School of Doctoral Studies in Biological Sciences

University of South Bohemia in České Budějovice

Faculty of Science

SYSTEMATICS OF COCCAL GREEN ALGAE OF THE CLASSES CHLOROPHYCEAE AND TREBOUXIOPHYCEAE

Ph.D. Thesis

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České Budějovice 2020

This thesis should be cited as:

Štenclová L., 2020: Systematics of coccal green algae of the classes Chlorophyceae and Trebouxiophyceae. Ph.D. Thesis Series, No. 20. University of South Bohemia, Faculty of Science, School of Doctoral Studies in Biological Sciences, České Budějovice, Czech Republic, 239 pp.

Annotation

Aim of the review part is to summarize a current situation in the systematics of the green coccal algae, which were traditionally assembled in only one order: Chlorococcales. Their distribution into the lower taxonomical unites (suborders, families, subfamilies, genera) was based on the classic morphological criteria as shape of the cell and characteristics of the colony. Introduction of molecular methods caused radical changes in our insight to the system of green (not only coccal) algae and green coccal algae were redistributed in two of newly described classes: Chlorophyceae a Trebouxiophyceae. Representatives of individual morphologically delimited families, subfamilies and even genera and species were commonly split in several lineages, often in both of mentioned classes.

For the practical part, was chosen two problematical groups of green coccal algae: family Oocystaceae and family Scenedesmaceae - specifically its subfamily Crucigenioideae, which were revised using polyphasic approach. Based on the molecular phylogeny, relevance of some old traditional morphological traits was reevaluated and replaced by newly defined significant characteristics.

Declaration [in Czech]

Prohlašuji, že svoji disertační práci jsem vypracovala samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury. Prohlašuji, že v souladu s § 47b zákona č. 111/1998 Sb. v platném zněni souhlasím se zveřejněním své disertační práce, a to v nezkrácené podobě elektronickou cestou ve veřejně přístupné časti databáze STAG provozované Jihočeskou univerzitou v Českých Budějovicích na jejich internetových stránkách, a to se zachováním mého autorského práva k odevzdanému textu teto kvalifikační práce. Souhlasím dále s tím, aby toutéž elektronickou cestou byly v souladu s uvedeným ustanovením zákona č. 111/1998 Sb. zveřejněny posudky školitele a oponentů práce i záznam o průběhu a výsledku obhajoby kvalifikační práce. Rovněž souhlasím s porovnáním textu mé kvalifikační práce s databází kvalifikačních prací Theses.cz provozovanou Národním registrem vysokoškolských kvalifikačních prací a systémem na odhalovaní plagiátů.

České Budějovice 3. 11. 2020

Lenka Štenclová

This thesis originated from Faculty of Science, University of South Bohemia, supporting doctoral studies in the Botany study program.

Financial support:

Financial support came from the budged of the Department of Botany (RVO) and partly from Assumption College Faculty Development Grant.

Acknowledgements:

On the very first place I thank to my supervisor Hanys for not stopping believing at this project, for his support and great ideas. I owe my thanks to Karolina Fučíková as well, for her friendly cooperation on Assumption college. I thank my colleagues and friends at the same time from České Budějovice for valuable advices and all their funny talks that this is thesis full of small green balls looking all alike. My special thanks belong to my parents for their kind support.

List of papers and author's contribution

The thesis is based on the following papers (listed according to the thematic chapters):

Štenclová, L., Fučíková, K., Kaštovský, J. and Pažoutová, M. (2017) Molecular and morphological delimitation and generic classification of the family Oocystaceae (Trebouxiophyceae, Chlorophyta). Journal of Phycology. 53(6):1263-1282. (IF 2017 = 3.000)

Lenka Štenclová cultivated strains, performed molecular methods and molecular phylogeny, morphological and ultrastructural (TEM) observations, and was main author of the manuscript.

Silva, T. G., **Štenclová, L.,** Archanjo, N.C.P. and Bagatini, C. L. Revised phylogenetic position of genus *Nephrocytium* Nägeli (Sphaeropleales, Chlorophyceae), with description of Nephrocytiaceae *fam. nov*. and *Nephrocytium vieirae sp. nov.* (Manuscript)

Lenka Štenclová cultivated one strain, performed molecular methods, morphological and ultrastructural (TEM) observations, and was second author of the manuscript.

Štenclová, L. Distribution of the Crucigenioid algae inside the classes Chlorophyceae and Trebouxiophyceae. (Manuscript)

Lenka Štenclová cultivated strains, performed molecular methods and molecular phylogeny, morphological observations, and was only author of the manuscript.

Štenclová, L. and Fučíková, K. (2019) *Dispora speciosa*, a new addition to the genus *Parallela* and the first coccoid member of the family Microsporaceae. Phytotaxa. 419(1):63-76. DOI:10.11646/phytotaxa.419.1.4 (IF 2018/19 = 1.168)

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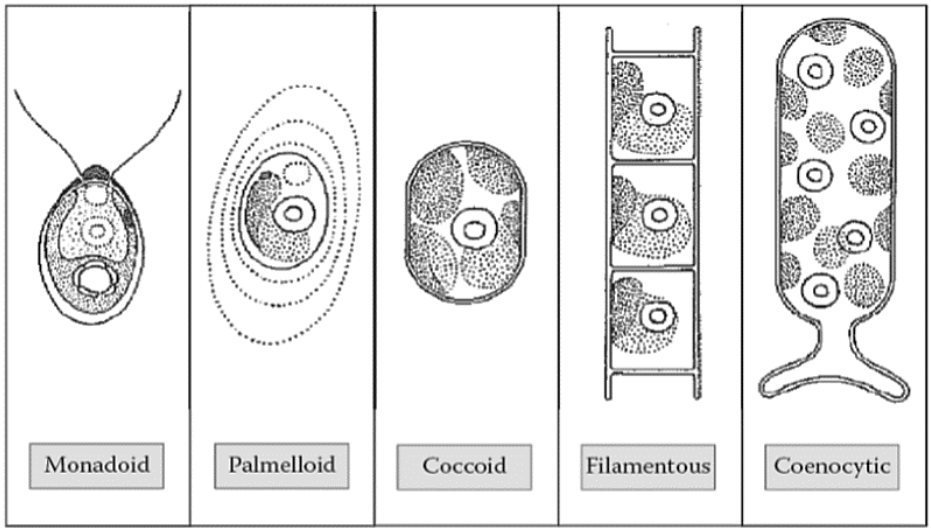
Chapter 1: Introduction: Situation in current systematics of green coccal algae



Pröschold and Leliaert 2007

Chapter 1: Situation in current systematics of green coccal algae

1.1 Traditional morphological system

Coccal thallus is the simplest organization of the algal body because the entire organism consists of simply one cell. Coccal thallus covered by visible mucilage wrap is called palmeloid or also capsal. Both types can agglomerate into regular or irregular colonies and coenobia. All the coccal and palmeloid green algae were, according to the traditional morphological system summarized in the last comprehensive morphological monograph by Komárek and Fott (1983), assembled in a single order Chlorococcales, just on the basis of the common body organization Komárek and Fott (1983), expecting its one time evolution from a green flagellate ancestor common for all green algae (Pröschold and Leliaert 2007).

Individual genera were distributed into the suborders, families and subfamilies according the cell cycle, mainly the way of their propagation: zoospores (sometimes accompanied by aplanospores), versus autospores, and according manner of the occurrence: single celled types or taxa forming colonies and coenobia (Komárek and Fott 1983). Concerning the family and subfamily level of the systematics, the attention was focused on the colonial or coenobial morphology (Figure 1). For the generic level was relevant in particular the shape of the cell and unique structures as spines or granules and partly also the character of the colony or coenobia (Komárek and Fott 1983, Figure 1). The above mentioned characteristics seems nowadays quite general and that is the reason why in the bottom view we can find the former genera especially wide; genera comprising many dozens of species (and subspecial taxa) were quite often (Komárek and Fott 1983). Also, it has commonly happened that several authors focused on a different morphological characteristic when defined species into genera (shape of the cell versus some of the characteristics of the colony) and therefore occurred quite big synonymity especially in some groups of taxa e.g. Crucigenioideae (Bock et al. 2013, Chapter 4).

Overall, all the old systems were not phylogenetic, but phenetic. Many taxonomical groups were later proved to be defined on the basis of too general traits as a result of homoplasy – an adaptation to the environmental conditions, separately formed multiple times e.g. typical colony of Crucigenioid taxa (Bock et al. 2013, Chapter 4), Radiococcaceae (Wolf et al. 2003, Pažoutová 2008, Fučíková et al. 2014a, Zhang et al. 2018), and *Dictyosphaerium* morphotypes (Krienitz et al. 2010, Bock et al. 2010, Bock et al. 2011a, Song et al. 2017), or as a plesiomorphic state – an old trait which were common for all algae which did not develop a novel form.

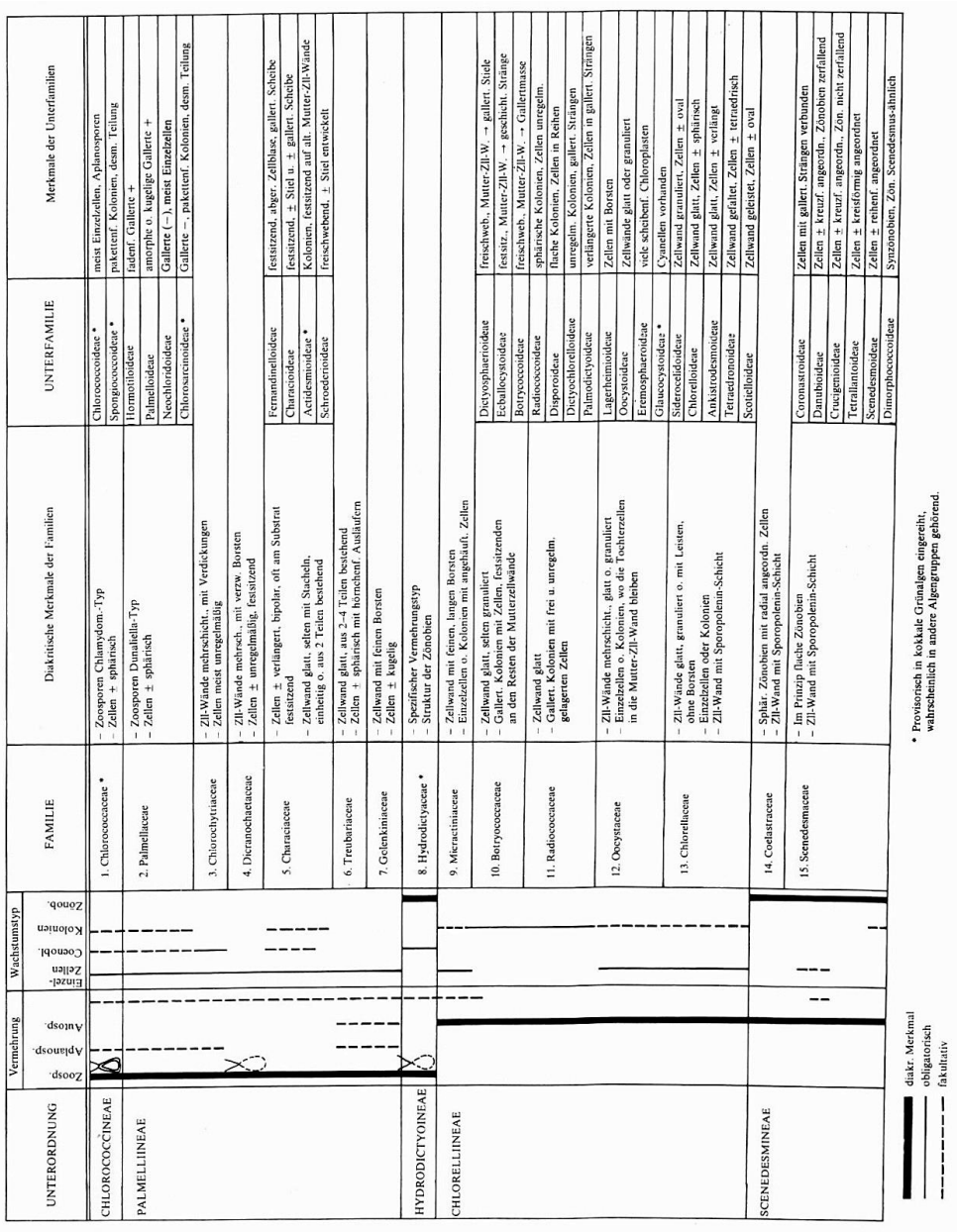


Figure 1: Traditional system according to Komárek and Fott (1983): single order Chlorococcales divided into 5 suborders, 15 families and 32 subfamilies, and morphological traits relevant for each systematic level.

1.2. Ultrastructural research challenging the morphological system

With modern microscopic methods came attempts to provide more natural system. Microscopic methods as electron microscopy – (EM both transmission – TEM and scanning – SEM) and fluorescent microscopy (FM) brought up more detailed view inside cells of examined green coccal algae, compared to the to the traditional light microscopy.

Fine section of the biological material used for TEM observations allowed us to peek inside the cells. More detailed observations of cell contents provided clear evidence of some morphological characteristics and moreover their ultrastructure, which resulted in re-valuation of these traditional morphologic structures and determination of relevant ones.

On the highest taxonomic level, instead of the on the thallus based groups, new clades were defined on the basis of arrangement of the basal bodies of flagella apparatus, distinguishing multilayered structure (MLS) of Streptophyte lineage and several structures in Chlorophyta, namely clock-wise (CW) and direct opposite (DO) clades of Chlorophyceae, counter clock-wise (CWW) structure of Trebouxiophyceae and Ulvophyceae accompanied by irregular (mono-flagellar or quadri-flagellar) Prasinophyceae (Mattox and Stewart 1984, Pröschold and Leliaert 2007). However, the classification was not applicable for many strictly autosporic coccal green algae taxa (Fučíková et al. 2015). Round (1984) and Van den Hoek et al. (1988) improved the system by including as key characteristics also life cycle, the way of cell division and the composition of the cell wall and proposed seven classes – five inside the Chlorophyta: Chlamydophyceae, Chlorophyceae, Prasinophyceae, Ulvophyceae and Trentepohliophyceae and two of the Streptophyte lineage: Charophyceae and Zygnematophyceae. Molecular concept (Lewis and McCourt 2004) some of this ultra-structurally defined classes confirmed (see Chapter 1.5).

Furthermore, ultrastructural data help us to define some units on lower levels of systematics. On the basis of ultrastructure of the cell wall was defined family Oocystaceae (Chapter 2). Ultrastructure of fine spines of green coccal algae was examined under the

transmission electron microscope (Schnepf et al. 1980, Hegewald and Schnepf 2002) and differentiated composition was determined, what supported its separated position and distribution in the system of green algae (Pröschold et al. 2010). The more detailed research of ultrastructure chloroplasts of green coccal algae removed doubts about bearing or not bearing of the pyrenoid. On the basis of presence or non-presence of pyrenoid were defined and distinguished numerous genera and species. *Oocystis* (with pyrenoid) were separated from *Oocystella* (without) (Hindák 1988) as well as *Nephrocytium* (with) from *Nephrochlamys* (without) (Edelstein and Prescott 1964 and Korshikov 1953). As not possessing pyrenoid was described genus *Makinoella* (Okada 1949). TEM research discovered pyrenoid presence in each of mentioned taxa (Hegewald et al. 1999, Krienitz et al. 2011a, Chapter 2) and on the other hand, confirmed its not existence e.g. in Microsporaceae family (Chapter 5) and genus *Chromochloris* (Kalina and Punčochářová 1987, Liu et al. 2017).

SEM allowed us to simulated 3D structure of the algal material and clearly see the cell distribution and the character of colony and what is also important, the cell surface with all structures as a placement of spines, ribs or granules. Specific ribs were detected in the genera *Scotiellopsis* and *Coellastrella* and both species were merged (Kalina and Punčochářová 1987, Pröschold et al. 2010, Kaufnerová and Eliáš 2013). *Chromochloris zofingiensis* presents cell wall surface with a network of irregular ribs (Kalina and Punčochářová 1987, Liu & al. 2017) what distinguished it from relative algae.

Fluorescent microscopy enabled researchers better understand the shape of chloroplasts utilizing natural autofluorescence of chlorophyll pigments. To determine the shape of the chloroplast by chlorophyll autofluorescence is helpful in case of alga with confusing cell content with presence of numerous granules and inclusions inside the cell (Chapter 5). Combination of chlorophyll autofluorescence with confocal microscopy lead to fine reconstruction of the complicated 3D shape of chloroplasts of *Asterochloris* species (Škaloud et al. 2015). As well useful is fluorescent visualization of shape of hyaline mucilage or cell wall structures holding the colony together, when observing colonial species as *Dictyosphaerium* taxa (Song et al. 2017).

Benefits of modern methods of microscopy combined with photographical material (compared to basic light microscopy with linear drawings) are besides other accessible fair visualization of observed material, enabling researchers to effectively compare morphological and ultrastructural characteristics and find out novel trait more relevant for the systematics of green algae.

1.3. Modern system of green algae based on the polyphasic approach

The real revolution in the taxonomy of green algae has started with the introduction of the modern method of the molecular phylogeny, reflecting the evolution of examined taxa. It has been stated as a most crucial research tool in this field and further together with additional approaches as morphology, ultrastructure, biochemistry and ecology make a polyphasic approach (Pröschold and Leliaert 2007, Leliaert et al. 2012, Leliaert et al. 2014). Big part of the old system of green algae has been already revised using polyphasic approach and replaced by the modern one (Figure 2).

The novel system has a form of tree clearly divided into two main branches - two divisions: Streptophyta including the land plants, and Chlorophyta ´blind´ line of algae (Figure 2) (Lewis and McCourt 2004, Leliart et al. 2012).

Streptophyta contains basal small classes Mesostigmophyceae, Chlorokybophyceae and Klebsormidiophyce but also Coleochaetophycea, Zygnematophyceae and Charophyceae, large classes of most probably the closest relatives to the ´higher´ land plants, and land plants themselves (Leliaert et al. 2012). Streptophytean coccal algae - except basal monotypic genus *Chlorokybus* (Lemieux et al. 2007) are withouth any doubts assigned to the monophyletic order Desmidiales (Zygnematophyceae), typical by its specific two-tailed thallus (McCourt et al. 2000) in accordance with its traditional designation (Round 1971) (Figure 2).

On the other hand, situation is much more complicated for the coccal Chlorophyta. This division contains the taxa previously assigned to ´our´ order Chlorococcales. Being considered as polyphyletic, traditional Chlorococcales have been dissolved, and

subsequently its coccal green algae have been distributed through big part of Chlorophytean tree of life (Leliaert et al. 2012). Some basal Chlorophytes possesses the coccoid thallus, (*Picocystis*, *Prasinococcus*, *Pycnococcus*), (Lewin et al. 2001, Latasa et al. 2004, Guillard et al. 1991, Leliaert et al. 2016), nevertheless, the main diversity of the green coccal algae is found within the classes Chlorophyceae and Trebouxiophyceae (Figure 2) (Friedl and Rybalka 2012, Leliaert at al. 2012). Chlorophyceae, Trebouxiophyceae and also class Ulvophyceae are three largest classes and form UTC clade (Leliaert et al. 2012). All three classes, for long time well defined by molecular phylogeny and supported by morphological and ultrastructural characteristics (Friedl 1995, Lewis and McCourt 2004, Leliart et al. 2012) and commonly accepted, were questioned in some recent studies based on whole chloroplast genome sequences (Lemieux et al. 2014, Fučíková et al. 2014b). Challenged was monophyly of the class Trebouxiophyceae as individual clades of Chlorophyceae and Trebouxiophyceae clustered together within the Ulvophyceaean algae. On the other hand, most recent study of whole genomic and transcriptomic data, though including limited number of taxa, supported monophyletic Trebouxiophyceae and Chlorophyceae and polyphyletic Ulvophyceae (Del Cortona et al. 2020 (Figure 3). The monophyly of the algae previously assigned to the UTC clade is still supported, but the term was not recommended, because of doubts of the existence of classes (Lemieux et al. 2014, Fučíková et al. 2014b, Del Cortona et al. 2020). The group was newly designed as Core Chlorophyta extended by two smaller classes Chlorodendrophyceae and Pedinophyceae (Marin 2012, Fučíková et al. 2014b). Despite the uncertainty of classes, in present thesis, I still hold the traditional designation Chlorophyceae and Trebouxiophyceae (and also Ulvophyceae) for the purposes of less confusing outputs of the work.

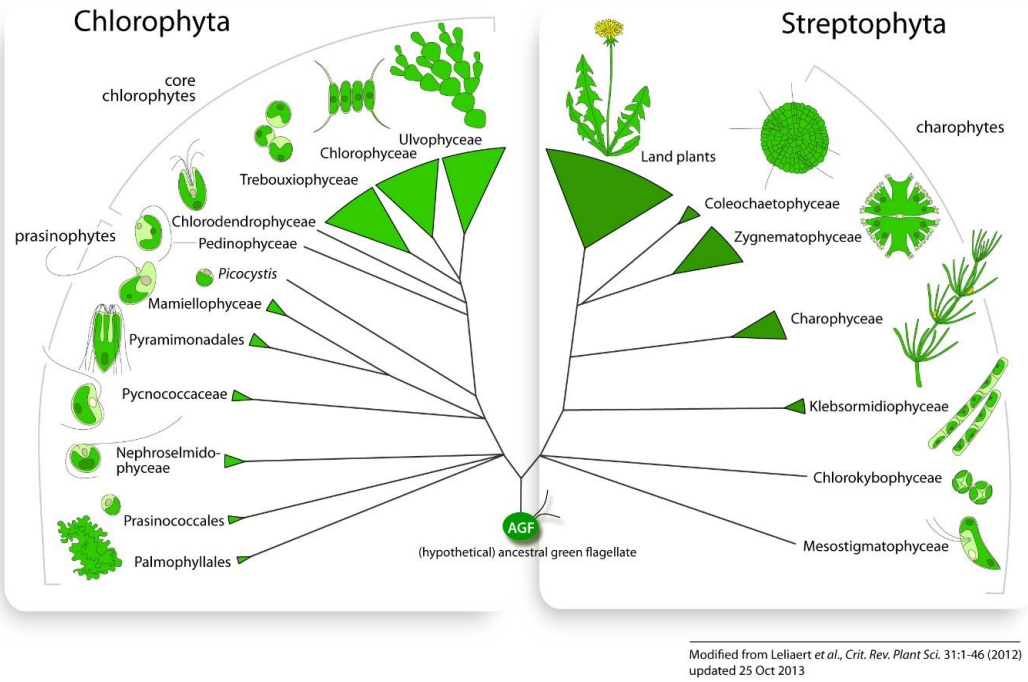


Figure 2: Overview phylogeny of the green lineage (Viridiplantae) (Leliaert at al. 2012).

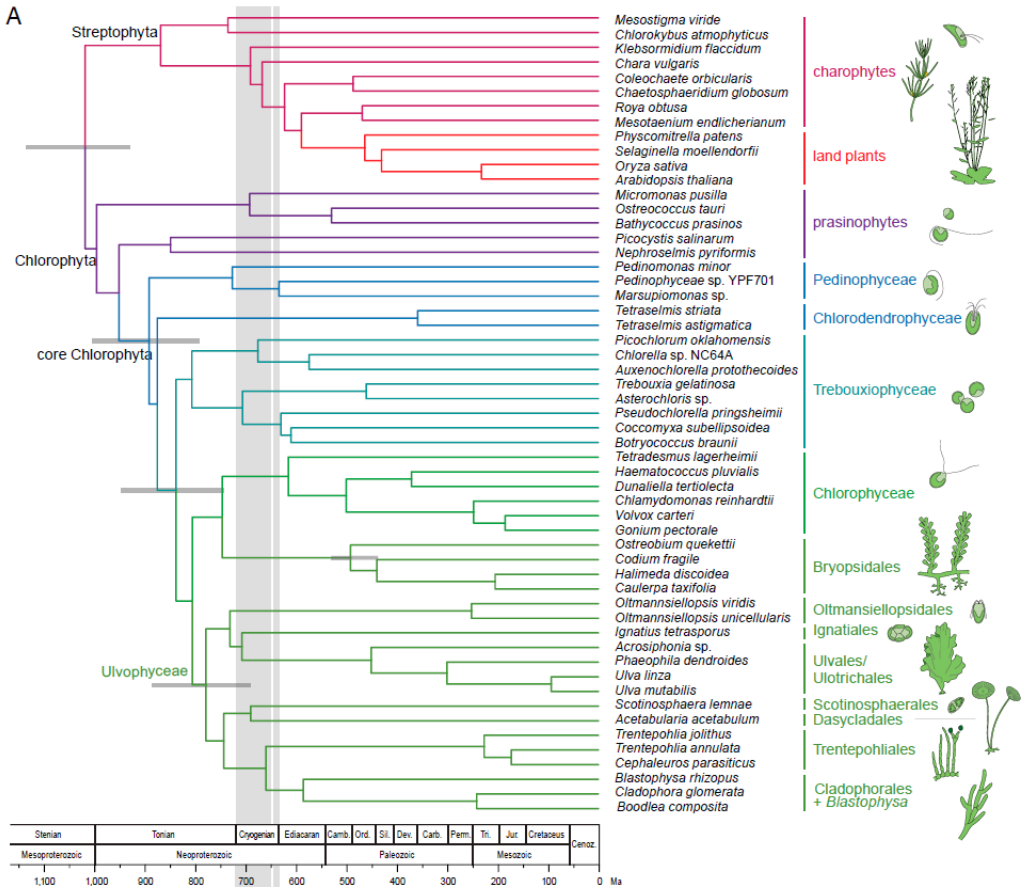


Figure 3: Time-calibrated phylogeny of the green algae. The topology of the tree is based on the ML analysis inferred from a concatenated amino acid alignment of 539 nuclear genes (supermatrix analysis of the core GF scaffolded untrimmed dataset). Branch lengths are based on are laxed molecular clock analysis of the 10 most clock-like genes from the scaffold trimmed dataset, and excluding *Proterocladus* as calibration point. Error bars are indicated for a number of key nodes. (Del Cortona et al. 2020)

1.4. Coccal algae inside classes Trebouxiophyceae a Chlorophyceae

Distribution of green coccal algae of former Chlorococcales *sensu* Komárek and Fott (1983) (Figure 1) into families according to morphological characteristics was not supported by molecular phylogeny. Delimitation of the families drastically changed, which, in some cases, caused its disintegration or novel much narrower definition e.g. in case of the family Radiococcaceae Wolf et al. 2003, Pažoutová 2008, Fučíková et al. 2014a, Zhang et al. 2018). Many families were erected *de novo* (Fučíková et al. 2014a). Also a lot of traditionally accepted genera was shown to be polyphyletic, and split to several more or many narrower ones. Example provides numerous studies the genera *Chlorella* or *Dictyosphaerium* (e.g. Hus et al. 1999, Luo et al. 2010, Bock et al. 2010, Bock et al. 2011a, Krienitz et al. 2010, Fučíková and Lewis 2012, Song et al. 2017). On the contrary taxa not assigned together were found to be closely related (examples found in all the system, therefore just for ilustration: Hepperle et al. 2000, Hegewald and Schnepf 2002, Krienitz et al. 2010, Bock et al. 2011b, Pegg et al. 2015). Moreover, modern taxonomical units often do not contain just coccal forms, but coccal algae are mixed together with monadoid or filamentous green algae (Friedl and Rybalka 2012). Significant amount of changes was already submitted, however, enough question marks persist in the in the system of green algae.

The elaborate system of Trebouxiophyceae have not been defined and except some of the accepted designation (Chlorellales, Microthamnionales and Trebouxiales) most of the authors prefer division of Trebouxiophyceae into clades rather than orders and families (*Choricystis/Botryococcus* clade, *Elliptochloris* clade, *Lobosphaera* clade, *Prasiola* clade *Watanabea* clade) and the system, is full of taxa with *incertae sedis* (Neustupa et al. 2011, Fučíková et al. 2014c - Figure 4, Li et al. 2020). Phylogenetic analyses determined coccal Trebouxiophyceae algae mixed with multicellular types, mostly (pseudo-)filamentous with various level of connection of the individual cells, in most of the clades of Trebouxiophyceae. Chlorellales contains simply filamentous genera *Geminella*, *Planctonema*, and *Ecballocystopsis* (Mikhailyuk et al. 2008, Chapter 2). *Prasiola* clade includes multicellular genera *Prasiola*, *Prasionella*, *Prasionema*, *Rosenvigiella* and *Ekerewekia* (Moniz et al 2012, Heesch et al. 2016, Kaštovský et al. 2016),

Microthamnionales branching genus *Microthamnion* (Neustupa et al. 2011), and Trebouxiales simply filamentous genus *Stichococcus* (Neustupa et al. 2011). Simple multicellularity occurs in the morphology of genus *Leptosira* with *incertae sedis.* (Neustupa et al. 2011, Gaysina et al. 2013).

