Katedra botaniky
Univerzita Karlova v Praze - Přírodovědecká fakulta

Phylogenetic diversity and generic concept in the family Radiococcaceae, Chlorophyta

(Druhový koncept a molekulární diverzita čeledi Radiococcaceae, Chlorophyta)

Marie Pažoutová

Diplomová práce
Praha 2008

Vedoucí diplomové práce: Pavel Škaloud

Prohlašuji, že jsem předkládanou práci vypracovala samostatně s použitím citované literatury.

Marie Pažoutová

This work was fully supported by the grant GA UK 149207/2007.

Můj dík patří na prvním místě mému školiteli Pavlovi Škaloudovi bez jehož nadšení, důvěry a nekonečné trpělivosti by se tato práce neobešla. Děkuji Pavlovi Přibylovi za velkorysou nabídku pomoci s kultivacemi v zázemí třeboňské sbírky a inspirativní klučičí přístup. Děkuji Tomášovi Hauerovi za všechno: za pomoc, za trpělivost, za přátelství. Děkuji Janici za přátelství a specifický vztah k Ottonovi. Děkuji všem kolegům v pražské laborce, že jsem se těch několik let mohla těšit z jejich společnosti a učit se a Jiřímu za zajímavé nápady. Děkuji kolegům z Budějc za vlídné přijetí.

Abstract:

The family Radiococcaceae, defined broadly as coccoid green algae with mucilaginous cover reproducing only by autospores, is one of the most taxonomically problematic groups among green algae. Radiococcaceae are common organisms of freshwater as well as terrestrial habitats worldwide and they have been studied for more than 100 years, yet their taxonomy remains unclear. There was never stable generic concept for this group. Some of the traditional morphological traits, like the presence of mucilage itself, proved to be unreliable.

I examined the phylogenetic position of 25 strains of Radiococcaceae from several culture collections representing different traditional species with different morphology. According to the analysis of the 18S rRNA gene the strains are placed within two classes, 10 in Chlorophyceae and 15 in Trebouxiophyceae. I distinguished 7 distinct clades in the former and 5 in the latter and found new well suported phylogenetic lineages of green algae. The morphology and reproduction strategies of strains were studied in different culture conditions. These characters were compared with the results of phylogenetic analysis. The relevance of morphological criteria is discussed and taxonomical revisions concerning the strains are proposed.

1. Introduction

1.1 Algae and Mucilage

From the beginning of studies on algae, the appearance of the organisms, its morphology, bore great importance. The arrangement of algal body – the thallus – was for more than a century perhaps the primary criterion to take into account when one was to distinguish different entities, different taxons (Silva 2007).

The simplest form of algal body, a singe immobile cell without flagella or rhizopodia is called "coccoid". Sometimes simple cells envelop themselves within a mucilaginous cover. Then it is often referred to as a capsal thallus. Another term in use for quite a similar form is "palmelloid stage". This stands for a more or less temporary capsal stage in a more complex life cycle (like for example in *Chlamydomonas*).

For many species and genera, the mucilaginous sheats have been a distinguishing criterion. Thus, for anyone involved in taxonomy of green algae, the presence of mucilage has been a thing to take into account.

1.2 Radiococcaceae

1.2.1 Why Radiococcaceae

There were many genera of green algae described with mucilaginous sheaths around cells. Some of them bore conspicuous traits that helped to place them in (more or less) well defined taxonomical units (like for example *Dictyosphaerium*, where the cells are joined together by equally branched mucilaginous stalks originating from the old mother cell wall; Nägeli 1849). Other genera, simple green balls, were hard to sort out. These organisms were usually to be found in various families like Palmellaceae, Tetrasporaceae, Oocystaceae, Chlorelaceae, Coccomyxaceaeor Protococcaceae.

To solve out the problem of simple-shaped capsal algae and to make the system workable, the family Radiococcaceae was erected (Fott 1959) to accomodate all the genera of unstable position into one common group. The idea was nice, but in the practice it

never worked out. The generic concept kept changing from author to author and failed to give a reliable tool for everyday determining routine.

1.2.2 Definition

The name Radiococcaceae was first given by Fott in a german translation of his textbook (Algenkunde, 1959). Unfortunately, there was no description, neither in latin nor in german, so it was not published validly according to the International Code of Botanical Nomenclature (the Code; version in use at that time was probably that by Lanjouw et al. 1954).

The name Radiococcaceae was correctly validised by Komárek (1979) with this description:

Cellulae sphaericae, globosae, ovoideae, ellipsoideae vel fusiformes, plus minusve asymmetricae, in colonias mucosas plus minusve irregulariter dispositae, non conjunctae. Chloroplastum parietale, cum vel sine pyrenoideo. Propagatio autosporibus, zoosporae vel hemizoosporae absunt.

(Cells spherical, globose, ovoidal, ellipsoidal or fusiform, more or less asymmetrical, arranged more or less irregularly /irreg or not/ in mucilaginous colonies, not connected together. Chloroplast parietal, with or without pyrenoid. Reproduction through autospores, zoospores or hemizoospores absent.)

As one can see from the description, Komárek (1979) stressed the absence of zoospores, not following the studies of Fott (1959), Fott (1974) and Hindák (1977), but rather adhering to Koršikov's point of view (Koršikov 1953).

Komárek's taxonomical concept of Radiococcaceae was then adopted in all relevant works (e.g. Komárek & Fott 1983, Ettl & Gärtner 1994, Kostikov et al. 2002).

The most up-todate definition was given by Kostikov et al. 2002 as follows: "colonial autospore-producing green algae with spherical, regularly or irregularly ellipsoid cells with a smooth cell wall, lacking vegetative cell division, lying in a more or less thick and more or less strong mucilage."

In the next part, I present a brief outline of history of the genera and generic concepts connected with the family Radiococcacae. I will follow the description by Komárek (1979) and discuss the autosporic genera preferably, with few important zoosporic genera, too. I will omit genera that do not fit the condition "cells free, not connected together" (e. g. subfam. Dictyochlorelloideae in Fott 1974 and Komárek & Fott 1983).

1.2.3 Histories of Genera

First descriptions of the genera of capsal green algae date the 19th century. Here, the works of Küetzing (1843, 1845, 1849) and Nägeli (1849) are the most important – and within them mainly genera *Palmella, Palmogloea, Tetraspora, Gloeocystis, Palmodictyon* and *Palmodactylon.* Obviously, the depth of the descriptions – the information provided and the quality of the drawings – is often not sufficient for a taxonomist today. Some of the early genera were rather a mixture of unrelated organisms: for example several species of *Palmella* and *Palmogloea* were later recognized as cyanobacteria and placed to the genus *Aphanothece*, two members of *Palmogloea* were moved to genus *Mesotaenium,* Zygnematophyceae. On the contrary, few species later recognized as members of Radiococcaceae were described in the cyanobacterial genus *Gloeocapsa* (e.g. *Coccomyxa confluens*, *Gloeocystis polydermatica*).

The main feature of ***Palmella*** LYNGBYE 1819, probably the oldest of genera of interest, is an indeterminate, shapeless mass of mucilage, in which the cells are embedded. Although Nägeli (1849) stated there was no evidence of motile stages, probably all following authors congruently regarded the genus as zoosporic. Chodat (1902) made an emendation of the genus, accepting only single "well characterized" species (the type species of the emended genus, *P. miniata*).

Another capsal genus ***Tetraspora*** LINK 1820 was characterized by the presence of two "gelatinous flagella", later called pseudocilliae. Although it was often put in a relationship with *Gloeocystis* and other radiococcacean genera, *Tetraspora* species themselves were not confused with Radiococcaceae.

The description of ***Palmogloea*** KÜTZING 1843 was quite brief (original in Latin see in the picture XYZ) and accompanied by no picture. Usually *Palmogloea* was taken as somewhat similar to *Palmella*, but without zoospores. The genus was re-established by Fott and Nováková (1971) and subsequently rejected by Hindák (1978) (as mentioned further on).

When establishing ***Gloeocystis*** NÄGELI 1849, the author put emphasis on the form of multilayered mucilaginous envelopes. The morphology of this genus strongly resembles that of palmelloid stages of *Chlamydomonas*, though in original Nägeli's description the organism lacks motile cells. However, there is particular similarity of some characters as well as drawings with Palmella. This will be dicussed in more detail in this work.

The genera ***Palmodictyon*** KÜTZING 1845 and ***Palmodactylon*** NÄGELI 1849 are different from the others by a specific feature: the cells are lying within a mucilaginous tube, often quite long and branched (the colony is not indeterminate or rounded). The difference between these two genera is a structureless mucilage (*Palmodactylon*) versus stratified mucilage with envelopes around individual cells or small groups of cells (*Palmodictyon*). For West (1904), it was a reason to place these taxa into two different subfamilies. On the contrary, the fine distinction was not accepted by Lemmermann (1915), who united the two genera in one under the older name *Palmodictyon*.

Dactylothece LAGERHEIM 1883 resembles the genus *Gleocystis* in the layered form of mucilaginous colony, but there are some intristing differences: cells are more ellipsoid and West (1904) also points out plate-like chloroplast occupying only about 2/3 of the cell and the cell division taking place only in one direction. While *Gloeocystis* resembles much of palmelloid stages of *Chlamydomonas* (Chlorophyceae), the characteristics of *Dactylothece* rather reflect that the type material was a specimen of Trebouxiophyceae. The genus has been often mistaken (or synonymized) either with *Coccomyxa* or *Gloeocystis* and encompassed in *Palmogloea* according to Drouet & Daily (1956) and Fott & Nováková (1971). On the other hand, the arrangement of cells (more or less in rows with small distance to each other) was a reason for Komárek & Fott (1983) to keep this genus and encompass it in the subfamily Palmodictyoideae.

