010
KR 1991/4	*Catena viridis*	Oxidation pond Neuglobsow, Brandenburg, Germany	GU592792	Bock et al. 2011
ACOI 311	*Chlorella* sp.	M.F. Santos 865, 1984, Mira, trout nursery, Portugal	GQ176853	Krienitz et al. 2010
SAG 211-6	*Chlorella* sp.	Endosymbiont of the ciliate *Paramecium bursaria*, USA	FM205849	Luo et al. 2010
SAG 211-11b	*Chlorella vulgaris*	M. W. Beijerinck 1889, eutrophic pond near Delft, Netherlands, authentic strain	FM205832	Luo et al. 2010
CCAP 211/81	*Chlorella vulgaris*	Pond "Salzteich" near Trebbichau, Sachsen-Anhalt, Germany	FM205854	Luo et al. 2010
SAG 11.86	*Closteriopsis acicularis*	Hegewald 1977-126, Plankton of Grömitzer See, Germany	FM205847	Luo et al. 2010
UTEX LB1382	*Coronastrum ellipsoideum*	F. Hindák Fall 1965, Edafon, St. Tereza, Cuba	**GQ507370**	This study
UTEX 75	*Dictyosphaerium ehrenbergianum*	E.G. Pringsheim, 1940, Pond near Cambridge, UK	GQ176856	Krienitz et al. 2010
CCAP 222/23	*Dictyosphaerium ehrenbergianum*	KR 2006/305, Lake Victoria, Homa Bay, Kenya	GQ176859	Krienitz et al. 2010
CCAP 222/14	*Dictyosphaerium ehrenbergianum*	KR 2005/31, Sewagepond, Jerba, Tunesia	GQ176858	Krienitz et al. 2010
CCAP 222/1A Authentic strain	*Dictyosphaerium ehrenbergianum*	E.G. Pringsheim, 1940, Pond near Cambridge, UK	GQ176854	Krienitz et al. 2010
CB 2008/108	*Dictyosphaerium* morphotype	CB 2008/108, Lake Schwandter See, Mecklenburg-Western Pomerania, Germany	**GQ507371**	This study
CCAP 222/9	*Dictyosphaerium* morphotype	LW 2003/181, Lake Geron, Gransee, Brandenburg, Germany	**GQ507369**	This study
UTEX 731	*Dictyosphaerium pulchellum*	R.A. Lewin (T1/3), 1952, Nova Scotia, Canada	GQ176861	Krienitz et al. 2010
SAG 70.80	*Dictyosphaerium sphagnale* *(Dictyosphaerium ehrenbergianum)*	G. Paris, Bourrelly 141, origin unknown	GQ176860	Krienitz et al. 2010
ACOI 1988	*Dictyosphaerium* sp.	Origin unknown, Portugal ?	GQ176863	Krienitz et al. 2010
CCAP 222/25	*Dictyosphaerium* sp.	KR 2006/310, Kazinga Channel, near Katunguri, Uganda	GQ176862	Krienitz et al. 2010
CCAP 222/1C	*Dictyosphaerium* sp.	G. Jaworski (FBA L141), 1966, Windermere, Cumbria, UK	GQ176864	Krienitz et al. 2010
SAG 41.98	*Dicloster acuatus*	Hegewald 1998–13, Pond at Krasne, Ukraine	FM205848	Luo et al. 2010
SAG 18.91	*Didymogenes anomala*	Hegewald 1990/11, River Rhein, near Linz, Germany	FM205839	Luo et al. 2010
SAG 30.92	*Didymogenes palatina*	Hegewald 1982/83, Water tank, Jülich, Germany	FM205840	Luo et al. 2010
CCAP 222/29	*Hindakia fallax*	KR 2006/317, Lake Victoria, Dunga Beach, Kenya	GQ487223	Bock et al. 2010
CCAP 222/73	*Hindakia tetrachotoma*	CB 2007/27, Lake Waldstichsee Zehdenick, Brandenburg, Germnay	GQ487231	Bock et al. 2010
CCAP 222/2D	*Heynigia dictyosphaerioides*	G. Jaworski (FBA L246), 1972, Windermere, Cumbria, England	GQ487221	Bock et al. 2010
CCAP 222/47	*Heynigia riparia*	KR 2007/12, Kunene, Angola	GQ487225	Bock et al. 2010
CCMP 2258	*Meyerella planctonica*	Arrowwood Lake, Arrowwood National Wildlife Refuge, Stutsman County, North Dakota, USA	AY543039 AY543043	Fawley et al. 2005
CCMP 2446	*Meyerella planctonica*	Itas224S1w, Lake Itasca, Itasca State Park, Minnesota, USA	AY543040 AY543045	Fawley et al. 2005
SAG 42.98	*Micractinium belenophorum*	Berlin, plankton in Lietzensee, Germany	FM205879	Luo et al. 2010
CCAP 248/5	*Micractinium pusillum*	Nakuru Sewage Pond, Kenya	FM205836	Luo et al. 2010
SAG 2046	*Parachlorella beijerinckii*	KR 1999/1, Nonnenbach brook, a tributary of Lake Tollense, Mecklenburg-Western Pomerania, Germany	FM205845	Luo et al. 2010
ACOI 473	*Parachlorella hussii* sp. nov. *(Coronastrum aestivale)*	O. Lourenço 1991, Montemor-o-Velho, Vala de Maiorca, Portugal	**HM126550**	This study
ACOI 938	*Parachlorella hussii* sp. nov. *(Coenochloris hindakii)*	M. F. Santos 1994, rock pool, Serra do Gerȩs, Rio Homem,	**HM126549**	This study