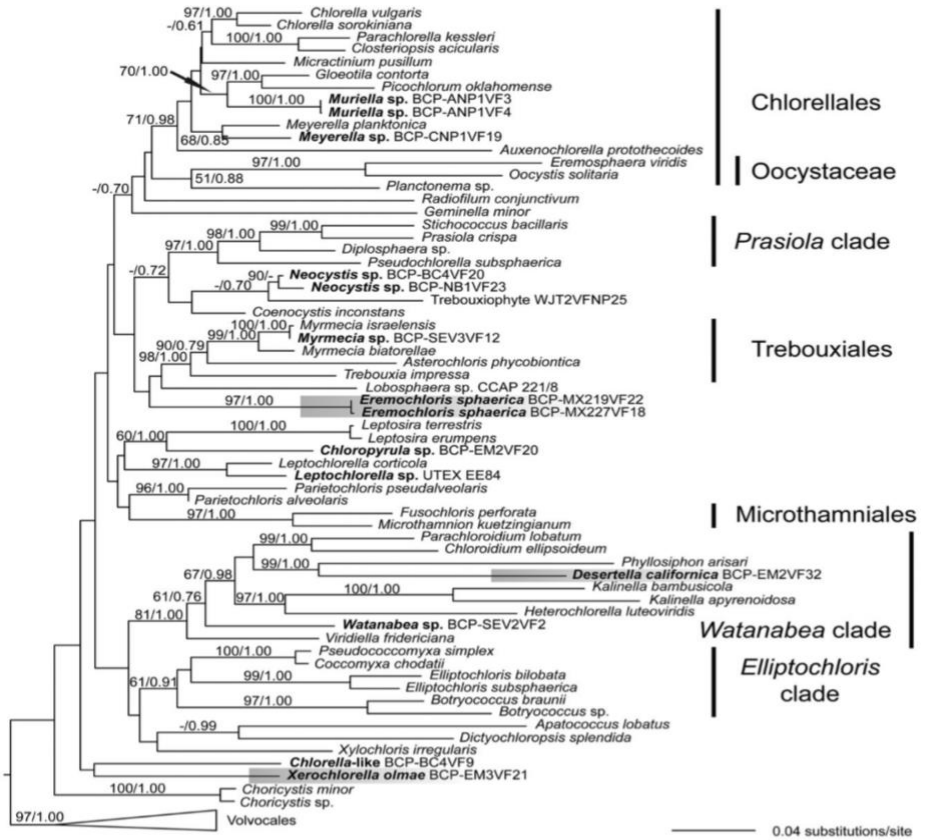


Figure 4: ML tree based on analyses of the combined 18S rRNA and *rbc*L dataset. Numbers on branches represent ML bootstrap support values and Bayesian posterior probabilities, respectively. Dashes (–) indicate support lower than 50 or 0.50. Sequences obtained in this study are highlighted in bold font. Newly characterized taxa are highlighted in grey boxes. Scale bar represents the expected number of substitutions per site. (Fučíková et al. 2014c).

Inside Chlorophyceae, coccal algae are associated in clearly defined SV clade containing orders Sphaeropleales and Volvocales. In Volvocales, coccal forms are mixed with monadoid algae and all Sphaeropleales is predominantly coccal, except small filamentous family Microsporaceae (Chapter 5) (Turmel et al. 2009, Buchheim et al. 2012, Tippery et al. 2012). The rest of Chlorophyceae includes orders Chaetophorales, Chaetopeltidales and Oedogoniales commonly designed as OCC clade (Turmel et al. 2009, Buchheim et al. 2012, Tippery et al. 2012) consists of algae with more complex thallus. Divergence of SV and OCC clades dispose of robust support from molecular, morphological and ultrastructural data (Fučíková et al. 2014b, Fučíková et al. 2019, Del Contorta 2020 - Figure 3).

However, most recent study suggested, that definition of Volvocales and Sphaeropleales itself is not so distinct (Fučíková et al. 2019). Molecular analyses of protein coding genes of chloroplast genomes confirmed monophyletic (though adjusted) clade of Volvocales, nevertheless, Sphaeropleales was designed as possible paraphyletic, because of unstable position of Sphaeropleaceae (Figure 5). Also, exact position of the clade of Microsporaceae and Treubarinia clade is uncertain. Both problematic groups contain simple filamentous algae.

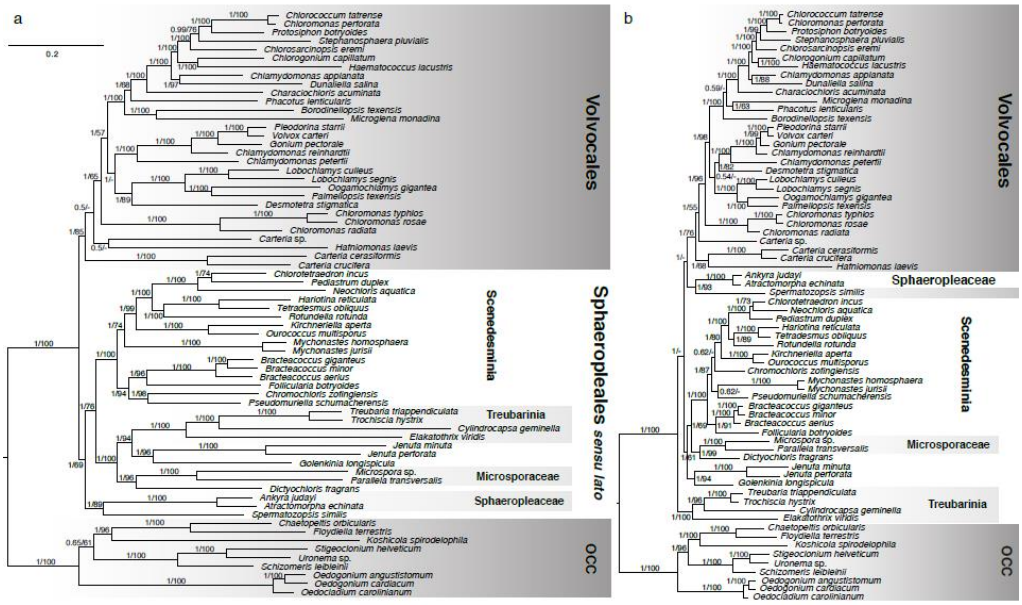


Figure 5: Bayesian consensus trees inferred from analyses of concatenated (A) nucleotide and (B) amino acid chloroplast data (58 protein-coding genes). Taxon groups of interest are designated with boxes and tentative clade names. Scale bar represents expected number of substitutions/site for both trees. Numbers at nodes represent, respectively, Bayesian Posterior Probabilities (BPP) and Maximum Likelihood bootstrap values (BS) derived from 200 pseudo-replicates. BPP values lower than 0.5 and BS values lower than 50 are reported as dashes (-). (Fučíková et al. 2019).

1.5. Ongoing problems in current systematics of green coccal algae

Unanswered questions still remain in the system of green algae. Its systematics has been problematic from its very beginning – the definition of species. Traditional biological concept is widely not accepted, because green coccal algae, despite having meiotic genes (Fučíková et al. 2015), are mostly asexual. It was proved that using either only morphological data is insufficient, especially in the case of as simple forms as are coccal green algae. Thallus of the coccal algae possesses limited amount of morphological traits, and most of them evolved much more than once as advantageous ecological adaptation to living conditions (Krienitz et al. 2010, Pröschold et al. 2010). Differences between taxa are often not clearly visible, and its detection complicate small dimensions of the cells (Krienitz et al. 2010). In that case, modern microscopical methods as electron and fluorescent microscopy are more helpful. Much more suitable molecular phylogenetic based species (generic etc.) concept (Mishler and Theriot 2000) has also its limitation. Molecular phylogeny is useful tool to describe the natural variability, which is more or less continual. Borders of species, genera and higher taxonomic unites are still quite arbitrary. Finding the optimal marker, sometimes different for each group and each level is in the scope of recent studies (see further in Chapter 1.6.2). For distinguishing individual species is the most accepted marker internal transcribed spacer 2 (ITS2) and its compensatory bases changes (CBC) approach which deals with reproduction incompatibility and so supplies the biological concept (Luo et al. 2010, Darienko et al. 2016). However, it has been doubted in some taxonomic groups (Caisová et al. 2011, Caisová et al. 2013).

Molecular approach often reveals that from our point of view morphologically identical species should be separated species. So-called cryptospecies revealed among the commonly known algal genera as *Bractaecoccus*, *Chlorella*, *Dictyosphaerium*, *Mychonastes* or *Pseudomueriella* (Bock et al. 2010, Bock et al. 2011b, Fučíková et al. 2011 Fučíková et al. 2013, Krienitz et al. 2010, Krienitz et al. 2011b, Song et al. 2017) contribute to the underestimation of the real variability of green coccal algae. On the other hand, overestimation occurs when, the same alga is described multiple times under several names. This situation is hard to prove, because it is necessary to find enough clearly

determined natural material, ideally including the original material, which has rarely been preserved until today, while many of taxa were described decades ago and usually without strain isolation. When we found the sufficient material, it is necessary to confirm the identity of some hypervariable region of the DNA (mostly ITS 2 region, which is commonly used on species level) (Eliáš et al. 2013, Kawasaki et al. 2015).

Generic conception follows the species ones. Molecular phylogenetic concept accepts only monophyletic genera; therefore, a lot of previously morphologically delimited genera shown as paraphyletic (e.g. *Scenedesmus s.l.*, *Pediastrum s.l.*, (Buchheim et al. 2005, Hegewald et al. 2010, Hegewald et al. 2013, Jena et al. 2014) or deeply polyphyletic (e.g. above mentioned *Chlorella* and *Dictyosphaerium*) were split. It resulted in more precisely defined, much smaller genera (Chapter 2), in contrast of the traditional, bigger genera, based just on few diacritic morphological characteristics (Komárek and Fott 1983).

Traditional system was based on hierarchy of individual taxonomic units (subfamilies, families, suborders, orders) and each unit was strictly defined according to the key morphological characteristics (Chapter 1.1., Figure 1). Each species was incorporated in the system and its taxonomical categories were defined, thus this placement varied in some authors. In modern system morphology in its traditional understanding usually fails and more crucial is molecular phylogeny. Nevertheless, researchers cannot completely exclude morphology, because it gives fair amount of data and finally, morphology and molecular phylogeny are not distant entities, each morphological trait is a result of expressing multiple genes. It is necessary to search for relevant morphologic structures, which are in accord with phylogeny of examined group of algae.

Delimitation of higher systematic unites based on molecular phylogeny is quite challenging. The position of already described taxa without molecular data (Chapter 1.6.) is automatically doubtful. Some taxa dispose of just one marker, (mostly 18S rRNA gene) which cannot resolute each taxonomical level (Chapter 1.6.2). There is hope, that adding more locus data for such taxa refine the resolution ability. Problematic situation occurs, when several markers gives contradictory hypothesis, which is usually caused by separated evolution of nuclear and chloroplast genomes as happened in case of

controversial Treubarinia clade (Fučíková et al. 2019), or when despite of data from numerous genes is support of individual branches of phylogenetic tree still missing. Attempts to resolute the unified system in ordinal and familiar level based on the multigene analyses were applied in the class Chlorophyceae with just some particular uncertainties (Fučíková et al. 2014a, Fučíková et al. 2019), but are mostly missing in the class Trebouxiophyceae.

On the higher level of systematics of green algae, similarly as on the generic level, researchers accept only the monophyletic taxonomic unites. On the contrary, natural usually result in dichotomy evolution (except rare fast radiation of the species, which is caused by specific evolutionary events). Asymmetric evolution occurs, when one of the newly formed groups is more successful and more diverse and the second group evolve just one or few forms. When this kind of event happens repeatedly, paraphyletic group formed next to the core group. Those lonely taxa or small groups of taxa often on the basis of taxonomical unites dispose of long branches, which complicates the reconstruction of the phylogeny (Pröschold and Leliaert 2007, Chapter 2). Solution for paraphyletic groups is to define the higher taxonomical unites for each small monophyletic group separately, even for small one containing a few taxa, as happened on the class level for basal lineages of both main group of green algae, in Chlorophyta in the case of Prasinophyte algae and for early diverging Streptophyta (Marin and Melkonian 1999, Leliaert et al. 2016) or continue with defining monophyletic groups as clade without the hierarchy determination (Fučíková et al. 2019).

Traditional system of green algae expected that green coccal algae evolved all at once from an ancestral green flagellate separately from multicellular algae (Pröschold and Leliaert 2007). Modern system denied the theory and tries to understand the distribution of coccal algae along multiple types of multicellular algae in evolutionary context. Evolution usually expect more complicated structures evolved from easier ones. Nevertheless, complicated macroscopic thallus occurred surprisingly even among early diverging ´Prasinophyte´ algae in the class Palmophyllophyceae (Leliaert et al. 2016). Several types of macroscopic thallus with diverse type of multicellularity evolved independently in (Ulvophyceae), which was matched to the specific sequence of climate

changes in the Earth history (Del Cortona et al. 2020). Among Chlorophyceae, filamentous forms include OCC clade and the more derived orders Sphaeropleales and Volvocales contains mostly unicellular algae, though colonial and coenobial (Turmel et al. 2009, Buchheim et al. 2012, Tippery et al. 2012). Inside Volvocales occur both coccal and monadoid forms, nevertheless some flagellated genera derived differentiated coenobia in the half way to real multicellularity (typically the genus *Volvox*). Sphaeropleales contain Microsporaceae in which was developed multicellularity and its possible conversion back to the coccal form in the genus *Parallela* (Chapter 5). In peculiar Treubarinia clade with unresolved position among CS clade, alga of the genus *Cylindrocapsa* possesses simply pseudo-filamentous morphology, unlike its close relatives in the clade, including the sister taxon coccal colonial alga *Oonephris obesa* (Chapter 2, Chapter 1.10.3). Trebouxiophyceae algae rarely developed macroscopic thallus, exceptions can be found in the its most diverse Prasiola clade, where multicellularity developed several times (Moniz et al 2012, Heesch et al. 2016). In the case of *Ekerewekia* plays the role probably its isolation and separated evolution for long time (Kaštovský et al. 2016), in the rest of genera their polar distribution (Moniz et al 2012, Heesch et al. 2016). Numerous other multicellular Trebouxiophyceae algae, possess more likely pseudo-filamentous thallus, which is easy to break, with cells connected just by mother cell wall or mucilage (Chapter 1.4.). Their phylogeny and even some position are rather not well known therefore stays unresolved even their evolution background.

1.6. Phylogenetic methods as the backbone for modern systematics

Algae communities are often examined by the new generation sequencing, which bring a lot of metagenomic data useful for detection of existing diversity but without better understanding of the taxa, therefore more applicable for ecology then for systematics. More useful knowledge for systematics of green algae brings research based on the cultivation of individual strains because we also gain more type of additional data of the strains and not only a pure sequence (Fučíková 2014c). Therefore, in present thesis, I deal with individual isolated strains and variable scale of data. Most crucial data in the systematics of green coccal algae is molecular data (Pröschold and Leliaert 2007). Despite the trend of sequencing whole genomes, in the case of green algae primarily chloroplast

genomes (e.g. Fučíková et al. 2019) and secondary mitochondrial ones (Fučíková et al. 2014d, Žihala and Eliáš 2019), which stays still too expensive, for main part of the thesis were chosen only specific parts of the genome. The research presented in papers included in the thesis was based on the data provided using regular molecular methods consisting of DNA extraction, PCR reaction and Sanger sequencing of the chosen part of the genome of the strains of green algae.

The appropriated gene selection is necessary for each level of resolution. Finding the right gene is a part of process, and it is essential to define it for each systematic level and commonly for each specific group, because some groups can possess different mutation rate = speed of evolution then others. E.g. for most parts of phylogeny of the family Oocystaceae is informative 18S rRNA gene, however inside the family Chlorellaceae dispose of poor resolution ability (Bock et al. 2013). Researchers choose some from the group of housekeeping genes, which possess all or most of the examined taxa.

From the nuclear genes, the most commonly used is gene coding small subunit ribosomal RNA (SSU rRNA) or 18S rRNA gene, which is useful for the primary molecular characterization of the taxa with not distinguished phylogenetic position, because the GenBank dispose of a huge amount of 18S rRNA gene data for the comparison among the various taxa (Hall et al. 2010, Chapters 2-5). For more detailed analyses and commonly species delimitation are suitable other more variable parts of nuclear rRNA operon, namely: internal transcribed spacer 1 (ITS1) and 2 (ITS2) and the 5,8S rRNA gene (Hall et al. 2010). Secondary structure of ITS2 enables search for compensatory bases changes (ITS2/CBC) concept, which is most commonly used concept for species delimitation in green coccal algae systematics (Neustupa et al. 2011, Eliáš et al. 2013, Darienko et al. 2018, Darienko et al. 2019). Gene for large subunit ribosomal RNA (LSU rRNA or 26S rRNA) is still less used though contain informative sequences (Buchheim et al. 2002, Buchheim et al. 2005, Buchheim et al. 2013, Pegg et al. 2015).

Chloroplast genes are easier to get, in case of non-axenic strain with some fungal contamination of in case of lichen algae, when the alga occurs in low density in collected sample, because fungi and other non-photosynthetic organisms do not have a chloroplast

to be amplified. Moreover, it is not necessary to work with the secondary structure as in the case of nuclear genes coding ribosomal rRNA and in 18S rRNA genes of some groups of green algae were found big number of introns complicating its amplification (Pažoutová et al. 2010, Chapter 2, author´s unpublished data). Chloroplast also dispose of its own rRNA genes, but they are rarely used for phylogenetic analyses. The *rbcl* gene is a most commonly used chloroplast marker used on the species and generic level (Rindi et al. 2007, Hall et al. 2010, Fawley et al. 2010, Fučíková et al. 2014c, Li et al. 2020, Chapters 2-5), followed by variable *tufA* (Hall et al. 2010, Vieira et al. 2016, Liu et al. 2017, Wang et al. 2019, Chapter 3). Comparatively lower mutation rate occurs in the phylogeny of *atpA, atpB, psaA, psaB, psbA, psbC,* therefore are rather used for the systematics on higher taxonomical levels (Fučíková et al. 2014a, b, genes *atpB, psaB* were analyzed in Chapter 5). With growing number of whole genome sequence available in the GenBank, analyses of supermatrix containing big number of chloroplast gene came more often, 73 chloroplast genes were analyzed by Fang et al. (2018), 56 genes in Fučíková et al. (2016), 58 genes in Liu et al. (2020).

Mitochondrial genomes are at least explored ones from the three genomes of green algae. No mitochondrial genes are widely used for molecular phylogeny of green algae so far, because databases still do not contain much mitochondrial data, thus promising variable locus is cytochrome c oxidase I (COI) gene (Hall et al. 2010), moreover genomes of mitochondrion are a reservoir of lot of housekeeping genes and in case of green algae possess unusual phylogenetic code (Fučíková et al. 2014d, Žihala and Eliáš 2019).

1.7. Aims of the thesis

This dissertation thesis aims to resolve some chosen parts of the system of green coccal algae. I chose family Oocystaceae and family Scenedesmaceae subfamily Crucigenioideae. Both taxonomical unites were poorly examined and dispose of unresolved phylogeny. Some data were obtained during the master study *Systematic revision of the family Oocystaceae* (Štenclová 2013) and are published in (Chapter 2) and remaining other problems (Chapters 3-5) more or less follow the topic of the master thesis.

Both Oocystaceae and Scenedesmaceae Crucigenioideae are colonial groups of green coccal algae defined on the basis of strong morphological characteristics highlighting specifically their unique colonial organization of the cell. Traditional morphological definitions of each group follow.

The family Oocystaceae was defined on the basis of the oval to elliptical shape of the cell, sometimes lemon like, sometimes (quite rarely) asymmetric or spherical with or without apical thickness, and propagation by autospores which are retained in the mother cell wall for longer time, sometimes even all their life cycle making composed multigeneration colonies. Typical is its ultrastructure of the multilayered cell wall with the network of cellulose fibril in each layer arranged cross-wise to the previous one. Spines are characteristic for subfamily Lagerheimioideae, smooth cell wall and small amount of chloroplast for Oocystoideae and big cells and numerous chloroplasts for Eremosphaeroideae (Komárek 1979, Komárek and Fott 1983).

Crucigenioid four-celled coenobial morphology of Scenedesmaceae Crucigenioideae is characterized as 4 celled colony of cells of one generation living together for all their live. Coenobia are flat and cells in tetrads are not arranged in a row (Scenedesmus-like) or any other different shape, but in cross shape (see schematic figure in Komárek 1974) – one each cell touch at least two other cells, sometimes the gap in the middle is present (*Willea*) sometimes not (*Tetrastrum*) and therefore are in touch all 4 cells. Composed coenobia developed when more generation of coenobia stay together (Komárek and Fott 1974).

1.8. Results

Most crucial results of preset thesis are adjusted parts of the system of green algae and taxonomical changes, both described and summarized in following chapters. Each chapter summarized most important results of single paper. Each paper overlaps the topic and bring some insights to the general systematics of green algae. Together present papers interfere nearly to every common problems of systematics of algae described in the Chapter 1.5.

1.8.1 Molecular and morphological delimitation and generic classification of the family Oocystaceae

The traditional morphological delimitation and generic classification of the family Oocystaceae varied in several previous studies (Smith 1950, Fott 1967, Komárek 1979, Komárek and Fott 1983 and Melkonian 1983) Finally, Komárek (1979) and Komárek and Fott (1983) stated as most characteristic attribute cell wall ultrastructure that is multi-layered with the cellulose fibrils in each layer perpendicular to that of the adjoining layer. However, the ultrastructural data is available only for limited amount of genera (Chapter 2). Molecular phylogeny proved the morphological generic conception of Oocystaceae (Komárek and Fott 1983) to be inaccurate, when representatives of genera with atypical or irregular shape of the cell included to the family by Komárek and Fott (1983) *Elakatothrix*, *Nephrochlamys*, *Nephrocytium*, *Rhombocystis*, spherical spiny *Trochiscia* nevertheless also ovoid *Oonephris* were transferred to the class Chlorophyceae (Buchheim et al. 2001, Krienitz et al. 2011a, Štenclová 2013 (Chapter 2). Additionally some previously outside Oocystaceae categorized taxa, newertheless with similar shape of the cell as *Oocystis* were included to the family: representatives of colonial genera *Amphikrikos*, *Coenochloris*, *Quadricoccus* and *Schizochlamydella* (Hepperle et al. 2000, Wolf et al. 2003, Pažoutová et al. 2010, Krienitz and Bock 2011), coenobial *Crucigeniella*, *Makinoella*, *Tetrachlorella* and *Willea* from the Scenedesmaceae subfamily Crucigenioideae and pseudo filamentous *Ecballocystis and Ecballocystopsis* from the Botryococcaceae (Hepperle et al. 2000, Krienitz et al. 2003, Krienitz and Bock 2011, Xia et al. 2013, Chapter 2).

Significant changes in delimitation of the family led to its adjusted definition, more strict in the cell shape but wider in the cell organisation including not just oocys-like colonial taxa but also coenobial and pseudo-filamentous form. On the basis of the phylogeneetic tree of the family occurred crucigeniod coenobial genus *Tetrastrum* and simply filamentous *Planctonema lauterbornii,* both lacking the characteristic structure of the cell wall and therefore designed as *incertae sedis* (Chapter 2).

Family Oocystaceae was divided by Komárek and Fott (1983) into the subfamilies Eremosphaeroideae, Lagerheimioideae and Oocystoideae. Molecular phylogeny combined with newly evaluated morphology support different concept consisted of subfamilies Eremosphaeroideae, Oocystoideae and Makinelloideae *subf. nova* (Chapter 2). Nevertheless, Eremosphaeroideae is paraphyletic or polyphyletic and dispose of really long branches and a poor sampling complicating its phylogeny. Subfamily Makinelloideae is distinct and well supported by molecular phylogeny and represent coenobial and pseudo-filamentous clade. Oocystoideae consisted of former Oocystoideae and Lagerheimioideae. Subfamily was divided into 5 morphologically and phylogenetically well-defined clusters accompanied by residual strains of the genera *Oocystis* and *Tetrachlorella* (*Oocystis sensu lato* group) with unresolved relations (Chapter 2).

Taxonomic changes in several genera including morphologically well-defined genus *Oocystis* were executed, other proposed or expected to be done in future. Obtained findings supports the theory of smaller more strict defined genera compared to the big ones in traditional morphological generic conception (Chapter 2).

1.8.2. Revised phylogenetic position of genus *Nephrocytium* Nägeli (Sphaeropleales, Chlorophyceae), with description of Nephrocytiaceae *fam. nov*. and *Nephrocytium vieirae sp. nov.*

Green algal genus *Nephrocytium* traditionally classified inside the family Oocystaceae share with the family similar colonial morphology and some other characteristics e.g. propagation by autospores, number and shape of chloroplasts, nevertheless, differs in the shape of the cell, which is crescent or lunar (Komárek and Fott 1983) and never lemon like, oval to elliptical what is fairly characteristic for all its confirmed members of Oocystaceae (Chapter 2) and especially in the ultrastructure of the cell wall, which was defined as a diacritical character for the family Oocystaceae. Recently, this traditional position of *Nephrocytium* was questioned by molecular phylogeny, when *Nephrocytium* taxa clustered in the class Chlorophyceae (Liu et al. 2017, Štenclová 2013, Chapter 2), whereas Oocystaceae was placed in different class Trebouxiophyceae (Hepperle et al. 2000, Štenclová et al. 2013, Chapter 2). Previous studies did not resolve the exact place

and *Nephrocytium* was designed as *incertae sedis* inside the order Sphaeropleales. Phylogenetic analyses of two molecular markers (18S rDNA and *tuf*A) support position of taxa of the genus *Nephrocytium* distinct from other described families which led to the definition of new family Nephrocytiaceae *fam. nov.* inside Sphaeropleales (Chapter 3). Results are consistent with detailed morphological and ultrastructural observation performed in present study referring unique combination of morphological and ultrastructural characteristics differing from any other described family (Chapter 3). Comparison of phylogeny and morphology of studied strains together with the morphology of previously described *Nephrocytium* species resulted in description of novel species *Nephrocytium vieirae spec. nova*.

1.8.3. Distribution of Crucigenioid algae in classes Chlorophyceae & Trebouxiophyceae

Subfamily Crucigeniodeae (family Scenedesmaceae) so typical for its taxa sharing cross-shaped coenobial morphology also called crucigenioid, traditionaly contained genera *Crucigenia, Crucigeniella, Hofmania, Tetrachlorella, Tetrastrum, Westella,* and *Willea* (Komárek 1974) and was later expanded by genera *Didymogenes, Gilbertsmithia, Makinoella* and *Suxenella* (Komárek and Fott 1983). These genera were expected to belong together for long time. However even first molecular analyses suggested, that its systematics is more complicated (Hepperle et al. 2000). In following studies, examined crucigenioid taxa were redistributed into several lineages inside both the classes Chlorophyceae (Chapter 5) and Trebouxiophyceae (Hepperle et al. 2000, Bock et al. 2013, Chapter 2). Most of the representatives clustered inside or in the proximity of the family Oocystaceae (Hepperle et al. 2000, Bock et al. 2013, Chapter 2). Sister to the Botryococcus clade clustered another part of the species and they were recombined into re-established genus *Lemmermania* (Bock et al. 2013). Species *Crucigenia lauterbornii* was included in Chlorellaceae (Bock et al. 2013).

In present study, 16 strains of representatives of traditional Scenedesmaceae Crucigenioideae were studied morphologically and by molecular analyses of nuclear gene for 18S rRNA and chloroplast marker *rbcL* gene. Two strains determined as *Crucigenia*

lauterbornii and *Komárekia rotundata* clustered together inside the family Chlorellaceae (Bock et al. 2013, Chapter 4). Both taxa were primarily described as species of the genus *Hofmania* and subequently recombined with species *Komarekia appendiculata* as novel genus *Komarekia* (Fott 1981). Newertheless, Komárek and Fott (1983) recombined species *Komárekia lauterbornii* as *Crucigenia lauterbornii*. Based on the results of phylogenetic analyses accompanied by morphological survey in present study, the original genus *Komárekia* was restored *sensu* Fott (1981), including all three here established *Komárekia* species: *Komarekia appendiculata, Komárekia lauterbornii* and *Komárekia rotundata*.

Rest of the investigated strains clustered together in the family Scenedesmaceae, distinct from all other previously analyzed crucigenioid taxa and constituted separated well-supported clade designed as novel Scenedesmaceae Crucigenioideae, with adjusted definition. 14 analyzed strains represented traditional genera *Crucigenia* (including species *Crucigenia mucronata* and the type species of *Crucigenia*: *Crucigenia quadrata*) and *Crucigeniella* (including species *Crucigeniella apiculata* and *Crucigeniella saguei*). *Crucigeniella apiculata* and *Crucigeniella saguei* were recently combined in the genus *Willea* (John et al. 2014), nevertheless, our analyses showed their phylogenetic position distant from other recently analyzed *Willea* taxa (Chapter 2) and discovered their close relations with species of the genus *Crucigenia* including the type species of the genus, *Crucigenia quadrata*. Therefore, taxonomical adjustments were made and species were combined to the genus *Crucigenia* as *Crucigenia apiculata* and *Crucigenia saguei comb. nov.* (Chapter 4).

