Phylogenetic position of *Ooplanctella planoconvexa*, gen. et comb. nova and *Echinocoleum elegans* (Oocystaceae, Trebouxiophyceae, Chlorophyta).

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Abstract: The coccoid green algae *Coenochloris planoconvexa* and *Echinocoleum elegans* are assigned to different families (Radiococcaceae and Oocystaceae, respectively), but display similar ray–like mucilaginous envelopes. Molecular analysis of the 18S rRNA gene of *C. planoconvexa* CAUP H 5502 and *E. elegans* SAG 37.93 revealed that they both belong to Oocystaceae. However, the two strains were not a part of a monophyletic cluster. Furthermore, their morphology differed, namely in the shape of their mucilaginous envelopes, and in their ability to form four–celled units in a broadened sporangial wall. Because the name *Coenochloris* cannot be used for members of *Oocystaceae*, a new genus *Ooplanctella,* with the type species *O. planoconvexa* comb. nova, is proposed.

Key words: 18S rDNA, *Coenochloris, Echinocoleum*, molecular phylogeny, Oocystaceae, *Ooplanctella* gen. nov., Trebouxiophyceae

Introduction

Since the 1990's, molecular methods have become increasingly important in the taxonomy of green algae. The traditional generic concept, based predominantly on morphological characters, has often been challenged (Krienitz et al. 2001; Krienitz et al. 2004; Luo et al. 2006). Whilst some genera or families have been split into different lineages (Huss et al. 1999, Wolf et al. 2003), the family Oocystaceae has constituted a monophyletic lineage within Trebouxiophyceae retaining high bootstrap support in most published trees (Hepperle et al. 2000; Pröschold & Leliaert 2007).

The family Oocystaceae is either reserved for algae that somewhat resemble the genus *Oocystis* with its characteristic oval cell–shape (Brunnthaler 1915), or a much broader concept is applied and the group comprises diverse autosporine algae such as *Chlorella* or *Kirchneriella* (Smith 1950). Since the work of Komárek (1979) the definition of the family has been based upon the ultrastructure of the multilayered cell wall.
The status of Oocystaceae as a discrete family within Trebouxiophyceae was confirmed with molecular data by Hepperle et al. (2000). In this work, the genera *Amphikrikos* and *Tetrachlorella,* not previously attributed to this family, were included in the Oocystaceae. Also, paraphyly of the genus *Oocystis* itself was revealed.

We investigated the phylogenetic position of two strains, *Coenochloris planoconvexa* CAUP H 5502 and *Echinocoleum elegans* SAG 37.93. *Echinocoleum elegans* was described by Jao & Lee (1947) as a genus close to *Oocystis*, but possessing extraordinary ray–shaped mucilaginous envelopes. *Coenochloris planoconvexa* was originally proposed as a member of the family Radiococcaceae (Hindák 1977), but it exhibits mucilaginous structures similar to those of *Echinocoleum*. The objective of our study was to test whether these two species represent the same genus.

Materials and methods

Strains of *Coenochloris planoconvexa* CAUP H 5502 and *Echinocoleum elegans* SAG 37.93 were kindly provided by the Culture Collection of Algae of

Charles University of Prague (CAUP) and the Culture Collection of Algae at the University of Göttingen (SAG). The strains were maintained in test tubes on BBM medium (BISCHOFF & BOLD 1963) solidified by 1% agar under a constant light source of 5–15 μmol.m^{-2}.s^{-1} and a temperature of 15 °C. To induce mucilage production, the strains were transferred to 100% and 50% liquid BBM medium in aerated 50ml tubes and kept at room temperature.

For morphological observations and microphotography an Olympus BX 51 light microscope equipped with Nomarski DIC optics and an Olympus Camedia C–5060 digital camera were used. To detect the mucilaginous structures, samples were stained with Methylene blue immediately prior to examination under light microscopy.