Table 1. (continued).

Strain number	Species	Origin$^{a)}$	Accession Number	Reference
ACOI 939	*Parachlorella hussii* sp. nov. *(Coenochloris hindakii)*	M. F. Santos 1994, pond , Serra do Gerȩs, Rio Homem	**HM126551**	This study
ACOI 1508	*Parachlorella hussii* sp. nov. *(Dispora speciosa)*	M. F. Santos 2003, plant squeezing, Abrantes, Campo Militar de Sta Margarida, lake north of Lagoa da Murta	**HM126548**	This study
SAG 211–11g	*Parachlorella kessleri*	Pond at state New York, USA	FM205885	Luo et al. 2010
CCAP 206/1	*Parachlorella* morphotype	KR 2005/233, Lake Victoria, Kenya	**GQ502287**	This study

[a] For own isolates the initials of the isolator were given: CB = Christina Bock, KR = Lothar Krienitz

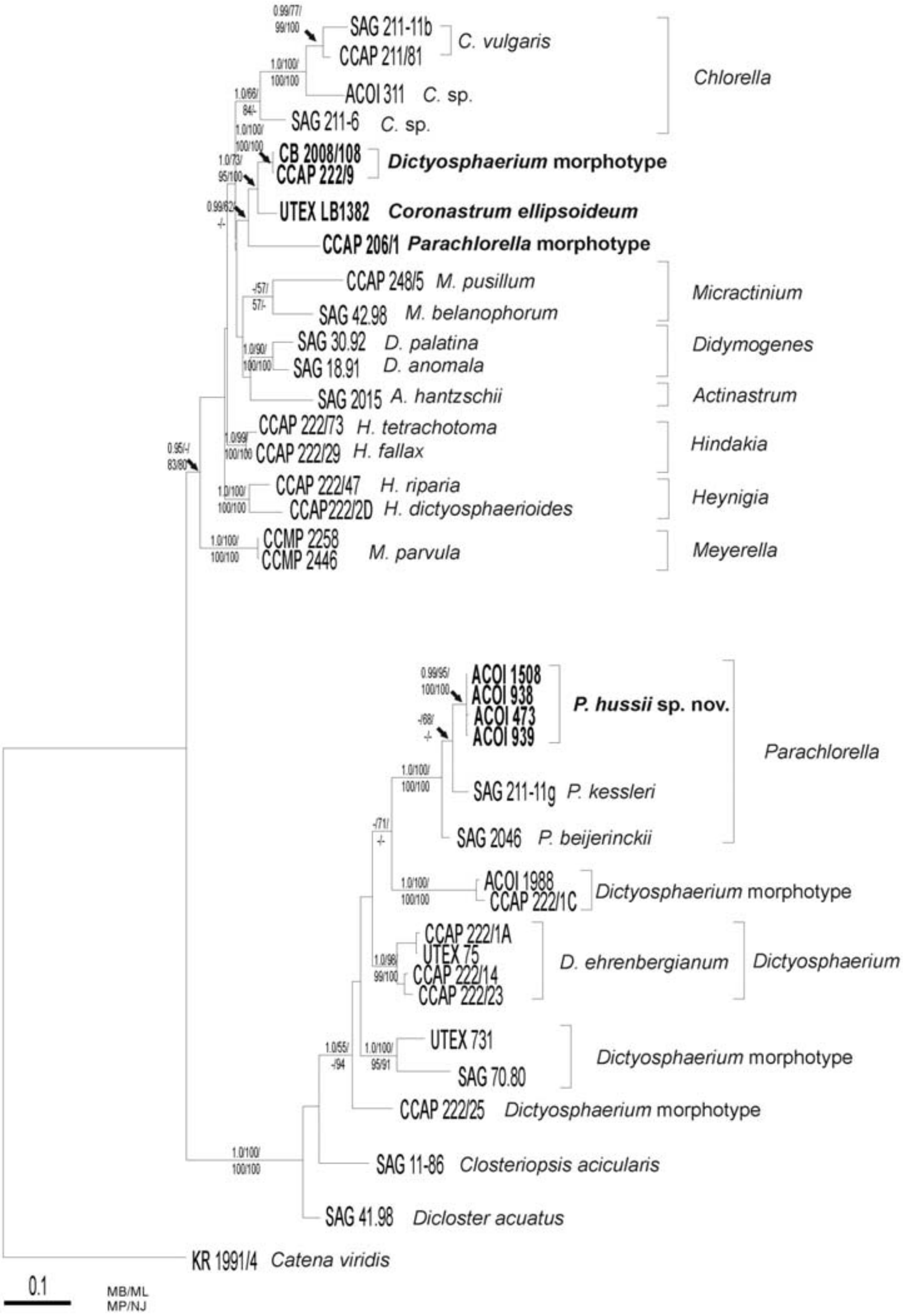
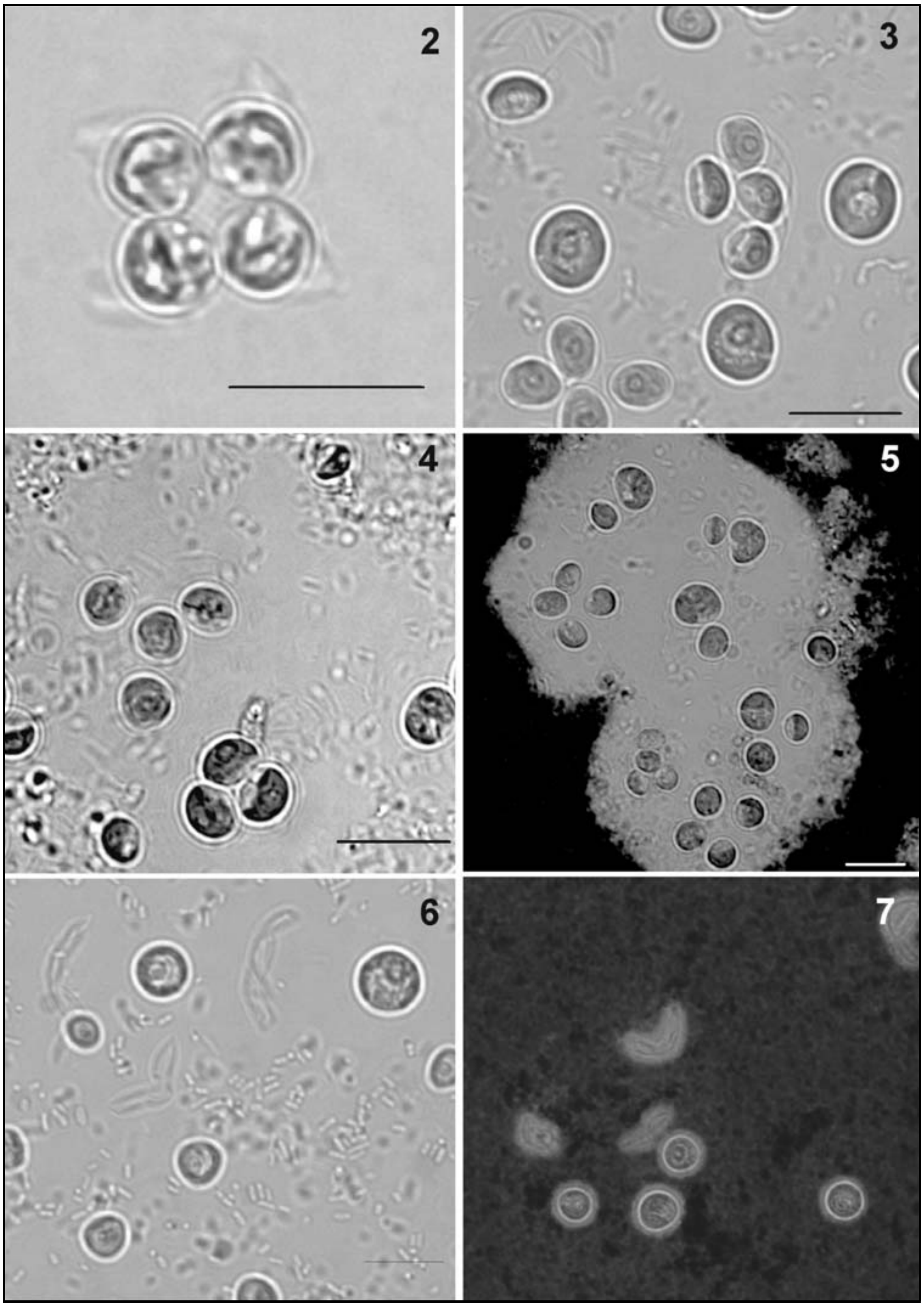