Sphaerocystis CHODAT 1897 has globose cells aggregated together in groups of 1-16 cells in a spherical mass of mucilage; daughter colonies are embedded within the mother colony until it breaks and sets them free. It was described having biciliate zoospores, but was later emended as autosporic genus by Koršikov (1953). Not all authors accepted the emendation.

Under a name ***Coccomyxa***, SCHMIDLE 1901 green alga with following characters was described: cells single or in groups of two or four, enlongated, longer than wide, assymetrical („unevenly curved sides"), with rounded or narrowed ends, with parietal chloroplast lying by one side of the cell, lacking pyrenoid, that divides by transversally usually forming four autospores. Contrary to Nägeli (1849), the layered form of mucilage was not emphasized and some later species (e.g. *Coccomyxa subglobosa*), it was not structured at all.

Radiococcus (DE WILDEMAN) SCHMIDLE 1902 was a new name for *Pleurococcus*, later *Tetracoccus nimbatus*. Its characteristics according to Schmidle (1902): freshwater algae forming microscopic colonies with cells arranged strictly in fours (not less, not more) and embedded within ray-like structured mucilaginous envelope; has chloroplast that covers only part of the cell volume, with one pyrenoid. Produces four autospores that are released after the sporangial cell wall breaks, than usually stay in tetrads surrounded by irregularly distributed fragments of the mother cell wall. The authenticity of ray-like mucilage was later doubted by Fott (1974), whose emendation of the genus placed emphasis on tetraedrical arrangement of both vegetative cells and autospores.

Pseudotetraspora WILLE 1906 is probably the first radiococcacean genus from marine habitat. The main character different from all the previous genera is that the mucilaginous colony is flat, plate-like with only one layer of cells. Usually consists of daughter colonies and the cells, oval to spherical and sometimes slightly asymmetrical, group in twos or fours. Chloroplast shape is of interes here: lobate to stellate, with pyrenoid. Reproduces by four or eight autospores.

Dispora PRINTZ 1914 is another genus with plate-like colonies of more faint and homogenous mucilage; its cells group in fours and reproduce by four autospores, chloroplast is cup-shaped, without pyrenoid.

According to some authors (e.g. Hindák 1988) the description of ***Eutetramorus*** WALTON 1918 was quite weak. Its main characteristic is the arrangement of collony, four groups of four cells lying on the perifery of the mucilaginous sphere; cells posses central pyrenoid. The author supposed this specimen was related to *Coelastrum*, he did not discuss the difference between the new genus and for example *Radiococcus*. The main difference from this older taxon would be the lack of ray-like structure in the mucilage and probably the position of cells on the perifery of the mucilaginous sphere (not in the centre).

Planktosphaeria G. M. SMITH 1918 has spherical cells with one central and later (in mature cells) many peripheral chloroplasts, each possessing one pyrenoid. It was originally described from the freshwater habitat and supposed to reproduce by autospores. However, later a production of zoospores was reported from soil isolates (Starr 1954). The zoosporic form was moved to genus *Follicularia* (Lukešová 1994) and the authority of both of the names is still in question.

Sporotetras BUTCHER 1932 was described with emphasis on the overall shape of colony as epilithic, attached specimen „obviously related to *Tetraspora*". In early stages it resembles genera *Pseudotetraspora* and *Dispora* in the flat form of colonies (but with more cell layers), than the colony develops into a rounded mass with individually enveloped cells in groups of four or eight on the mucilage surface. The shape of the cells is somewhat pyriform, with apices towards the centre of colony, chloroplast lobed with large and distinct pyrenoid.

The placement of ***Thorakochloris*** PASCHER 1932 into Radiococcaceae is questionable, this taxon was not included in latest revisions. The reason is that, although it reproduces through „successive division of protoplast" that results in immobile daughter cells, the young spores possess contractile vacuoles and sometimes also a stigma. This indicates a close affinity to chlamydomonads. Cells of *Thorakochloris* group in 16 or less often in

four, characteristically arranged in layered mucilage, also typical is the placement of fragments of sporangial cell wall. Chloroplast is massive, without pyrenoids.

Phacomyxa SKUJA 1956 falls into the group of genera with flattened colonies. The cells are of diverse shape, embedded in more or less layered mucilage, in one plane, or in packet-like or irregular groups. Each cell posses several parietal chloroplasts without pyrenoid, but with small starch grains. It reproduces by two or four (occasionally eight) autospores that are released by mucilaginisation of the sporangial cell wall.

Pascher's first edition of Süsswasser-flora Deutschlands, Österreichs und der Schweiz could be considered as a milestone in the 20th centrury phycology. In chapters on Chlorophyceae the author kept the traditional family Palmellaceae with, among others, genera *Gloeocystis* and *Palmodictyon* (regarded as zoosporic, despite of original descriptions; Lemmermann 1915). *Radiococcus* was placed in Chlorellaceae, whilst *Coccomyxa* and *Dactylothece* in the provisional group of uncertain taxonomical position (Lemmermann 1915, Pascher 1915).

Smith (1950) brought in the family Coccomyxaceae, which, remarkably, he excluded from Chlorococcales because of „their multiplication by vegetative cell division". Here, *Dactylothece, Coccomyxa* and *Dispora* were placed. Smith also retained the zoosporic family Palmellaceae with *Gloeocystis, Palmodictyon* and *Sphaerocystis*. *Radiococcus* and *Planktosphaeria* were included in Oocystaceae.

An intrinsic progress in the knowledge of capsal green algae was brought by O. A. Koršikov (1953). Although his monograph was concerned solely with algae found in Ukraine, it added a lot of new information on the diversity and morphology of green algae in general. Koršikov described a great portion of the (later) radiococcacean taxa. Most of these he placed in the family *Protococcaceae*, where, except *Protococcus* itself, all genera were of capsal thalus. He included only autosporic algae in this group. In this context, he emended the genus *Sphaerocystis* as autospore producing (as was already mentioned, not all authors respected the emendation).

Because of the author's tragical death, his work was never finished and the monograph was issued incomplete. Apart from some minor cavities in the data, there are no latin diagnoses and no typification of the genera. However, this does not violate the validity of description according to the Code (McNeill 2006), and Koršikov's new descriptions were widely accepted (e.g. Fott 1959, Hindák 1977).

Koršikov added four new genera of autosporic capsal green algae, three newly described, one only a new name. The most delimiting feature was for him the arrangement of cells in colony.

The cells of ***Coenochloris*** KORŠIKOV 1953 should be arranged in tight accumulations in the centre of the colony. However, for authours of later papers, rather the oval or globose cell shape and the breakage of mother cell wall was of importance. According to the author, the species may or not posses a pyrenoid.

The characteristic of ***Coenocystis*** KORŠIKOV 1953 is: cells not globose, arranged in fours or eights, with pyrenoid and showing remnants of the mother cell wall for a period of time (shorter than in case of *Coenochloris*).

Coenococcus KORŠIKOV 1953 was defined by spherical cells grouped in fours, production of four autospores and a complete gelatinisation of the mother cell wall. This genus brought rather controversy, being not clearly delimited in relation to two older genera, *Radiococcus* and *Eutetramorus*. It was synonymized with *Eutetramorus* (Bourrelly 1966, Komárek & Fott 1983, Kostikov et al. 2002) and with *Radiococcus* (Fott 1974).

Schizochlamydella KORŠIKOV 1953 was a new name for *Schizochlamys delicatula*. The older genus was placed near *Tetraspora* and was supposed to have pseudociliae, which are lacking in *S. delicatula*. According to Koršikov's diagnosis, here cells are scattered in a structureless mucilage without any particular arangement, cells altering with empty sporangia that rupture and stay in one piece. Cells have cup-shaped chloroplast with or without pyrenoid (this trait was not discernible in the original description of *S. delicatula*). Reproduction takes place by two autospores.

After Koršikov (1953) most important events were the efforts to encompass all supposingly related capsal genera into a consistent family.

Fott's first edition of phycological textbook (in Czech, 1956), rather mirrored the old system of Pascher (Lemmermann 1915, Pascher 1915) and Smith (1950), with *Gloeocystis* in Palmellaceae and *Radiococcus* in Oocystace and many other (more problematic) genera simply omitting. (Koršikov's new taxons were not included yet.) But the second version of the textbook (in German, 1959) came with a new concept and a group of genera under the name Radiococcaceae. As was mentioned before, the propre description of the family was not given. Fott included folowing genera into the new family: *Radiococcus*, *Coenococcus*, *Coenocystis*, *Schizochlamydella* and *Thorakochloris*. Radiococcaceae were characterized by the lack of zoospores in contrary to otherwise rather similar algae in Gloeocystidaceae.

The system of Bourrelly (1966) seems to combine main concepts of previous authorities. He adopted the new family Radiococcace, which he understood in much broader sense and added to it few more genera (often with unknown mode of reproduction and three even without pigmentation). Apart from this family, he included some more „radiococcacean" genera in Coccomyxaceae (*Coccomyxa, Dactylothece, Dispora*), Gloeocystaceae (not Gloeocystidaceae; *Gloeocystis*), Hormotilaceae (*Palmodictyon*) and Chlorococcaceae (*Planktosphaeria*).

Inspired by Drouet and Daily (1956), Fott and Nováková (1971) later revised the taxonomy of aerophytic mucilaginous genera *Palmogloea* and *Gloecystis*. They synonymized these two taxa together, with some members of two other genera, *Dactylothece* and *Coccomyxa*, and concluded that the name *Palmogloea*, the oldest, should hold the priority. In following review of the whole group Fott (1974) left the name Radioccaceae and used a new name Palmogloeaceae. Again, the delimitation of the family was broader than in Fott's Algenkunde (1959), combining autosporic as well as zoosporic genera together. A new subfamily *Dictyochlorelloideae* was added (cells in mucilaginous colonies conected by mucilaginous strands, the connectives do not originate from sporangial cell wall).