Total DNA was extracted from either lyophilized (*C. planoconvexa*), or untreated (*E. elegans*) biomass, using the CTAB method according to DOYLE & DICKSON (1987), or the Invisorb Spin Plant Mini Kit (Invitek) following the manufacturer's protocol, respectively.

The polymerase chain reaction (PCR) was carried out with Jump Start Red Taq Polymerase (Sigma), using 0.5 μm of the enzyme, and 5 ng of the extracted DNA in a 20 μl total reaction volume. Samples were processed on an XP thermal cycler (Bioer) with the following cycle: initial denaturation 95 °C, 5 min – [denaturation 95 °C, 1 min – annealing 54 °C, 3 min] – elongation 72 °C, 1 min – final elongation 72 °C, 10 min; the cycle of denaturation, annealing and elongation performed 35x. The amplified fragments were visualized by staining with ethidium bromide following electrophoresis in 1% agarose.

For the amplification of the 18S rRNA gene we used either universal eukaryotic, algal specific or Oocystaceae–specific primers (Table 1). To obtain the complete sequence of *C. planoconvexa,* two overlapping PCR reactions were performed with 34F + 1650R vivi and 1500 AF + ITS4 primers. Common universal primers were unsuccessful with *E. elegans*, and for this reason specific primers were designed according to conservative areas from known 18S rRNA sequences of Oocystaceae.

The PCR product was purified with the QIAquick Gel Extraction Kit (Quiagen) and sent to Macrogen Inc., Korea, for sequencing. The sequences were completed with Seqassem (HEPPERLE 2004).

The initial alignment of Trebouxiophyceae was obtained from the European Ribosomal RNA database (VAN DE PEER et al. 2000). This alignment was then improved upon by the addition of several sequences not included in the database, and aligned using ClustalX 1.83 (THOMPSON et al. 1997) and MUSCLE (EDGAR 2004), with the assistance of a predicted secondary structure model for *Micractinium pusillum* (LUO et al. 2006). Introns and 5′ and 3′ terminal regions were removed, resulting in an alignment comprising 1895 positions. To further improve alignment quality, the stability of alignment was assessed through comparison of ClustalW alignments produced under different gap opening/extension penalties using SOAP v.1.2 alpha 4

Table 1. PCR and sequencing primers used in this study.

primer name	sequence	authority	specificity
34F	GTCTCAAAGATTAAGCCATGC	FRIEDL (unpubl.)	Eukaryota
1650R vivi	TCACCAGCACACCCAAT	KIPP (2004)	algae
1500 AF	GCGCGCTACACTGATGC	HELMS et al. (2001)	algae
ITS 4	TCCTCCGCTTATTGATATGC	WHITE et al. (1990)	Eukaryota
370R	AGGCTCCCTCTCCGGAATCRAACCC	FRIEDL (unpubl.)	Eukaryota
402–23F	GCTACCACATCCAAGGAAGGCA	KATANA et al. (2001)	Eukaryota
1122F	GGCTGAAACTTAAAGGAATTG	FRIEDL (unpubl.)	Eukaryota
1263R	GAACGGCCATGCACCACC	FRIEDL (unpubl.)	Eukaryota
18L	CACCTACGGAAACCTTGTTACGACTT	HAMBY et al. (1988)	Eukaryota
895–916F	GTCAGAGGTGAAATTCTTGGAT	KATANA et al. (2001)	Eukaryota
898–919R	TAAATCCAAGAATTTCACCTCT	KATANA et al. (2001)	Eukaryota
OOF	CTGTGGCTTAATTTGACTCAACACG	This study	Oocystaceae
OOR 1	GAGCTCTCAATCTGTCAATCCTCAC	This study	Oocystaceae
OOR 2	AGCCACAGGCTCCC-GTTCATTTCGGGC	This study	Oocystaceae
OOR 3	ACGCCTGGTGGTGCCCTTCCGT	This study	Oocystaceae