Fig. 1. Maximum-likelihood (ML) phylogenetic tree (50% majority rule consensus) as inferred from combined SSU, ITS1, 5.8S and ITS2 rRNA sequences. Numbers indicate Bayesian values (MB) and bootstrap support from maximum likelihood (ML, 100 replicates), maximum parsimony (MP, 1000 replicates) and neighbour joining (NJ, 1000 replicates).



Figs 2–7. Microphotographs. 2 – *Coronastrum ellipsoideum* (UTEX LB1382); 3 – *Dictyosphaerium* morphotype (CB 2008/108); 4 – *Parachlorella* morphotype (CCAP 206/1); 5 – *Parachlorella* morphotype (CCAP 206/1), stained with drawing ink (scale bars 10 μm); 6 – *Parachlorella hussii* (ACOI 473); 7 – *Parachlorella hussii* (ACOI 473), stained with drawing ink.

100 replicates) criteria. Bayesian analyses were performed by using MrBayes version 3.1. (Huelsenbeck & Ronquist 2001). Two runs with four chains of Markov chain Monte Carlo (MCMC) iterations were performed for 2,000,000 generations using GTR+I+G with covarion settings. The first 25% of the calculated trees were discarded as burn-in. A 50% majority-rule consensus tree was calculated for posterior propabilities (PP).