Study also discussed the traditional morphological approach in the light of modern molecular analyses. Traditional on the morphology well-defined subfamily Crucigenioideae was split in multiple independent lineages, as was common for many on morphology based taxonomic unites defined in past two centuries. Its deeply polyphyletic status added one more such example to the current situation of systematics of green coccal algae. Overall, many of old morphological traits considered as taxonomically relevant, have been recently revealed to evolved multiple times as a result of common advantageous

adaptations to the ecological conditions and in conformity of molecular phylogeny are being found novel significant morphological synapomorphies.

1.8.4 *Dispora speciosa*, a new addition to the genus *Parallela* and the first coccoid member of the family Microsporaceae

Another colonial coccal green algal species *Dispora speciosa* has traditionally been classified into the Radiococcaceae family due the its mucilaginous envelopes (Komárek and Fott 1983) or to relatives of the Scenedesmaceae family (subfamily Crucigenioideae) due to the formation of four-cell coenobia mainly reminiscent coenobia of the genus *Willea* (Komárek (1974). The phylogenetic analysis of four molecular markers surprisingly determined its position in the Microsporaceae family, so far consisted of only multicellular representatives. A detailed morphological and ultrastructural study (Chapter 5) showed congruent features with the genus *Parallela* and subsequently was the algae moved to the genus as *Parallela speciosa*. Probable position of other *Dispora* species according its morphology is discussed. The study also valorized relevant traditional morphological features in green algae systematics and demonstrates example of coevolution of colonial and filamentous forms of green algae, an exemplary evidence that the morphological features (in this case filamentous versus coccal types) are results of convergent evolution.

1.9. Conclusion

Though big boom of the modern systematics of green (coccal) algae already passed, there still exist groups with unresolved relations and commonly unsuspected phylogeny. Elaborated systems of individual classes are missing, and even the definition of the classes is questionable/doubted. Same situation occurs among the individual orders. Sometimes, analyses of different (usually chloroplast *versus* nuclear) genes propose several hypotheses, which are in conflict. Detection of phylogeny complicated incomplete taxon sampling, long branches and paraphyly of solitary taxa on the basis of taxonomic unites or other taxa with *incertae sedis*. Still many already described species and even genera missing relevant material or just molecular data to resolve its position. Researches tend to

split traditional wide genera into smaller more precisely defined ones and tend to improve species delimitations including the resolution of cryptospecies. With the systematics revisions, also relevant morphological criteria for individual taxonomic unites are found, therefore we can better understand their evolution.

1.10. Following problems

The aim of the study was to clarify some of the unknown parts of the systematics of the green coccal algae. In above chapters, the new findings concerning the families Oocystaceae and Scenedesmaceae (subfamily Crucigenioideae) have been presented. Nevertheless, it also has brough some partial results and indicates direction of future prospects.

1.10.1. Delimitation and species concept of the genus *Oocystis*

As indicated above, traditional big genera defined on the basis of one or several morphological traits (usually, the shape of the cell or some characteristic of the colony formation or some specific structures as spines or granules on its surface) are recently tendent to be split in smaller monophyletic clades defined as novel genera. Previous and present (Chapter 2) studies demonstrated polyphyletic status of the genus *Oocystis* (Hepperle et al. 2000, Krienitz and Bock 2011). Still just part of the numerous *Oocystis* species (28 accepted and 16 incompletely described species, according Komárek and Fott (1983) or 48 accepted species according Guiry and Guiry (2020) were analyzed by molecular phylogeny so far. *Oocystis solitaria* clustered far away from the rest of the *Oocystis*, along with *Neglectella*, (both with numerous chloroplast and bigger cells than convention *Oocystis* species) and its transfer was submitted (Chapter 2). *´Oocystis´ nephrocytioides* (Chapter 2) belonging to the coenobial clade is obviously not relative to other *Oocystis* species, new placement is supported by its long elongated shape of the cell. Granulated *Oocystis bispora* differs from other *Oocystis* spp. additionaly to the phylogenetic possition also by granules on the cell surface, therefore should be excluded. Outside Oocystacea and inside Scenedesmaceae acording to the preliminary data cluster *Oocystis minuta* a and *Oocystis polymorpha* and position of *Oocystis borgeii* was not

determined yet. The rest of the examined *Oocystis* taxa was classified as inside Oocystaceae in *Oocystis sensu lato* group with mostly unresolved relations. Group contain also reference strain designed as *Oocystis naegelii*, the type of the species. Mentioned analyses indicates, that true *Oocystis* is rather smaller and more specifically defined genus than expected in the studies dealing with the traditional systematics.

1.10.2. Phylogenetic position of *Juraniella javorkae* and new insides in the phylogeny of the genus *Kirchneriella*

Another chapter of the systematics of genera previously assigned to Oocystaceae is the classification of its crescent shaped taxa. As demonstrated in Chapter 3, taxonomy around the genus *Nephrocytium* dispose of some question marks resulting from limited data available so far, and so does the problematic around the genus *Nephrochlamys*. Despite five (Komárek and Fott 1983) respective six (Nygaard et al. 1987) validly described species, only one strain (*Nephrochlamys subsolitaria*, the type species of the genus) have been analyzed (Krienitz et al. 2011a). Consequently, we obtained strain CCALA 392 designated as *Nephrochlamys rotunda*. The strain shares with the genus *Nephrochlamys* morphological traits as shape of the cell, propagation by autospores and a parietal chloroplast with hardly visible pyrenoid (Krienitz et al. 2011a). Nevertheless, considering the very clearly visible granulated surface I propose the designation *Juranyiella javorkae*. Preliminary analyses have shown, that the strain belongs to one of the lineages of polyphyletic genus *Kirchneriella* (Silva et al. 2017). By sequencing additional six strains isolated of Lipno reservoir I aim to resolve the polyphyletic status of the genus *Kirchneriella* – most probably by defining several smaller monophyletic genera as in many previous studies.

1.10.3. Characterizing the chloroplast and mitochondrial genomes of a microscopic alga *Oonephris obesa* (Chlorophyceae)

So far poorly studied coccal algae *Oonephris obesa* traditionally considered as a member of Oocystaceae family was according to the molecular phylogeny assigned to the atypical Treubarinia clade (Chapter 2), far from the family Oocystaceae. The clade was defined

inside the order Volvocales based on the phylogeny of the 18S rRNA (Buchheim et al. 2001, Chapter 2), nevertheless, its position inside the order was not specified. The clade and especially *Oonephris obesa* are characteristic by its long branches of 18S rRNA phylogenetic tree, which can introduce to the phylogeny unwanted artefacts. Long branches are caused by limited taxa of the clade and also the accelerated evolution of particular DNA inside the group. Nevertheless, this position is in conflict with the chloroplast analyses. Most recent publication dealing with chloroplast genomes of the representatives of Chlorophyceae algae moved Treubarinia algae into the order Sphaeropleales (Fučíková et al. 2019). According to the study, exact position of Treubarinia inside the order is unstable. We performed sequencing and assembling chloroplast genome of *Oonephris obesa*. To determine the chloroplast gene and non-coding parts present in chloroplast genome and compare sequences with chloroplast genomes of closest relatives can potentially strengthen the position of Treubarinia clade. Besides the chloroplast sequence, also mitochondrial genome, less studied but not less interesting genome is in the scope of the project. Mitochondrial genomes dispose of potentially informative hypervariable genes (Hall et al. 2010) and often unusual phylogenetic code in case of green algae of Sphaeropleales (Fučíková et al. 2014d, Žihala and Eliáš 2019). *Oonephris obesa* shares with her closest sequenced neighbor algae *Cylindrocapsa geminella* similar cell morphology, including distribution of organelles and characteristic multilayered cell wall (Hoffman & Hofmann 1975, Chapter 2) still with unknown composition. Both taxa differ basically just by their cell arrangement. *Oonephris obesa* occurs in 2-4 celled (*Oocystis*-like) colonies, whereas *Cylindrocapsa geminella* make multicellular pseudo-filaments. The project also aims to describe the chemical characterization of the cell walls and the 3D shape of the chloroplasts of these two close relatives using fluorescence microscopy.

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Chapter 2 Molecular and morphological delimitation and generic classification of the family Oocystaceae (Trebouxiophyceae, Chlorophyta)

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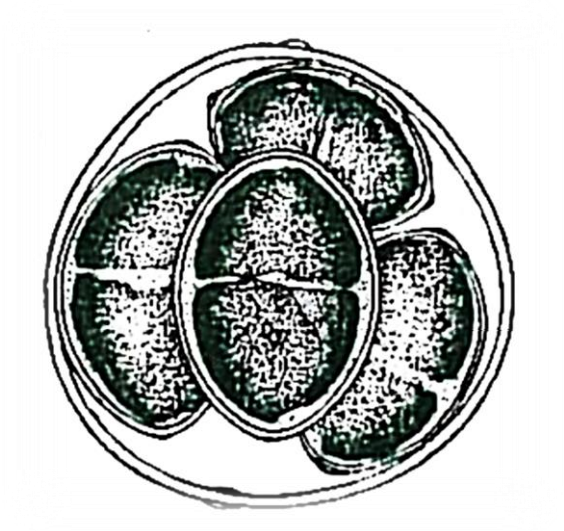

MOLECULAR AND MORPHOLOGICAL DELIMITATION AND GENERIC CLASSIFICATION OF THE FAMILY OOCYSTACEAE (TREBOUXIOPHYCEAE, CHLOROPHYTA)[1]

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Abstract

The family Oocystaceae (Chlorophyta) is a group of morphologically and ultrastructurally distinct green algae that constitute a well-supported clade in the class Trebouxiophyceae. Despite the family's clear delimitation, which is based on specific cell wall features, only a few members of the Oocystaceae were examined using other data than morphological. In previous studies of Trebouxiophyceae, after the establishment of molecular phylogeny, the taxonomic status of the family was called into question. The genus *Oocystis* proved to be paraphyletic and some species were excluded from Oocystaceae, whilst a few other species were newly redefined as members of this family.

We investigated 54 strains assigned to the Oocystaceae using morphological, ultrastructural and molecular data (the SSU rRNA and the *rbcL* genes) to clarify the monophyly of and diversity within Oocystaceae. *Oonephris obesa* and *Nephrocytium agardhianum* clustered within the Chlorophyceae and thus are no longer members of the Oocystaceae. On the other hand, we transferred the coenobial *Willea vilhelmii* to the Oocystaceae. Our findings combined with those of previous studies resulted in the most robust definition of the family to date. The division of the family into three subfamilies and five morphological clades was suggested. Taxonomical adjustments in the genera *Neglectella*, *Oocystidium*, *Oocystis*, and *Ooplanctella* were established based on congruent molecular and morphological data. We expect further taxonomical changes in the genera *Crucigeniella*, *Eremosphaera, Franceia, Lagerheimia*, *Oocystis*, and *Willea* in the future.

Keywords: Chlorophyceae, Crucigenioideae, morphology, Oocystaceae, phylogeny, *rbcL*, Scenedesmaceae, SSU, Trebouxiophyceae, ultrastructure.

Introduction

Green algae with a coccal thallus were associated with the order Chlorococcales for decades. With the introduction of molecular phylogenetics, green algal taxonomy has undergone significant changes. The order Chlorococcales dissolved and subsequently most coccal Chlorophytes were distributed in the classes Chlorophyceae and Trebouxiophyceae along with the multicellular green algae (e.g., Lewis et al. 1992).

Traditional definitions of the families, genera and species have been based on morphology. Microscopic coccal green algae possess only a limited number of morphological traits, some of which occurred far more than once in the evolutionary history of the group. In some cases, more detailed ultrastructural examination can help to recognize monophyletic and polyphyletic origins of some features, e.g. spines (Hegewald and Schnepf 2002, Pröschold et al. 2010). However, other features are doubtful, e.g. cell shape (Luo et al. 2010). Therefore, the morphological species (and generic) concept can hardly work for the unicellular algae. Biological species concept by Mayr (1942), the most frequently used criterion for species delimitation in eukaryotes, cannot be applied either, because many of the coccal green algae are considered asexual, especially in Trebouxiophyceae. Cryptic sexual reproduction, however, was found in some cases in Trebouxiophyceae, through genome analyses (Blanc et al. 2010, Fučíková et al. 2015). Nevertheless, only a few species were directly observed to propagate sexually (Kies 1967, Gonzalves and Mehra 1959, Iyengar and Ramanathan 1940, Iyengar and Ramanathan 1974, summarized in Fučíková et al. 2015) and the required circumstances are not understood. The phylogenetic species concept based on the reconstruction of evolutionary relationships, established by Mishler and Theriot (2000), has proved to be useful for systematics of asexual green coccal algae. Molecular phylogeny is currently an essential part of the modern polyphasic approach in algal taxonomic research (Pröschold and Leliaert 2007, Leliaert et al. 2012).

The traditional morphological delimitation and generic classification of the family Oocystaceae Bohlin varied in several previous studies (Smith 1950, Fott 1976, Komárek 1979, Komárek and Fott 1983, Melkonian 1983). The family Oocystaceae according to Komárek (1979) and Komárek and Fott (1983) included species with typically oval or

elliptical, sometimes (atypical) spherical, rhombic, spindle-shaped, bean-shaped or slightly irregular cell shape. Each cell possesses one, a few or many chloroplasts, mostly cup-shaped or girdle–shaped, sometimes radial or spongiomorph, with or without a pyrenoid. Oocystacean algae reproduce by autospores and daughter cells usually stay enclosed in the mother cell wall for a prolonged period. Cell wall, often with polar thickenings, can be smooth or bear warts or spines. The surface of the cell wall, together with the dimensions of the cell and number of chloroplasts, were traditionally used as determination traits for distribution of the Oocystean algae into subfamilies: Eremosphaeroideae with large cells, numerous chloroplast and smooth cell wall, small-celled and few-chloroplasts containing Lagerheimioideae with spiny cell walls, and Oocystoideae with smooth cell walls (Komárek 1979, Komárek and Fott 1983).

The above-described definition was broad and corresponded with 31 genera. Komárek (1979) and Komárek and Fott (1983) identified as the most characteristic attribute the cell wall ultrastructure that is multi-layered with cellulose fibrils in each layer perpendicular to those of the adjoining layer. However, out of numerous oocystacean genera, ultrastructural data are available only for five traditional genera: *Eremosphaera*, *Franceia*, *Lagerheimia*, *Neglectella*, *Oocystis* and for seven genera recently included in Oocystaceae: *Amphikrikos*, *Ecballocystis*, *Ecballocystopsis*, *Makinoella* and *Siderocystopsis*, (Bowen 1965, Crawford and Heap 1978, Hegewald et al. 1978, Quader et al. 1978, Hegewald et al. 1980, Hegewald et al. 1999, Schagerl 1993, Schnepf et al. 1980 and Xia et al. 2013).

More helpful for the definition of the Oocystaceae was the establishment of the molecular phylogeny that showed the morphology-based generic concept of Oocystaceae (Komárek and Fott 1983) to be inaccurate and the family's internal taxonomic structure was called into question. Four species with atypical or irregular cell shape, included in the family by Komárek and Fott (1983), *Elakatothrix viridis*, *Nephrochlamys subsolitaria*, *Rhombocystis complanata,* and the spherical and spiny *Trochiscia hystrix* were transferred to the class Chlorophyceae (Buchheim et al. 2001, Krienitz et al. 2011b). On the other hand, a cell shape similar to *Oocystis* is found in the recently included *Amphikrikos* sp., *Quadricoccus ellipticus, Schizochlamydella capsulata* (Hepperle et al. 2000, Wolf et al.

2003, Krienitz and Bock 2011) and *Coenochloris planoconvexa* (now known as *Ooplanctella planoconvexa* (Pažoutová et al. 2010)), which are new to the expanded family. These taxonomic changes suggest that the shape of the cell is rather specific and potentially characteristic of the family.

The inclusion of the coenobial strains *Crucigeniella rectangularis* (recently *Willea rectangularis* (John et al. 2014)), *Makinoella tosaensis*, *Tetrachlorella alternans* from the Scenedesmaceae subfamily Crucigenoideae and the pseudo-filamentous *Ecballocystis hubeiensis* and *Ecballocystopsis dichotomus* from the Botryococcaceae, newly redefined as members of the family (Hepperle et al. 2000, Krienitz et al. 2003, Krienitz and Bock 2011, Xia et al. 2013) indicates that the family Oocystaceae has a wider definition with more variable cell arrangement than previously expected (Krienitz et al. 2003), and suggests that there may be additional candidates for moving into Oocystaceae. So far some authors considered including the coenobial genus *Tetrastrum*, previously Crucigenoideae (Komárek 1974, Komárek and Fott 1983), which was shown to phylogenetically cluster at the base of the Oocystean tree (Bock et al. 2013), as well as the difficult-to-classify, simply filamentous *Planctonema lauterbornii* (Krienitz and Bock 2011).

The most controversial finding of the oocystean molecular phylogeny was the polyphyletic status of the morphologically well-defined genus *Oocystis* (Hepperle et al. 2000). Only four sequences of the numerous *Oocystis* species were analyzed so far, and they formed three lineages (Krienitz and Bock 2011). A new genus, *Elongatocystis* (Krienitz and Bock 2011), was erected to accommodate the former *Oocystis ecballocystiformis*, but the remaining two *Oocystis*-like lineages were not taxonomically treated.

All previous studies have brought new insights to the phylogeny and systematics of the Oocystaceae, but the main questions about diversity, delimitation and generic classification of the family Oocystaceae remained unresolved. A comprehensive study using multi-approach taxonomical revision of the family is still needed.

The present study focused on the delimitation of the family Oocystaceae through a polyphasic approach and the provision of a coherent definition of Oocystaceae. We also

aimed to describe the morphological and molecular variability inside the family and to compare it with the within-family structure proposed by Komárek and Fott (1983). We considered the importance of the following morphological characters: spines, mucilage covers with or without projections, granules on the surface of the cell wall, coenobial character of cell arrangement, cell dimension and number of chloroplasts, for the structure of Oocystaceae and also the generic concept in the family.

Materials and Methods

Algae strains

We obtained 54 unialgal strains from the public collections Culture Collection of Autotrophic Organisms at the Academy of Sciences of the Czech Republic in Třeboň (CCALA), National Centre for Marine Algae and Microbiota (NCMA formerly CCMP), Culture Collection of Algae of the Charles University of Prague (CAUP), Culture Collection of Algae at the University of Gottingen (SAG), Coimbra Collection of Algae (ACOI), Culture Collection of Algae and Protozoa (CCAP) and the private collections of Marvin W. Fawley, Christina Bock and Lothar Krienitz. One strain was isolated by the authors (Table S1). Unidentified strains from the private collections were taxonomically assigned following Komárek and Fott (1983). Determined strains from the public collections were morphologically verified according to Komárek and Fott (1983). Appropriateness of the names of the investigated taxa was checked by Index Nominum Algarum and the forms of authors' names by The International Plant Names Index. We kept strains in tubes with solid BBM medium (Bischoff and Bold 1963) (solidified with 1.5% agarose) under standard cultivating conditions: irradiance 22 μmol m^{-2} s^{-1} and constant temperature 16°C. Selected strains were additionally cultivated in a liquid medium, because of better conservation of significant morphological traits: mucilage covers and spines.

Morphology

All strains were repeatedly observed using the light microscope Olympus BX, equipped with an Olympus DP71 camera and DP software, to capture all stages of their life cycle. Magnifications of 400x without and 1000x with immersion oil were used. We stained the strains with methylene blue to detect potentially ornamented cell walls and mucilage covers. Picture plates were constructed using CorelDraw X6.

Ultrastructure

Twelve strains of species with no ultrastructural data from previous studies were chosen for transmission electron microscopy (TEM), to observe cell wall ultrastructure. Samples were prepared by staff at the Electron Microscopy Laboratory, Institute of Parasitology, Academy of Sciences, Czech Republic. Samples were washed with 0.05 M phosphate buffer and postfixed with 2% osmium tetroxide in 0.05 M phosphate buffer at room temperature for 2 hours. Samples were then repeatedly washed with 0.05 M phosphate buffer. Cells were dehydrated with a concentration gradient of isopropanol solutions and embedded in the Spurr's resin (Spurr 1969) afterward, with propylene oxide as an intermediate stage. Thin sections were stained with uranyl acetate and lead citrate. We observed the prepared samples in a Jeol JEN 1010 transmission electron microscope at an accelerating voltage of 80 kV. Results of the examination are documented and figure plates created in CorelDraw X6.

Molecular analyses

DNA was extracted using Invisorb® Spin Plant Mini DNA extraction kit (Invitek, Berlin, Germany) following the manufacturer's instructions, and by modified xanthogenate-SDS buffer extraction protocol with the addition of 3% PVPP and PEG-MgCl2 precipitation (Yilmaz et al. 2009). We chose the SSU rRNA gene and the *rbcL* gene for molecular analysis. Both genes are considered housekeeping genes, and therefore conserved and appropriate for family and genus level phylogenetics. There are also a large number of

sequences of the SSU rRNA gene in the public database GenBank, NCBI. For the *rbcL* gene, the database GenBank, NCBI contains a fair amount of data as well.

We amplified both genes with PCR reactions consisting of 10 ng of the template DNA with 2.5 pmol of forward and reverse primer and 10 µl Plain PP Master Mix (Top Bio, Vestec, Czech Republic) using cyclers XP Cycler - Bioer T 300 Thermocycler (Biometra, Göttingen, Germany). The primer combination for amplification SSU rRNA was NS1F – ITS4R or 1650R when obtaining the entire gene was possible, and the combination of NS1F – 1150R and 1170F – ITS4 or 1650R, if the first attempt was not successful. Program for PCR reaction was started by initial denaturation (95°C, 1 min), followed by 35 cycles of denaturation (95°C, 1 min), annealing (52-55°C, 1min) and elongation (72°C, 3 min) and completed by final elongation (72°C, 10 min). The annealing temperature was estimated from the Tm of the used pair of primers (Checked by OligoAnalyzer 3.1 - Integrated DNA Technologies). We used the combination of newly designed primers ORB1F and ORB1R for the amplification of the *rbcL* gene. The program started with an initial denaturation step (95°C, 1 min), continued with 35 cycles consisting of denaturation (95°C, 1 min), annealing (52°C, 1 min) and elongation (72°C, 3 min), and was concluded by elongation (72°C, 10 min). Successfully amplified DNA was verified by gel electrophoresis on a 1% agarose gel in TBE buffer. DNA stained by GEL RED was visualized by UV transilluminator ULTRA LUM. INC – gel imager with software Scion VisiCapture. PCR products were refined JetQuick PCR Purification Kit - Genomed (Qiagen, Hilden, Germany). The manufacturer's instructions were followed. Samples for sequencing were analyzed by the Laboratory of Genomics, Biology Centre of the Academy of Sciences of the Czech Republic, České Budějovice (using the sequence analyzer ABI PRISM 3130 XL, Applied Biosystems, Life Technologies Corp., CA, USA) or processed by commercial companies Macrogen (Seoul, Republic of Korea) and SeqMe (Dobris, Czech Republic). Primer information is listed (Table 1).

We assembled the reads of each gene sequence using SeqAssem (Hepperle 2004). The approximate phylogenetic affinity of each strain was checked by BLAST against all sequences contained in the database GenBank - NCBI. All new sequences were posted in the public database GenBank - NCBI and the accession numbers were assigned (Table 2).

Alignments consisted of authors' sequences and sequences obtained from the public database GenBank - NCBI. Alignments of Chlorophyceae - for taxa that our study excluded from Oocystaceae - were assembled following previous studies (Fučíková et al. 2011, Fučíková and Lewis 2012, Fučíková et al. 2014 - Sphaeropleales and Buchheim et al. 2001, Nakada et al. 2008 - Volvocales) to select suitable sequences that cover the main lineages of each examined group and reasonable outgroup. SSU rRNA gene was sufficient to determine the approximate positions of these taxa. The alignment of the family Oocystaceae was assembled using all suitable available sequences from GenBank - NCBI, longer than 1500bp except introns in case of SSU rRNA gene (Table S2) and longer than 1000bp in case of *rbcL* gene, to cover the phylogenetic diversity of the family. BLAST search of all newly obtained sequences and also sequences from previous studies was used for finding all suitable sequences. The concatenated SSU rRNA + *rbcL* dataset was combined of both sequences of the Oocystaceae taxa, if both are available. Members of Chlorellaceae were used as outgroup. All analysed sequences are listed (Tables 2-4). Datasets were aligned using ClustalW (Larkin et al. 2007) and edited manually in Mega 5.2.2 (Tamura et al. 2011). All alignments were tested by jModelTest 2 (Darriba et al. 2012) to find the optional evolution model for the phylogenetic analyses. For all four alignments generalized time-reversible (Tavaré 1986) model of evolution with gamma distribution and invariable sites (GTR+Γ+I) was determined. The gamma shape parameter α, as well as the proportion of invariable sites were estimated from the data set. The phylogenetic trees were inferred for all datasets using Maximum Likelihood (ML) in PHYML 3.0 (Guindon et al. 2010). Nonparametric bootstrap support was calculated (1000 repetitions) to determine ML branch support. Secondly, we used Bayesian inference using Mr. Bayes 3.2.2. (Ronquist et al. 2012). Two runs with four MCMC chains each were executed with default parameters for 2,000,000 (simple dataset) or 3,000,000 (concatenated dataset) generations. Two analyses of concatenated alignment were executed: the first without partitions and the second with partitions. For the second one, four partitions were established, one for 18S and three for *rbcL*, separated by codon position. Posterior probabilities of branches were recorded.

Results

Morphology

Names of five strains: CCALA 396, SAG 2085, SAG 81.80, SAG 1194 and CAUP H 1110, were revealed as incorrect when authenticated according to Komárek and Fott (1983) and appropriate corrections were made (Table S1). Three names were completed by determining the correct specific epithet (SAG 30.96, SAG 2074 and CCALA 515) and nine unknown strains were identified (AN9-1, AN2/29-4, CB 99, CB 210, MP STE7, Tow 6/3 P-1ou, W Twin SlisT, MDL6-7 and As7-C) (Table S1). Three strains (SAG 81.80, CAUP H 1110 and CCALA 396) were identified only to genus level, when one or more determining traits were missing or uncertain (Table S1). Two picture plates were constructed: first documents the morphology of individual morphological clades (Figure 1) and second includes taxa to which we pay particular attention in this study because they are subject to taxonomic changes (Figure 2). Tables with relevant morphological characteristics were made to enable synoptical comparisons of the strains for spiny and granulated clades (Tables 5-6).

Ultrastructure

The cell wall ultrastructure of twelve strains representing taxa not previously examined ultrastructurally was observed with a transmission electron microscope (Figure 3). Cell walls of strains SAG 34.81 *Nephrocytium agardhianum* and CCALA 398 *Oonephris obesa* possessed clearly different fine structure than expected in Oocystaceae (Figure 3). The cell wall of *Planctonema lauterbornii* was composed of several layers. However, no characteristic arrangement of fibrils was detected. The cell walls of *Tetrastrum* formed three layers, inner, middle and outer, also without the regular arrangement of the cellulose fibrils (Figure 3). All remaining investigated strains (SAG 2081 *Willea rectangularis*, SAG 56.81 *Granulocystis verrucosa* (, Tow 6/3 P-1ou *Oocystis parva*, SAG 42.81 *Tetrachlorella alternans* and CCALA 515 *Willea vilhelmii* possessed the *Oocystis*-like ultrastructure (Figure 3).

Molecular phylogeny

In total, we obtained 30 new sequences of the SSU rRNA gene and 45 new sequences of *rbcL* (Table S1). The remaining sequences were retrieved from the public database GenBank (Tables 2-4). Overall, five datasets were aligned. One SSU alignment of the Sphaeropleales (1661bp) and one of the Volvocales (1710bp) were analyzed to classify two taxa excluded from the family Oocystaceae. Three datasets were made of sequences of Oocystaceae. The final alignment of SSU rRNA gene included 1568bp, the *rbcL* alignment 1108bp and the concatenated alignment of both genes 2676bp. Phylogenetic trees were constructed for each dataset, and phylogenetic position of all strains was determined. Sample sorting and level of resolution differed between the two genes, yet topologies did not strongly conflict except for the base of the trees. Therefore, the concatenated tree likely shows the best representation of relationships in Oocystaceae.

Phylogenetic delimitation of Oocystaceae

Two of the examined strains turned out to be phylogenetically distant from the family Oocystaceae. The strain SAG 34.81 *Nephrocytium agardhianum* was placed in the Sphaeropleales *incertae sedis* as sister to the taxon *Pseudomuriella* sp. (Figure 4). The strain CCALA 398 *Oonephris obesa* clustered as a sister to *Cylindrocapsa geminella* within the volvocalean Treubarinia clade *sensu* Nakada et al. (2008) (Figure 5). All remaining strains composed a monophyletic group with strong support according to all three trees based on the SSU rRNA data, the *rbcL* data, and the concatenated alignment of both genes (Supplementary materials 1 and 2, Figure 6).