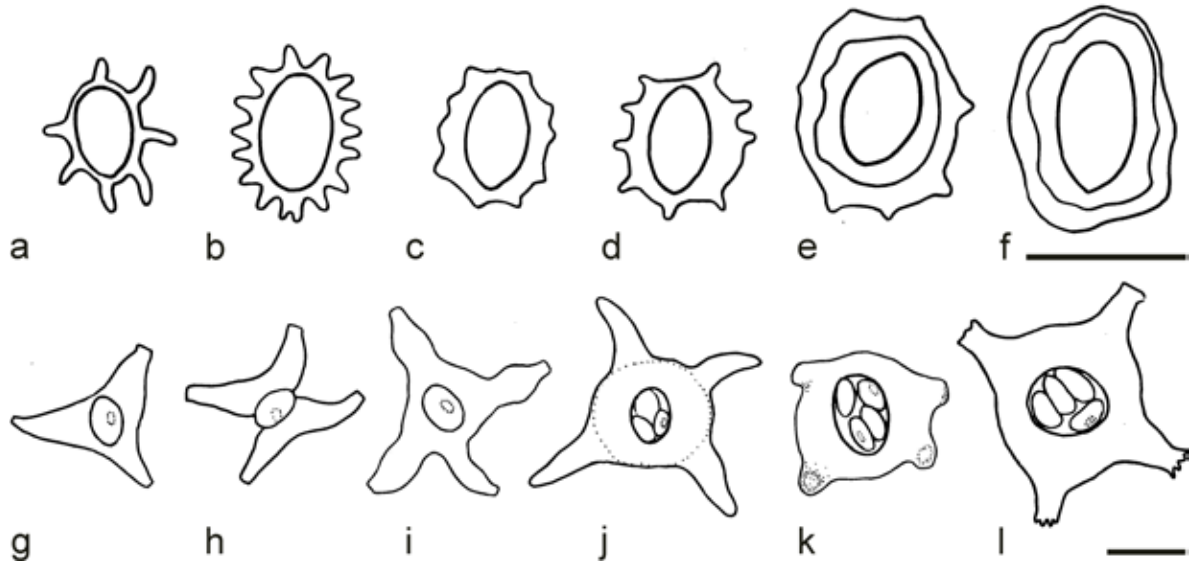

Fig. 1. Mucilaginous envelopes of *Ooplanctella planoconvexa* (a–f) and *Echinocoleum elegans* (g–l). Scale bar 10μm.

(Löytynoja & Milinkovitch 2001). Gap penalties were incrementally adjusted from 7 to 17 by steps of 2, and extension penalties were adjusted from 4 to 9 by steps of 1. Regions of instability were deleted by computing to a 90% consensus among the thirty–six different alignments, leaving an alignment of 1797 positions (alignment available from authors upon request).

The phylogenetic trees were inferred by maximum likelihood (ML) and weighted parsimony (wMP) criteria using PAUP*, version 4.0b10 (Swofford 2002), and by Bayesian inference (BI) using MrBayes version 3.1 (Ronquist & Huelsenbeck 2003). For ML and BI analyses, a suitable model for the process of DNA substitution was chosen using the Akaike Information Criterion (AIC) with PAUP/MrModeltest 1.0b (Nylander 2004). The best model was found to be GTR+Γ+I. Maximum likelihood analyses consisted of heuristic searches with 1,000 random sequence addition replicates and Tree Bisection Reconnection swapping. Reliability of the resulting topology was tested using bootstrap analysis (100 replications) consisting of heuristic searches with 10 random sequence addition replicates, tree bisection reconnection swapping, and a rearrangement limit of 5,000 for each replicate. The wMP bootstrapping was performed using heuristic searches with 100 random sequence addition replicates, tree bisection reconnection swapping, random addition of sequences (the number limited to 10,000 for each replicate), and gap characters treated as a fifth character state. In BI analysis, two parallel MCMC runs were carried out for 2 million generations, each with one cold and three heated chains. Trees and parameters were sampled every 100 generations. Convergence of the two cold chains was checked and burn–in was determined using the "sump" command.