Analyses of the secondary structure

We analyzed the secondary structure of the ITS1 and ITS2 according to Marin et al. (2003) and Coleman (2003, 2007) to locate non-homoplasious synapomorphies (NHS), hemi-compensatory base changes (h-CBCs) and compensatory base changes (CBCs). Structures were calculated with the help of mfold (Zucker 2003). To locate NHS, h-CBCs and CBC in the ITS1 and ITS2, the program 4Sale and the ITS2 database was used (Schultz & Wolf 2009; Seibel et al. 2006, 2008). Structures were drawn by Pseudoviewer (Byun & Han 2006).

Results

Molecular phylogeny

In the combined SSU and ITS rRNA gene tree

(Fig. 1) the new sequenced strains cluster within the *Chlorella* and *Parachlorella* clades of the Chlorellaceae (Trebouxiophyceae). *Coronastrum ellipsoideum* (UTEX LB1382) clustered in a new lineage within the *Chlorella* clade, together with the new sequenced strains CB2008/108, CCAP 222/9 and CCAP 206/1. The new phylogenetic lineage showed moderate statistical support in Bayesian and maximum likelihood analyses (0.99 PP/62BP). The strain ACOI 473 (predesignated as *C. aestivale*) formed a new lineage within the *Parachlorella* clade. The strain clustered together with ACOI 938, 939 and 1508 next to members of the genus *Parachlorella* with high statistical support in all analyses (0.99PP/95–100BP).

Light microscopically observations
The strain UTEX LB1382, showed the typical morphology of *C. ellipsoideum* with elliptic cells in four-celled coenobia. The cells within the coenobia are 4–6.5 × 3–4.5 µm, smaller then previously reported, bearing narrow, horn-like appendages of cell wall remnants (Fig. 2). The cells are lying closely together, connected by short colourless strands of cell wall substances. The coenobia often become single celled and almost spherical in culture with 6–8.3 × 4–6 µm. Reproduction with 4 autospores. The related strains CB2008/108 and CCAP 222/9 showed the typical *Dictyosphaerium*-like morphology with spherical to oval cells, 5.7–6.7 × 3.9–5.0 µm, connected by mucilaginous strands of mother cell wall remnants at the broader side of the cells and surrounded by a mucilage envelope (Fig. 3). The cells of strain CCAP 206/1 resembled more the *Chlorella*/*Parachlorella* morphotype, but with broadly oval to spherical cells and a single, parietal chloroplast containing a pyrenoid. Connecting strands or cell wall appendages were lacking. The cells formed loose colonies with four or more cells within a hyaline gelatinous sheath (Figs 4–5).

Light microscopically observations of ACOI 473 (predesignated as *C. aestivale*) showed not the typical coenobium-structure of *C. aestivale* with cell appendages, but spherical single cells with 4.5–6.5 µm in size. The cells contained a parietal chloroplast with an ellipsoid pyrenoid, covered by two starch grains. The reproduction occurred by 2–4 (8) autospores. The mother cell wall ruptured mainly in two, but sometimes in four flaps. The strain lacked the connecting strands between the cells and no appendages could be observed. Adult cells were surrounded by a thin mucilage envelope (Figs 6–7). ACOI 938, 939 and 1508 match the morphology of ACOI 473. The morphology corresponded best to the genus description of *Parachlorella*.

Comparison of the secondary structure of Parachlorella
The ITS1 and ITS2 of the strains belonging to *Parachlorella* showed the typical branching pattern of the Chlorellaceae with four unbranched helices each (Fig. 8). Within the new lineage comprising of the strain ACOI 473, 938, 939 and 1508 no compensatory base changes could be observed. This new lineage differed in comparison to *Parachlorella beijerinckii* (SAG 2046) in the ITS1 in three additional base pairs within the Helix 3, and in the ITS2 with three CBCs and one h-CBC within the Helix II, one CBC and one h-CBC in the Helix IV. Furthermore, the new lineage differed from *P. kessleri* (SAG 211–11g) in the ITS1 in three CBCs within the Helix 3 and in the ITS2 in five CBCs, two h-CBCs in Helix II and one CBC in Helix IV.