Definition of the family Oocystaceae

Molecular phylogeny excluded members with different shape of the cell and in turn included coenobial strains. The new delimitation requires an emended definition of the family, reflecting the newly included taxa and their characteristics:

Cells solitary or arranged in 2-4-8-16-32-celled groups or coenobia or connected in pseudo-filaments. Cells oval to elliptical or spindle-shaped, sometimes nearly spherical,

or slightly asymmetrical. One, a few or numerous chloroplasts present in a single cell, parietal or nearly so, with pyrenoid that is sometimes not clearly visible. Cell wall smooth, with or without thickened ends, or covered with granules or spines. Cell wall characterised by special ultrastructure (multilayered with several layers of crystalline cellulose microfibrils arranged in each layer perpendicularly to the next layer). Mucilage cover may be present. Propagation by autospores, sometimes by oogamy. Daughter cells usually remain inside mother cell wall for a prolonged amount of time, sometimes for several generations.

Internal structure of Oocystaceae

The phylogeny of the family Oocystaceae shows the distribution of its taxa to three subfamilies Eremosphaeroideae, Oocystoideae, and Makinoelloideae *subf. nov.* Figures 6, S1 and S2.

Makinoelloideae *subf. nov.* Štenclová (newly defined)

Diagnosis: Cells relatively small (width 3-18 μm, length 4-30 μm), oval to elliptical to elongated, arranged in pairs or tetrads to multicellular coenobia or pseudo-filaments. Cells with 1-4 parietal chloroplast with a big clearly visible pyrenoid. Two to four autospores remain inside the mother cell wall for a prolonged time. Cell wall is smooth.

Type genus: *Makinoella* Okada

Eremosphaeroideae (emended)

Diagnosis: Cells oval to elliptical, occurring solitary or in pairs or tetrads (less commonly in 8-celled colonies). Each cell with 8 to more than 20 chloroplasts each with one pyrenoid. Propagation by 2, 4, 8 or 16 autospores, which sometimes stay in mother cell wall for a few generations. Autospores with 2-8 chloroplasts. Dimensions of the cells are up to several times larger than those of other subfamilies (width 4,9-200 μm, length 12-200 μm). The cell wall is smooth.**Oocystoideae** (emended)

Diagnosis: Cells usually small (width 1,5-22 μm, length 3,2-40 μm), oval to elliptical. Cells are found solitary or in pairs or tetrads or 8-16 celled colonies. Each cell with 1-2-4-8 chloroplasts. Propagation by 2-4-8 autospores, which sometimes remain in the mother cell wall for a longer time.. Cell wall can bear diverse ornamentation, such as spines or granules and can produce wide mucilage covers.The subfamily Oocystoideae is divided into five well-supported clades (Figures 6, S1 and S2). Distinguishing of these clades is well-supported by morphological and molecular data (see discussion below).

Generic concept

The following taxonomic changes are based on a combination of phylogenetic, ultrastructural, and morphological analyses presented in this study.

Neglectella solitaria* (Wittrock) Štenclová & Kaštovský *comb. nov.

Basionym: *Oocystis solitaria* Wittrock in Wittrock & Nordsted, *Botaniska Notiser* 1879, p. 24, figs 1-5, 1879). Homotypic synonym: *Oocystella solitaria* (Wittrock) Hindák, Heterotypic Synonyms: *Oocystis solitaria* var. *notabile* West & G.S.West, *Oocystis crassa* Wittrock, Heterotypic synonym: (established in the present study) *Oocystis solitaria* var. *major* (Wille) P.M.Tsarenko.

Oocystidium planoconvexum* (Hindák) Štenclová & Pažoutová *comb. nov.

Basionym: *Coenochloris planoconvexa* Hindák, *Studies of the chlorococcal algae (Chlorophyceae). I. – Biol. práce*, Veda, Bratislava, p. 22, pl. 5 fig. 1, 1977. Epitype: The strain CAUP H5502 permanently cryopreserved at the Culture Collection of Algae of the Charles University in Prague, Czech Republic (CAUP) (Pažoutová et al. 2010). Homotypic synonym: *Ooplanctella planoconvexa* (Hindák) Pažoutová, Škaloud & Nemjová.

Discussion

Delimitation of the family Oocystaceae

Two examined taxa SAG 2082 *Nephrocytium agardhianum* and CCALA 398 *Oonephris obesa* were excluded from the family based on the molecular phylogeny. *Nephrocytium agardhianum* was placed in the Sphaeropleales *incertae sedis* as the sister to the strain '*Pseudomuriella*' sp. These two taxa represent a distinct lineage within Sphaeropleales and are not closely related to the genus *Pseudomuriella* (Figure 4). Our results are consistent with Vieira et al. (2016), whose phylogenetic study recently also placed *Nephrocytium* in Sphaeropleales based on a different chloroplast gene, *tufA*. Cell characteristics of *Nephrocytium agardhianum* are similar to the other Sphaeropleales. *Oonephris obesa* clusters as a sister taxon to *Cylindrocapsa geminella* within the volvocalean clade Treubarinia, a peculiar, morphologically heterogeneous group with disproportionately long branches in the phylogeny of 18S rRNA gene (Nakada et al. 2008). *Oonephris obesa* and *Cylindrocapsa geminella* both possess very similar cell characteristics and differ from each other by cell arrangement. The cells of *Oonephris obesa* occur in spherical colonies whereas the cells of *Cylindrocapsa geminella* are stacked in rows and form pseudo-filaments. The phylogenetic placement of *N. agardhianum* and *O. obesa* outside of Oocystaceae is also supported by their cell wall ultrastructure, which is not *Oocystis*-like (Figure 3).

All other examined strains clustered as a monophyletic clade sister to Chlorellaceae inside Trebouxiophyceae. Near the base of the phylogeny, two lineages crystallized: the monotypic genus *Planctonema* and the genus *Tetrastrum* with two analysed species *T. heteracanthum* and *T. staurogeniiforme*. Whether to include these two deeply-diverging genera into the family Oocystaceae remains unclear, because they do not exhibit the characteristic ultrastructure (Figure 3). Accordingly, there is no reason other than phylogenetic to assign them to the family Oocystaceae as suggested in Bock et al. (2013), and they may thus become *incertae sedis*. Both genera also exhibit gross morphological differences from Oocystaceae. *Planctonema lauterbornii* has a filamentous thallus and does not reproduce by autospores but rather by fragmentation of the filament (Schmidle

1903). Its parietal chloroplast and cylindrical cell shape are, however, in agreement with the definition of Oocystaceae. In contrast, *Tetrastrum* species propagate via autospores and make crucigenoid coenobia similar to some taxa in the coenobial clade of Oocystaceae. The arrangement of the cells is similar to the rest of crucigenoid algae *sensu* Komárek (1979), Komárek and Fott (1983), and morphology is *Chlorella*-like with one parietal chloroplast filling the cell and containing one small rounded pyrenoid. In our study, phylogenetic positions of both genera differed depending on the genes used: SSU rRNA gene analysis showed *Tetrastrum* closer to the family Oocystaceae than *Planctonema*, whereas *rbcL* gene and concatenated trees proposed *Planctonema* closer than *Tetrastrum* Figures 6, S1 and S2. These differences may be resulting from long branch attraction or other phylogenetic artefacts.

The rest of the strains constitute a well-supported Oocystaceae clade. We newly included the coenobial *Willea vilhelmii* originally placed in Scenedesmaceae (Komárek and Fott 1983). Its ultrastructure of the cell wall together with the cell and chloroplast shape, both similar to the typical members of the Oocystaceae, grant strong support to the position of *Willea vilhelmii* (Figures 2-4). All studied strains of Oocystaceae had the multi-layered structure with cellulosic fibrils arranged crosswise to those in the adjoining layer (Figure 3). It is evident that the number of wall layers corresponds with cell dimensions; cell wall of large-celled taxa such as *Eremosphaera*, *Oocystis solitaria* (Quader et al. 1978) and *Neglectella peisonis* Schagerl (Schagerl 1993) are composed of larger numbers of layers than cell walls of small-celled algae such as *Willea vilhelmii* or *Granulocystis verrucosa* and especially *Oocystis parva* (Figure 3), and *Amphikrikos nanus* (Crawford and Heap 1978).

Definition of the family Oocystaceae

In accordance with previous molecular studies (Buchheim et al. 2001, Krienitz et al. 2011b), changes presented in this study indicate that the definition of the family regarding the shape of the cell is rather robust. All analysed members of the family possess lemon-like, oval, cylindrical or nearly spherical cell shape. No irregular-shaped taxa stayed inside the family, so far. Conversely, coenobial and pseudo-filamentous members, new to the

family, show a larger diversity of cell arrangement than accommodated by the previous definition of the family. Oocystacean algae remain variable in traits like cell dimensions and number of chloroplasts. However, such traits seem to be informative for internal classification within the family.

Structure of the family Oocystaceae

Traditional morphological studies proposed that the family consisted of up to four subfamilies (Smith 1950, Fott 1976, Komárek and Fott 1983, Melkonian 1983). Fott (1976) included the subfamily Scotiellopsioideae. However, his proposition was rejected by molecular studies that proved its members close to Scenedesmaceae spp. (Hanagata 1998). Komárek and Fott (1983) divided the family Oocystaceae into the subfamilies Eremosphaeroideae, Lagerheimioideae, and Oocystoideae and tentatively Glaucocystoideae, which was more recently recognized as a separate phylum (Bhattacharya 1995). In the present study, molecular phylogeny combined with cell morphology support a concept comprising three subfamilies: Eremosphaeroideae, Makinoelloideae *subf. nov.*, and Oocystoideae.

Among the three subfamilies, a large disproportion of branch lengths is visible especially in the case of the SSU rRNA tree (Figure S1). Basal taxa of the family Oocystaceae affiliated with the subfamily Eremosphaeroideae are placed on apparently long branches, which is probably caused by incomplete taxon sampling. Therefore, relations among the subfamilies have still not been resolved with certainty. The SSU rRNA tree showed all subfamilies as a paraphyletic grade, whereas trees of *rbcL* gene and concatenated dataset showed them as monophyletic. However, neither state was well supported. Long branches (suggesting high substitution rates) entail numerous homoplasies and therefore a long branch attraction may have affected the constructed phylogenetic tree, especially in the maximum likelihood analyses. Additional taxon selection would be useful to break the long branches and provide more explicit results. The whole clade of the subfamily Makinoelloideae is subtended by an extremely long branch. In contrast, the branches inside Oocystoideae are multiple times shorter and exhibit low molecular variability among some strains in both SSU rRNA and *rbcL*, as well as the concatenated tree. A more

variable molecular marker such as the ITS region or another chloroplast housekeeping gene may provide additional resolution inside Oocystoideae.

The newly defined subfamily Eremosphaeroideae contains genera with large cells and numerous chloroplasts (Figure 1). The subfamily Eremosphaeroideae *sensu* Komárek and Fott (1983) included only three genera: *Eremosphaera*, *Excentrosphaera*, and *Oocystaenium*, notwithstanding the similar morphology of genus *Neglectella* described in Vodenicarov and Benderliev (1971). According to the molecular phylogeny, the *Neglectella* clade is closely related to the *Eremosphaera* spp. clade. Monophyly of the subfamily is not significantly supported, and mutual relations among genera poorly resolved, possibly because of limited species sampling and long branch attraction. Therefore, sequencing of additional taxa is recommended, though finding candidate strains may be problematic. However, the subfamily is clearly morphologically delimited based on the presence of numerous chloroplasts and large size of the cells, which distinguish the subfamily as a separate classification unit.

Subfamily Makinoelloideae is well supported by molecular phylogeny Figures 6, S1 and S2. Three of its taxa were previously classified as members of the scenedesmacean subfamily Crucigenoideae (*Willea rectangularis*, *Makinoella tosaensis* and *Willea wilhelmii*, Komárek and Fott 1983) typical by its crucigenoid coenobial morphology (Figure 1). Another one ex-crucigenoid coenobial alga SAG 42.81 *Tetrachlorella alternans* was not classified as a member of the coenobial clade but is closely related to species of the genus *Oocystis* (Hepperle et al. 2000, present study). The genus *Tetrachlorella* differs from all taxa in Makinoelloideae by its spindle-shaped cells; the rest of the strains possess oval to elliptical cells with rounded ends. In addition to the coenobial strains, four noncoenobial strains with similar cell characteristic were included into the subfamily Makinoelloideae based on phylogenetic results: *Ecballocystis hubeiensis*, *Ecballocystopsis dichotomus*, *Elongatocystis ecballocystiformis* and *Oocystis nephrocytioides.* All four strains possess long cylindrical cell organized in tetrads and enclosed in the mother cell wall (Krienitz and Bock 2011, Xia et al. 2013). The tetrads of *Ecballocystis hubeiensis* and *Ecballocystopsis dichotomus* are arranged into simple filaments (Xia et al. 2013).

The newly defined monophyletic subfamily Oocystoideae consisted of previously included Oocystoideae (except *Neglectella*), as well as Lagerheimioideae *sensu* Komárek and Fott (1983) and received significant support (Figures 6, S1 and S2). The subfamily was divided into five morphologically and phylogenetically well-defined clusters: spiny clades 1 and 2, granulated clade 1 and 2 and *Oocystidium* clade, accompanied by *Oocystis* spp. and *Tetrachlorella alternans* arranged into the *Oocystis* group (Figures 6, S1 and S2).

Spiny clades

All spiny strains of Oocystaceae were traditionally associated with the subfamily Lagerheimioideae (Komárek and Fott 1983), which comprised 11 genera. Molecular phylogeny excluded the genera *Trochiscia* (now placed in Treubarinia, Buchheim et al. 2001), and *Diacanthos* (now Chlorellaceae Krienitz et al. 2004, Pröschold et al. 2010 and confirmed the genera *Lagerheimia* and *Franceia* (Krienitz et al. 2003, this study) as members of the family. Spine ultrastructure of both *Lagerheimia* and *Franceia* also differs from spines of other algae in their unique composition where a fibrillary axis is covered by amorphous matter. (Hegewald et al. 1980). Some analyses show spiny strains investigated here as monophyletic (Figure S1), others as paraphyletic (Figures 6,S2) and neither state is well-supported. The spineless mucilaginous *Oocystidium* clade clusters inside or sister to the former Lagerheimioideae Figures 6, S1 and S2.

We propose two spiny clades that differ in the number and position of the spines. Four species *Franceia amphitricha* (Lagerheim), *L. ciliata* (Lagerheim), *L. subsalsa,* and *L. longiseta* cluster together as spiny clade 1. These taxa form a monophyletic clade according to all three trees (Figures 6, S1 and S2). The clade is characterised by plurality of spines, and different species are distinguished by their length, number, and placement. Spiny clade 2 consisted of *Lagerheimia genevensis* and *L. hindakii* (Figure S2). The clade's synapomorphy is the arrangement of spines, two on each pole of the cell placed subpolar. The placement of *Lagerheimia marssonii* received poor support. This alga differs from both spiny clades by a few spines arranged polarly and equatorially (Figure 1, Table 5). It is clear that the apparently paraphyletic genus *Lagerheimia* will require further revisions (see discussion below).

Granulated clades

Only two granulated genera, *Granulocystis* and *Granulocystopsis*, belong to the family Oocystaceae according to Komárek and Fott (1983). Additionally, Hindák (1988) described a granulated species *Oocystella oogama* and Heynig (1991) established a new genus *Oocystopsis* on the basis of the granulated species *Oocystis granulata* inside the family. *Siderocystopsis* previously in the Micractiniaceae, *Amphikrikos*, previously in the Chlorellaceae Siderocelidoideae, and *Quadricoccus* the Botryococcaceae (Komárek and Fott 1983) were assigned to the family by molecular phylogeny (Hepperle et al. 2000, Pröschold et al. 2010, Krienitz and Bock 2011) and ultrastructure (Crawford and Heap 1978, Schnepf et al. 1980). Granulated strains inside Oocystaceae formed two monophyletic clades differentiated by the type of granulation, therefore the type of granulation is a systematically informative trait for Oocystaceae (Table 6, Figures 6, S1 and S2). Granulated clade 1 consisted of strains with spindle-shaped or elliptical cells with granule irregularly arranged on all cell surfaces (Table 6). This clade included granulated genera *Siderocystopsis* and *Granulocystis*, together with two granulated species *Oocystis bispora* and *Oocystella oogama*. Further taxonomical changes in this group are expected in the future. The monophyly of the clade is not supported by maximum likelihood analysis of *rbcL* (Figure S2). Bayesian inference put the three strains CCALA 396 *Siderocystopsis sp.*, SAG 28.81 *Siderocystopsis punctifera* and SAG 56.81 *Granulocystis verrucosa* together with the posterior probability of 0.97. Differences may be caused by long branch attraction as a result of poor taxon sampling. Granulated clade 2 consisted of strains with oval to nearly spherical shape of the cell and subpolar arrangement of granules (Table 6). It contained three strains of the genus *Quadricoccus*, two strains of the species *Amphikrikos nanus* and CCMP 245 *Schizochlamydella capsulata* with strong support. Strain CCMP 245 *Schizochlamydella capsulata* was not authenticated. Description of the species does not mention granule on the surface of the cell visible on the photo of the strain from NCMA (CCMP). The clear morphological affiliation of the strain SAG 33.81 *Granulocystopsis coronata* to the clade stays without molecular support.

Oocystidium clade

The well supported (Figures 6, S1 and S2.) *Oocystidium* clade includes species of the genus *Oocystidium* and *Ooplanctella planoconvexa*. Their remarkably close relationship predicted by Krienitz and Bock (2011) and confirmed by our analyses and the distinct separation of the clade from the rest of the Oocystaceae suggests that this clade represents a single genus and we propose the name *Ooplanctella* to recognize as synonymous to *Oocystidium* (Figures 6, S1 and S2.). The representatives of the clade are characterized by wide mucilaginous envelopes (Korshikov 1953) that can be structured in several layers (Hortobágyi 1973) or can make projections (Pažoutová et al 2010). The strain SAG 37.93 *Echinocoleum elegans* possesses a similar mucilaginous envelope (Pažoutová et al 2010) and cell characteristics as *Oocystidium*. Therefore, its unresolved relationship with the *Oocystidium* clade is surprising. Similar to Pažoutová et al (2010) we could not determine the position of *Echinocoleum elegans* despite the phylogenetic analyses based on sequences of two genes. Analyses of SSU rRNA gene show its position close to the *Oocystidium* clade (Figure S1), though without significant bootstrap support and the result of the *rbcL* phylogeny does not support this placement (Figure S2). Similar mucilage cover with projections was also described for material labelled as *Oocystis lacustris* (Řeháková 1969) and *Lagerheimia ciliata* (Hindák 1978). *Lagerheimia* strains are close to the *Oocystidium* clade according to the molecular phylogeny, however we did not see any mucilage covers during our observations of *L. ciliata* (SAG 1194 and SAG 2083). The relationship between *Oocystis lacustris* and the genus *Oocystidium* has not been investigated yet.

Oocystis group

The remaining strains were assigned to the *Oocystis sensu lato* group. This assemblage contains *Oocystis* strains *sensu* Komárek and Fott (1983) with one strain of the genus *Tetrachlorella* (Hepperle et all. 2000, present study). Most of the branches are missing bootstraps (i.e, the values were below 50%; Figures 6, S1 and S2.). The relationships between the strains remain unclear except two clades: *Oocystis heteromucosa* clade and *Oocystis parva* clade (Figure S1). The first clade contains the sister strains SAG 82.80

Oocystis parva and Tow 6/3 P-1ou *Oocystis parva*. The latter cluster consisted of four strains. Two strains designated as *Oocystis parva* and *Oocystis* sp. (GenBank - NCBI) cluster together with two strains of *Oocystis heteromucosa.* This clade contained the authentic strain SAG 1.99 and therefore can represent *Oocystis heteromucosa*

Generic Issues

Neglectella

Genus *Neglectella* was established by Vodenicarv and Berdenliev (1971) to define the large-celled algae with oval cell shape and numerous chloroplasts arranged peripherally and radially. Subsequently, *Neglectella* was divided into the genera *Neglectella*, *Neglectellopsis* and *Skujaster* (Vodenicarov 1989). In the present study, *Neglectella peisonis* and *Oocystis solitaria* cluster together in Eremosphaeroideae (Figures 6, S1 and S2.). The close relationship of the two taxa proposed by molecular phylogeny is in agreement with shared morphological characteristics such as numerous chloroplasts, large cell dimensions, the lemon-like shape of the cells and daughter cells remaining for a prolonged time in the mother cell wall (Hepperle et al. 2000, Schagerl 1993). The ecology of *Oocystis solitaria* - littoral of acidic freshwaters - is also closer to that of *Neglectella* (also littoral of acidic freshwater) than to the freshwater planktonic *Oocystis* (Schagerl 1993). The new combination as *Neglectella solitaria* is proposed. *N. solitaria* differs from other *Neglectella* species by relatively smaller cells and smaller number of peripheral chloroplasts which are loosely organized and not radially arranged.

Eremosphaera

Eremosphaera, a traditional genus of extremely large oocystoid algae with numerous chloroplasts, has not been shown as monophyletic in any of our analyses (Figures 6, S1 and S2.). Long branch attraction is a potential problem causing the uncertain placement of the strain ACOI 1819 *Eremosphaera gigas*. Its position varied according to the type of analyses, but was always widely separated from other *Eremosphaera* strains. ML analysis assigned it to the Makinoelloideae but without bootstrap support; BI to *Neglectella* with

moderate support. None of the analysis suggested being related to the type species of *Eremosphaera* - *E. viridis* clustering on the very base of the family Oocystaceae. The cell structure, especially chloroplast arrangement, of the two original *Eremosphaera* species differs. Cells of *E. viridis* contain a large central vacuole traversed by radial strands of cytoplasm with chloroplasts which connect the central nucleus to the peripheral part of the cell with numerous irregularly dispersed chloroplasts (De Bary 1858). No such structure was found in *E. gigas* whose arrangement of the chloroplast is rather *Neglectella*-like: stacked in the surface layer of the cell (Shagerl 1993). Simultaneously, *E. gigas* differ from *Neglectella* by its nearly spherical to widely oval cell shape - unlike the clearly elliptical *Neglectella* (Figures 1-2) - and its enormous cell dimensions (up to 130 µm according to Komárek and Fott (1983)).

Eremosphaera gigas, as described by Archer (1877), currently does not have a type strain designated. Several strains bearing this name exist in culture collections and match *E. gigas* morphologically and ecologically, including the strain ACOI 1819. None of the available strains are authentic, and it is unknown whether or not they form a monophyletic group - a question that will be addressed in future studies, which may result in the description of further new taxa. This study should comprise more strains of the genus *Eremosphaera*, primarily all available strains of the species *E. gigas* accompanied by a new isolate from the type locality in Ireland (Archer 1877).

Crucigeniella Lemmermann

The strains of former Scenedesmaceae members *Crucigeniella rectangularis* and *Willea vilhelmii* cluster inside Oocystaceae as sister taxa with moderate support and both share similar cell morphology and arrangement into crucigenoid coenobia. Our results support their close relationship proposed by John et al. (2014) who transferred seven *Crucigeniella* species including *C. rectangularis* (as *Willea rectangularis*) into the genus *Willea*. The status of the remaining six renamed *Crucigeniella* species stays speculative because *Crucigeniella* is polyphyletic: *C. apiculata* together with some *Crucigenia* species have been placed inside Scenedesmaceae (Chlorophyceae) (Bock et al. 2013). The entangled

taxonomy of the genera *Crucigeniella* and *Crucigenia* has yet to be resolved through a prospective study, likely one including the key type species *Crucigenia lunaris*.

Lagerheimia

The spine-bearing genus *Lagerheimia*, which currently comprises twenty accepted species (Guiry and Guiry 2016), is not monophyletic and should be split into two or three genera. This would be in agreement with a recent trend to establish small genera that differ from each other only in a handful of features and include a small number of species (Luo et al. 2010). Spiny clade 2 contains the type species of *Lagerheimia*: *L. genevensis*; therefore, it will remain *Lagerheimia.* Spiny clade 1 represents another genus, presumably *Franceia*, according to one of the included strains (SAG 10.81 *Franceia amphitricha*). Sequencing more species of both genera *Lagerheimia* and *Franceia*, including the type species *Franceia ovalis*, is recommended for the resolution of this taxonomical issue. More data can also help to resolve the position of *Lagerheimia marssonii* (Figure S2, Table 5). According to our preliminary results, the redefined monophyletic units will also share morphological synapomorphies, namely in the number and position of spines (Table 5).

Oocystidium

Korshikov (1953) described new genus *Oocystidium* with the type species *O. ovale* for *Oocystis*-like algae with a wide mucilage cover around the cell. Hortobágyi (1973) considered the shape and structure of the cover taxonomically important and distinguished *O. ovale* with a smooth and elliptical cover and *O. polymammilatum* with irregular-shaped and structured mucilage. On the basis of the molecular phylogeny and morphological similarity of the cell structure and wide mucilage covers, we suggest a new combination, *Oocystidium planoconvexum*. Genus *Ooplanctella* and species *Ooplanctella planoconvexa* as well as *Coenochloris planoconvexa* is proposed to be recognized as synonymous to *Oocystidium* and *Oocystidium planoconvexum* respectively. Inside the clade, two strains of *Oocystidium polymammilatum* clustered together with two strains labelled as *Oocystidium* sp. Therefore, we rejected the suggested combination of *Oocystidium polymammilatum* into the genus *Echinocoleum* as *Echinocoleum*

polymammilatum (Hindák and Horecká 1987) and we recommend the original combination *Oocystidium polymammilatum*.

Oocystis

Oocystis previously proved to be a wide genus with enormous variability including all lemon-shaped algae Komárek and Fott (1983). Its delimitation has been subject to changes since the genus was established; therefore, species originally assigned to *Oocystis* are now scattered in various genera, e.g. *Eremosphaera*. *Skujaster* Vodenicarov, *Franceia*, *Lagerheimia*, *Granulocystis*, *Granulocystopsis*, *Siderocelis*, *Elongatocystis* (Krienitz and Bock 2011, Komárek and Fott 1983). A large albeit somewhat mechanical reorganization was suggested by Hindák (1988), who followed the concept of Lemmermann (1903) and proposed to split the genus on the basis of presence/absence into the pyrenoid-bearing *Oocystella* (type species *O. natans*) and the pyrenoid-less *Oocystis* (type species *O. naegelii*). Hindák (1988) took into account the original description of *Oocystis naegelii*, which mentioned chloroplasts without pyrenoid (Braun 1855) and transferred 13 *Oocystis* species that possessed a pyrenoid to *Oocystella*. However, the step is controversial, because the type material of (Braun 1855) was re-examined by Skuja (1964), who found chloroplasts with a pyrenoid. Therefore, in some later literature (e.g. John and Tsarenko 2002) only *Oocystis* is still recognized.

The present study classified *Oocystis solitaria* outside the genus *Oocystis*, in accord with previous studies (Schagerl 1993, Hepperle et al. 2000). Hepperle et al. (2000) suggested to exclude *O. solitaria* on the basis of distant phylogenetic position from other *Oocystis* species and different morphology, namely the marked morphological dissimilarity with the type species of *Oocystis*, *O. naegelii*. Here we have transferred *Oocystis solitaria* into the genus *Neglectella*. Additionally, we found that *Oocystis bispora*, *Oocystis nephrocytioides* and also *Oocystella oogama* do not cluster with most other *Oocystis* species. Our study does not have enough data to come to a definitive taxonomic conclusion regarding the complex problems of *Oocystis*, and therefore the taxonomic treatment of such putative *Oocystis*-affiliated taxa will be subject of future studies.

For the future revision of the genus *Oocystis*, we found that the strain MP STE 7 may be of special interest. Its characteristics correspond to the characteristics of *Oocystis naegelii*, which is still recognized as the type of the genus *Oocystis*, although Řeháková (1969) suggested *O. lacustris* as a new type for the genus. Subsequent studies, namely Komárek and Fott (1983) recognize *O. naegelii* as the type, advocating the thorough examinations of Skuja (1964). The position of *Oocystis lacustris* remains to be verified with molecular data, especially its relationship to the morphologically close genus *Oocystidium*.

Conclusion

Green coccal algae comprise a wide diversity of numerous described (and undescribed) species. Only a fraction of them has been examined with molecular phylogenetic data so far. With the aid of molecular tools, monophyletic units can be defined, natural taxa better delimited, and informative morphological traits that correspond to the phylogeny can be determined. In the present study, an updated suite of morphological characteristics was set for the definition of the family Oocystaceae as well for its internal taxonomic structure. The most remarkable result of molecular phylogenetics in systematics of green algae is the recognition of only monophyletic units, resulting in the establishment of fairly small genera. Current oocystacean genera will likely be further divided into smaller taxa as demonstrated here on the genera *Eremosphaera*, *Lagerheimia* and *Oocystis*.