In 1977, Hindák published first of his monumental works on green algae (Biologické práce) with a chapter of studies on Radiococcaceae. He adhered to first Fott's concept of Radiococcaceae, but mixed zoosporic and autosporic taxa together. He also omitted *Thorakochloris* and added *Sphaerocystis* and *Planctococcus*.

In the same publication Hindák described a new genus ***Catenococcus*** HINDÁK 1977 in the family Hormotilaceae, which was later transferred to Radiococcaceae, subfam. Palmodictyoideae (Komárek & Fott 1983) and finaly synonymized with *Radiococcus* (Kostikov et al. 2002).

Regarding *Palmogloea* and *Gloeocystis,* Hindák (1978) had different opinion than Fott and Nováková (1971). According to the Article 70 of the actual edition of the Code (Stafleu et al. 1978; this article is not present in late versions), Hindák rejected the genus *Palmogloea*, becouse the description did not allow to be interpreted unambiguously. On the contrary he accepted the genus *Gloeocystis*.

Ten years later Hindák established two new genera, ***Neocystis*** HINDÁK 1988 for former *Coenochloris* species with oval cells and no pyrenoid and ***Sphaerochloris*** HINDÁK 1988 for those species of *Coenochloris* that showed special way of autospore development and release, with young spores arranged in one layer in sporangium and only later after their liberation shifting to tetrahedric position (Hindák 1988).

In 2000 ***Garhundacystis*** KOSTIKOV et HOFFMANN 2000 was erected for specimens that lack spherical cells, have parietal chloroplast with pyrenoid and reproduce by two autospores that are released after the rupture of mother cell wall.

Kostikov et al. (2002) made the last extensive review of the family (excluding subfam. *Dictyochlorelloideae*), where they also discussed the use and credibility of traditional morphological characteristics. Their aim was to establish firm and logical system, where the criteria have always the same relevance. In that intent they made a lot of taxonomical changes among genera, and added ten new generic names: ***Coenobotrys***, ***Coenodispora***, ***Diplosphaeropsis***, ***Korshikoviobispora***, ***Palmococcus***, ***Planktococcomyxa***, ***Schizochloris***, ***Sphaerochlamydella***, ***Sphaerococcomyxa*** and ***Sphaeroneocystis***.

Nowadays it seems almost surprising to approach to taxonomical issues without the employment of phylogeny. In case of Radiococcaceae, however, only single work with molecular analysis on several strains has been published so far (Wolf et al. 2003). With one sequence of *Planktosphaeria gelatinosa*, *Sphaerochlamydella capsulata* and three of *Radiococcus polycoccus*, they clearly showed polyphyly of the group (with representants in both Chlorophyceae and Trebouxiophyceae) and called for furter studies. Apart from this work sequences of several *Coccomyxa* species and *Coenocystis inconstans* (authentic strain that has been lost in the collection) are available, accompanied by too short fragments of unknown organisms labelled *Gloeocystis* spp.

1.3 Morphological criteria

The morphological traits used as taxonomical criteria in the taxonomy of Radiococcaceae were discussed in detail mainly in Kostikov (2002) and works of Hindák (e.g. Hindák 1984). Here I put a brief list with examples how the criteria were applied in Radiococcaceae. Characters that were observed during my work are further discussed in chapter four in this thesis.

- shape of the whole colony – important on the generic level since the early taxonomy, distinguished indeterminate colonies (*Palmella, Palmogloea*), spherical colonies (e.g. *Sphaerocystis, Radiococcus, Eutetramorus*), tubular colonies (*Palmodictyon, Palmodactylon, Catenococcus*) or flat plate-like colonies (subfamily *Disporoideae*: *Dispora, Pseudotetraspora, Phacomyxa* and *Sporotetras*; plate-like with mucilaginous thorns in *Crucigloea*).
- arragement of cells in the colony – for some genera there is no visible arrangement of cells in the mucilage, but there are many other possibilities: aggregated group of cells (1-16 or more), lying towards the edge of the colony (*Sphaerocystis*); tetrads (*Radiococcus, Eutetramorus, Coenococcus*); groups of four or eight (*Coenocystis*); tight accumulations of cells in the centre of colony (*Coenochloris*), uniseriate chain of cells (*Catenococcus*) etc.

• form of mucilage – in case of most of the genera the mucilage is weak, sometimes diffluent without distinct margin, on the other side thick layered mucilaginous envelopes discriminate the genera *Gloeocystis, Dactylothece* and *Coccomyxa* and according to few older authors also *Palmodactylon*.

As summarized by Kostikov et al. (2002), the mucilage could be of different consistency, strong or diffluent or sometimes even disappearing; originating from secretion or from gelatinisation of the mother cell wall.

• mode of reproduction – in older taxonomical systems, autosporic and zoosporic organisms were by some authors put together, since Komárek's validization of the family Radiococcaceae, only autosporic reproduction was comprised by definition. Hindák (1982) distinguished between autospore production and true vegetative division, where the mother cell wall takes part in the newly built daughter cell (applies to genera that produce only two spores).

• number of autospores – after succesive divisions, the cells usually produce 2, 4, 8, 16 or even 32 autospores, the lower numbers being more common. For some genera only one possibility was given (for exapmle strictly four autospores in the descritption of *Radiococcus*), others could produce different number in spores.

• position of autospores – tetrahedrical, parallel or serial arrangement of spores was used in Komárek & Fott (1983); this trait was doubted by Hindák (1984) and Kostikov et al. (2002). This character was not mentioned in most of the generic descriptions.

• way of autospore release – basically, the mother cel wall can either rupture or gelatinize. In can rupture with one crack and stay undivided (*Schizochlamydella*) or break into several pieces (*Thorakochloris*), regularly or irregularly, or it first enlarges and than dissolves, or ruptures and later the fragments dissolve (*Coenocystis*); the sporangium cell wall can also dissolve as a whole or only on one side etc. The complete mucilaginisation leads to layered form of *Gloeocapsa*-like clusters in *Gloeocystis, Dactylothece* or *Coccomyxa*. The evidence of sporangium rupture was taken from presence of the cell wall remnants in the sample.

- cell shape – usually spherical and ellipsoidal, sometimes elongated cells are distingushed; this marker being used partly on the generic level (e.g. *Eutetramorus, Coenococcus*), in other cases on the species level (*Gloeocystis vesiculosa* and *Gloeocystis polydermatica*; genus *Coenochloris*); rather exceptional is pyriform cell shape in *Sporotetras*; species *Coenocystis obtusa* with unusually prolonged cells was later moved to *Kirchneriella*.
- number of chloroplasts – the majority of radiococcacean taxa have only one chloroplast per cell, the exceptions are only few: genus *Planktosphaeria*, *Phacomyxa sphagnophila* and *Palmodictyon varium*.
- type of chloroplast – this trait was commonly used as a diagnostic feature at the genus level for non motile green algae, but usually not in case of Radiococcaceae, where simple parietal chloroplast was – with the exception of e.g.*., Phacomyxa spahgnophila* and *Palmodictyon varium ...and some zoosporic algae in the older systems*
- presence or absence pyrenoid – another common marker that was used on different taxonomical level, either generic or species (genera without pyrenoids – *Coccomyxa, Neocystis*; with pyrenoids – *Eutetramorus*, with and without together – *Coenochloris* in the original Koršikov's conception); more than one pyrenoid was reported form *Sphaerocystis schroeteri* or *Radiococcus polycoccus* (described as *Sphaerocystis*).

1.4 Aim of this work

The aim of this work was to obtain more molecular data (sequences of 18S rRNA gene) from members of Radiococcaceae, to explore their phylogenetic diversity and in the same time to characterize all the sequenced strains with traditional morphological tools. The focus of this work lies in the comparison of molecular, morphological and life cycle data. Evaluation of the traditionally used taxonomical criteria and an application of polyphasic approach in the taxonomy, of the studied group should be the result, so that the morphological criteria respect the framework of phylogeny.

2. Materials and Methods

2.1 Strains

The strains of algae investigated in this work were kindly provided by the following culture collections: The Culture Collection of Algae of Charles University of Prague (CAUP), The Culture Collection of Algal Laboratory in Třeboň (CCALA), The Culture Collection of Algae and Protozoa (CCAP), The Culture Collection of Algae at the University of Göttingen (SAG) and The Culture Collection of Algae at The University of Texas at Austin (UTEX). The list of strains with more information is shown in the tab. 3.

2.2 Isolation

In addition, I isolated two new strains (*Gloeocystis polydermatica* NP 20-02 and *Gloeocystis polydermatica* NP 20-04) from the sandstone rock in the Bohemian Switzerland National Park. The material was aseptically scratched down from the rock surface, placed in the sterile microtube and processed in the laboratory within few days. For the isolation, part of the sample was mixed with distilled water and a small amount of glass bullets (0,5mm in diameter, Sigma) and moved to agar plates with BBM medium (Bischoff & Bold 1963).

2.3 Cultivation

The algal cultures were maintained in test tubes on BBM medium (Bischoff & Bold 1963) solidified by 1,5 % agar. The tubes were provided with constant source of light of 5-15 $\mu mol.m^{-2}.s^{-1}$ and kept at a temperature of 15 °C. To compare the morphology under different conditions, the strains were also cultivated in aerated liquid cultures. In this case medium ½ SŠ was used (Zachleder & Šetlík 1982) and the temperature was higher,

approximately 25 °C After five days the algae in liquid medium were provided with 2 % CO_2. On selected strains I also tried the cultivation in liquid medium without aeration, but with constant movement of the medium. Here Erlanmayer flasks with ½ BBM medium were placed on a rocking platform (Rocker 25, Labnet, 100rpm) in 22 °C.