Discussion

The genus *Coronastrum* was erected in 1930 as a monospecific genus and considered to be a rare member of the Scenedesmaceae (Thompson 1938). Since the description of the type species *C. aestivale* four more *Coronastrum* species were included and the genus was three times reemended (Fott 1946; Komárek 1974; Thomspon 1950). Its generic characters are now as follows: cells in four-celled coenobia or syncoenobia, connected by hyaline strands, each cell bears appendages of remnants of the mother cell wall. The cell size, shape and arrangement of coenobia and syncoenobia are considered to be of species-specific value (Komárek & Fott 1983). Beside the type species *C. aestivale*, the genus comprises *C. elipsoideum* Fott, *C. lunatum* Thompson and *C. chodatii* Komárek (Fott 1946; Komárek 1974; Thomspon 1950). The status of *C. anglicum* Flint as an independent species was since its description in 1950 dubious (Flint 1950). Already Flint pointed out, that the species bears a high similarity to *C. aestivale* (Flint 1950). Williams (1972) discussed, that it may be a variety of *C. aestivale* nevertheless he kept it as a separate species. However, Komárek & Fott (1983) placed *C. anglicum* as a synonym of both *C. ellipsoideum* and *Komarekia appendiculata* Chodat which reduced the genus *Coronastrum* to four species.

Whereas *C. aestivale* forms syncoenoebia with spherical cells, *C. elipsoideum* is characterized by mainly four celled coenobia with elliptic cells and appendages attached at the cell poles (Komárek & Fott 1983; Fott 1946). The species are easily distinguished from *C. lunatum* which has lunate to triangular cells with awl-shaped, straight or slightly curved appendages (Jose et al. 1990; Kim & Takamura 2000). The fourth species *C. chodattii* Komárek shows straight, oval to broad ellipsoid cells with thick, hardly visible connecting strands (Komárek 1974; Komárek & Fott 1984). The main distribution of *Coronastrum* covers Europe, America and Asia with reports from Germany, Great Britain, Sweden, Switzerland, Czech Republic/Slovakia ("Czechoslovakia"), the Ukraine, Maryland, Oklahoma, Cuba, India and Japan (Bürgi 1985; Comas 1992; John & Tsarenko 2002; Kim & Takamura 2000; Komárek & Fott 1983; Pfiester 1977; Thompson 1950).

At the moment, there are only three strains of *Coronastrum* deposited in public culture collections. The strain SAG 2079 is lost due to contamination of *Scenedesmus* sp. (Bock pers. observation). *Coronastrum ellipsoideum* (UTEX LB1382) evolved within the