Results

Morphology

Coenochloris planoconvexa CAUP H 5502. Cells mostly ellipsoid to broadly oval, *Oocystis*–like, often asymmetrical; cell breadth 4–6–(8) μm; cell length 5–9 μm. Reproduction by 2 or 4 autospores. On agar plates the cells showed no regular arrangement, and no mucilage was visible. When grown in aerated liquid medium, faint mucilaginous envelopes were visible after staining with Methylene blue, usually ray– shaped with numerous small mucilaginous "spines", otherwise irregular, or loosely undulating (Fig. 1: a–f). Sometimes, two separate envelope layers were present around a single cell. On the stained material polar thickenings of the cell walls were visible. Tetrads of young cells enclosed within a broadened sporangial wall were not observed.

Echinocoleum elegans SAG 37.93. Cells ellipsoid, with a typical *Oocystis*–like shape and with distinct cell wall thickenings clearly visible on at least one end of the cell without staining. Cell breadth 3–5 μm; cell length 6–8 μm. Reproduction by 4 autospores. Both on agar and in liquid medium, large mucilaginous envelopes were visible after staining with Methylene blue, and were of various distinct shapes, typically with three or four mucilaginous arms (Fig. 1: g–l). Single cells or groups of four cells in the broadened sporangial wall were found within individual envelopes.