The chlorophycean strains were treated to induce zoospore production. This was done during the cultivation in aerated liquid medium by cutting the light off in the exponential phase; the method is described in Přibyl & Cepák (2007).

2.4 Observation, Documentation, Staining

Morphological observations were done with the Olympus BX 51 light microscope equipped with Nomarski DIC optics and Olympus DP 71 digital camera or Olympus Camedia C-5060WZ microphotographic equipment. To detect the mucilage, the algae were stained with methylene blue and Indian ink.

2.5 Molecular methods

The total genomic DNA was isolated from either fresh or lyofilized biomass following the Invisorb Spin Plant Mini Kit protocol (Invitek). Obtained DNA was amplified by polymerase chain reaction (PCR) with universal of algal-specific (Vivi) 18S rRNA primers (see tab. 1) and with Jump Start Red Taq Polymerase (Sigma). It was processed on the XP thermal cycler (Bioer) using following cycle: initial denaturation 95°C, 5min – [denaturation 95°C, 1 min – annealing 54°C, 3 min] – elongation 72°C, 1 min – final elongation 72°C, 10 min; the cycle of denaturation and annealing being performed 35x. The amplified fragments were visualised stained with ethidium bromide by electrophoresis in 1% agarose gel. Than the PCR product was purified either with the JetQuick PCR Purification Kit (Genomed), when there was clean algal PCR-product, or with the QIAquick Gel Extraction Kit (Quiagen), when also contaminating foreign DNA was amplified. Than, for part of strains, a 1/4 sequencing reaction and purification with

ethanol/ sodium acetate precipitation was performed using the ABI Prism Big-Dye terminator cycle sequencing ready reaction kit (Applied Biosystems) and the product proccessed on the ABI 3100 Avant automated sequencer (Applied Biosystems). The rest of strains was sent to the Macrogen company in the form of purified PCR product. The primers used in sequencing reaction are presented in tab 1.

Tab. 1: **Primers used in PCR and seguencing reactions.**

(o. = orientation: forward/reverse)

primer name	o.	sequence	PCR	seq.	citation
Katana F	F	AACCTGGTTGATCCTGCCAGT	✓		Katana et al. (2001)
34F	F	GTCTCAAAGATTAAGCCATGC	✓	✓	Friedl (unpubl.)
NS1	F	GTAGTCATATGCTTGTCT	✓		Hamby et al. (1988)
402-23F	F	GCTACCACATCCAAGGAAGGCA		✓	Katana et al. (2001)
1122F	F	GGCTGAAACTTAAAGGAATTG		✓	Friedl (unpubl.)
370R	R	AGGCTCCCTCTCCGGAATCRAACCC		✓	Friedl (unpubl.)
1263R	R	GAACGGCCATGCACCACC		✓	Friedl (unpubl.)
vivi (1650)	R	TCACCAGCACACCCAAT	✓	✓	Kipp (2004)
Katana R	R	TGATCCTTCTGCAGGTTCACCTACG	✓		Katana et al. (2001)
18L	R	CACCTACGGAAACCTTGTTACGACTT	✓		Hamby et al. (1988)

2.6 Analysis of molecular data

Sequence data reads were assebled together to complete sequences with Seqassem (Hepperle 2004). For each strain, a hundred of most similar sequences was searched using Blast algorythm (http://blast.ncbi.nlm.nih.gov/Blast.cgi, Basic Local Alignment Search

Tool; Altschul et al. 1990). All other sequences of representants of green algae were obtained form the on-line NCBI sequence database.

Sequences were edited using programs BioEdit (Hall 1999) and Mega (Tamura et al. 2007) and aligned with Muscle (Edgar & Robert 2004), Mega and Clustal X (Thompson et al. 1997). Phylogenetic analysis were computed with Paup, version 4.0b10 (Swofford 2000), PhyML (Guindon & Gascuel 2003) and Mr Bayes (Huelsenbeck & Ronquist 2001) the program MrMt Gui (Posada & Crandall 1998) was used to choose the appropriate substitution model. The topology of the final tree was taken from the Maximum Likelihood analysis made with Paup, bootstrap support was computed in PhyML (Maximum Likelihood) and Paup (Maximum Parsimony), and posterior probabilities in MrBayes. The GTR+Γ+I model was chosen as the best.

3. Results

3.1 Molecular analysis of 18S rRNA

In total, rRNA sequences of 23 strains were obtained. The lenght of the sequences spans from 1299 base pairs to 2144 base pairs, with the exception of the strain *Coenochloris koshikovii* CAUP, where only partial sequence of 543 bp was gained so far. In the final alignment, data sets of 1792 (Chlorophyceae) and 1569 (Trebouxiophyceae) nucleotide positions were analyzed (from these 323 and 316 were parsimony-informative).

The phylogeny as revealed by Maximum Likelihood analysis, is shown in figs. 1 and 2. Representants of Radiococcaceae are distributed within two classes of green algae, Chlorophyceae (10 strains) and Trebouxiophyceae (13 strains). Within these two groups the strains are scattered among a number of different phylogenetic lineages. I distinguished 7 clades in the first and 5 clades in the second class. The bootstrap values supporting the lineages vary from no or weak support to quite reliable numbers and are discussed individually for each lineage.

Regarding the sequences previously published and available from the NCBI database, no strain is related to *Coccomyxa* species in Trebouxiphyceae. The *Neocystis*-clade was put in close relativity to *Coenocystis inconstans* by some analysis, but with very poor bootstrap support, so that this relationship is not reliable. *Coenochloris planoconvexa* CAUP 5502 lies in Oocystaceae, as does *Schizochlamydella capsulata*, but they are not sister to each other. Not a lot can be assumed regarding the position of *Radiococcus polycoccus* Kr 98/4. It does not cluster with strains of *Radiococcus polycoccus* from SAG analyzed here.

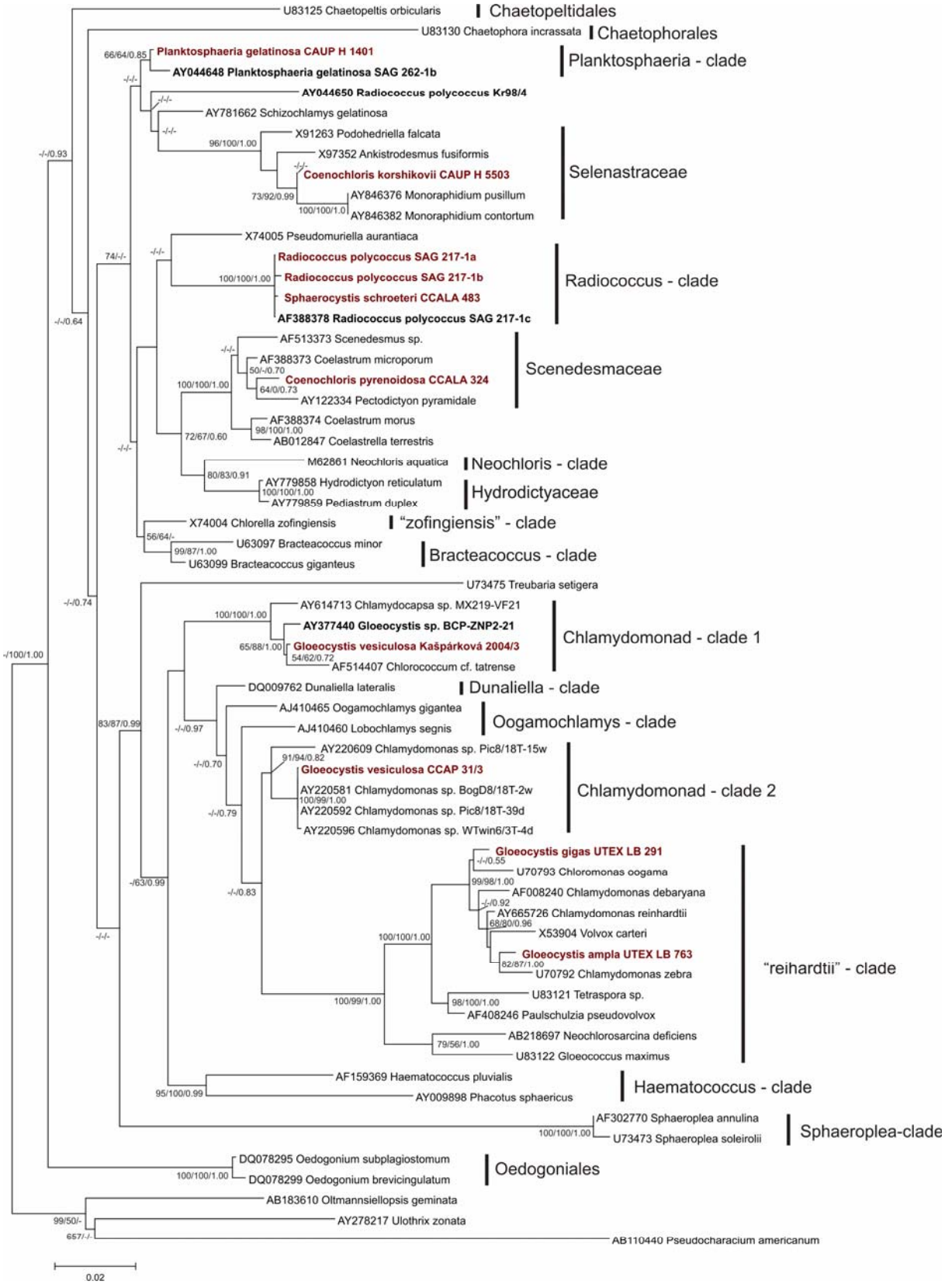


Fig. 1: Maximum likelihood tree of Chlorophyceae inferred by partial 18S rRNA sequences. ML/MP bootstrap values greater than 50% and Bayesian posterior probabilities greater than 0.5 are indicated.