The phylogenetic approach has its drawbacks, however. Long branch attraction and other artefacts resulting from systematic error in the data are some of the most problematic, as many green algal groups suffer from incomplete sampling and long branches. It may be hard to distinguish whether long branches are a result of poor sampling or a reflection of reality – accelerated rates of evolution. In Oocystaceae, especially at the deeper nodes, long branches occurred - for example in Eremosphaeroideae. Therefore, our molecular phylogeny did not resolve the question of monophyly or paraphyly of the subfamily. Despite the possibility that Eremosphaeroideae is paraphyletic, we considered the morphology of its members as diagnostic and Eremosphaeroideae is recognized as a taxon in the present study.

Phylogenetic relationships that remain uncertain or unresolved will be subject to further studies. Additional taxon sampling and data from multiple more genes will help solidify the taxonomy within Oocystaceae. We expect that new species and genera will be erected in the future to accommodate the phylogenetic diversity within the family.

Acknowledgements

Firstly, we thank Jiří Košnar for his guidance in the molecular lab. We are grateful to the kind members of the Laboratory of Electron Microscopy for their assistance with the transmission electron microscope. Special thanks belong to Marvin W. Fawley, Christina Bock and Lothar Krienitz for providing some Oocystaceae strains from their private collections.

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Table 1: Primers used in the present study. Primers used only for sequencing are marked by an asterisk. Tm was checked by OligoAnalyzer 3.1 (Integrated DNA Technologies).

GEN	**NAME**	**SEQUENCE**	**F/**	**T**	**REFERENC**
rbcL	ORB1F	CCACAAACTGAAACAAAAGCA	F	48.	present study
rbcL	ORB1R	CTGGAGCATTACCCCAAGG	R	53.	present study
SSU	NS1F	GTAGTCATATGCTTGTCTC	F	47.	Friedl
SSU	402F*	GCTACCACATCCAAGGAAGGC	F	59.	Katana et al.
SSU	1150R	ACGCCTGGTGGTGCCCTTCCGT	R	68.	Pažoutová et
SSU	1170F	CTGTGGCTTAATTTGACTCAAC	F	56.	Pažoutová et
SSU	1500AF	GCGCGCTACACTGATGC	F	57.	Helms et al.
SSU	1650R	TCACCAGCACACCCAAT	R	54.	Kipp 2004
ITS	ITS1F*	TCCGTAGGTGAACCTGCGG	F	59.	White et al.
ITS	ITS4R	TCCTCCGCTTATTGATATGC	R	52.	White et al.

Table 2.: Sequences of the SSU rRNA and *rbcL* genes used for molecular analysis of Oocystaceae in present study. New sequences published in present study are highlighted by bold font. The column marked as C bears information whether both sequences were included into the concatenated alignment.

STRAIN	**NAME**	**SSU rRNA**	***RbcL***	**C**
SAG 96	*Amphikrikos nanus*	-	**KY710891**	-
SAG 2074	*Amphikrikos nanus*	AF228690*	**KY710892**	YES
SAG 37.93	*Echinocoleum elegans*	FM881776	**KY710878**	YES
CCAP 274/3	*Elongatocystis*	HQ008713	-	-
ACOI 1819	*Eremosphaera gigas*	**KY013478**	**KY710899**	YES
SAG 228-1	*Eremosphaera viridis*	**KY006556**	**KY710888**	YES
SAG 39.92	*Eremosphaera viridis*	**KY006557**	**KY710889**	YES
SAG 10.81	*Franceia amphitricha*	**KY013473**	**KY710893**	YES
SAG 56.81	*Granulocystis verrucosa*	**KY006562**	**KY710867**	YES
SAG 33.81	*Granulocystopsis coronata*	-	**KY710868**	-
SAG 11.94	*Lagerheimia ciliata*	**KY013469**	**KY710885**	YES
SAG 2083	*Lagerheimia ciliata*	**KY013470**	**KY710886**	YES
SAG 48.94	*Lagerheimia genevensis*	AY122336	**KY710866**	YES
SAG 11.92	*Lagerheimia hindakii*	-	**KY710884**	-
SAG 57.81	*Lagerheimia longiseta*	**KY013471**	**KY710887**	YES
CCALA 365	*Lagerheimia marssonii*	**KY006561**	**KY710858**	YES
SAG 2084	*Lagerheimia subsalsa*	**KY047577**	**KY710897**	YES
SAG 28.97	*Makinoella tosaensis*	**KY006566**	**KY710890**	YES
CCALA 961	*Makinoella tosaensis*	AF228691	**KY710879**	YES
SAG 37.96	*Neglectella peisonis*	**KY013476**	**KY710898**	YES
SAG 83.80	*Neglectella solitaria*	AF228686	**KY710862**	YES
CAUP H 1106	*Neglectella solitaria*	**KY014642**	**KY710876**	YES
SAG 3.96	*Oocystella oogama*	**KY013474**	-	-
CAUP H 5502	*Oocystidium planoconvexum*	FM881777	**KY710877**	YES
AN9-1	*Oocystidium polymammilatum*	**KY006565**	**KY710873**	YES

AN2/29-4	*Oocystidium polymammilatum*	AY195966	**KY710874**	YES
SAG 81.80	*Oocystidium* sp.	**KY006559**	**KY710863**	YES
CB 99	*Oocystis bispora*	**KY013467**	-	-
SAG 1.99	*Oocystis heteromucosa*	AF228689	**KY7108**	YES
CB 210	*Oocystis heteromucosa*	**KY013466**	**KY710880**	YES
SAG 2085	*Oocystis* cf. *marssonii*	**KY014640**	**KY710900**	YES
MP STE7	*Oocystis naegelii*	**KY047576**	**KY710882**	YES
CCALA 397	*Oocystis nephrocytioides*	-	**KY710860**	-
SAG 82.80	*Oocystis parva*	**KY006560**	**KY710864**	YES
Tow 6/3 P-1ou	*Oocystis parva*	AY197635	**KY710869**	YES
W Twin SlisT.	*Oocystis rhomboidea*	**KY006563**	**KY710870**	YES
CAUP H 1110	*Oocystis* sp.	**KY038331**	**KY710875**	YES
SAG 11.95	*Planctonema lauterbornii*	-	-	-
SAG 68.94	*Planctonema lauterbornii*	**KY013475**	**KY710896**	YES
MDL6-7	*Quadricoccus verrucosus*	AY197626	**KY710871**	YES
As7-C	*Quadricoccus verrucosus*	**KY006564**	**KY710872**	YES
CCMP 245	*Schizochlamydella capsulata*	**KY013468**	**KY710881**	YES
SAG 28.81	*Siderocystopsis punctifera*	**KY014641**	**KY710901**	YES
CCALA 396	*Siderocystopsis sp.*	**-**	**KY710859**	-
SAG 24.81	*Tetrastrum heteracanthum*	JQ356709	**KY710894**	YES
SAG 45.81	*Tetrastrum staurogeniiforme*	JQ356703	**KY710895**	YES
KR 1996/3	*Tetrastrum staurogeniiforme*	JQ356702	**KY710883**	YES
SAG 42.81	*Tetrachlorella alternans*	AF228687	**KY710865**	YES
SAG 2081	*Willea rectangularis*	AH012990	-	-
CCALA 515	*Willea vilhelmii*	**KY006555**	**KY710857**	YES
	GENBANK			
J.C.Han_32	*Amphikrikos*_sp.	KP013378	-	-
J.C.Han_43	*Amphikrikos*_sp.	KP013379	-	-
NIES 3911	*Chlorella*_sp.	LC129521	-	-
NIES 3912	*Chlorella*_sp.	LC129522	-	-
-	*Ecballocystis hubeiensis*	JX018185	JX018187	YES

-	*Ecballocystopsis dichotomus*	JX018184	JX018186	YES
CCAC 0071	*Eremosphaera viridis*	HE610127	-	-
UTEX LB 34	*Eremosphaera viridis*	AF387154	-	-
NIES_382	*Lagerheimia_ciliata*	LC192142	-	-
KMMCC	*Lagerheimia longiseta*	JQ315525	-	-
CCAP 222/49	*Oocystidium* sp.	HQ008711	-	-
LN1	*Oocystis borgeii*	KU720481	-	-
KRI. 96/10	*Oocystis marssonii*	AF228688	-	-
KMMCC 443	*Oocystis parva*	JQ315649	-	-
KMMCC 356	*Oocystis* sp.	JQ315800	-	-
FACHB_1429	*Oocystis_sp.*	KF928745	-	-
FACHB_1427	*Oocystis_sp.*	KJ522683	-	-
GR35	*Planctonema lauterbornii*	-	EF113462	-
M110-1	*Planctonema* sp.	AF387148	EF113463	YES
CCAP 286/1	*Quadricoccus ellipticus*	HQ008712	-	-
NKS72	Uncultured_Chlorophyta_clone	JX296619	-	-
KRL03E76	Uncultured_eukaryote_clone	KC315825	-	-
KRL03E42	Uncultured_eukaryote_clone	KC315819	-	-
KRL01E35	Uncultured_eukaryote_clone	HQ008711	-	-
NKS72	Uncultured_Chlorophyta_clone	JX296619	-	-
	OUTGROUP (GENBANK)			
-	*Auxenochlorella protothecoides*	FN29893	EU038285	YES
-	*Chlorella variabilis*	AB206549	AB260903	YES
-	*Chlorella vulgaris*	FR865658	AB260909	YES
-	*Micractinium pusillum*	AF364101	EF113451	YES

Table 3.: Sequences of the SSU rRNA gene used for molecular analysis of Sphaeropleales. A new sequence published in present study is highlighted by bold font.

SPHAEROPLEALES	
Ankistrodesmus bibraianus Y16938	*Neochloris vigenis* M74496
Ankistrodesmus fusiformis X97352	**SAG 34.81 *Nephrocytium agardhianum* KY013477**
Ankistrodesmus gracilis Y16937	*Pediastrum duplex* JQ315560
Asterarcys-quadricellulare AF388375	*Planktosphaeria gelatinosa* AY044648
Bracteacoccus aerius JQ259915	*Polyedriopsis spinulosa* AY780667
Bracteacoccus minor JQ259944	*Pseudomuriella aurantiaca* AB005748
Bracteacoccus pseudominor JQ259953	*Pseudomuriella cubensis* HQ292770
Bracteacoccus ruber JQ259919	*Pseudomuriella engadinensis* HM852442
Bracteacoccus sp. JQ259940	*'Pseudomuriella' sp.* AY195974
Chlorella zofingiensis X74004	*Pseudoschroederia antillarum* AF277649
Chlorotetraedron bitridens AY663043	*Radiococcus polycoccus* AF388378
Coelastrum astroideum var. rugosum	*Selenastrum bibraianum* HM483514
Coelastrum morus AF388374	*Scenedesmus bajacalifornicus* HQ246321
Coelastrum sphaericum AF388376	*Scenedesmus obliquus* X56103
Dictyococcus schumacherensis HM852439	*Scenedesmus regularis* FR865732
Dictyococcus schumacherensis HQ292769	*Scenedesmus rubescens* X74002
Enallax acutiformis AB037089	*Schizochlamys gelatinosa* AY781662
Follicularia texensis JN630516	*Sorastrum spinulosum* AY663041
Graesiella emersonii FR865687	*Schizochlamys gelatinosa* AY781662
Graesiella vacuolata FR865685	*Sorastrum spinulosum* AY663041
Hydrodictyon reticulatum HE610123	**OUTGROUP**
Kirchneriella obesa HM483513	*Characium vacuolatum* M63001
Monoraphidium contortum AY846382	*Chlamydomonas reinhardtii* JX888472
Monoraphidium saxatile AY846385	*Chlamydomonas monadina* JN903976
Mychonastes zofingiensis GU827478	*Dunaliella salina* EU589200

Table 4.: Sequences of the SSU rRNA gene used for molecular analysis of Volvocales. A new sequence published in present study is highlighted by bold font.

VOLVOCALES	
Carteria crucifera D86501	*Hafniomonas reticulata* AB248250
Carteria eugametos U70595	*Lobocharacium coloradoense* AF395436
Carteria lunzensis JN904001	*Lobochlamys culleus* AJ410461
Carteria radiosa D86500	*Lobochlamys segnis* AB701525
Characiochloris sasae AB360741	*Oogamochlamys ettlii* AJ410469
Characiosiphon rivularis AF395437	*Oogamochlamys gigantea* AJ410466
Chlamydomonas culleus U70594	*Oogamochlamys zimbabwiensis* AJ410472
Chlamydomonas fimbriata U70784	**CCALA 398 *Oonephris obesa* KY006558**
Chlamydomonas monadina JN903976	*Phacotus lenticularis* AY009897
Chlamydomonas noctigama AB701503	*Polytoma uvella* U22943
Chlamydomonas reinhardtii N903984	*Tetracystis aeria* U41175
Chlorogonium euchlorum AB278610	*Tetracystis pampae* JN903997
Chloromonas brevispina AF517092	*Tetracystis vinatzeri* JN903998
Chloromonas reticulata GU117583	*Treubaria schmidlei* U73474
Chlorosarcinopsis arenicola AB218701	*Treubaria setigera* U73475
Cylindrocapsa geminella U73471	*Trochiscia hystrix* AF277651
Cylindrocapsa geminella AF387159	**OUTGROUP**
Dunaliella lateralis DQ009762	*Bracteacoccus minor* JQ259943
Dunaliella salina EU589200	*Bracteacoccus ruber* JQ259919
Elakatothrix viridis AY008844	*Scenedesmus obliquus* AJ249515
Golenkinia longispicula JN968588	*Pediastrum duplex* JQ315560
Hafniomonas conica AB248251	

Table 5.: Morphological characteristics of investigated strains of spiny clades. Aut = number of chloroplasts before autosporulation. C = clade.

STRAIN	**C**	**DIMENSIONS (**μm)	**CHLOROPLASTS**	**SPIN. NUMBER**	**SPIN. POSITION**
1 SAG 10.81 *Franceia amphitricha*	S1	4-8 x 7-13	1-2-(4-aut)	Numerous	All surface
2 SAG 1194 *Lagerheimia ciliata*	S1	7-15 x 8-17	1-2-(4-aut)	4-5	On each pole
3 SAG 2083 *Lagerheimia ciliata*	S1	6-15 x 7-17	1-2-(4-aut)	4-5	On each pole
4 SAG 57.81 *Lagerheimia longiseta*	S1	6-11 x 7-15	1-2-(4-aut)	6-8	On each pole
5 SAG 2084 *Lagerheimia subsalsa*	S1	3-7 x 8-15	1-2-(4-aut)	3-4	On each pole
6 SAG 48.94 *Lagerheimia genevensis*	S2	2-6 x 3-11	1-(4-aut)	2+2	Slightly subpolarly
7 SAG 11.92 *Lagerheimia hindakii*	S2	2-4 x 3-7	1-(4-aut)	2+2	Slightly subpolarly
8 CCALA 365 *Lagerheimia marssonii*	?	4-8 x 6-12	1-(2-4-aut)	1,1+3-4	On poles + equatorial

Table 6.: Morphological characteristics of investigated strains of granulated clades. C = clade.

STRAIN	**C**	**DIMENSIONS (μm)**	**CHLOROPLASTS**	**GR. NUMBER**	**GR. POSITION**
1 SAG 56.81 *Granulocystis verrucosa*	G1	4-15 x 7-21	1-2	Numerous	All surface
2 SAG 3.96 *Oocystella oogama*	G1	4-6 x 6-9	1-2	Numerous	All surface
3 SAG 28.81 *Siderocystopsis punctifera*	G1	3-4 x 5-7	1	Numerous	All surface
4 CCALA 396 *Siderocystopsis sp.*	G1	3-9 x 6-15	1-4	Numerous	All surface
5 SAG 30.96 *Amphikrikos nanus*	G2	2-4 x 3-7	1	Several	Slightly subpolarly
6 SAG 2074 *Amphikrikos nanus*	G2	2-4 x 3-6	1	Several	Slightly subpolarly
7 CH 99 *Oocystis bispora*	G2	2-4 x 4-7	1	Several	Slightly subpolarly
8 MDL6-7 *Quadricoccus verrucosus*	G2	2-4 x 4-6	1-2	Several	Mainly on poles
9 As7-C *Quadricoccus verrucosus*	G2	3-4 x 4-8	1-2	Several	Mainly on poles
10 CCMP 245 *Schizochlamydella capsulata*	G2	?	?	?	?
11 SAG 33.81 *Granulocystopsis coronata*	G2?	3-5 x 5-8	1	Several	Slightly subpolarly

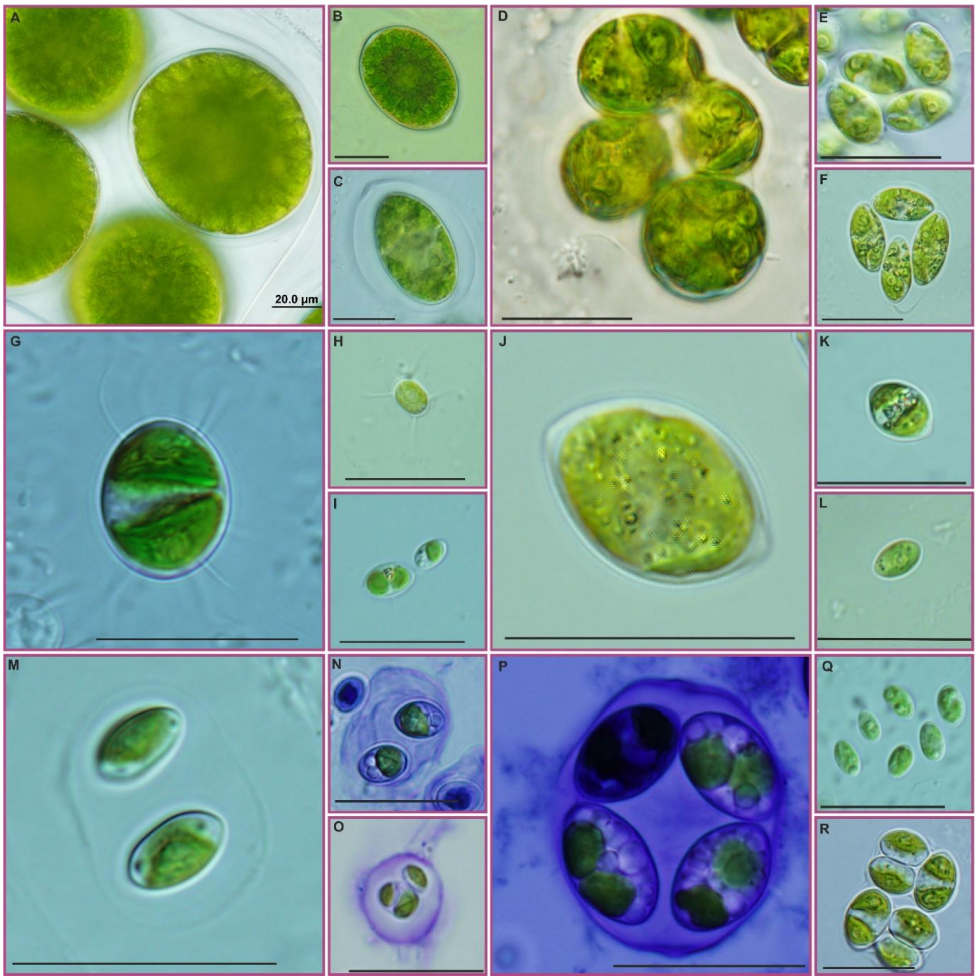


Figure 1: Morphological traits characteristic for each subfamily and morphological clade. A-C: Eremosphaeroideae – large cells with numerous chloroplasts. A: ACOI 1819 *Eremosphaera gigas*, B: SAG 37.96 *Neglectella peisonis*, C: SAG 83.80 *Neglectella solitaria*. D-F: Makinoelloideae – coenobia. D: SAG 28.97 *Makinoella tosaensis*, E: CCALA 397 '*Oocystis*' nephrocytioides, F: CCAP 274/3 *Elongatocystis ecballocystiformis*. G-I: Oocystoideae – spines. G: SAG 57.81 *Lagerheimia longiseta*, H: SAG 48.94 *Lagerheimia genevensis*, I: CCALA 365 *Lagerheimia marssonii*. J-L: Oocystoideae – granules. J: SAG 56.81 *Granulocystis verrucosa*, K: SAG 3.96 *Oocystella oogama*, L: CB 99 '*Oocystis*' *bispora*. M-O: Oocystoideae – mucilage covers (stained with methylene blue). M: AN9-1 *Oocystidium polymammilatum*, N: SAG 81.80 *Oocystidium* sp., O: CAUP H 5502 *Oocystidium planoconvexum comb. nov*. P-R: Oocystoideae – *Oocystis s.l.* P: SAG 2085 *Oocystis* cf. *marssonii*, Q: SAG 82.80 *Oocystis parva*, R: SAG 42.81 *Tetrachlorella alternans*. The scale bars indicate 20µm.

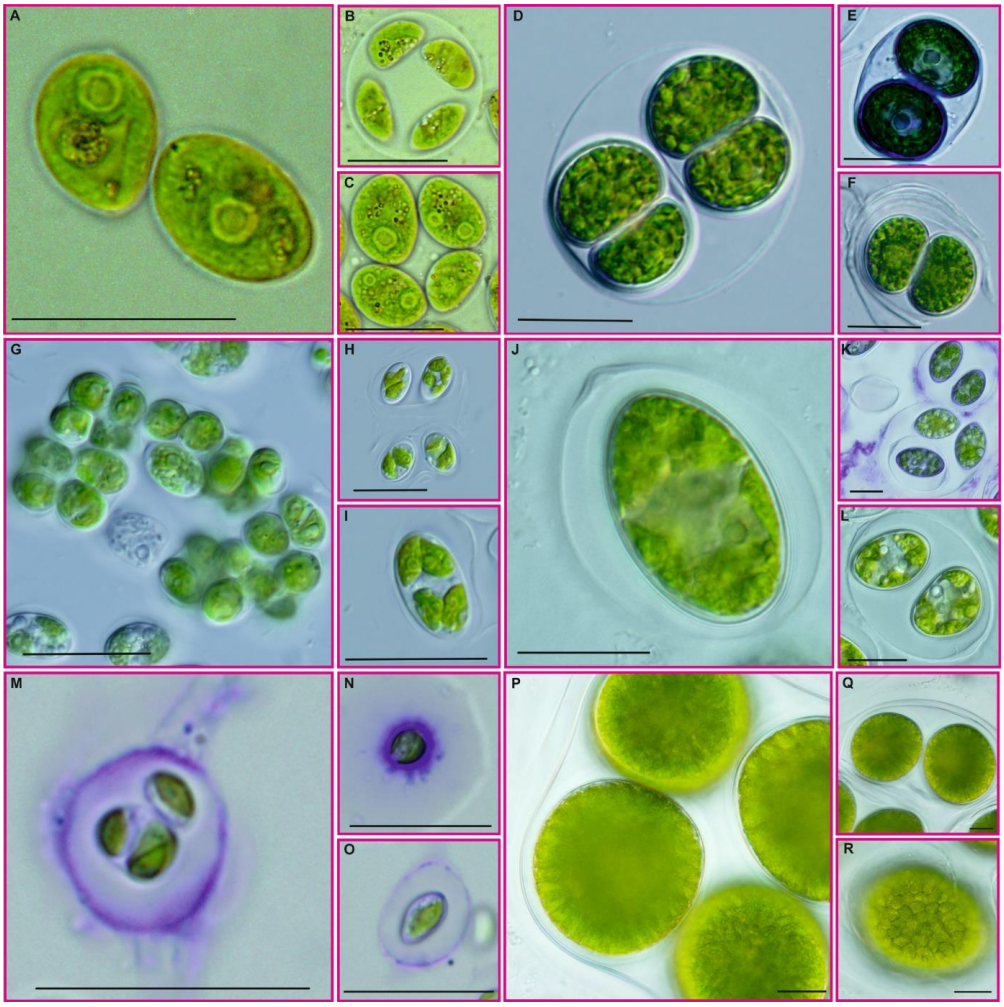


Figure 2: Morphology of taxa subject to taxonomic changes in the present study A-C: SAG 34.81 *Nephrocytium agardhianum*, D-F: CCALA 398 *Oonephris obesa*, G-I: CCALA 515 *Willea vilhelmii*, J-L: SAG 83.80 *Neglectella solitaria comb. nov.*, M-O: CAUP H5502 *Oocystidium planoconvexum comb. nov*, P-R: ACOI 1819 *Eremosphaera gigas*. The scale bars indicate 20µm.

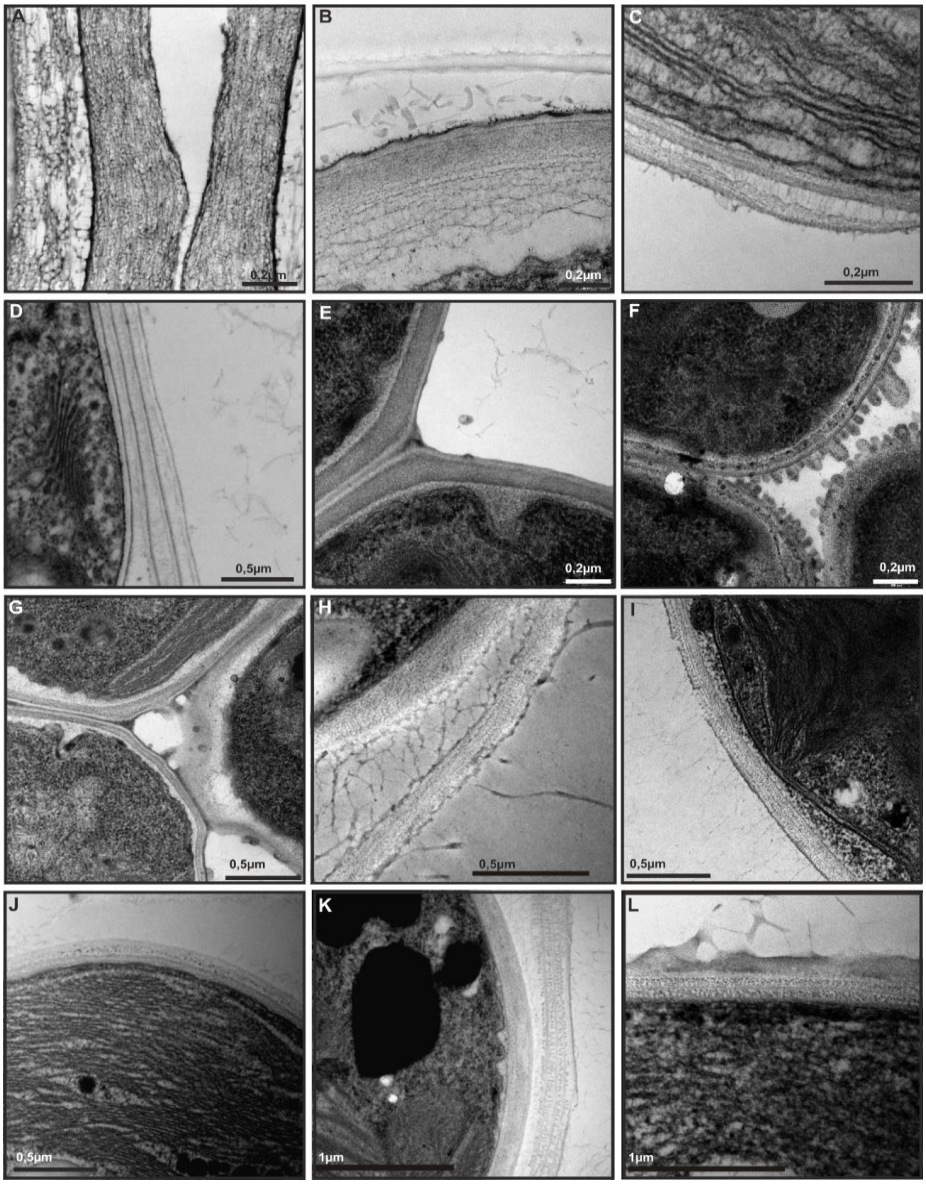


Figure 3: Ultrastructure of the cell wall of 12 investigated strains (TEM). A: SAG 34.81 *Nephrocytium agardhianum*, B: CCALA 398 *Oonephris obesa*, C: SAG 11.95 *Planctonema lauterbornii*, D: SAG 68.94 *Planctonema lauterbornii*, E: SAG 24.81 *Tetrastrum heteracanthum*, F: KR 1996/3 *Tetrastrum staurogeniiforme*, G: SAG 45.81 *Tetrastrum staurogeniiforme*, H: SAG 2081 *Willea rectangularis*, I: SAG 56.81 *verrucosa*, J: Tow 6/3 P-1ou *Oocystis parva*, K: SAG 42.81 *Tetrachlorella alternans*, L: CCALA 515 *Willea vilhelmii*.