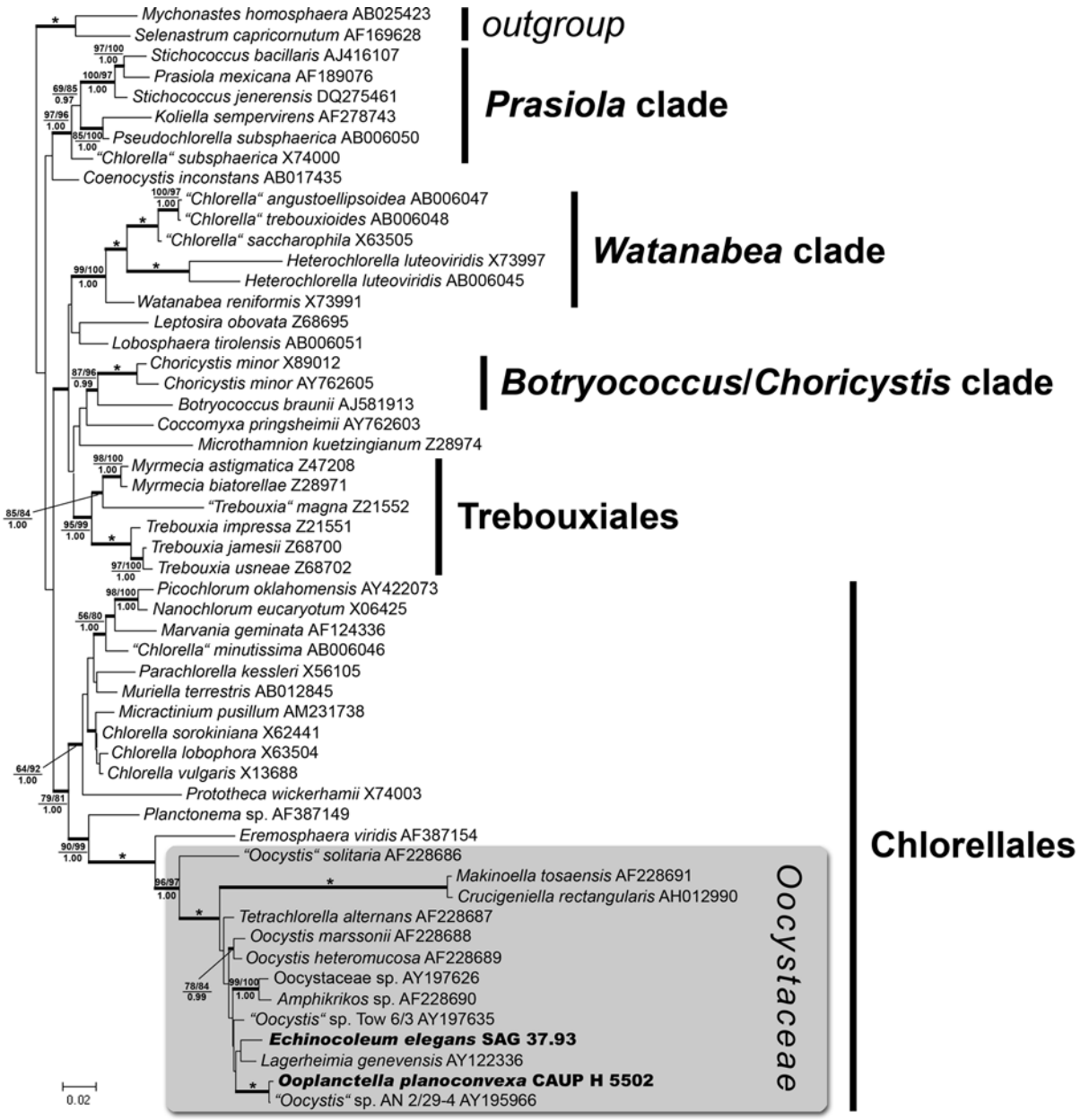

Fig. 2. Maximum likelihood tree of a representative set of 18S rRNA sequences from the class Trebouxiophyceae, with emphasis on the Oocystaceae diversity. Evolutionary model: GTR + Γ + I. Values at the nodes indicate statistical support estimated by three methods: maximum likelihood bootstrap (top left), maximum parsimony bootstrap (top right), and MrBayes posterior node probability (lower). Only values greater than 80% (ML/MP) and 0.95 (BI) are shown. Full support (100/100/1.00) is marked with an asterisk. Thick branches represent nodes receiving high Bayesian support (≤ 0.99). New 18S rRNA sequences determined in this study are shown in bold face. Scale bar represents substitutions per site.

Phylogeny

The PCR amplicon of the 18S rRNA gene of *Echinocoleum elegans* was significantly larger than that of *Coenochloris planoconvexa*. Sequencing of the PCR product of *E. elegans* produced seven 18S rDNA primary exon regions and six intervening sequences (introns). The sequenced part of 18S rRNA gene, including introns, comprised 4257 bp. Excluding all introns, we analyzed a segment of 1629 bp. The sequence of 18S rRNA gene of *C. planoconvexa* was 2266 bp long and contained one intron segment of 615 bp. According to BLAST searches against the nr database at NCBI (as of January 2009), the sequences were most similar to 18S rRNA gene sequences of trebouxiophycean algae classified in the family Oocystaceae (namely *Amphikrikos* sp. AF228690 and *Oocystis* sp. AY195966, for *E. elegans* and *C. planoconvexa*, respectively). To determine the position of *E. elegans* and *C.*