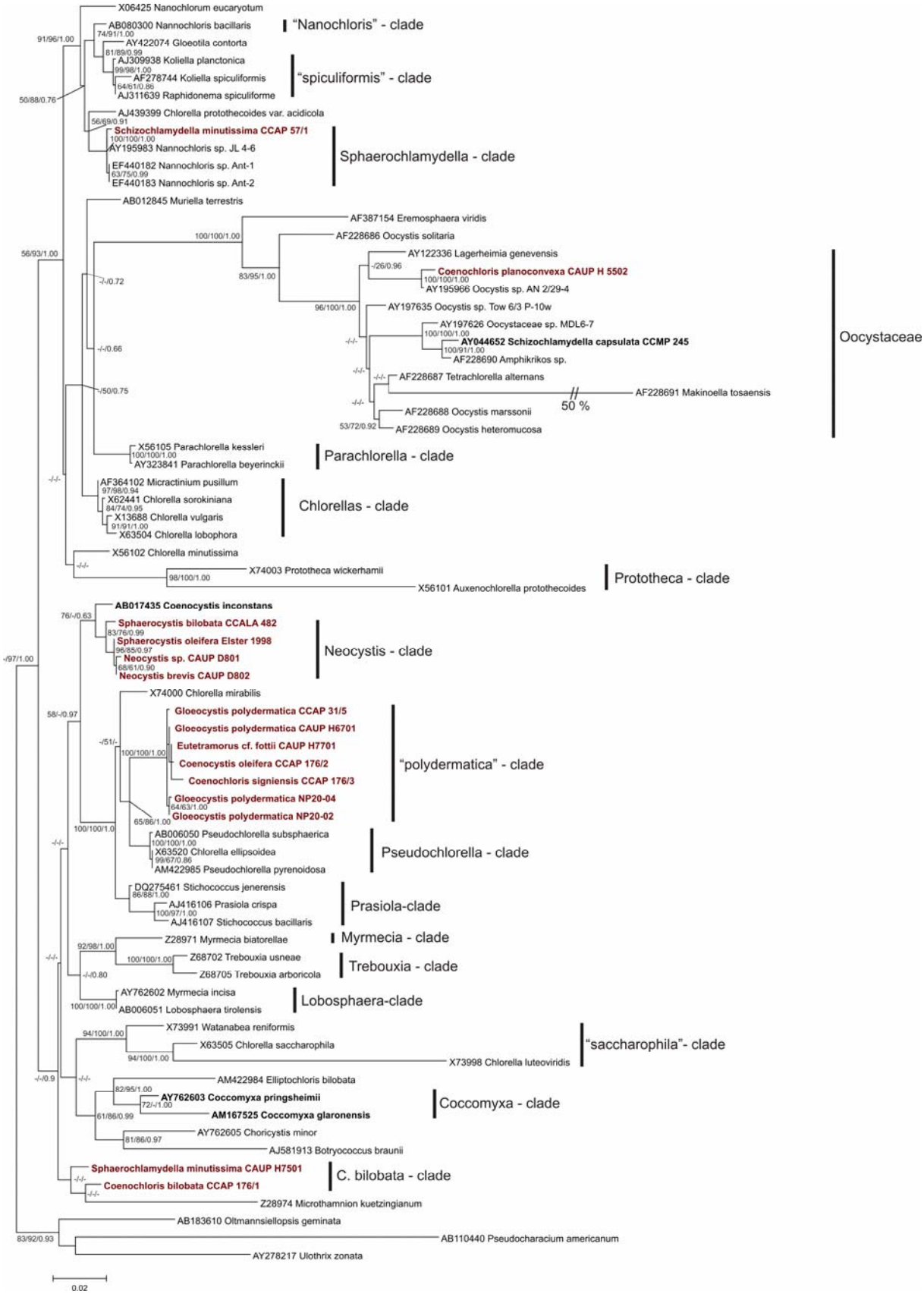


Fig. 2: Maximum likelihood tree of Trebouxiophyceae inferred by partial 18S rRNA sequences. ML/MP bootstrap values greater than 50% and Bayesian posterior probabilities greater than 0.5 are indicated.

3.2 Phylogenetic position of strains

3.2.1 Chlorophyceae

(***Planktosphaeria*-clade.**)

Planktosphaeria gelatinosa CAUP H 1401 pairs with *Planktosphaeria gelatinosa* SAG 262-1b (data published by Wolf et al. 2003). The position of this pair within Chlorophyceae is unclear and there is no other sequence in close relationship to them.

(***Sphaerocystis* -clade.**)

Strains of *Coenochloris polycocca* SAG 217-1a, SAG 217-1b, *Sphaerocystis schroeteri* CCALA 483 and *Coenochloris polycocca* 217-1c sequenced as *Radiococcus* by Wolf et al. (2003) cluster together constituting a new, well supproted clade within Chlorophyceae. There is not a relevant support for any group sister to them.

(**Selenastraceae.**)

Coenochloris korsikovii CAUP H 5503 is with high bootstrap support placed in the group Selenastraceae. Somewhat distant from other members of the clade, its closest relatives are *Monoraphidium pusillum* and *Monoraphidium contortum*.

(**Scenedesmaceae.**)

Coenochloris pyrenoidosa CCALA 324 lies with 100 % bootstrap values within Scenedesmaceae. In its closest neighbourhood *Coelastrum microporum* and *Pectodictyon pyramidale* is placed, but the bootstrap values inside the cluster are quite poor.

(***Chlamydomonas*-clade.**)

There are four strains marked as *Gloeocystis* spp. lying within the CW group of *Chlamydomonas* s.l. However, these strains do not constitute a single phylogenetic lineage.

a. *Gloeocystis vesiculosa* Kašpárková 2004/3 clusters with *Chlorococcum* cf. *tatrense*, *Gloeocystis* sp. and *Chlamydocapsa* sp.
b. *Gloeocystis vesiculosa* CCAP 31/3 clusters together with three unknown chlamydomonad species sequenced from environmental samples.

c. *Gloeocystis ampla* and *Gloeocystis gigas* are placed within the clade of „true chlamydomonas“ (Pröschold et al. 2001). *G. ampla* pairs with *Chlamydomonas zebra* in the neighbourhood of *C. reinhardtii* and *Volvox carteri*. The closest relative of *G. gigas* may be *Chloromonas oogama* or *Chlamydomonas debaryana*, but there is no bootstrap support for either of the two.

3.2.2 Trebouxiophyceae

(**Oocystaceae.**)

Coenochloris planoconvexa CAUP H 5502, together with previously sequenced *Schizochlamydella capsulata* CCMP 245 are members of the well delimited group of Oocystaceae. The two strains do not cluster together, however. The closest organism to *C. planoconvexa* is uncultured species of *Oocystis* from environmental sample. *S. capsulata* groups with *Amphikrikos* sp. and another unrecognized organism.

(***Sphaerochlamydella*-clade.**)

Schizochlamydella minutissima forms a tight group with unknown „pico-“ coccoid organisms (*Nannochloris* sp.) from environmental samples. This clade is probably a sister to *Chlorella protothecoides* var. *acidicola*.

(***Neocystis*-clade.**)

A new clade of soil trebouxiophytes is formed by four strains, *Neocystis brevis* CAUP D 802, *Neocystis* sp. CAUP D 801, *Sphaerocystis bilobata* CCALA 482 and *Sphaerocystis oleifera* Elster 1998/26. In some analysis *Coenocystis inconstans*, another radiococcacean strain, lies as a sister taxon to this group, but this topology has not a robust bootstrap values and is not supported by Maximum Parsimony. The two *Neocystis* strains group in a sufficiently supported pair together.

(***„polydermatica“*-clade.**)

Another new clade consists of seven analyzed strains: *Gloeocystis polydermatica* CAUP H 6701, *Gloeocystis polydermatica* CCAP 31/5, *Eutetramorus* cf. *fottii* CAUP H 7701, *Coenocystis oleifera* CCAP 176/2, *Coenocystis signiensis* CCAP 176/3 and two new

isolates of *Gloeocystis polydermatica* (NP 20-02, 20-04). It is a sister group of the *Pseudochlorella* clade. The inter-relationships within the group were not sufficiently solved, the strains are genetically very close.

(**Trebouxiophytes of uncertain position.**)

The position of *Sphaerochlamydella minutissima* CAUP H 7501 and *Coenochloris bilobata* CCAP 176/1 remains unclear. In the consensus tree computed with Maximum Parsimony the two strains clustered together, but the pair did not have a strong support and it was not featured in Maximum Likelihood tree either,

3.3 Morphology

In the following text, I summarize the information on morphology for all the strains analyzed. The strains are listed according to the order in previous chapter, with phylogenetically related strains grouped together. For authentic strains the remarks on morphological data from the original description (o.d.) are added. Taxonomical changes done in recent revisions are mentioned.

The morphology is definitely not the same in Chlorophyceae and Trebouxiophyceae. Though it is sometimes not possible to draw the strict line („always/never"), the different tendencies are summarized in tab. 2.

Tab. 2. Differences in morphology between Chlorophyceae and Trebouxiophyceae.