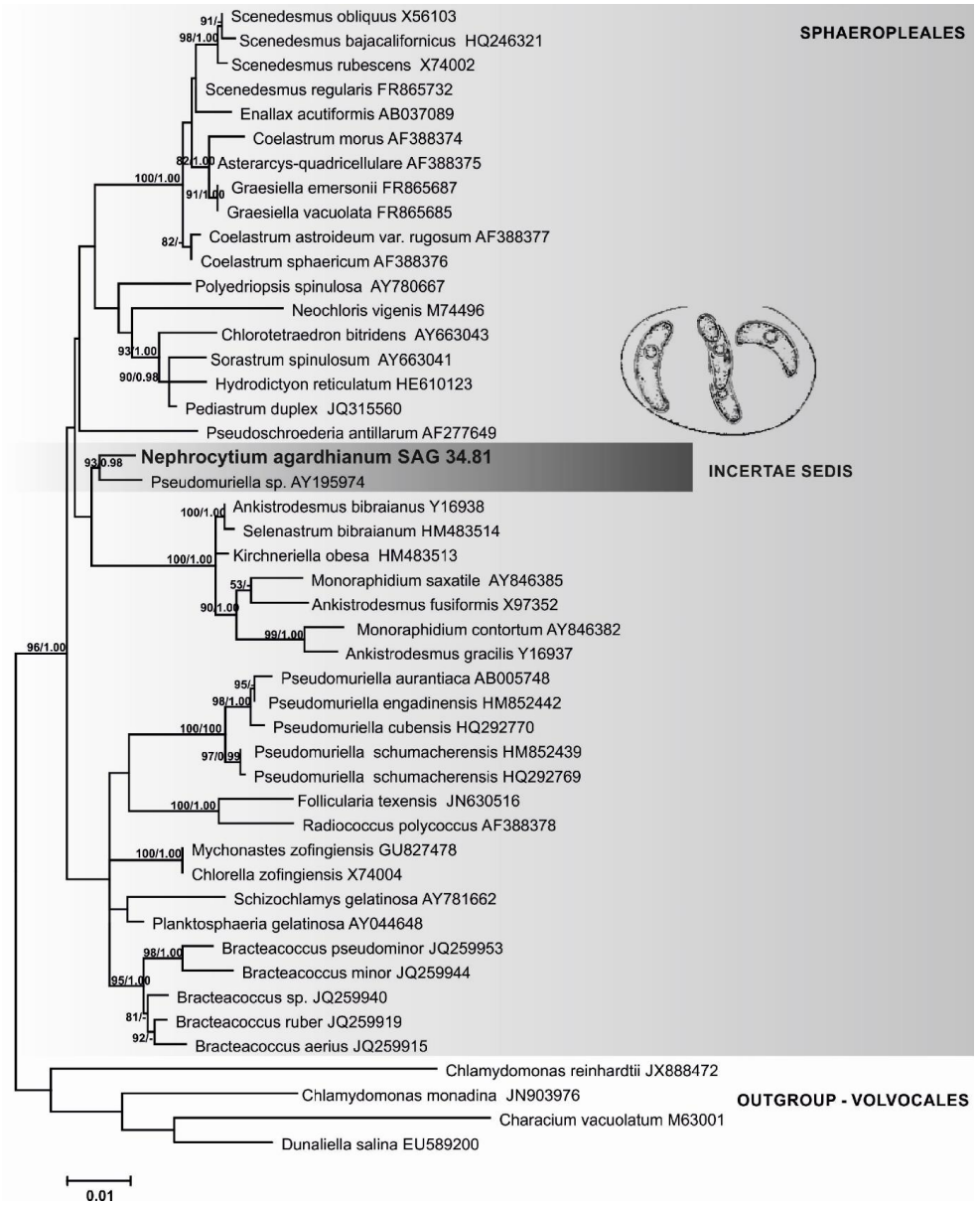


Figure 4: Phylogenetic analyses of SSU rRNA gene sequences of members of Sphaeropleales with Volvocales as an outgroup. Topology represents the best ML tree. A new sequence of *Nephrocytium agardhianum* is highlighted. Numbers at the branches indicate bootstrap support from maximum likelihood (ML, 1000 replicates) and Bayesian posterior probabilities (BI). Support ≥50% for ML and ≥0.95 for MB is shown. ML/BI. Drawing of *Nephrocytium agardhianum* according to Hortobágyi (1973) is shown.

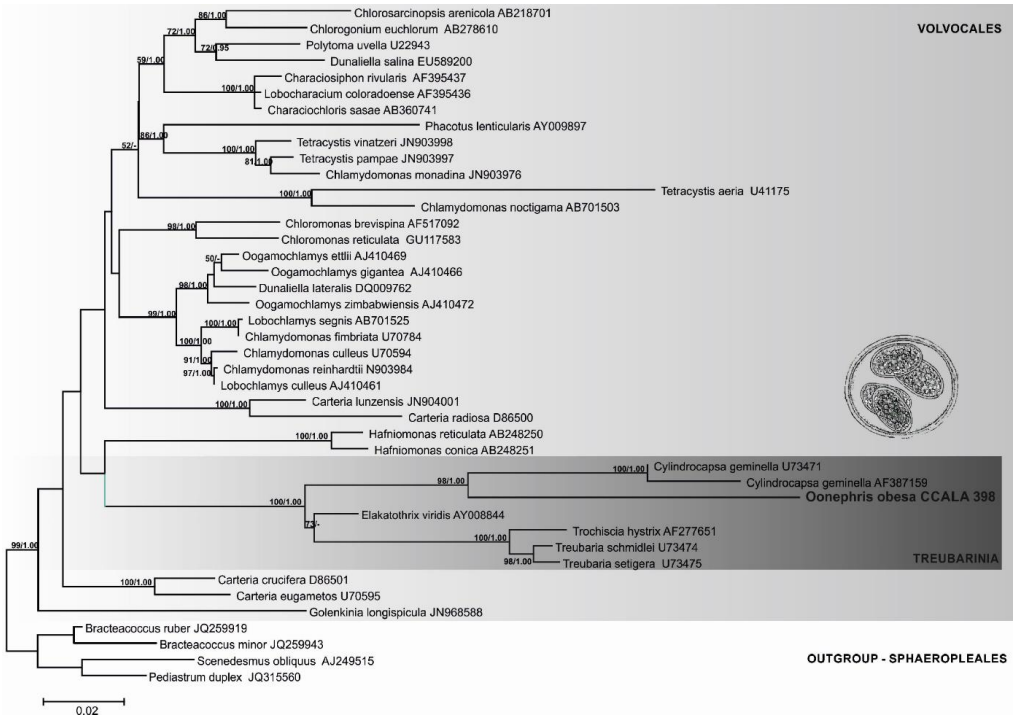


Figure 5: Phylogenetic analyses of SSU rRNA gene sequences of members of Volvocales with Sphaeropleales as an outgroup. Topology represents the best ML tree. A new sequence of *Oonephris obesa* is highlighted. Numbers at the branches indicate bootstrap support from maximum likelihood (ML, 1000 replicates) and Bayesian posterior probabilities (BI). Support ≥50% for ML and ≥0.95 for MB is shown. ML/BI. Drawing of *Oonephris obesa* according to Skuja (1964) is shown.

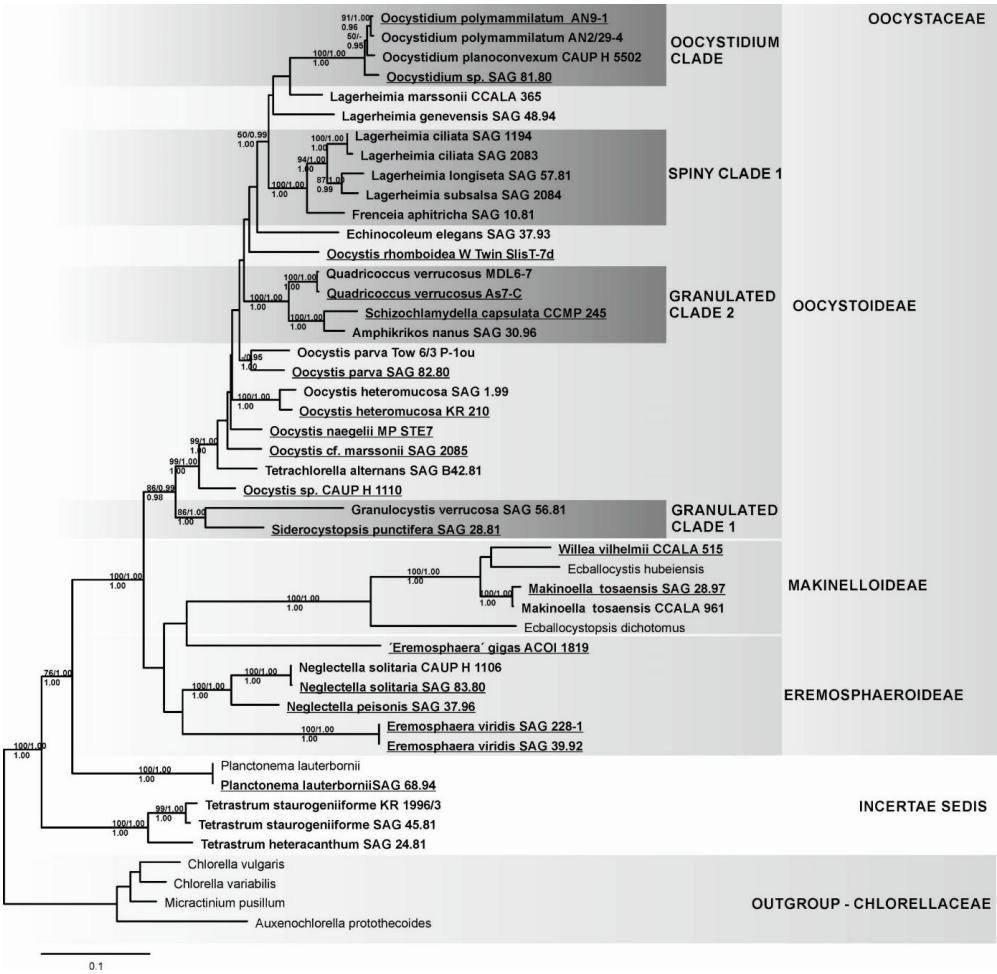


Figure 6: Phylogenetic analyses concatenated dataset of SSU rRNA and *rbcL* sequences of members of Oocystaceae with Chlorellaceae as an outgroup. Topology represents the best ML tree. Sequences are highlighted by bold font, when one sequence is new and underlined, when both sequences are new. Numbers at the branches indicate bootstrap support from maximum likelihood (ML, 1000 replicates) of unpartitioned alignment and Bayesian posterior probabilities of unpartitioned (BI) and partitioned (PBI) alignment. Support ≥50% for ML and ≥0.95 for MB is shown ML/BI/PBI.

Table S1.: Strains examinated in present study. Mor. = morphology, Ultra = examined ultrastructure of the call wall, SSU = obtained SSU rRNA sequence, *rbcL* = obtained *rbcl* gene sequence. P = present study.

STRAIN	**NAME / AUTHENTIC**	**MOR.**	**ULTRA.**	**SSU**	***RBCL***
SAG 30.96	*Amphikrikos nanus* NO	P	-	-	P
SAG 2074	*Amphikrikos nanus* NO	P	-	-	P
SAG 37.93	*Echinocoleum elegans* NO	-	-	-	P
CCAP 274/3	*Elongatocystis ecballocystiformis* YES	P	-	-	-
ACOI 1819	*Eremosphaera gigas* YES	P	-	P	P
SAG 228-1	*Eremosphaera viridis* NO	P	-	P	P
SAG 39.92	*Eremosphaera viridis* NO	P	-	P	P
SAG 10.81	*Franceia amphitricha* YES	P	-	P	P
SAG 56.81	*Granulocystis verrucosa* NO	P	P	P	P
SAG 33.81	*Granulocystopsis coronata* YES	P	-	-	P
SAG 11.94	*Lagerheimia ciliata* NO	P	-	P	P
SAG 2083	*Lagerheimia ciliata* NO	P	-	P	P
SAG 48.94	*Lagerheimia genevensis* NO	P	-	-	P
SAG 11.92	*Lagerheimia hindakii* YES	P	-	-	P
SAG 57.81	*Lagerheimia longiseta* NO	P	-	P	P
CCALA 365	*Lagerheimia marssonii* NO	P	-	P	P
SAG 2084	*Lagerheimia subsalsa* YES	P	-	P	P
SAG 28.97	*Makinoella tosaensis* NO	P	-	P	P
CCALA 961	*Makinoella tosaensis* NO	P	-	-	P
SAG 37.96	*Neglectella peisonis* NO	P	-	P	P
SAG 83.80	*Neglectella solitaria* NO	P	-	-	P
CAUP H 1106	*Neglectella solitaria* NO	P	-	P	P
SAG 34.81	*Nephrocytium agardhianum* NO	P	P	P	P
SAG 3.96	*Oocystella oogama* YES	P	-	P	-
CAUP H 5502	*Oocystidium planoconvexum* YES	-	-	-	P

AN9-1	*Oocystidium polymammilatum* NO	P	-	P	P
AN2/29-4	*Oocystidium polymammilatum* NO	P	-	-	P
SAG 81.80	*Oocystidium* sp. NO	P	-	P	P
CB 99	*Oocystis bispora* NO	P	-	P	-
SAG 1.99	*Oocystis heteromucosa* YES	P	-	-	P
CB 210	*Oocystis heteromucosa* NO	P	-	P	P
SAG 2085	*Oocystis* cf. *marssonii* NO	P	-	P	P
MP STE7	*Oocystis naegelii* NO	P	-	P	P
CCALA 397	*Oocystis nephrocytioides* NO	P	-	-	P
SAG 82.80	*Oocystis parva* NO	P	-	P	P
Tow 6/3 P-1ou	*Oocystis parva* NO	P	P	-	P
W Twin SlisT	*Oocystis rhomboidea* NO	P	-	P	P
CAUP H 1110	*Oocystis* sp. NO	P	-	P	P
CCALA 398	*Oonephris obesa* NO	P	P	P	P
SAG 11.95	*Planctonema lauterbornii* NO	P	P	-	-
SAG 68.94	*Planctonema lauterbornii* NO	P	P	P	P
MDL6-7	*Quadricoccus verrucosus* NO	P	-	-	P
As7-C	*Quadricoccus verrucosus* NO	P	-	P	P
CCMP 245	*Schizochlamydella capsulata* NO	-	-	P	P
SAG 28.81	*Siderocystopsis punctifera* NO	P	-	P	P
CCALA 396	*Siderocystopsis sp.* NO	P	-	-	P
SAG 24.81	*Tetrastrum heteracanthum* NO	P	P	-	P
SAG 45.81	*Tetrastrum staurogeniiforme* NO	P	P	-	P
KR 1996/3	*Tetrastrum staurogeniiforme* NO	P	P	-	P
SAG 42.81	*Tetrachlorella alternans* NO	P	P	-	P
SAG 2081	*Willea rectangularis* NO	P	P	-	-
CCALA 515	*Willea vilhelmii* NO	P	P	P	P

Table S2. All sequences belonging to the Oocystaceae family used for molecular analyses of SSU rRNA. Each newly obtained sequence (and additionally the sequences from previous studies) was checked by BLAST to find all sequences belonging to the family Oocystaceae. For the final analyses were chosen only sequence with required quality (sufficient length without introns, over 1500bp for SSU rRNA). Simultaneously we used only one sequence of each taxa (strain or clone) if more than one was available. We skipped sequences of taxa examined in another paper (in prep, in press). We covered as much molecular variability of the Oocystaceae and some lineages with uncertain positions (*Planctonem*a, *Tetrastrum*) as possible and used four species of Chlorellaceae as outgroup. The closest relations of Chlorellaceae was confirmed by previous studies and also by preliminary analyses made by authors.

STRAIN	**NAME**	**SSU rRNA**
SAG 96	*Amphikrikos nanus*	-
SAG 2074	*Amphikrikos nanus*	AF228690*
SAG 37.93	*Echinocoleum elegans*	FM881776
CCAP 274/3	*Elongatocystis ecballocystiformis*	HQ008713
ACOI 1819	*Eremosphaera gigas*	**KY013478**
SAG 228-1	*Eremosphaera viridis*	**KY006556**
SAG 39.92	*Eremosphaera viridis*	**KY006557**
SAG 10.81	*Franceia amphitricha*	**KY013473**
SAG 56.81	*Granulocystis verrucosa*	**KY006562**
SAG 33.81	*Granulocystopsis coronata*	-
SAG 11.94	*Lagerheimia ciliata*	**KY013469**
SAG 2083	*Lagerheimia ciliata*	**KY013470**
SAG 48.94	*Lagerheimia genevensis*	AY122336
SAG 11.92	*Lagerheimia hindakii*	-
SAG 57.81	*Lagerheimia longiseta*	**KY013471**
CCALA 365	*Lagerheimia marssonii*	**KY006561**
SAG 2084	*Lagerheimia subsalsa*	**KY047577**
SAG 28.97	*Makinoella tosaensis*	**KY006566**
CCALA 961	*Makinoella tosaensis*	AF228691
SAG 37.96	*Neglectella peisonis*	**KY013476**
SAG 83.80	*Neglectella solitaria*	AF228686

CAUP H 1106	*Neglectella solitaria*	**KY014642**
SAG 3.96	*Oocystella oogama*	**KY013474**
CAUP H 5502	*Oocystidium planoconvexum*	FM881777
AN9-1	*Oocystidium polymammilatum*	**KY006565**
AN2/29-4	*Oocystidium polymammilatum*	AY195966
SAG 81.80	*Oocystidium* sp.	**KY006559**
CB 99	*Oocystis bispora*	**KY013467**
SAG 1.99	*Oocystis heteromucosa*	AF228689
CB 210	*Oocystis heteromucosa*	**KY013466**
SAG 2085	*Oocystis* cf. *marssonii*	**KY014640**
MP STE7	*Oocystis naegelii*	**KY047576**
CCALA 397	*Oocystis nephrocytioides*	-
SAG 82.80	*Oocystis parva*	**KY006560**
Tow 6/3 P-1ou	*Oocystis parva*	AY197635
W Twin SlisT.	*Oocystis rhomboidea*	**KY006563**
CAUP H 1110	*Oocystis* sp.	**KY038331**
SAG 11.95	*Planctonema lauterbornii*	-
SAG 68.94	*Planctonema lauterbornii*	**KY013475**
MDL6-7	*Quadricoccus verrucosus*	AY197626
As7-C	*Quadricoccus verrucosus*	**KY006564**
CCMP 245	*Schizochlamydella capsulata*	**KY013468**
SAG 28.81	*Siderocystopsis punctifera*	**KY014641**
CCALA 396	*Siderocystopsis sp.*	-
SAG 24.81	*Tetrastrum heteracanthum*	JQ356709
SAG 45.81	*Tetrastrum staurogeniiforme*	JQ356703
KR 1996/3	*Tetrastrum staurogeniiforme*	JQ356702
SAG 42.81	*Tetrachlorella alternans*	AF228687
SAG 2081	*Willea rectangularis*	AH012990
CCALA 515	*Willea vilhelmii*	**KY006555**
	GENBANK	
J.C.Han_32	*Amphikrikos*_sp.	KP013378
J.C.Han_43	*Amphikrikos*_sp.	KP013379

NIES 3911	*Chlorella*_sp.		LC129521
NIES 3912	*Chlorella*_sp.		LC129522
-	*Ecballocystis hubeiensis*		JX018185
-	*Ecballocystopsis dichotomus*		JX018184
CCAC 0071	*Eremosphaera viridis*		HE610127
UTEX LB 34	*Eremosphaera viridis*		AF387154
NIES_382	*Lagerheimia_ciliata*		LC192142
KMMCC 1544	*Lagerheimia longiseta*		JQ315525
CCAP 222/49	*Oocystidium* sp.		HQ008711
LN1	*Oocystis borgeii*		KU720481
KRI. 96/10	*Oocystis marssonii*		AF228688
KMMCC 443	*Oocystis parva*		JQ315649
KMMCC 356	*Oocystis* sp.		JQ315800
FACHB_1429	*Oocystis_sp.*		KF928745
FACHB_1427	*Oocystis_sp.*		KJ522683
GR35	*Planctonema lauterbornii*		-
M110-1	*Planctonema* sp.		AF387148
CCAP 286/1	*Quadricoccus ellipticus*		HQ008712
NKS72	Uncultured_Chlorophyta_clone		JX296619
KRL03E76	Uncultured_eukaryote_clone		KC315825
KRL03E42	Uncultured_eukaryote_clone		KC315819
KRL01E35	Uncultured_eukaryote_clone		HQ008711
NKS72	Uncultured_Chlorophyta_clone		JX296619
	OUTGROUP (GENBANK)		
-	*Auxenochlorella protothecoides*		FN29893
-	*Chlorella variabilis*		AB206549
-	*Chlorella vulgaris*		FR865658
-	*Micractinium pusillum*		AF364101
	NOT USED SEQUENCES		REASON
SAG 9.86	*Chlorella stigmatophora*	KM020186	Part of another paper
LN1	*Oocystis* sp.	KJ713151	Same as KU720481

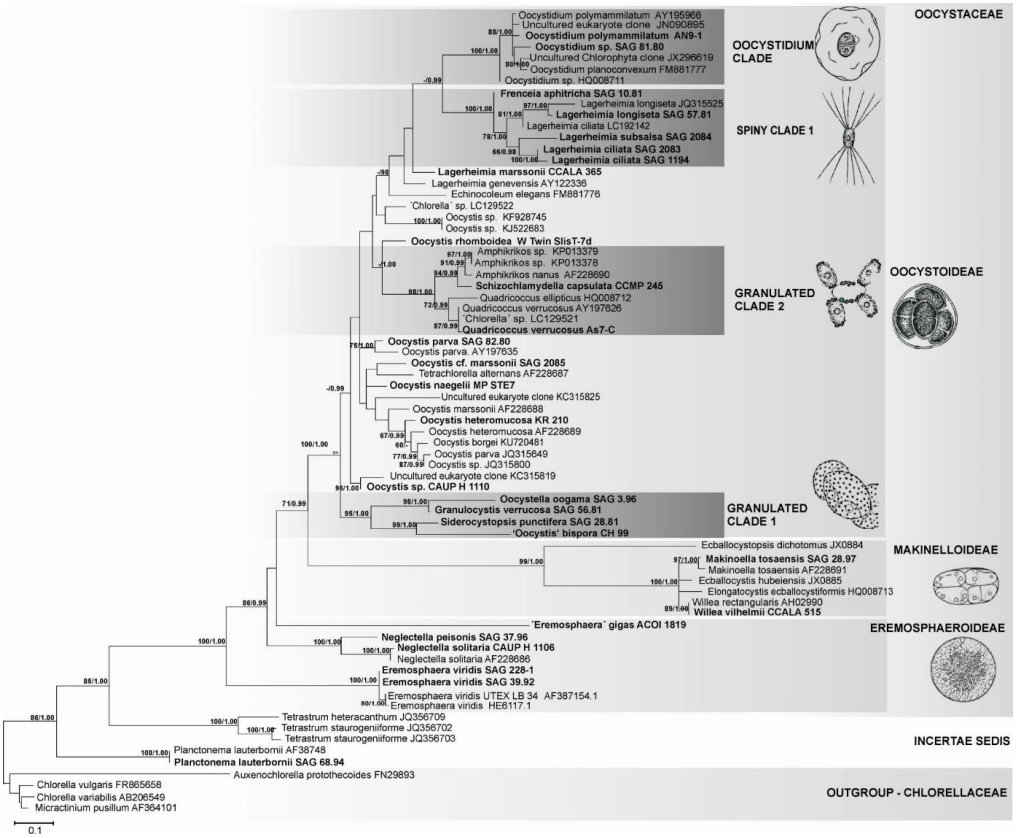


Figure S1: Phylogenetic analyses of SSU rRNA gene sequences of members of Oocystaceae with Chlorellaceae as an outgroup. Topology represents the best ML tree. Taxa with a new sequence are highlighted by bold font. Numbers at the branches indicate bootstrap support from maximum likelihood (ML, 1000 replicates) and Bayesian posterior probabilities (BI). Support ≥50% for ML and ≥0.95 for MB is shown. ML/BI Drawings according to Fott and Kalina (1962), Hindák (1977), Hortobágy (1973), Skuja (1956) and Skuja (1964) demonstrate typical morphology of the subfamilies and of the individual clades.

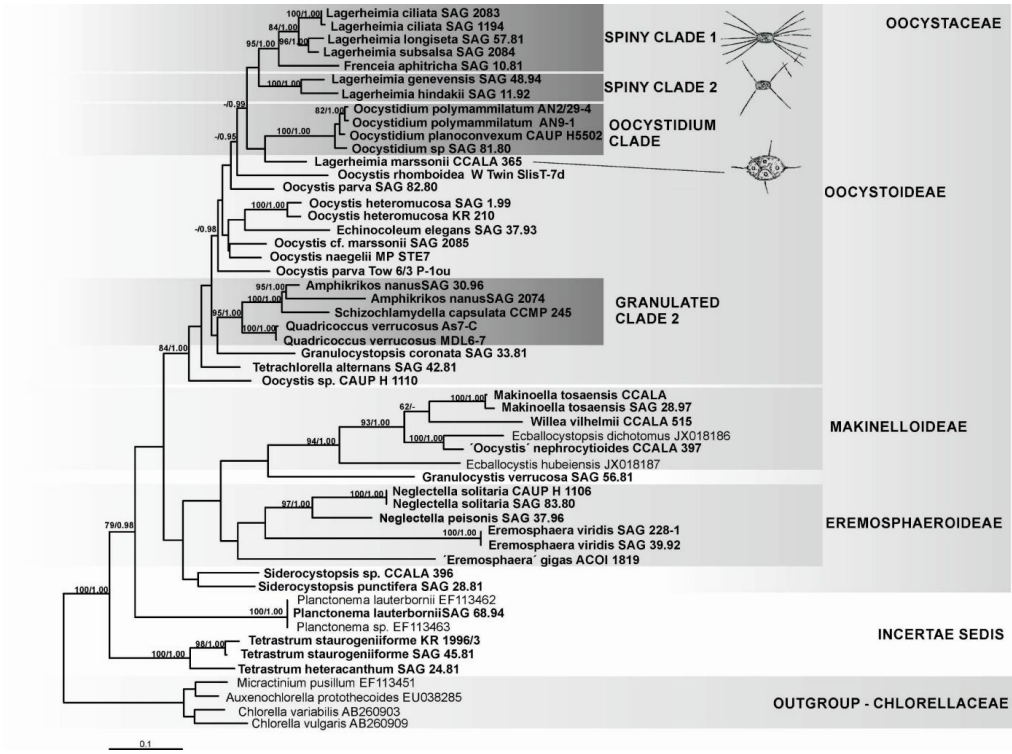
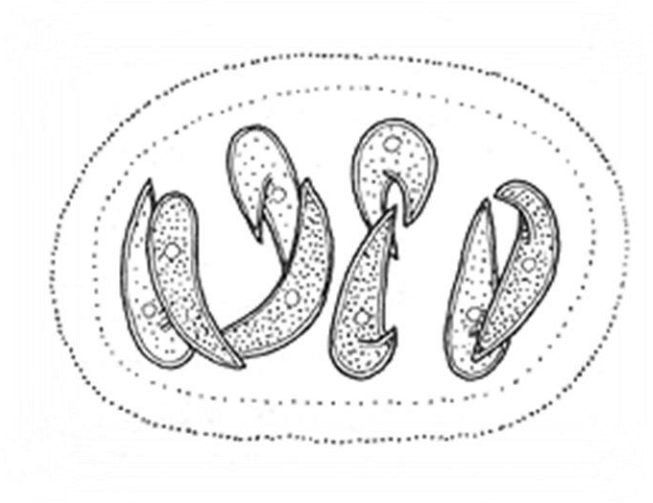


Figure S2: Phylogenetic analyses of *rbcL* gene sequences of members of Oocystaceae with Chlorellaceae as an outgroup. Topology represents the best ML tree. Taxa with a new sequence are highlighted by bold font. Numbers at the branches indicate bootstrap support from maximum likelihood (ML, 1000 replicates) and Bayesian posterior probabilities (BI). Support ≥50% for ML and ≥0.95 for MB is shown. ML/BI. Drawings according to Hortobágyi (1962) and Skuja (1956) demonstrate typical morphology of the individual *Lagerheimia* lineages.

Chapter 3 Revised phylogenetic position of genus *Nephrocytium* Nägeli (Sphaeropleales, Chlorophyceae), with description of Nephrocytiaceae *fam. nov*. and *Nephrocytium vieirae sp. nov.*

Silva, T. G., **Štenclová, L.,** Archanjo, N.C.P. and Bagatini, C. L. Revised phylogenetic position of genus *Nephrocytium* Nägeli (Sphaeropleales, Chlorophyceae), with description of Nephrocytiaceae *fam. nov*. and *Nephrocytium vieirae sp. nov.* Submitted 27. 12. 2019 in Taxon.