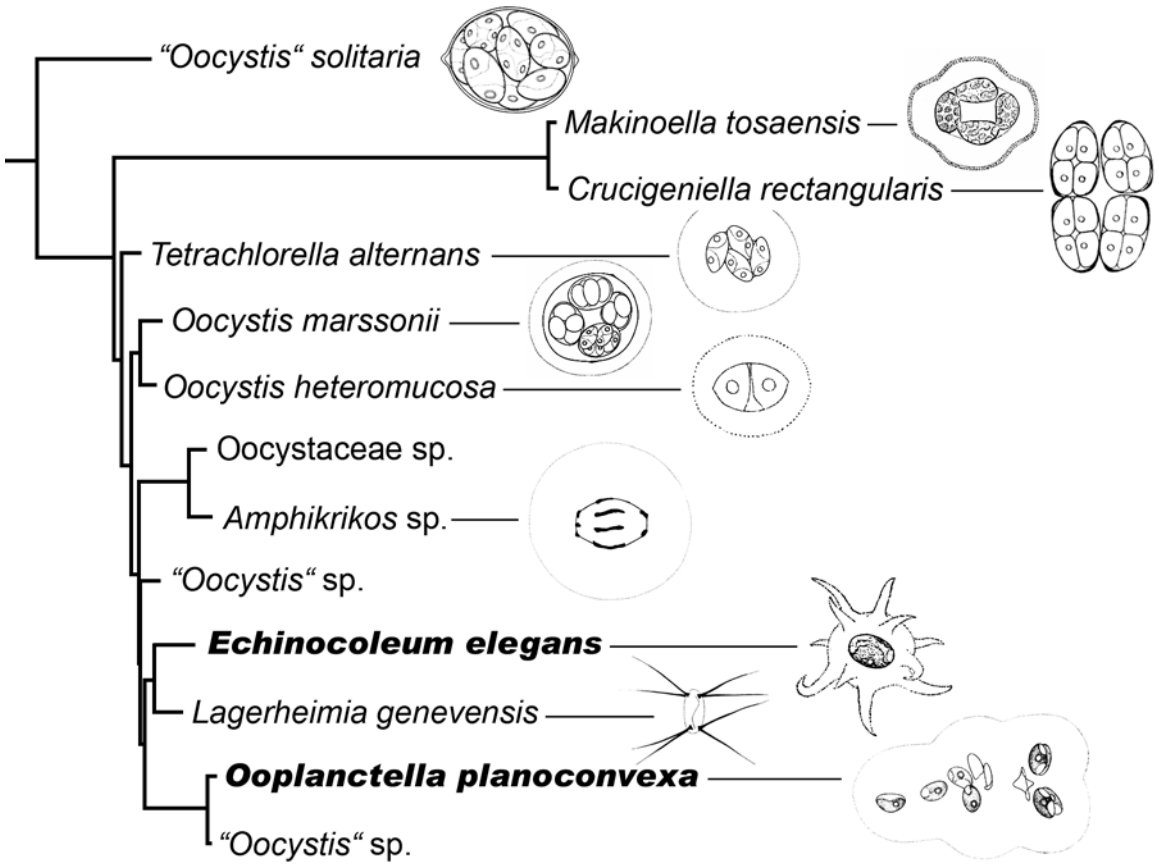

Fig. 3. Maximum likelihood tree of the Oocystaceae (a detailed view of the 18S rRNA tree presented in Fig. 2), on which morphological variability has been mapped. Names of organisms considered in this study are shown in bold face. Each drawing illustrates general diacritical features for the presented species (with emphasis to cell arrangement and mucilage structure). Drawings modified after Jao & Lee (1947), Hindák (1977, 1984, 1988) and Komárek & Fott (1983).

planoconvexa in more detail, we performed a phylogenetic analysis using a broad sample of Oocystaceae diversity that included all published sequences of organisms belonging to this family, except four (AF267867, AF267868, AM072917 and DQ887507) which were excluded due to their extremely short length (less than 620 bp).

Phylogenetic analysis (Fig. 2) of sequences from *E. elegans* and *C. planoconvexa* established these as members of the Oocystaceae, and the monophyly of the family was substantiated with 96/97% ML/MP bootstrap support and 1.00 posterior probability. The analysis demonstrated the paraphyly of the genus *Oocystis*, as the gene sequence of *Oocystis solitaria* is positioned outside of a clade formed by the remaining members of *Oocystis*. The analysis revealed the close relationship of *C. planoconvexa* to *Oocystis* sp. AY195966. These two strains form a distinct crown lineage within the Oocystaceae. *E. elegans* occupies a discrete position in the family, with an unresolved relationship to other taxa.