Chlorophyceae	Tebouxiophyceae
cells generally bigger	cells generally smaller
vegetative cells always spherical (lack of ellipsoidal stages; except monadoid cells in *Chlamydomonas*-clade or zoospores)	spherical or ellipsoidal or, quite often, both
chloroplast massive, complex, structured, occasionally many peripheral chloroplasts	chloroplast smooth, parietal, cup-shaped, band-shaped or trough shaped
if present, pyrenoid large and prominent, in some cases more than one per cell	if present, pyrenoid smaller and simple, with smooth margin; only one
zoospores and motile stages observed in some cases	no motile stages

Planktosphaeria gelatinosa CAUP H 1401

→ *Follicularia starii* (Lukešová 1993)

- no regular arrangement of cells, mucilage not visible in agar culture, fine mucilage observed in aerated culture after stainig with Indian ink
- cells spherical
- cell diameter 5–16 μm; extremities 25-27 μm
- chloroplast massive, structured, parietal, with one or more prominent pyrenoids, in old cells many chloroplast distributed on the periphery of the cell
- reproduction: probably 2+4+8+16 autospores – depends on culture conditions; 4–8 and only sporadically 16 autospores observed by Hindák (1984); zoospores observed when grown in aerated culture without light (confirmation of data of Hindák 1984)
- remnants of mother cell wall present
- thick cell walls, chlorophyl-free central area with nucleus

Coenochloris polycocca SAG 217-1a

→ *Radiococcus polycoccus* (Kostikov et al. 2002)

- cells envelopped in massive mucilagious covers, lying in a common mucilage, either solitary or in groups of 16 or more (or less) cells – several generations together
- cells spherical, huge
- cell diameter (12)21–36(42) μm
- chloroplast massive, filling the whole cell, with one or several pyrenoids
- reproduction by 16 or more autospores
- remnants of mother cell wall not observed

Coenochloris polycocca SAG 217-1b

→ *Radiococcus polycoccus* (Kostikov et al. 2002)

- morphology similar to the strain *Coenochloris polycocca* SAG 217-1a

Sphaerocystis schroeteri CCALA 483

→ genus zoosporic, not a member of Radiococcaceae (Kostikov et al. 2002)

- cells not arranged in liquid culture, on agar somewhat distributed in a mucilage
- cells spherical
- cell diameter (5,5–)8–19(–45) µm
- chloroplast parietal, filling the whole cell, with 1 or more (up to 9) massive pyrenoids
- polysporic sporangia of (8) 16 or 32 cells
- empty sporangia present
- old cultures bright orange

Coenochloris korsikovii CAUP H 5503 – Authentic Strain

- no regular arrangement of cells, mucilage not visible in agar culture, traces of mucilage present in aerated liquid culture
- cells spherical, or almost spherical
- cell breadth 5–13 µm; cell length 6–14 µm; smaller (about 7 µm) in liquid culture
- chloroplast massive, structured, parietal, with no visible pyrenoid but conspicuous chlorophyl-free central area
- reproduction: 2 or 4 or 8 autospores, polysporic sporangia (aplanosporangia?) in aerated liquid culture
- remnants of mother cell wall not observed

Coenochloris pyrenoidosa CCALA 324

- no regular arrangement of cells, mucilage not visible, neither in agar nor in aerated culture
- cells spherical
- cell diameter 5–12–(16) µm
- chloroplast massive, structured, parietal, in old cells many chloroplast attached to the cell wall; with one or two pyrenoids

• reproduction: very scarce in agar culture (2 autospores solely), frequent in shaked culture with 2, 4 or 8 autospores

• mother cell wall cracks and opens in two halves

Gloeocystis vesiculosa isol. Kašpárková 2004/3

• no regular arrangement of cells, mucilage not visible (even when cultivated in liquid culture, only sporangial envelopes react with methylene blue)

• cells mainly spherical, or broadly oval, autospores oval

• cell diameter 6–12µm, sporangium up to 17µm

• chloroplast massive, structured, in some cells cup shaped or lobate with thickened basal part, with massive pyrenoid

• reproduction: (2) or 4 autospores

• remnants of mother cell wall present

Gloeocystis vesiculosa CCAP 31/3

• cells in envelopes in groups of 2 or 4 or many (up to 20), after staining with methylene blue layered mucilage was visible in both agar and aerated cultures

• cells spherical or broadly ellipsoidal to pyriform or monadoid

• cell breadth (5)7–9,5 µm, min. 5 µm (autospore); cell length (6)8–9,5 µm

• chloroplast structured, with massive pyrenoid, sometimes trough-like (in monadoid cells), sometimes with two lobes and a cavity that is surrounded by tiny vacuoles

• reproduction: 2 or 4 autospores

• remnants of mother cell not observed

• motile cells not observed, but apical papila and perhaps contractile vacuoles present

Gloeocystis ampla UTEX LB 763

→ *Chlamydocapsa ampla* (Fott 1972)

• layered mucilage in agar culture, but suprisingly, mucilage not present in aerated culture

• cells spherical or monadoid, motile monadoid cells present (in both agar/aerated c.)

• cell breadth 6–10 μm; cell lenght 7–9 μm (spherical cells) to 9–11 μm (monadoid cells), sporangium 7,5×11 μm

• chloroplast parietal, cup-shaped with massive pyrenoid in the thickened basal part

• reproduction: division in two or four, in all directions

• remnants of broken mother cell not observed, but sometimes an empty cell wall as undivided shell was visible

Gloeocystis gigas UTEX LB 291

• no regular arrangement of cells, mucilage not visible in agar culture

• cells spherical or monadoid

• cell diameter 9–11–12 μm (sphaecial cells), 8×11 μm (monads)

• chloroplast parietal with a massive pyrenoid in the basal part (huge starch grains)

• one or two vacuoles near the apex of the monadoid cell

• reproduction unknown (probably 2 autospores)

• remnants of mother cell not observed

Coenochloris planoconvexa CAUP H 5502 – Authentic Strain

• no regular arrangement of cells, mucilage not visible in agar culture, but unusual ray-like shaped mucilaginous cover observed in aerated culture

• cells mostly ellipsoid to broadly oval, oocystis-like, often assymetrical

• cell breadth 4–6–(8) μm; cell length (4,5) –8–9 μm

• chloroplast parietal, trough-shaped with smooth, faint but clearly visible pyrenoid surrounded by small starch grains

• reproduction: only scarcely 2 autospores observed; 4 autospores according to o.d., ocasional production of 2 or 8 autospore noted by later observation of the author (Hindák 1980)

• remnants of mother cell wall present, mcw splits and into two or several parts (Hindák 1977)

Schizochlamydella minutissima CCAP 57/1 – Authentic Strain

→ *Sphaerochlamydella minutissima* (Kostikov et al. 2002)

- no regular arrangement of cells, mucilage not visible, neither in agar nor in aerated culture
- cells strictly spherical, very small
- cell diameter 2–3,5–6 μm; (exceptional cell 9 μm)
- chloroplast parietal, bilobate, often with a single large vacuole between the lobes
- tiny vacuoles on the surface of the cell (almost regularly)
- reproduction: autospores 4 or 8, scarcely observed; usually 4, but also 2 and 8 autospores according to o.d.
- remnants of mother cell wall not observed

***Neocystis* sp.** CAUP D 801

- no regular arrangement of cells, mucilage not visible in agar culture, fine mucilage observed in aerated culture after stainig with Indian ink
- cells mostly oval, some broadly oval or spherical
- cell breadth 2–6 μm; cell length 4–8 μm;
- chloroplast parietal, lobate
- reproduction: 2–(4) autospores; ellipsoidal sporangia
- remnants of mother cell wall present

Neocystis brevis CAUP D 802 – Authentic Strain

- no regular arrangement of cells, mucilage not visible in agar culture, fine mucilage observed in aerated culture after stainig with Indian ink
- cells elongate, ellipsoid, broadly oval to spherical
- cell breadth 2–5,8 μm; cell length 3,5–8 μm
- chloroplast parietal, bilobate
- reproduction: (2)–4–(8) autospores, broadly ellipsoidal sporangia

- remnants of mother cell wall present

Sphaerocystis bilobata CCALA 482

→ *Radiococcus bilobatus* (Kostikov et al. 2002)

- no regular arrangement of cells, mucilage not visible in agar culture
- cells spherical or broadly oval
- cell breadth 3–8 µm; cell length 4,5–8,5 µm
- chloroplast smooth, parietal, cup-shaped, with a pyrenoid surrounded by a ring of starch grains
- reproduction by (2), 4 and 8 autospores
- remnants of mother cell not observed

Sphaerocystis oleifera strain Elster 98/26

→ *Coenochloris oleifera* (Kostikov et al. 2002)

- no regular arrangement of cells, mucilage not visible
- small cells ellipsoidal, almost oocystoid, big cells spherical
- cell breadth 3,5–7 µm; cell length 5–9 µm
- chloroplast parietal, bilobate, pyrenoid hardly visible
- reproduction: 4 or 8 autospores
- remnants of mother cell present

Gloeocystis polydermatica CAUP H 6701 – Authentic Strain

→ *Sporotetras polydermatica* (Kostikov et al. 2002)

- no regular arrangement of cells, mucilage not visible in agar culture
- cells ellipsoidal, broadly ellipsoidal to spherical
- cell breadth 3,5–7 µm; cell length 6–9 µm
- chloroplast smooth, parietal, band shaped with fine pyrenoid
- reproduction: 2 or 4 autospores, ellipsoid sporangia
- remnants of mother cell wall not observed

Gloeocystis polydermatica CCAP 31/5

→ *Sporotetras polydermatica* (Kostikov et al. 2002)

- no regular arrangement of cells, faint mucilage visible in agar culture after staining with methylen blue: mucilaginous envelopes around individual cells and sporangia, thin, not layered
- cells elongated ellipsoidal, broadly ellipsoidal to spherical
- cell breadth 4–10 µm; cell length 5–11 µm
- chloroplast parietal, cup-shaped, with easily visible, sometimes prominent pyrenoid
- reproduction: autospores (2) or 4, parallel or tetrahedrical arrangement
- remnants of mother cell not observed

Gloeocystis polydermatica NP 20.04

→ *Sporotetras polydermatica* (Kostikov et al. 2002)

- cells in a common mucilage, often arranged in groups of eight (after release of autospores)
- cells mainly ellipsoidal or broadly ellipsoidal, or less often spherical
- cell breadth 5–11 µm; cell length 5–11 µm
- chloroplast parietal with pyrenoid
- reproduction by 8 autospores
- remnants of mother cell wall not observed

Gloeocystis polydermatica NP 20.02

→ *Sporotetras polydermatica* (Kostikov et al. 2002)

- no regular arrangement of cells, mucilage not visible
- cells ellipsoidal or broadly ellipsoidal, spherical cell observed only exceptionally
- cell breadth 4–7(–10) µm; cell length 7–10 µm
- chloroplast parietal with simple but clearly visible pyrenoid
- reproduction by 2 or 4 autospores

• remnants of mother cell wall not observed

Eutetramorus* cf. *fottii CAUP H 7701

→ *Radiococcus* sp. according to T. Darienko (pers. comm.)