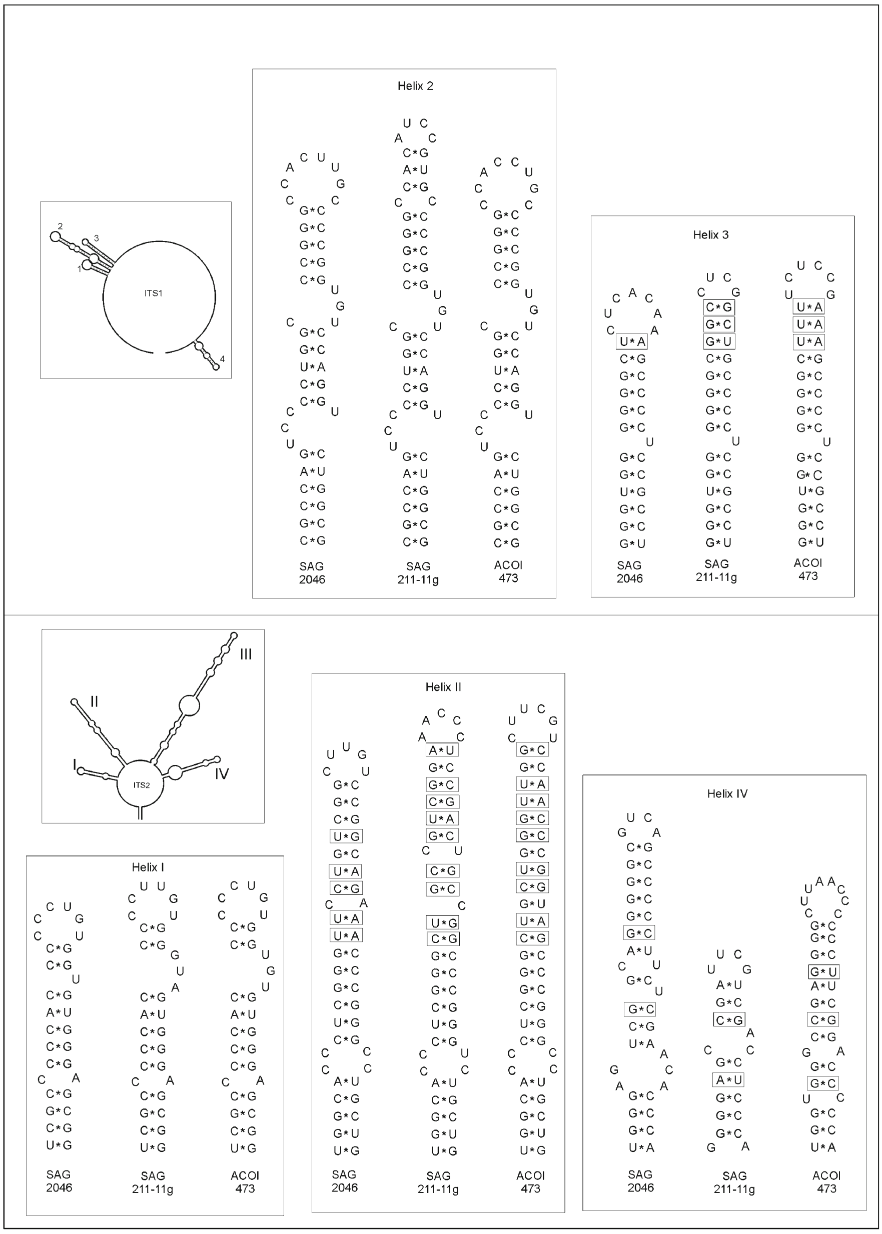


Fig. 8. Comparison of the secondary structure of *Parachlorella* species (SAG 2046 = *P. beijerinckii*, SAG 211-11g = *P. kessleri*, ACOI 473 = *P. hussii*).

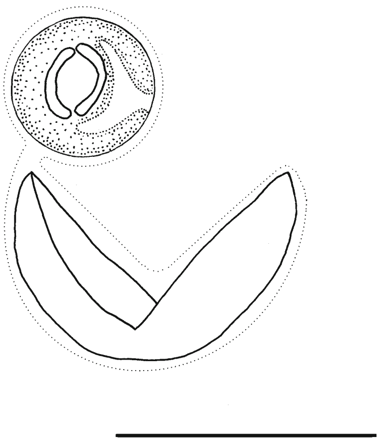


Fig. 9. Type drawing of *Parachlorella hussii*. Scale bar 10 µm.

Chlorella clade of the Chlorellaceae (Fig. 1). Surprisingly, it clustered in close relationship of two strains of the *Dictyosphaerium* morphotype. Another strain with *Chlorella*/*Parachlorella*-like morphotype could be observed next to *C. ellipsoideum* (Figs 4–5). The polyphyletic origin of the *Dictyosphaerium* morphotype was already reported by Krienitz et al. (2010). Independent lineages based on former *Dictyosphaerium* morphospecies form new genera within the *Chlorella* and *Parachlorella* clades (Bock et al. 2010). More diverse morphotypes were found within the Chlorellaceae due to molecular revisions (Fawley et al. 2005; Luo et al. 2010; Pröschold et al. 2010). Among others, the former Scenedesmaceae genera *Didymogenes* and *Dicloster* belong now to the Chlorellaceae. Also members of genera like *Dictyosphaerium* (formerly

Botryococcaceae), *Micractinium* Fresenius (former Micractiniaceae), *Actinastrum* Lagerheim (former Coelastreaceae) and *Closteriopsis* Lemmermann (Selenastraceae) cluster within this relationship. These molecular results showed, that former morphological criteria like coenobia, autosporulation or mucilage production cannot be used in differentiating families. A clear morphological criterion in seperating the Scenedesmaceae from the Chlorellaceae is still missing.