Discussion

Nowadays, the taxonomy of green algae relies heavily on molecular data. The new molecular phylogenetic insights allow us to reevaluate the morphological criteria on which taxonomy has relied for more than a century. In the case of Oocystaceae, the broad definition conceived, for example, by Smith (1950) and Melkonian (1983) has been refuted by Hepperle (2000) and other recent works. However, some of the morphological and ultrastructural criteria of the more recently defined Oocystaceae *sensu stricto* (Komárek 1979; Komárek & Fott 1983), notably the specific cell shape, can still be applied successfully to recognize this family. Yet, the distinctions are not as clear at the generic and species levels. For example, an extraordinary

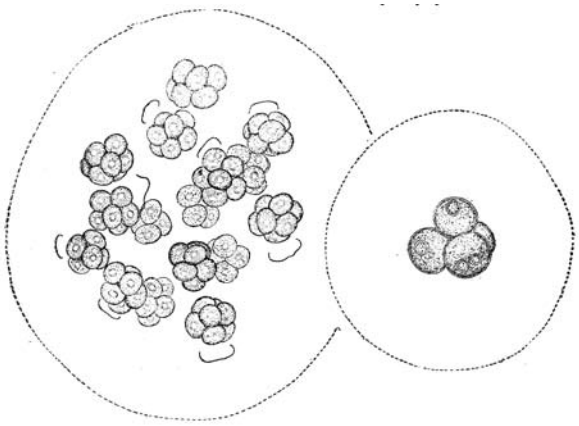

Fig. 4. Iconotype of *Coenochloris pyrenoidosa*, orig. after KORŠIKOV (1953).

shaped "spinuous" mucilage was reported to be a characteristic trait of the genus *Echinocoleum* (JAO & LEE 1947; KOMÁREK & FOTT 1983). However, a similar feature was also described for *Oocystis lacustris* (CHODAT 1902; ŘEHÁKOVÁ 1969), *Coenochloris planoconvexa* (HINDÁK 1977, 1980) and *Lagerheimia ciliata* (HINDÁK 1978). A notable morphological plasticity of a single strain was detailed especially in the last–mentioned species, including spinuous, as well as spineless stages and stages with or without mucilage of simple or ray–like shape. Interestingly, *Echinocoleum* and spiny *Lagerheimia* may be closely related according to our data (Fig. 2). It is therefore highly doubtful whether the shape of mucilage itself, or the presence of spines, can distinguish a single genus, as has already been shown for *Scenedesmus* or *Micractinium* (LÜRLING & BEEKMAN 1999; VERSCHOOR et al. 2004; LUO et al. 2006).

Gaining insight into the phylogeny of Oocystaceae is complicated due to the highly divergent gene sequences of some of the included species, combined with the presence of several rapidly evolving organisms. According to current knowledge, the genus *Oocystis* itself is paraphyletic, with one species in a basal position, and two others located in a moderately supported lineage within the Oocystaceae. As was proposed by KRIENITZ et al. (2001) in the case of *Ankistrodesmus,* when traditional morphological criteria fail to distinguish real monophyletic groups, we could possibly (re)establish the "large" genera for whole clusters. The family Oocystaceae would than be comprised of the single genus *Oocystis*, redefined to encompass all the previously "smaller" taxa. Alternatively, we could use more narrow, "smaller" genera that would differentiate every distinct phylogenetic lineage and respect at least some of the morphological differences among them.

A large phenotypic plasticity is found within the family of Oocystaceae (Fig. 3). Cells can be either solitary or colonial, with or without mucilage, and with different numbers of spines, etc. Traditionally, this plasticity was mirrored by a number of different genera (KOMÁREK & FOTT 1983). Only a small number of Oocystaceae members has been sequenced so far, therefore we can not presume to evaluate its morphological criteria, and we refrain from large taxonomical revisions in this group. Concerning the two species *Echinocoleum elegans* and *Coenochloris planoconvexa*, due to the high morphological variability within Oocystaceae, and in deference to its traditional perception, we prefer to adhere to the concept of "small" genera.