• cells not regularly arranged, but often grouping in four or eight, mucilage not easily visible, but present at least in aerated cultures

• cells mostly spherical or broadly oval

• cell breadth 5–12 µm; cell length 5–12 µm, extreme cell up to 15 µm

• chloroplast smooth, parietal, characteristic(ally) band-shaped (*Chlorella luteoviridis* – like), with fine small pyrenoid (not surrounded by starch grains)

• reproduction: 4–(8) autospores

• remnants of mother cell wall present and quite easily visible

Coenocystis oleifera CCAP 176/2 – Authentic Strain

→ *Coenochloris oleifera* (Kostikov et al. 2002)

• no regular arrangement of cells, mucilage not visible in agar culture

• cells both ellipsoidal and spherical

• cell breadth 5–10,5 µm; cell length 7–11,5 µm (up to 11 µm according to o.d.)

• chloroplast parietal, band-shaped or cup shaped to lobed or deeply curved in adult sphaerical cells, with a fine pyrenoid

• reproduction by 4 – 8 autospores (rarely 2 and 16 according to o.d.), ellipsoidal sporangia

• remnants of mother cell not observed clearly, but may be present according to o.d.

Coenochloris signiensis CCAP 176/3 – Authentic Strain

→ *Radiococcus signiensis* according to the latest revision (Kostikov et al. 2002)

(unfortunately, during all observations the strain was not in good condition)

• no regular arrangement of cells, mucilage not visible in agar culture

• cells spherical to broadly ellipsoidal

• cell breadth 4–7 µm; cell length 6–8 µm

- chloroplast parietal with a simple pyrenoid, may be surrounded by starch grains
- reproduction: only 2 autospores observed; 2 or 4 or rarely 8 according to o.d.
- remnants of mother cell present

Sphaerochlamydella minutissima CAUP H 7501

- cells regullarly arranged in pairs or in fours in a common mucilage, with ± similar distances among them, sometimes with individual envelopes around groups of 2 or 4; suprisingly different appearance in aerated culture: not regular, tight clusters of at least 8 cells)
- cells ellipsoidal to spherical, strikingly small
- cell breadth 2–5 µm; cell length 3–6 µm
- chloroplast parietal, cup-shaped, trough-shaped or bilobed
- reproduction: 2 or 4, rarely 8 autospores
- remnants of mother cell wall not observed so far

Coenochloris bilobata CCAP 176/1 – Authentic Strain

→ *Radiococcus bilobatus* (Kostikov et al. 2002)

- no regular arrangement of cells, mucilage not visible in agar culture
- young cells elongated, adult cells spherical
- cell breadth 4–9(–11) µm; cell length 6–12 µm; (usually 6 µm and up to 8,5 µm according to o.d.)
- chloroplast parietal, often thin and deeply bilobed, pyrenoid not observed in this study but should be present according to the original description
- reproduction by 4 + 8 autospores
- remnants of mother cell not observed but should be present acording to o.d.

4. Discussion

The primary aim of this work was to assess the phylogenetic diversity of representants of the traditional family *Radiococcacae*. The need of molecular data in taxonomy of green algae was stressed elsewhere (e.g. Pröschold & Leliaert 2007) and in our particular case by Kostikov et al. (2002). It was already shown by Wolf et al. (2003) that the family is polyphyletic. With more molecular data available, this work bears a robust evidence, that the group *Radiococcaceae* is rather a mixture of many unrelated taxa. New lineages of trebouxiophyte and chlorophyte algae were also discovered.

The taxonomy of the family Radiococcaceae was based on common morphological characters – in the same way as the majority of green algal taxa was classified (Komárek & Fott 1983, Ettl & Gärtner 1994). With the introduction of molecular techniques, since about 20 years ago, the traditional taxonomical concept was challenged. New analysis discovered for example fragmentation of genera into different classes (e.g. *Chlorella*, Huss et al. 1999, *Botryococcus*, Senousy et al. 2004, *Muriella*, Hanagata 1998, *Pediastrum,* Buchheim et al. 2005), or gathered morphologically dissimilar taxa into a single lineage of close relatives (e.g. *Micractinium* and *Chlorella*, Krienitz et al. 2004, Luo et al. 2006). This work bears new examples of both cases – distributing the species within Chlorophyceae and Trebouxiophyceae and unifying strains of different labels in well supported clades on the other hand – and follows the line of revealing hidden diversity among green algae (Fawley et al. 2004, Lewis & Flechtner 2004, Vanormelingen et al. 2007).

4.1 molecular and morphological approach in taxonomy and determination

The unexpected number of clades involved in this analysis of Radiococcaceae supports the strong need of revision of the point of view we used to take when dealing with the taxonomy of green algae. For example, in our case, a use of mucilage production as a taxonomical criterion on the level of family is untenable.

It is more than evident that the family Radiococcaceae as it was understood does not exist. It is therefore necessary to find a new concept to sort out the former radiococcacean taxa. This cannot be done without molecular tools. Isolation of more strains, covering as much morphological variability as possible, together with phylogenetic analysis of the strains, seems necessary to allow a general revision of the group. This process would comprise two steps: first, we need to sort out the specimens of Chlorophyceae and Trebouxiophyceae and secondly to define each new clade by specific combination of characters.

I am convinced that for the use in every-day practice (at least before handy all-in-minute barcoding machine is invented and widely available), we should keep the traditional tools of determination , i. e. a simple system based on morphological characters with the support of ecology or perhaps physiology – but the criteria should be revised and updated according to latest progress in molecular taxonomy.

Not only for every-day determination the morphology is important. Thorough morphological observations and studies of life cycle should not be replaced by sequencing even in taxonomy. Molecular taxonomists often use strains from culture collection without revising its identity, which could lead to misinterpretation of molecularr data. For example, Senousy et al. 2004 discussed the species concept in *Botryococcus*, where the species *B. sudeticus* was by some authors recognized as *Botryosphaera* or *Botryosphaerella*. While molecular data clearly showed that the *B. sudetica* is totally unrelated to most representants of *Botryococcus*, the issue was left open and no taxonomic conclusion was made. The strain and its sequence are still labeled as *Botryococcus* which could possibly cause confusion in other studies. Further evidence was than added by Přibyl & Cepák (2007) who reported zoospore production in the same strain of *B. sudetica*. On the basis of that observation they finally removed *B.sudetica* from the autosporic genus *Botryococcus*.

4.2 evaluation of morphological criteria

The most serious issue in the generic system of Radiococcaceae is the stability of morphological traits when comparing fresh material with cultivated one.

In general, the applicability of a particular morphological trait on a particular taxonomical level, is limited by its variability on that level. Only markers of certain stability can be chosen as taxonomical criteria (for example, when the number of pyrenoids, hypothetically, spans from two to four, no one would chose the character „three pyrenoids“ as a relevant marker). In case of Radiococcaceae, however, the morphology often changes significantly when the alga is removed from natural sample to culture and also when different cultivation conditions are applied (like for example the shape of colony and form of mucilage, as will be discussed later).

Most descriptions of the radiococcacean genera were based on observation of fresh material (e.g. Koršikov 1953), only few authentic strains are available from more recent studies (Kostikov & Hoffmann 2000; Broady 1976, 1982; Hindák 1977, 1978,1984). But to describe the morphology thoroughly – to see the shape of the chloroplast or to observe the whole life cycle for example – we usually need the cultivation and a long-term observation. Because of the lack of authentic cultures, for most of the radiococcacean taxa it is impossible to verify the morphology of the type species and to amend the eventual cavities in the description.

Moreover, the morphology of authentic strains does not necessarily correspond with the original description after decades of cultivation. This can be a result of either mutations or adaptations to distinct environmental conditions in cultivation (more constant, free of predators, free of water movement etc.). The AFLP data obtained by Muller et al. (2005) showed no differences in genetic material of several clones kept in different collections for many years: it seems, supprisingly, that strains in culture collections remain (relatively) genetically stable. Than the possibility of adaptive morphological changes should be examined.

The importance of culture collection increases with further development of taxonomy as well as applied biosciences and it is necessary to make sure we put an accurate label on the organism kept in cultivation (Silva 2007). For these reasons it is better to use criteria that are present in both fresh and cultivated material.

In the next part, the morphological criteria studied in this work are discussed.