Revised phylogenetic position of genus *Nephrocytium* Nägeli (Sphaeropleales, Chlorophyceae), with description of Nephrocytiaceae *fam. nov.* and *Nephrocytium vieirae sp. nov.*

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ABSTRACT

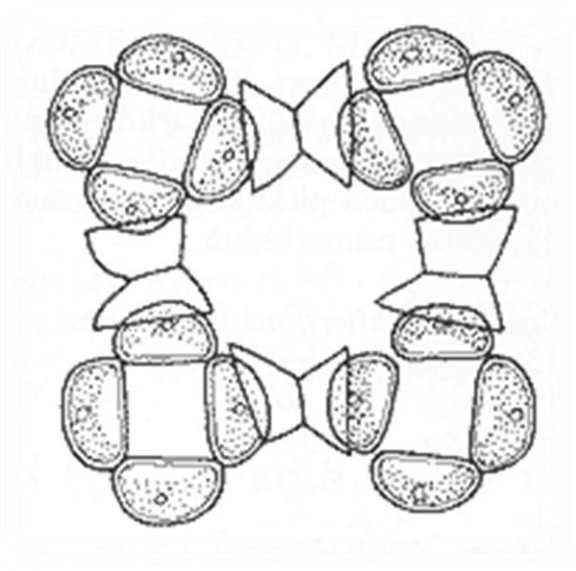
Commonly known planktonic green algal genus *Nephrocytium* was traditionally assumed to have a close relationship with the genus *Oocystis* and consequently has been included in the family Oocystaceae. Although *Nephrocytium* position inside the Oocystaceae differed according to some authors over the years, its inclusion in the family has not been questioned. With molecular studies of *Oocystis*, the position of the family Oocystaceae changed from the class Chlorophyceae to Trebouxiophyceae, and the genus *Nephrocytium* followed this classification. However, recent molecular studies of some of the former Oocystaceae members have assigned them back to Chlorophyceae. These studies suggested placement of *Nephrocytium* in Sphaeropleales, but no taxonomic positioning within the order has been determined for the genus. The relocation of *Nephrocytium* agrees with a strong morphological trait - it lacks the particular oocystaceaen multilayered ultrastructure of the cell wall. Based on molecular markers (18S rDNA and *tuf*A), optical and electron micrographs, the present study aimed to position the genus within Sphaeropleales. The results have assigned the genus *Nephrocytium* to a new family of Sphaeropleales, the Nephrocytiaceae. Furthermore, we have carried out a review of previously described *Nephrocytium* species, and based on the morphological and molecular data available so far, we proposed the description of *Nephrocytium vieirae spec. nova*.

KEYWORDS: 18S rRNA; coccoid green algae; Oocystaceae; *tuf*A; taxonomic revision.

(47 pages with unpublished manuscript are following in complete thesis)

Chapter 4 Distribution of Crucigenioid algae in classes Chlorophyceae & Trebouxiophyceae

Štenclová, L. Distribution of Crucigenioid algae in classes Chlorophyceae and Trebouxiophyceae. Submitted 2. 11. 2020 in Journal of Phycology.



DISTRIBUTION OF CRUCIGENIOID ALGAE IN CLASSES CHLOROPHYCEAE & TREBOUXIOPHYCEAE[1]

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[1] Running title: DISTRIBUTION OF CRUCIGENIOID ALGAE

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ABSTRACT

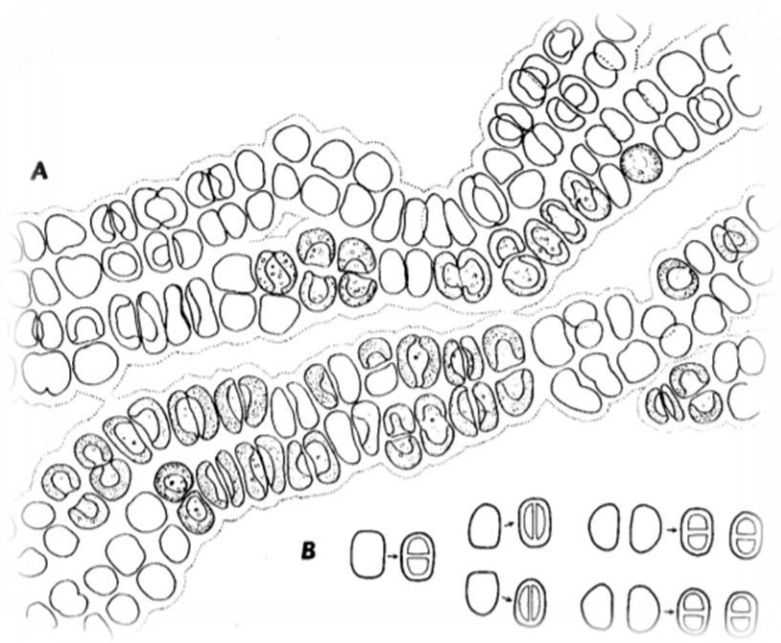
Crucigenioid algae had been traditionally assembled in the chlorophycean family Scenedesmaceae, subfamily Crucigenioideae. The taxa shared typical cross-shaped four-celled coenobia, which was considered a relevant morphological characteristic, strongly indicative of their relatedness. Nevertheless, the list of the genera belonging to the subfamily differed according to various authors. Moreover, taking in account different morphological traits for generic circumscriptions (e.g. position of autospores in daughter coenobia, surface and shape of the cells) led to the numerous corrections among the genera and subsequently to widespread synonymity. Sparse molecular studies proved that neither the subfamily's definition nor the delimitation of the genera reflect the phylogeny. Previous studies showed multiple polyphyletic status of members of Scenedesmaceae Crucigenioideae, brought up changes in the taxonomy of *Tetrastrum*, *Crucigenia* and *Crucigeniella* and resulted in the recovery of the genus *Lemmermania*. The extended genus *Willea* was intended to solve illegitimate designations of *Crucigeniella* species. In the present study, we propose the reintroduction of the name *Komarekia* for ex-crucigenioid taxa placed in Chlorellaceae (Trebouxiophyceae). We define a core *Crucigenia*-group in Scenedesmaceae as a novel delimitation of Crucigenioideae with an adjusted definition. Relevant changes are presented here and additional ones are expected in the taxonomy of genera *Crucigenia*, *Crucigeniella, Lemmermania*, and *Willea* in future studies. Additionally, we discuss the role of morphology in modern systematics of green coccal algae.

KEYWORDS: 18S rRNA, Chlorellaceae, Crucigenioideae, *Komarekia*, Oocystaceae, *rbcL*, Scenedesmaceae

(45 pages with unpublished manuscript are following in complete thesis)

Chapter 5 *Dispora speciosa*, a new addition to the genus *Parallela* and the first coccoid member of the family Microsporaceae

Štenclová, L. and Fučíková, K. (2019) *Dispora speciosa*, a new addition to the genus *Parallela* and the first coccoid member of the family Microsporaceae. Phytotaxa. 419(1):63-76. DOI:10.11646/phytotaxa.419.1.4



***Dispora speciosa*, a new addition to the genus *Parallela* and the first coccoid member of the family Microsporaceae.**

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Abstract

The clade that currently represents the green algal family Microsporaceae is one of the few filament-forming groups of Chlorophyceae. Molecular phylogenies show this clade containing the genus *Microspora* and the more recently circumscribed *Parallela*, whose filaments are loosely arranged and often multiseriate. We initially investigated the enigmatic bog-loving *Dispora speciosa* as a commonly accepted member of the mucilage-forming Radiococcaceae or a putative member of crucigenoid chlorophytes (a non-monophyletic group formerly placed in Scenedesmaceae) based on its two-dimensional colony formation. However, our plastid and nuclear ribosomal phylogenies confidently placed *Dispora* within the genus *Parallela* instead, and therefore distantly related to both Radiococcaceae and crucigenoids. Upon further examination of the cell morphology and ultrastructure, we found several corresponding features between *Dispora* and *Parallela*, despite *Dispora*'s apparent coccoid-colonial gross morphology. Both genera have cells with a parietal plastid positioned around a large central nucleus. The loose, multiseriate filament formation in *Parallela* can be interpreted as similar to *Dispora*'s flat colony formation in its natural state. Because we only present data from one non-type species and strain of *Dispora*, we cannot merge the entire genus with *Parallela*. We do however argue that *D. speciosa*, of which this strain is the sole available, morphologically and ecologically faithful representative, should be transferred into *Parallela*, and the specimen prepared from strain ACOI 1508 be designated as type. Our study also impacts the current view on evolution of multicellular (colonial and filamentous) forms in Chlorophyceae.

Key words: 18S rRNA, *atpB*, epitype, phylogeny, *rbcL*, *psaB*, TEM

Introduction

The ancient common ancestor of all green plants and algae likely was a single-celled flagellate (Leliaert *et al.* 2012 and references within). Within the different green algal classes, complex morphologies are thought to have evolved independently multiple times including colonial, coenobial, filamentous, thalloid and other forms. Coccal (single-celled, vegetatively non-motile) forms are common for example in the classes Chlorophyceae and Trebouxiophyceae (e.g., Fučíková *et al.* 2014a,b), and in some cases may represent repeated evolutionary reductions from more complex ancestors. In other cases coccoid forms may be ancestral. An accurate understanding of the diversity within these algal groups and a robust assessment of their phylogenetic relationships are critical to answering fundamental evolutionary questions about the evolution of complex body forms.

The green algal phyla Chlorophyta and Streptophyta contain numerous ancient lineages, the biodiversity of which is likely drastically underestimated. Recent studies have demonstrated time and again that the morphological diversity of microscopic green algae does not reflect their phylogenetic diversity. Similar, putatively convergent morphologies are common across distantly related groups (e.g., Fučíková *et al.* 2014a,b). Cases of morphological crypsis uncovered by molecular data are especially common in coccoid microalgae, but have been documented even in more complex taxa, such as the filamentous *Klebsormidium* P.C. Silva, K.R. Mattox & W.H. Blackwell (1972: 643) (Škaloud & Rindi 2013). In some cases, morphological, ultrastructural, ecological, or other species-delimiting features are discovered post-hoc, in light of a molecular phylogeny (e.g., Škaloud & Rindi 2013).

The fairly rare, peat pond inhabiting green coccal alga *Dispora speciosa* Korshikov (1953: 324) is characterized by its flat, four-celled coenobial organisation and a wide mucilage cover (Korshikov 1953). Cell organisation and presence of mucilage covers had been considered crucial morphological characters for categorization of green algae for the last two centuries (Lemmermann 1915, Smith 1950, Korshikov 1953, Fott 1959, Komárek & Fott 1983, Ettl & Gärtner 1988, Kostikov et al. 2002). The genus

Dispora Printz (1914: 32) was originally described in the family Pleurococcaceae (Printz 1914), and subsequently went through several different taxonomic placements (e.g. Bourrelly 1966, Fott 1974). Nevertheless, the latest complex morphological studies (Komárek 1979, Komárek & Fott 1983, Ettl & Gärtner 1988, Kostikov et al. 2002) all placed *Dispora* in the family Radiococcaceae, highlighting especially the presence of mucilage covers. The few available insights to the phylogeny of Radicococcaceae all uncovered that the family is considerably polyphyletic (Wolf *et al* 2003, Pažoutová 2008, Pažoutová *et al.* 2010, Fučíková 2014a, Zhang *et al.* 2018). Former Radicococcaceae members appeared scattered in the class Trebouxiophyceae (Hanagata & Chihara 1999, Wolf *et al.* 2003, Pažoutová 2008, Pažoutová *et al.* 2010) and in the class Chlorophyceae (Wolf *et al.* 2003, Pažoutová 2008, Fučíková 2014a, Zhang *et al.* 2018) in various lineages, which proves that extracellular mucilage is a rather common and circumstantial trait and thus offers limited taxonomic information.

Radiococcaceae taxa (including *Dispora*) with cells organized in flat tabelar coenobia have been grouped in the subfamily Disporoideae (Komárek & Fott 1983). The phylogenetic placement of some radiococcacean genera is now known, but not for any of the Disporoideae as yet. The flat four-celled coenobia of *Dispora speciosa* remarkably resemble the coenobia of algae assigned to the scenedesmacean subfamily Crucigenoideae *sensu* Komárek (1974) and Komárek & Fott (1983). A typical trait defining crucigenoid algae is the propagation by autospores, which have not been reported in *Dispora* spp. Further, much like the Radiococcaceae, crucigenoid algae also are demonstrably polyphyletic, and their members are distributed throughout the green algal phylogeny and inside both the classes Trebouxiophyceae (Hepperle *et al.* 2000, Bock *et al.* 2013, Štenclová *et al.* 2017) and Chlorophyceae (Hegewald *et al.* 2010, Bock *et al.* 2013). The relationship of the genus *Dispora* to radiococcacean and crucigenoid lineages is suspected but remains unexplored.

In recent years, there have been efforts to reconcile the traditional, morphology-based taxonomy with molecular approaches to describe biodiversity. By combining the two approaches, researchers strive to classify traditional and newly discovered taxa in a way that reflects their evolutionary history and relatedness. One of the challenges is

typification—the standards of type designation have changed over time, and many species described in the 19th and early 20th century are not accompanied with detailed (or any) illustrations, precise morphological descriptions, preserved specimens, and almost never with a living culture available for further examination and experimentation. Occasionally, modern phycologists have attempted to revisit type localities and establish new types for old species and genus names that would otherwise be taxonomically questionable or ambiguous (e.g., Fučíková *et al.* 2013). In some cases, an existing isolate is selected to serve as new type, ideally one collected near the type locality (e.g., Allewaert *et al.* 2015)—often this is the most practical solution, especially when the locality information is insufficient in the original species description, and it is thus impossible to find and revisit it. . The description of the type locality of *Dispora speciosa* (North part of the European part of the former USSR (Korshikov 1953)) is very broad and thus collecting material from the original site is not possible.

Given these limitations, we examined the only publicly available strain of *Dispora speciosa* (ACOI-1508) in order to determine the higher classification of this taxon. This strain originated from a locality distant to the original (Abrantes, Capo Militar de Sta Margarida, lake North of Lagoa da Murta in Portugal), but morphologically corresponded well with Korshikov´s description. The gross morphology of the species is rather unusual in Chlorophyta, and therefore an array of methods was used to pinpoint the species' taxonomic placement. Our assessment included morphological, ultrastructural, and molecular data analyses, exemplifying a modern polyphasic approach to taxonomy.

Materials & Methods

Strain information & culture conditions

The green algal strain ACOI 1508 *Dispora speciosa* was acquired from the public culture collection Coimbra Collection of Algae (ACOI), Portugal. The strain was cultivated on both solid and liquid medium LM-7 (prepared following the instructions of ACOI) and kept under the standard conditions: irradiance 22 μmol · m−2 · s−1 and constant temperature 16°C.

Light microscopy (LM)

Basic morphology was observed using an Olympus BX light microscope equipped with an Olympus DP71 camera and DP software (Olympus, Center Valley, PA, USA) under 1000x magnification using immersion oil. Methylene blue staining was used to detect the gelatinous covers around the cells.

Autofluorescence

Observations of chlorophyll autofluorescence were carried out on an Olympus BH-2 photomicroscope equipped with a mercury lamp at a 1000x magnification and micrographs were captured using an AmScope MU1000 digital camera (AmScope, Irvine, CA, USA).

Transmission electron microscopy (TEM)

For ultrastructural observation, ultrathin sections of the cell culture were prepared. Samples were processed by staff at the Electron Microscopy Laboratory, Institute of Parasitology, Academy of Sciences, Czech Republic. Samples were treated with 0.05 M phosphate buffer, postfixed with 2% osmium tetroxide in 0.05 M phosphate buffer at room temperature for 2 h and then repeatedly washed with 0.05 M phosphate buffer. Washed cells were dehydrated serially in isopropanol concentration gradient, dissolved in propylene oxide and finally embedded in Spurr's resin (Spurr 1969). Thin sections were prepared and stained with uranyl acetate and lead citrate. Specimens were observed using a Jeol JEN 1010 transmission electron microscope (JEOL, Peabody, MA, USA) at an accelerating voltage of 80 kV.

Picture plates documenting microscopic methods were constructed using CorelDraw 2018 (Corel Corporation, Ottawa, Canada).

Molecular data & analyses

Biomass was manually ground with sterile sand and DNA was subsequently isolated using the DNeasy PowerPlant Pro kit (Qiagen Inc., Germantown, MD, USA). The chloroplast genes *atpB*, *psaB* and *rbcL* were selected because of their availability for a wide sampling of Chlorophyceae, including various *incertae sedis* taxa (Fučíková *et al.* in press). The 18S nuclear ribosomal gene was also selected because of its common usage for phylogenetic systematics in green algae. Polymerase chain reaction (PCR) was run as described in McManus & Lewis (2011) for *rbcL*, according to Novis *et al.* (2010) for *atpB* and *psaB*, and according to Shoup & Lewis (2003) for 18S. Initially, after obtaining partial *rbcL* data, we used BLAST (Altschul 1990) to determine the approximate phylogenetic placement of *Dispora*. Based on this information, we refined the *atpB* and *psaB* primers of Novis *et al.* (2010) to be more taxon-specific and less degenerate, and also designed a new taxon-specific *atpB* primer based on alignments of *Parallela* E.A. Flint (1974: 358) and *Microspora* Thuret (1850: 222) sequences. Based on alignments we also selected 18S primers to fit the Microsporaceae clade and simultaneously circumvent amoebal contamination in the *Dispora* culture, which was otherwise preferentially amplified with most standard algal 18S primers. A nested PCR was necessary to obtain at least partial 18S data, initially using the primer pair SSU1 (Shoup & Lewis 2003) and 1650R and re-amplifying from the resulting product using the pair 1170F and 1650R—the only successful 18S amplification. Cycle sequencing and Sanger sequence analysis was done at Macrogen USA (Boston, MA, USA). Primers successfully used for PCR and sequencing are listed in TABLE 1. Genbank accession numbers of sequences used in all analyses are provided in TABLE 2; alignments and analysis specifications are available in Supplements.

Plastid gene sequences of *Dispora speciosa* were manually added to existing alignments (Fučíková *et al.* in press) and ambiguously aligned codons were manually removed prior to analyses. The Supplements contain the full untrimmed alignments, with asterisks designating nucleotide positions to be removed, as well as ready-to-analyze trimmed alignments and the resulting trees for full transparency and reproducibility. 18S sequences were aligned using ClustalW (Larkin *et al.* 2007) in MEGA v.4 (Tamura *et al.*

2007). Fast-evolving, unalignable 18S positions were eliminated using GBlocks (http://molevol.cmima.csic.es/castresana/Gblocks_server.html) with default settings.

The four gene alignments were concatenated and analyzed using MrBayes v3.2 (Ronquist *et al*. 2012), implementing the nucleotide GTR+I+Γ model and partitioning by codon position, with 18S as a separate partition. Two MCMC chains were run for 5,000,000 iterations, sampling every 500, and discarding the first 20% of the trees as burn-in. Analogously, a Maximum Likelihood (ML) analysis was carried out using RAxML (Stamatakis 2014) with 100 rapid bootstrap pseudoreplicates. An analysis of each single-gene alignment was also carried out as described above. The single-gene analyses are available in the Supplements, including the consensus trees and their underlying alignments.

Results

Morphology

Multiple microscopical methods were combined to fully assess the morphology and ultrastructure of *Dispora speciosa*. The strain's cells are arranged in multiples of 2 or, more commonly, of 4 in flat, *Crucigeni*a-like (Morren 1830: 426) coenobia. Tetrads are arranged rather irregularly in the algal culture. Staining by methylene blue shows wide gelatinous covers around the cell agglomerations (FIGURE 1: C). Cells are spherical or oval to elliptical, usually slightly asymmetric or flattened where adjacent to another cell. Cell wall is considerably robust. Individual cells or tetrads enclosed in wide mucilage cover. Inside the cell, one to two large cup-shaped parietal chloroplasts are visible. Chloroplasts along with the large nucleus fill most of the cell (FIGURE 1). The chloroplast shape appears indistinct under light microscope but is confirmed using both fluorescent and transmission electron microscopy as parietal and bowl- or cup-shaped (FIGURE 1). TEM also shows that individual chloroplasts contain numerous starch grains but no pyrenoid. Numerous granules or inclusions are present in the cell, likely outside the chloroplast (FIGURE 1). No process of propagation was observed in the present study.

Cell dimensions (6-7µm x 9-11 µm) also fit in the dimension range reported in the original description of the species (Korshikov 1953).

Molecular analyses

Concatenated analyses as well as analyses of individual plastid genes (the latter only shown in Supplements) all strongly supported *Dispora* inside the clade containing the genera *Parallela* and *Microspora* (Microsporaceae from here on after). *Dispora* was nested inside *Parallela* (FIGURE 2), with *Microspora* being sister to *Parallela* + *Dispora*. Only *atpB* supported *Dispora* as sister to *P. novae-zelandiae* E.A. Flint (1974: 359) (0.99 BPP, not shown). The remaining data sets containing both *Parallela* species supported *Dispora* as sister to *P. transversalis* (Brébisson) Novis, M. Lorenz, Broady & E.A. Flint (2010: 382) (0.93 BPP in *rbcL*, 0.78 BPP in *psaB*; trees available in Supplements). Concatenation of all four genes yielded low BPP for the *P. novae-zelandiae* + *D. speciosa* relationship (FIGURE 2) and low ML BS support of 45 for the *P. transversalis* + *D. speciosa* relationship, which nevertheless appeared in the best ML tree (Supplements). The 18S data set only contained *P. transversalis* (data for *P. novae-zelandiae* are not available), and therefore did not contribute to the resolution of the placement of *D. speciosa*. The uncertainty in placement can likely be attributed to the apparent signal conflict between *atpB* and the remaining two plastid genes.

Taxonomic changes

Though the exact position among other *Parallela* species received poor support, the placement into the genus is obvious. Therefore, the following taxonomic change is proposed, including the establishment of an epitype according to article 9.9 of the Shenzhen Code (Turland *et al.* 2018). We argue that Korshikov's (1953) illustration is detailed enough to confidently match to our live and preserved material, but due to the cryptic, simple-bodied nature of most microalgae, and the rampant polyphyly of many morphotypes, any figure is ultimately ambiguous (the main criterion for establishing epitypes) and attaching names to physical material and live cultures is therefore of great importance.

***Parallela speciosa comb. nov.* (Korshikov) Štenclová & Fučíková**

Basionym and heterotypic synonym: *Dispora speciosa* Korshikov 1953: 334, Fig. 308 a, b. Epitype: Formaldehyde-fixed specimen kept at University of South Bohemia in České Budějovice, Czech Republic, found under the serial number CBFS A-107-1.

Discussion

Dispora in historical context

One clear conclusion from our analyses is that the strain ACOI 1508, from here on referred to as *Parallela speciosa* (unless historical context dictates otherwise), is phylogenetically distant from all previously analyzed lineages of the former, morphologically-defined Radiococcaceae. This is not surprising, considering the previously demonstrated polyphyly of Radiococcaceae (Pažoutová 2008, Pažoutová *et al.* 2010, Fučíková 2014a, Zhang *et al.* 2018). In light of the phylogeny, *Dispora's* mucilage could possibly be referred to as ´gelatinous matrix´ as it is called in *Parallela* in one case (Novis *et al.* 2010) rather than mucilage envelopes/covers of Radiococcaceae, to reinforce the taxonomic distinction. However, it is not currently known whether the two types of extracellular secretions are fundamentally different from each other, either chemically or developmentally.

Despite the similarity in coenobial shape and structure, our analyses also show ACOI 1508 as distant from all available lineages of the former Crucigenoideae, now known to be polyphyletic (Hepperle *et al.* 2000, Hegewald *et al.* 2010, Bock *et al.* 2013, Štenclová *et al.* 2017). Our own analyses only show *Crucigenia pulchra* West & G.S. West (1902: 63) (Scenedesmaceae, Sphaeropleales) (FIGURE 2), because it is the most likely candidate to represent the true *Crucigenia* lineage (*Crucigenia* itself being polyphyletic according to Bock *et al.* 2013), but also because the other crucigenoids are outside Chlorophyceae.

In terms of gross morphology, in ACOI 1508 we find noticeable similarity in coenobium shape and arrangement of the cells especially with the genus *Willea* Schmidle

(1900: 157). The cup-shaped chloroplast also occurs in both taxa. Fott (1933) noticed this resemblance and proposed *Willea vilhelmii* (Fott) Komárek (1974: 42) to be placed in the genus *Dispora*, but Komárek (1974) and Komárek & Fott (1983) rejected this idea and recognized both genera as distinct again. Our microscopical assessment confirmed the differences between *Willea* and *P. speciosa*—their individual cells are shaped differently (elongated in *Willea*) and their internal structures differ. Molecular phylogenies support the distinction unambiguously.

Willea belongs in the trebouxiophyte family Oocystaceae (Štenclová *et al.* 2017), and is therefore unrelated to *Parallella*. Consistently with this placement, the pyrenoid with a prominent starch sheath is often clearly visible in *Willea*, whereas in *Parallela* species it is not detectable. Even though presence or absence of pyrenoid likely supports our phylogenetic data, it should be noted that pyrenoids are a taxonomically problematic trait. Their visibility depends on the microscopic technique to some extent, may depend on sample preparation (e.g., staining), and the starch sheath around the pyrenoid may increase or decrease in robustness during a cell's life depending on conditions (e.g., Ramazanov *et al.* 1994).

In the original description of *Dispora* (*D. crucigenioides*, *D. cuneiformis* (Schmidle) Printz 1914: 33), Printz (1914) noted the absence of pyrenoid ("chromatophoro unico campanulato pyrenoide carente"—single bell-shaped chromatophore lacking a pyrenoid). Later, Korshikov (1953) noted in his circumscription of *D. speciosa*, "без піреноіда"—without a pyrenoid. Komárek & Fott (1983) interestingly mention "Pyrenoid fehlt (oder auch vorkommend?)."—pyrenoid lacking (or also occurring?). This note refers to the South American species *D. globosa* C.E.M. Bicudo & R.M.T. Bicudo (1970: 8), which however bears several features that sharply separate it from other *Dispora* species—spherical, rather than planar, colonies and the presence of pyrenoid, which could place it in the problematic Radiococcaceae according to Komárek & Fott (1983), further emphasizing the complicated nature of the taxonomy in these families and genera. The placement of *D. globosa* has not been resolved, but the taxon likely is not to be placed with the other species of the genus.

Dispora in modern phylogenetic context:

Our phylogenetic analyses confidently placed *Parallela speciosa* in Chlorophyceae, and in the phylogenetic proximity of the order Sphaeropleales. Nevertheless, its family-level classification remains somewhat uncertain due to taxonomic problems outside the scope of our study. Although the ACOI strain belongs to the genus *Parallela* in the family Microsporaceae, as pointed out in previous studies, Microsporaceae itself is a questionable taxon, as no type strain of *Microspora* exists (e.g., Fučíková *et al.* 2019). *Microspora* sp. strain UTEX LB472 has been used in various studies to exemplify the cellular structure of the genus (Pickett-Heaps 1973) and to represent the genus in molecular phylogenies (Buchheim & Buchheim 2001, Watanabe *et al.* 2016), even though it is not an authentic culture and does not even have a species-level identification in culture collections.

We did not observe motile cells in *P. speciosa*. However, the placement of Microsporaceae in the phylogenetic vicinity of Sphaeropleales is corroborated by the slightly uneven flagella and parallel flagellar basal body orientation in *Parallela* and *Microspora* respectively, and is also consistent with the sister placement to *Dictyochloris* (Novis *et al.* 2010, Lokhorst & Star 1999, Shoup & Lewis 2003).

Within the genus *Parallela*, the position of *P. speciosa* depends on which gene is used for phylogenetic inference. *AtpB* lends strong support to the sister relationship of *P. speciosa* and *P. novae zelandiae*, which also makes the most morphological sense: the multiseriate filaments of *P. novae zelandiae* shown in Novis *et al.* (2010) and Flint (1974) can be interpreted as similar to the planar colonies that *P. speciosa* forms in nature. The planar thalli were, however, not as obviously formed in our cultured sample, similar to Flint's (1974) observation that under culture conditions *P. novae-zelandiae* produces cell clusters but not the ribbon-like forms. Further, Flint (1974) describes "numerous, unidentified, oscillating granules" in *P. novae-zelandiae*, which are consistent with our observations in live cells of *P. speciosa*. Other cellular features, such as the large, centrally positioned nucleus, a single cup-shaped chloroplast, and the absence of pyrenoid (demonstrated via Lugol staining in *P. transversalis* by Novis *et al.* 2010) are also

consistent with our assessment of *P. speciosa*. Interestingly, Flint (1974) brings up the superficial similarity of *P. novae-zelandiae* to *Disporopsis* Korshikov (1953: 202), noting the important differences such as the presence/absence of pyrenoid. For some reason *Dispora* is not mentioned, even though it appears in the same publication by Korshikov (1953) and, at least in our opinion, bears greater morphological resemblance to *Parallela*. *Disporopsis* has since been reclassified as *Planochloris* Komárek (1979: 240) but molecular verification has not yet been attempted.

We examined the only available strain of the genus *Dispora* and without examination of additional live cultures and molecular data, we cannot confidently say whether any of the other *Dispora* species belong to the genus *Parallela*, or to the family Microsporaceae. However, based on morphological features such as cell shape and arrangement, the mucilage cover and the chloroplast characteristics of *D. crucigenioides* (Printz 1914) (which is the type species of *Dispora*) it is rather probable that the entire genus should be merged with *Parallela*. Komárek & Fott (1983) also noted that *D. crucigenioides* and *D. speciosa* may in fact be the same species, as the morphological differences between them are slight.