This study was set up to determine whether *Coenochloris planoconvexa* CAUP H 5502 and *Echinocoleum elegans* SAG 37.93 belong to the same genus. Our results, however, have indicated that the two strains involved in our study represent two different lineages (Fig. 2). This conclusion corroborates the morphological observations, notably differences in the shape of mucilaginous envelopes (Fig. 1), and lack of typical tetrads of cells enclosed within a broadened mother cell wall in the case of *C. planoconvexa*. Therefore, we conclude that these two species belong to different genera. In the case of *C. planoconvexa*, however, the generic name *Coenochloris* may not be used. The genus *Coenochloris* was first described by KORŠIKOV (1953) without a Latin diagnosis, but was accepted as valid and later typified by Koršikov's *C. pyrenoidosa* (Fig. 4). KORŠIKOV (1953) placed it in the family Protococcaceae, but later authors (e.g. FOTT 1959) assigned this genus to the Radiococcaceae, a family of autosporic algae with mucilaginous envelopes. *C. pyrenoidosa* comprises colonial algae with ellipsoid cells possessing pyrenoids, reproducing by four to eight autospores. Its cell shape bears no resemblance to that of *Oocystis,* and it has no polar thickenings of the cell wall. Considering the morphology of cells, *C. planoconvexa* could be classified in the genus *Oocystis*. However, there are several reasons to reject this approach: firstly, the formation of irregular mucilaginous colonies is not typical of *Oocystis*; secondly, this genus is paraphyletic and we are unable to establish which lineage includes its type species; and finally,

cell tetrads enclosed by a common cell wall, a characteristic feature for the genus *Oocystis*, were not observed in *C. planoconvexa*.

As the genus *Coenochloris,* with its type *Coenochloris pyrenoidosa,* does not belong to Oocystaceae, and because *C. planoconvexa* constitutes a discrete phylogenetic lineage within Oocystaceae with a specific combination of morphological traits, we propose to establish a new genus: *Ooplanctella,* with the type species *Ooplanctella planoconvexa*. Further, we presume that the sister sequence of *O. planoconvexa*, "*Oocystis"* sp. AY 195966, represents another member of this genus. However, as the sequence came from an ecological report (Fawley et al. 2004), we are unable to determine its morphology and therefore cannot verify its status.

As previously stated by Hepperle et al. (2000), a redefinition of the currently paraphyletic genus *Oocystis* is necessary. This will be guided predominantly by the phylogenetic position of the epitype, *O. lacustris* Chodat (Řeháková 1969). It is our prediction that, according to its morphological features, *O. lacustris* is more closely related to the *marssonii/heteromucosa* clade than to *O. solitaria* (Fig. 2), and that *O. solitaria* will need to be excluded from the genus *Oocystis*.

***Ooplanctella* gen. nov.**

Cellulae colonias formantes vel libere natantes, cum vel sine tegumento gelatinoso. Tegumentum gelatinosum amorphum vel spinosum. Cellulae oblongae, plerumque asymmetricae, membrana cellularis ad polos leviter incrassata. Chloroplastus parietalis, peripheriam cellulae non totam occupans, cupuliformis vel alveiformis, cum pyrenoide singulo. Propagatio 4 (etiam 2) autosporibus, cellulae adultae sine membrana matricali dilatata.

Cells in amorphous, structure–less, mucilaginous colonies, or solitary, sometimes with ray– shaped mucilaginous envelopes. Cells ellipsoid to broadly oval, often slightly asymmetrical, with polar thickenings of the cell wall. Chloroplast single, parietal, cup–shaped or trough–like, with pyrenoid. Reproduction by (2–)4 autospores, adult cells not enclosed within expanded mother cell wall.

Type species: *O. planoconvexa* (Hindák) Pažoutová, Škaloud & Nemjová comb. nova.

Basionym: *Coenochloris planoconvexa* Hindák (1977, p. 22, iconotype Pl. 5 fig. 1).

Etymology: "Ooplanctella" is a compound noun; "Oo" refers to the relationship to Oocystis, and "planctella" was chosen to reflect the ecology of the organism.

Epitype (here designated): The strain CAUP H5502 permanently cryopreserved at the Culture Collection of algae of the Charles University in Prague, Czech Republic (CAUP, http://botany.natur.cuni.cz/algo/caup.html).

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