We analyzed the strain ACOI 473 and could not confirm the predesignation as *C. aestivale.* The strain showed the typical morphology of *Parachlorella* with spherical cells of 4.5–6.5 (7.5) μm size (Figs 6–7). The genus *Parachlorella* was erected by Krienitz et al. (2004). Two species were included within the genus: *Parachlorella kesslerii* (Fott & Nováková) Krienitz, E. Hegewald, Hepperle, V. Huss, T. Rohr & M. Wolf and *P. beijerinckii* Krienitz, E. Hegewald, Hepperle, V. Huss, T. Rohr & M. Wolf. The latter is surrounded by a mucilage envelope, whereas *P. kessleri* lacks the indusium (Jahn & Kusber 2010; Krienitz et al. 2004). Only the adult cells of ACOI 473 showed mucilage envelopes whereas the young cells were not covered. Our molecular analyses based on SSU and ITS rRNA gene analyses confirmed this identification and placed this strain within the genus *Parachlorella,* establishing a new lineage together with the strains ACOI 938, 939, 1508 (Fig. 1). The analyses of the secondary structure revealed several CBCs in conserved regions of the ITS2 in comparison to the already known species *Parachlorella kessleri* (SAG 211-11g) and *P. beijerinckii* (SAG 2046). Already Coleman (2009) and Müller et al. (2007) pointed out, that CBCs in the conserved regions of the ITS2 are closely associated with an inability of sexual reproduction. We propose the new species *Parachlorella hussii* sp. nov. based on CBCs within the secondary structure of the ITS1 and ITS2.

Parachlorella hussii sp. nov. C. Bock, Pažoutová et Krienitz (Figs 6, 7, 9)
DIAGNOSIS: Cellulae solitariae, planctonicae; cellulae iuvenales ovoideae, cellulae adultae globosae, 4.5–6.5 (7.5) μm. Cellulae adultae tegumentis mucilagineis vestitae 1–3 μm latae. Chloroplastus unicus, parietalis vel poculiformis; pyrenoide late ellipsoidea granis amyli tecto. Reproductio asexualis 2, 4 vel 8 autosporum ope. A speciebus ceteris generis ordine nucleotidorum in 18S rRNA et ITS rRNA differt.

Cells solitary, planktonic, young cells oval, adult cells spherical to slightly oval, 4.5–6.5 (7.5) μm. Adult cells surrounded by a gelatinous indusium, 1–3 μm thick. Chloroplast single, parietal, cup-shaped, with broadly ellipsoid pyrenoid, covered by two starch grains. Reproduction by autosporulation with 2, 4 or 8 autospores. It differs from other species of the genus by the order of the nucleotides in SSU and ITS rRNA gene.
HOLOTYPE (here designated): An air-dried as well as a formaldehyde-fixed sample of strain ACOI 473 was deposited at the Botanical Museum at Berlin-Dahlem under the designation B400040719.
AUTHENTIC STRAIN: maintained at the Coimbra Collection of Algae under the designation ACOI 473.
TYPE LOCALITY: Montemor-o-Velho, Vala de Maiorca, Portugal.
ETHYMOLOGY: The species is named in honour of the phycologist V.A.R. Huss, (Erlangen, Germany) the pioneer of the molecular-phylogenetic studies in *Chlorella.*

Unfortunately, no material of the type species *C. aestivale* could be analysed. Therefore the phylogenetic position of the genus *Coronastrum* based on molecular analyses is still unknown. Our analyses could show that *Coronastrum ellipsoideum* belongs to the *Chlorella* clade of the Chlorellaceae. We could further observe a new species of the genus *Parachlorella*, *P. hussii* which differs from the other species of the genus by molecular criteria.

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