Several other strains of *Dispora speciosa* as well as *Dispora crucigenioides* are or were kept in the ACOI strain collection, but cannot be provided for future research (per ACOI website and correspondence). *Dispora globosa* appears anomalous within the genus, possessing colonies that are globular rather than flat and tabular, and also has distinct pyrenoids in chloroplasts. For this reason, Komárek & Fott (1983) suggested that this species may be better referred to as *Coenocystis* than *Dispora*. Moreover, the poorly known *Dispora cuneiformis* remains a questionable taxon in clear need of revision because of its incomplete original description (Komárek & Fott 1983). Another taxonomic problem would arise if *Dispora* and *Parallela* were merged, or even if just *D. crucigenioides* were shown as closely related to *P. speciosa*, because *Dispora* is the older name and thus takes priority. However, this cannot happen until a new generitype is established and sequenced. Until then, we believe that our re-classification of *P. speciosa* is an improvement on the current taxonomic situation in *Dispora*, and better reflects evolutionary relationships in

Chlorophyceae. Sinking the genus *Parallela* into the ill-defined *Dispora* would not be wise with the limited data that our study presents.

Insights into morphological evolution in Chlorophyceae:

We show that *Parallela speciosa* is a member of an otherwise filamentous clade representing the family Microsporaceae (FIGURE 2). However, filament formation in this group is not easy to interpret in evolutionary terms, though a careful look at the cellular structure and development helps find common features. The peculiar two-part cell wall structure of *Microspora* is initiated during cytokinesis (Ramanathan 1964) and superficially appears quite different from *Parallela*'s bipartite walls (Novis *et al.* 2010), but both are consistent with the Sphaeropleales-specific criterion established by Mattox & Stewart (1984) stating that new walls are deposited within the old filament wall during growth. In Microsporaceae *sensu* Mattox & Stewart (1984) the newly formed walls do not surround the entire surface of daughter cells, distinguishing the family from Sphaeropleaceae.

Fascinatingly, Skuja's (1956) illustration of *D. crucigenioides* includes a filament-like morphotype with clear bipartite character of the cell wall, strikingly reminiscent of *Parallela transversalis* in images by Novis *et al.* (2010). However, such bipartite cell wall is evident neither in *P. novae zelandiae* (Novis *et al.* 2010) nor in *P. speciosa*, indicating that this particular character may have been lost in some *Parallela* lineages. However, even in *P. speciosa* it is clear (e.g., in FIGURE 1) that the daughter cell wall is deposited within the mother wall. Similarly, the filamentous habit appears to have been 'loosened' in *Parallela* compared to its sister genus *Microspora*, and nearly completely disassembled into a coccoid-like colonial form in *P. speciosa*.

Such a reduction towards a coccoid or colonial form from a more complex, filamentous or coenobial ancestor, has been inferred in other green algal groups before. For example, in Chlorellaceae (Bock *et al.* 2010) or within the genus *Scenedesmus* (e.g., phylogeny of Lewis & Flechtner 2004) multiple shifts between unicellular and coenobial

forms may have occurred, although comprehensive analyses of trait evolution would be necessary to conclusively determine the directionality of these shifts.

The present study is an example of a small handful of known filament-to-coccoid transitions. On the other hand, the evolution of a complex form within a clade of otherwise simple-bodied, single-celled algae has been documented as well: for example in the recent study by Kaštovský *et al.* (2016), in which the branched filamentous genus *Ekerewekia* Kaštovský, Fučíková, Štenclová & Brewer-Carías (2016: 171) was clearly demonstrated to have arisen within an otherwise coccoid clade. Interestingly, in the broader context of the *Prasiola* (C.Agardh) Meneghini (1838: 360) clade (the group containing *Ekerewekia* and its closest relatives), another example can be found: *Prasiola* and *Rosenvingiella* P.C. Silva (1957: 41) form multiseriate filamentous to thalloid forms, and analogously to *Ekerewekia* are found within a clade comprising numerous coccoid lineages. Another example, recently documented by Štenclová *et al.* (2017), is the sister relationship between the coccoid *Oonephris* Fott (1964: 134) and the filamentous *Cylindrocapsa* Reinsch (1867: 66). In this case, however, it is unclear whether it represents a reduction or independent evolution of complexity, as the phylogenetic relationships in the morphologically diverse clade are problematic and taxon sampling sparse. While our understanding of morphological evolution in green algae is still incomplete, it is clear that switches between simple and complex body forms have been numerous across the green algal evolutionary history.

Conclusion

The genus *Dispora* exemplifies how tangled taxonomic histories can be, and how placing morphologically defined species and genera in a phylogenetic framework can be both enlightening and complicated. The delimitation and higher classification of *Dispora* is interwoven with other taxa - *Parallela* and *Microspora* in particular. Here we transfer one *Dispora* species into *Parallela* based on extensive review of literature, morphological and ultrastructural observations, and a multigene phylogeny. Despite this detailed evaluation of the former *D. speciosa*, without live material of other *Dispora* species,

especially the generitype *D. crucigenioides*, we cannot confidently make genus-level adjustments to the current, morphologically based taxonomy.

Our study also shows that molecular phylogenetics needn't be thought of as a replacement for traditional morphological taxonomy. Instead, a DNA-based phylogeny can be a useful tool to complement morphological approaches, and give them more evolutionary meaning. We use a phylogeny to re-evaluate morphological criteria for taxon classification, and re-interpret morphological characters in light of independently derived evolutionary relationships.

Acknowledgments

This study was supported by the Assumption College Faculty Development Grant. 18S sequences of *Chlorococcum tatrense* and *Palmellopsis texensis* were provided through the NSF grant DEB-1036448 awarded to Louise Lewis and Paul Lewis at the University of Connecticut. Analyses were carried out at the Computational Biology Core Facility of the University of Connecticut. Our special thanks belong to Jan ´Hanys´ Kaštovský for valuable advice and support.

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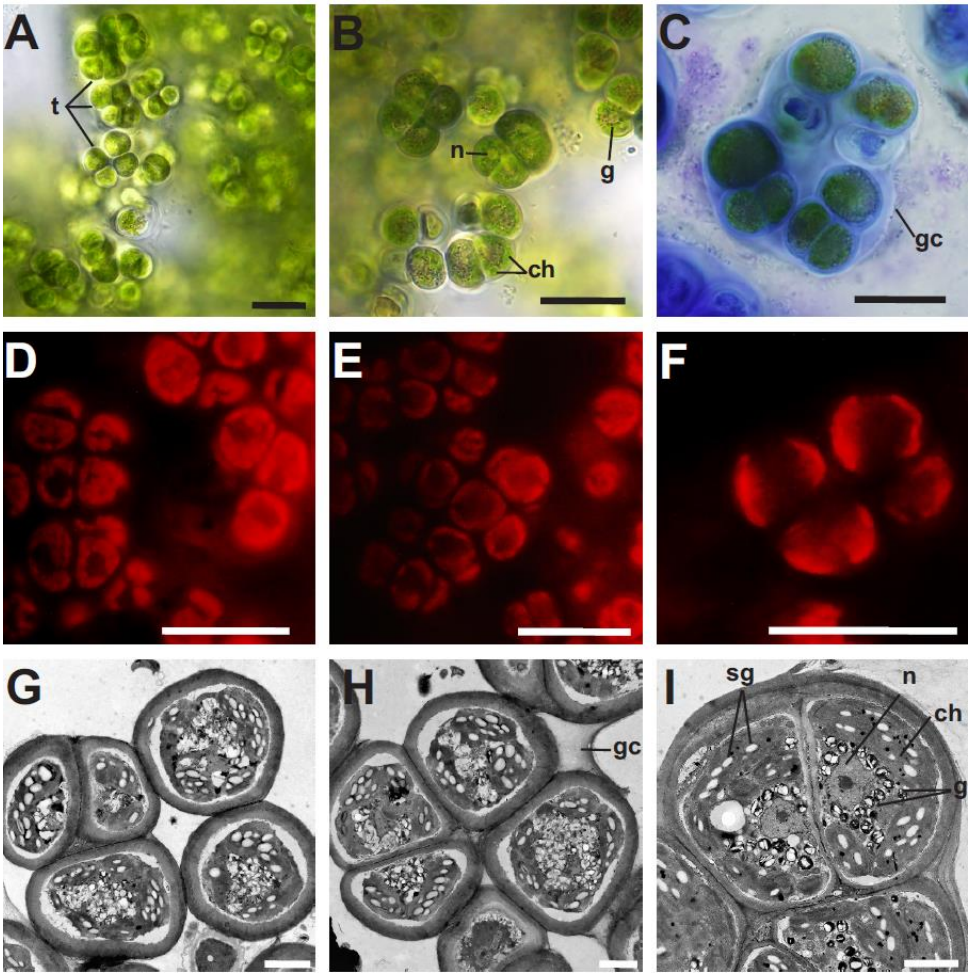

FIGURE 1. Gross morphology of *Dispora speciosa* strain ACOI 1508. Microscopical observation were carried out using: light microscopy (A–C), fluorescence microscopy: observing autofluorescence of chlorophyll (D–F) and transmission electron microscopy (G–I). A: arrangement of the cells into tetrads in the culture, B: distribution of individual organelles inside cells, C: gelatinous cover around the cell aggregation highlighted by methylene blue, D–F: shape of autofluorescent chloroplasts inside cells, G: dividing cells in a tetrade, H: tetrade conjoined to others by the mucilage cover, I: detailed content of the cell. Description: ch=chloroplast, g=granules, gc=gelatinous cover, n=nucleus, sg=starch grain, t=tetrads of cells. The scale bars indicate 20µm (A–F) or 2 µm (G–I).

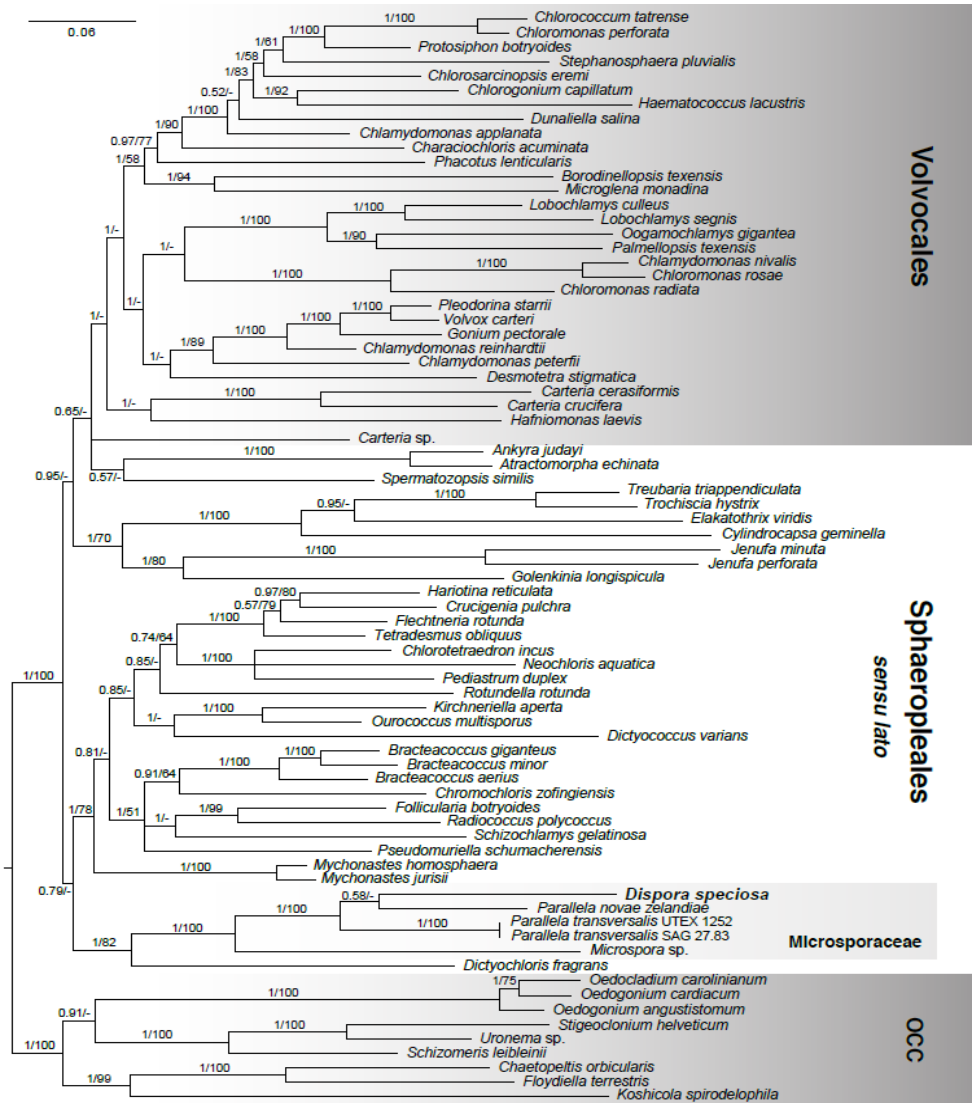

FIGURE 2. Bayesian consensus tree resulting from analysis of concatenated 18S, *atpB*, *psaB* and *rbcL* nucleotide sequences. The species of interest, *Dispora speciosa*, is highlighted in boldface and major taxonomic groups of Chlorophyceae are shown in shaded boxes. Numbers on branches indicate Bayesian posterior probability (BPP) and Maximum Likelihood bootstrap (BS) support, respectively. Only BPP > 0.5 and BS > 50 are shown. Scale bar represents the number of expected substitutions/site as estimated by MrBayes.

TABLE 1. Primers used to amplify plastid genes and 18S of *Dispora speciosa* and their sources. Taxon-specific primers designed for this study are highlighted in boldface font. Tm is as determined by Oligo Analyzer 3.1: Integrated DNA Technologies. * indicates modifications from published primer.

Gene	Name	F/R	Sequence	Position (bp)	Tm (°C)	Citation
18S	1170F	F	CTGTGGCTTAATTTGACTCAACACG	1170	56.6	Pažoutová et. al 2010
18S	1650R	R	TCACCAGCACACCCAAT	1650	54.2	Kipp 2004
AtpB	Pa2b	F	ATYTTTGAAACAGGWATTAAAGT	411	46–53	*Novis et al. 2010
AtpB	D_atpB_1345	R	GCTAAACTTACATATTTTCCAGG	1345	49.0	Present study
PsaB	Pp1b	F	TTCCAYGTAGCWTGGCAAGG	195	55–61	*Novis et al. 2010
PsaB	Pp3b	R	AAGAAAATRGCWCCRTGRGCAAA	1158	52–62	*Novis et al. 2010
RbcL	28F	F	GGTGTTGGATTWAAAGCTGGTGT	28	55.9	McManus & Lewis 2011
RbcL	650R	R	CGGTCTCTCCAACGCATGA	650	57.3	McManus & Lewis 2011

TABLE 2. Algal strains used in phylogenetic analyses and GenBank accession numbers for their 18S, *atpB*, *psaB*, and *rbcL* sequences. Strains are ordered to reflect their phylogenetic groupings. Newly obtained sequences highlighted in boldface font. In cases where information from multiple strains of the same species was used, both/all strain numbers are given. In species where two different names have recently been used in literature, both names are shown for easier comparison to other studies.

Strain	**Name**	**18S**	***atpB***	***psaB***	***rbcL***
Microsporaceae					
ACOI 1508	*Dispora speciosa*	**MG991819**	**MG991818**	**MG991820**	**MG991817**
Liffey	*Parallela novae-zelandiae*	N/A	GQ423922.1	GQ423927.1	GQ423930.1
SAG 27.83	*Parallela transversalis*	N/A	GU270868.1	GU270869.1	GU270870.1
UTEX LB 1252	*Parallela transversalis*	AF387161.1	EF113533.1	MG786420.1	EF113468.1
UTEX LB 472	*Microspora sp.*	AF387160.1	EF113517.1	KT693221.1	KT693222.1
Sphaeropleales *sensu lato*					
SAG 34.88	*Crucigenia pulchra*	KF673376.1	N/A	N/A	N/A
BCP SEV3VF 49	*Flechtneria rotunda*	HQ246317.1	N/A	KC145475.1	HQ246350.1
UTEX 393 UTEX 1450	*Tetradesmus obliquus*	AJ249515.1	NC008101	NC008101	NC008101

UTEX LB1365 SAG 8.81	*Hariotina reticulata*	AH012395.2	KY792693.1	JN630546.1	JQ394815.1
UTEX 2979	*Rotundella rotunda*	KC145434.1	KT369368.1	KT369353.1	KT369354.1
UTEX 138	*Neochloris aquatica*	M62861.1	KT199248.1	KT199248.1	KT199248.1
UTEX LB1364	*Pediastrum duplex* var. *asperum*	AY779859.1	MF536520.1	MF536515.1	MF536514.1
SAG 43.81	*Chlorotetraedron incus*	AF288363.1	KT199252.1	KT199252.1	KT199252.1
SAG 2137	*Pseudomuriella schumacherensis*	HQ292768.1	KT199256.1	KT199256.1	KT199256.1
UTEX LB62	*Dictyococcus varians*	GQ985408.1	N/A	KC145487.1	GQ985404.1
UTEX LB 951	*Follicularia botryoides*	KC145433.1	MG778401.1	KC145485.1	JQ259910.1
SAG 217-1c	*Radiococcus polycoccus*	AF388378.1	N/A	KC145490.1	HM852437.1
SAG 66.94	*Schizochlamys gelatinosa*	AY781662.1	N/A	KC145483.1	KC145516.1
UTEX 1250	*Bracteacoccus aerius*	U63101.1	KT199254.1	KT199254.1	KT199254.1
UTEX 1251	*Bracteacoccus giganteus*	U63099.1	KT625421.1	KT625421.1	KT625421.1
UTEX 66	*Bracteacoccus minor*	U63097.1	KT199253.1	KT199253.1	KT199253.1
UTEX 56	*Chromochloris zofingiensis*	HQ902933.1	KT199251.1	KT199251.1	KT199251.1
SAG 2004	*Kirchneriella aperta*	AJ271859.1	KT199250.1	KT199250.1	KT199250.1

UTEX 1240	*Ourococcus multisporus*	AF277648.1	JN630550.1	KT369443.1	KT369475.1
CAUP 6502	*Mychonastes homosphaera*	GQ477056.1	KT199249.1	KT199249.1	KT199249.1
SAG 37.98	*Mychonastes jurisii*	AF106074.1	KT625411.1	KT625411.1	KT625411.1
UTEX 127	*Dictyochloris fragrans*	AF367861.1	MG778236.1	KC145480.1	KC145513.1
UTEX LB 606	*Trochiscia hystrix*	AF277651.1	EF113543.1	MG778511.1	EF113480.1
SAG 38.83 NIES 394	*Treubaria triappendiculata*	LC192143.1	KT625410.1	KT625410.1	KT625410.1
SAG 3.87	*Cylindrocapsa geminella*	U73471.1	EF119849.1	MG778196.1	MG778214.1
SAG 9.94	*Elakatothrix viridis*	AY008844.1	MG778344.1	MG778308.1	MG778310.1
SAG 73.80	*Golenkinia longispicula*	AF499923.1	KT625129.1	KT625105.1	KT625127.1
CAUP H8102	*Jenufa minuta*	HM563744.1	KT625414.1	KT625414.1	KT625414.1
CAUP H8101	*Jenufa perforata*	HM563743.1	KT625413.1	KT625413.1	KT625413.1
SAG 17.84	*Ankyra judayi*	U73469.1	KT369399.1	KT369399.1	KT369399.1
UTEX 2309	*Atractomorpha echinata*	U73470.1	EF113487.1	JN630539.1	EF113412.1
SAG B 1.85	*Spermatozopsis similis*	X65557.1	EF113535.1	MG778500.1	MG778500.1
Volvocales					

UTEX 2227	*Chlorococcum tatrense*	MG991815.1	MG778173.1	MG778173.1	MG778173.1
SAG 11–43	*Chloromonas perforata*	U70794.1	KT625416.1	KT625416.1	KT625416.1
UTEX B99	*Protosiphon botryoides*	JN880460.1	KT693220.1	JN630554.1	JN880463.1
SAG 78-1a	*Stephanosphaera pluvialis*	LC066326.1	KT625300.1	KT625323.1	KT625343.1
UTEX 1186	*Chlorosarcinopsis eremi*	AB218706.1	MG778185.1	MG778185.1	HQ246342.1
UTEX 11 CCAP 12/2a	*Chlorogonium capillatum*	AB278612.1	KT625087.1	KT625086.1	KT625086.1
SAG 34-1b	*Haematococcus lacustris*	AF159369.1	KT625206.1	KT625227.1	KT625244.1
CCAP 19/18	*Dunaliella salina*	EF473745.1	GQ250046.1	GQ250046.1	GQ250046.1
SAG 11–9	*Chlamydomonas applanata*	FR865616.1	KT625417.1	KT625417.1	KT625417.1
SAG 31.95 UTEX 2095	*Characiochloris acuminata*	AF395435.1	KT625418.1	KT625418.1	KT625418.1
SAG 61-1 KR 91/1	*Phacotus lenticularis*	X91628.1	KT625422.1	KT625422.1	KT625422.1
UTEX 1593 SAG 17.95	*Borodinellopsis texensis*	KM020129.1	MG778120.1	MG778121.1	MG778126.1
SAG 31.72	*Microglena monadina*	JN903976.1	KT624718.1	KT624742.1	KT624766.1

SAG 19.72 SAG 18.72	*Lobochlamys culleus*	U70594.1	KT625172 .1	KT625186 .1	KT625162 .1
SAG 9.83	*Lobochlamys segnis*	U70593.1	KT624821 .1	KT624809 .1	KT624842 .1
SAG 44.91	*Oogamochlamys gigantea*	AJ410465. 1	KT625412 .1	KT625412 .1	KT625412 .1
UTEX 1708	*Palmellopsis texensis*	MG99181 6.1	MG77845 3.1	MG77848 2.1	MG77847 6.1
UTEX LB 1969	*Chloromonas nivalis/typhlos*	U57696.1	KT624652 .1	KT624641 .1	KT624639 .1
UTEX 1337	*Chloromonas rosae*	U70796.1	AB084315 .1	AB084350 .1 AB084351 .1	AB022536 .2
UTEX 966	*Chloromonas radiata*	U57697.1	KT625014 .1	KT625021 .1	KT625036 .1
NIES 1363 NIES 1362	*Pleodorina starrii*	LC086359. 1	JX977846. 1	JX977846. 1	JX977846. 1
UTEX 2908 UTEX 1885	*Volvox carteri* f. *nagariensis*	X53904.1	GU084820 .1	GU084820 .1	GU084820 .1
K3-F3-4 NIES 569	*Gonium pectorale*	LC066324. 1	AP012494. 1	AP012494. 1	AP012494. 1
CC-503 cw92 UTEX 90	*Chlamydomonas reinhardtii*	AB511834 .1	FJ423446. 1	FJ423446. 1	FJ423446. 1
SAG 70.72	*Chlamydomonas peterfii/asymmetrica*	U70788.1	KT624943 .1	KT624953 .1	KT624961 .1
UTEX 962	*Desmotetra stigmatica*	AB218711 .1	MG77823 2.1	MG77823 1.1	MG77823 2.1

NIES 425	*Carteria cerasiformis*	AB688624.1	KT625420.1	KT625420.1	KT625420.1
UTEX 432	*Carteria crucifera*	D86501.1	KT624917.1	KT624903.1	KT624910.1
NIES 257	*Hafniomonas laevis*	AB101517.1	KT625415.1	KT625415.1	KT625415.1
SAG 8-5 UTEX 2	*Carteria* sp.	AF182817.1	KT625419.1	KT625419.1	KT625419.1
OCC clade (Oedogoniales, Chaetophorales, Chaetopeltidales)					
UTEX LB 422	*Chaetopeltis orbicularis*	U83125.1	KT693210.1	KT693211.1	KT693212.1
UTEX 1709	*Floydiella terrestris*	D86498.1	NC014346.1	NC014346.1	NC014346.1
NIES 3575	*Koshicola spirodelophila*	KT693223.1	KT713390.1	KT713392.1	KT713390.1 KT713391.1 KT713392.1
CCAP 334/1	*Uronema sp.*	FN824391.1	MG778533.1	MG778533.1	MG778533.1
UTEX LB1228	*Schizomeris leibleinii*	AF182820.1	NC015645.1	NC015645.1	NC015645.1
UTEX 441	*Stigeoclonium helveticum*	U83131.1	NC008372.1	NC008372.1	NC008372.1
UTEX LB1686	*Oedocladium carolinianum*	U83135.1	NC031510.1	NC031510.1	NC031510.1
UTEX 1557	*Oedogonium angustistomum*	U83134.1	KT693216.1	KT693217.1	KT693218.1
UTEX LB40	*Oedogonium cardiacum*	U83133.1	NC011031.1	NC011031.1	NC011031.1

Supplementary data

S1: zipped folder containing raw and trimmed alignments, analysis specifications, and resulting trees in.tre format.

Authors CV

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Scientific interests

Ecology and diversity of green algae and Cyanobacteria

Evolution, systematics and taxonomy of green coccal algae and Cyanobacteria

Genomics of green algae and Cyanobacteria

Cultivation of green algae and Cyanobacteria

Skills

Cultivation, media preparation, light microscopy, electron (SEM and TEM) microscopy, fluorescent microscopy (autofluorescence and DAPI)

DNA isolation, PCR, ElFo, Sequencing, Cloning, common programs of molecular phylogenetic analyses, Single cell isolation, MDA

Basics of NGS: Oxford nanopore - MinION and Ilumina, cyanobacterial, chloroplast and mitochondrion genomics, bioinformatics

Basics of separation methods: high-pressure liquid chromatography (HPLC)

Education

1999-2007 Secondary grammar school in Teplice

2008–2011 Bachelor study: University of South Bohemia in České Budějovice, thesis: Phylogeny inside the family Oocystaceae

2011–2014: Master study: University of South Bohemia in České Budějovice, thesis: Systematic revision of the family Oocystaceae

2014 – present: Doctoral study: University of South Bohemia in České Budějovice, (present) thesis: Systematics of coccal green algae of the classes Chlorophyceae and Trebouxiophyceae

History of employment

2013–2014: University of South Bohemia in České Budějovice: cloning, Cyanobacterial sequencing

2015: Institute of Soil Biology CAS: cultivation, media preparation, strain cleaning

2016–2020: Institute of Hydrobiology CAS: cultivation, cyanobacterial sequencing, cyanobacterial genomic

2018: Institute of Microbiology CAS: HPLC

Publications

Štenclová, L. Distribution of the Crucigenioid algae inside the classes Chlorophyceae and Trebouxiophyceae. Submitted 2. 11. 2020 in Journal of Phycology.

Silva, T. G., **Štenclová, L.,** Archanjo, N.C.P. and Bagatini, C. L. Revised phylogenetic position of genus *Nephrocytium* Nägeli (Sphaeropleales, Chlorophyceae), with description of Nephrocytiaceae *fam. nov.* and *Nephrocytium vieirae sp. nov.* Submitted in Taxon 27.12.2019.

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Conferences

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Fučíková, K. and **Štenclová, L.** (2018) *Dispora Speciosa*, the first coccoid member of Microsporaceae. Northeast Algal Symposium, New Haven, CT, USA. Poster

Štenclová, L. (2015) Molecular and morphological delimitation and generic classification of the family Oocystaceae (Trebouxiophyceae, Chlorophyta). Annual meeting of the Czech phycological society, České Budějovice, Czech Republic and 6th European Phycological Congress in London. European Journal of Phycology 50(6):129-129. Poster

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E. Berrendero, M. Bohunická, **L. Štenclová,** J. H. Kaštovský. (2013) A taxonomic study on *Calothrix* (Cyanobacteria) based on morphology and analysis of the 16S rRNA gene fragment. 10th International Phycological Congress in Orlando, Florida. Phycologia 52(4):10-10. Poster

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Štenclová L. (2012) Systematická revize čeledi Oocystaceae. Annual meeting of the Czech phycological society, Ostrava, Czech Republic. Presentation (in Czech)

Štenclová, L. (2011) Fylogeneze zelených řas čeledi Oocysatceae. Annual meeting of the Czech phycological society, Praha, Czech Republic. Presentation (in Czech)

Stays abroad

2017: Laboratory of Dr. Karolina Fučíková, Assumption College Worcester, MA, USA, projects: *Dispora speciosa*, a new addition to the genus *Parallela* and the first coccoid member of the family Microsporaceae and Characterizing the chloroplast and mitochondrial genomes of a microscopic alga *Oonephris obesa* (Chlorophyceae)

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Systematics of coccal green algae of the classes Chlorophyceae and Trebouxiophyceae
Ph.D. Thesis Series, 2020, No. 20
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Printed in the Czech Republic by Typodesign Edition of 12 copies

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