4.2.1 presence of mucilage

Presence of mucilaginous envelope around the cell, underlying the whole existence of Radiococcaceae, is not a stable and reliable marker.

The ability to produce mucilage is not an exclusive character of few algal taxa, but rather a feature commonly observed in various groups of green algae, like for example Oocystaceae or even desmids (Komárek & Fott 1983). A problem is that the presence of mucilage is highly influenced by the environmental factors, in our case by culture conditions. When comparing the fresh and cultivated material, this trait is probably the most unstable one.

Even though all 23 strains in my project were determined as members of Radiococcaceae, less than half produced mucilage when growing on agar or in aerated liquid medium. Seven strains (one chlorophyte, six trebouxiophytes) did not show any mucilage at all during the cultivation. Four of them are authentic strains isolated in Antarctica by Broady (1976, 1982) whose morphology was rigorously characterized in the description. The strains *Coenocystis oleifera* (CCAP 176/2) and *Coenochloris signiensis* (CCAP 176/3) were even supposed to form slightly layered envelopes.

Coenochloris planoconvexa (CAUP H 5502) would not exhibit any mucilage growing on agar, but produced remarkable ray-like shaped mucilage when moved to aerated liquid medium. Comparable shape of mucilage was described only in *Echinocoleum elegans*, family Oocystaceae.

In some cases, the mucilage or its proper structure is not visible in the light microscope without stainig. For the layered structure, methylen blue is appropriate,

whilst for simple detection of a fine diffluent envelope, Indian ink works better. It is probable that without staining the mucilage would not be detected in many algae. This could lead to ambiguous determination, when a simple coccal cell is labelled for example either as *Chlorella* (when the mucilage was not detected) or as *Coenochloris.*

As was pointed out by Kostikov et al. (2002), we deal with different kind of mucilaginous material: thick or thin, strong or weak, distinct or diffluent, spherical or of various shapes or shapeless... This fact too was often negliged in the taxonomy. (For example, the envelope of the three *Coenochloris polycocca* strains from SAG reached about 2-3μm in thickness, was layered and prominent regardless the cultivation. This surely is not the same character like the unstable ray-like structure seen on *Coenochloris planoconvexa* or faint pall of mucilage around *Neocystis* spp. visible only after staining with Indian ink.)

The layered structure of mucilage, characterictic for *Gloeocystis* and some other genera, was only scarcely observed on cultured material (hints in *Gloeocystis polydermatica* CCAP 31/5), and never so distinct like in the fresh sample. This is not the case of chlamydomonad species, where the envelopes are often conspicuously layered, but here the structure is rather a layered sporangium cell wall which may eventually gelatinize. The change of mucilage character during cultivation of *Planktosphaeria gelatinosa* mentioned also Hindák (1984).

There was also a special form of radially structured mucilage described in the genus *Radiococcus*. Nothing like it I observed on the analyzed strains. As I mentioned in the introduction, the ray-like structure was doubted by Komárek 1974 and Hindák 1984. Without robust evidence by thorough microscopic analysis, their hypothesis that the ray-like structure was probably caused by associated rod-like bacteria is to be accepted.

Few years ago, *Parachlorela beijerinckii* Krienitz et al. (2004) was described as a new species in a sister group of *Chlorella*. It was a simple mucilage producing coccoid green alga, but because the description was strongly based on molecular data, its classification within Radiococcaceae was not considered.

4.2.2 shape of the colony and arrangement of cells

Because all the mophological observations were done on a cultured material, it was not possible to see the apperance of the taxa in the natural state. This refers especially to the shape of the whole mucilaginous colony, that should be spherical in many planctic taxa (e.g. genera *Radiococcus, Eutetramorus, Sphaerocystis, Coenochloris*). Unfortunately, a determined shape of colony was not seen in any agar or liquid culture in this work. For strains in culture collections it could be difficult or even impossible to obtain original morphological data on the material from which the strain was isolated. The use of such a marker, observable only on fresh material, is questionable.

The arrangement of cells is also an issue. Cells grouped in two, four or more within a sporangial wall were present in chlamydomonad strains, but this does not correspond to the character of arrangement as it was understood for example by Koršikov (1953). Groups of four or eight after autospore release were observed in some strains from the „*polydermatica*" clade. Cells of strains form the *Sphaerocystis* clade were in groups of 16 or more, several generations together. The most distinct arrangement was observed in agar culture of *Sphaerochlamydella minutissima* (CAUP H 7501), nice pairs of cells distributed in more or less even distance to each other. But this appearance changed dramatically when the strain was transferred to aerated liquid culture: the cells grouped in eights and were haphasardly assembled in clusters in the medium.

4.2.3 sporangial cell wall behaviour

Various ways how the autospores are released from the mother cell were thoroughly described by Kostikov et al. (2002). Basically, there are two possibilities: rupture (which leaves remnants of the cell wall around the young daughter cells) or gelatinisation (the mother cell wall turns in gelatinous sheath around the daughter cell). This model has many modifications, for example rupture of the mother cell wall (mcw) and later gelatinisation of the fragments. Some authors (Kostikov 1953, Komárek & Fott 1983) distinguished the „late gelatinization", Kostikov et al. (2002) relied only on the presence of mother cell wall fragments in the sample. Again, it brings the question of applicability of

the marker. It is obvious that one can easily misjudge this character based on a single observation.

During my observations, the question of mcw behaviour was not always succesfully solved and in few cases, for example only once a single fragment was observed. In the *Neocystis*-clade, the mother cell wall splits and its fragments are scattered around the cells in all strains. In the *„polydermatica"*-clade the remnants were not observed except *Eutetramorus* cf. *fottii* (CAUP H 7701) and only once a ruptured empty cell wall was seen in *Coenocystis signiensis* (CCAP 176/3). According to Broady (1976) the remnants should be visible in *C. signiensis* (CCAP 176/3) and *C. oleifera* (CCAP 176/2). Thus in this case, mcw behaviour is not specific for the phylogenetic lineage. An exceptionally clear form of cleavage was observed in culture of *Coenochloris pyrenoidosa* (CCALA 324). Here the sporangium wall splits in two more or less adequate portions.

As a good illustration, the strain *Eutetramorus* cf. *fottii* (CAUP H 7701) can be mentioned. It was isolated and subject to thorough microscopy analysis by Škaloud (2004) without an observation of mcw fragmentation. Few years later, the strain was revised by T. Darienko (unpubl.) and determined as *Radiococcus* sp. because of the presence of mcw remnants. During this study, mcw remnants were also clearly visible. However, this feature was not evident in the fresh material and in newly established culture (Škaloud 2004).

4.2.4 vegetative cell shape

In the group of Radiococcaceae sphaerical or ellipsoid vegetative cells were traditionally recognized. According to Kostikov et al. (2002), three types of morphology are to be seen: cells always spherical, cells sometimes spherical and cells never spherical. The first two groups were put together in the latest revision of the system (Kostikov et al. 2002). I suppose that this decision was not appropriate, leading to the underestimation of the general appearance of the organism. In almost all ellipsoid-celled taxa in this study it was possible to observe at least few spherical cells, or there were mainly ellipsoidal with the minority of spherical cells.

The best results were obtained when this particular marker was used on the level of classes. Only spherical cells were present in all chlorophyte strains (with the exception of the chlamydomonas group). The trebouxiophytes can have morphology of all three types, i. e. cells spherical (*Schizochlamydella minutissima* CCAP 57/1) or ellipsoidal (*Coenochloris planoconvexa* CAUP H 5502, more or less also *Gloeocystis polydermatica* NP 20-02 and 20-04) or both (most of trebouxiophyte strains). The cell shape give a good information when correlated with cell size (see below).

4.2.5 cell size

The vegetative cell size was not used on generic level in any relevant review /systems/ of Radiococcaceae (Koršikov 1953, Bourrelly 1966, Fott 1974, Komárek and Fott 1983, Kostikov et al. 2002), (sometimes it was used to distinguish species). However, the cell size corresponds more or less with the distinction of two classes, Chlorophyceae and Trebouxiophyceae.

4.2.6 pyrenoid

The presence/absence of pyrenoid was applied either on the generic or species level in green algae and in Radiococcaceae too (Hindák 1977, Komárek & Fott 1983). This ambiguous use was criticised by Kostikov et al. (2002), who established a system with pyrenoid as generic marker. There are several issues concening such a use.

Not every microscopic observation would detect the presence of pyrenoid. For example, in the description of *Coenocystis inconstans*, the authors state that „a pyrenoid is present, but hardly distinguished from the chloroplast stroma“ (Hanagata & Chihara 1999). In case of *Cenochloris korsikovii* (CAUP H 5503) in this study it was not possible to detect a pyrenoid with only the light microscopy, but the isolator and autor of the description stated the alga possessed a nude pyrenoid (Hindák 1984).

Nozaki et al. (1998) bore evidence that the precence of pyrenoid can depend on environmental conditions: 17 of 23 strains of *Chlorogonium* (all commonly with pyrenoids) lost or showed only reduced pyrenoids in medium with organic compounds.

Molecular data can even advocate the use of pyrenoid presence on the generic level – for example, in traditinal genus *Chlorella* s.l., the presence of pyrenoid was characteristic for about half of the species (Fott & Nováková 1969). After the revisions based on phylogenetic analysis, all species of „true chlorellas" have cells with pyrenoid (Krienitz et al. 2004). However, this was not the case of the *Neocystis*-clade in this work. On the other hand, the same marker has no taxonomical significance on the generic and even species level in *Chlamydomonas* and *Chloromonas*. The study of Pröschold et al. (2001) showed that species of both genera, that were traditionally recognized according to the presence resp. absence of pyrenoid, are mixed together in several phylogenetic lineages and the lost of pyrenoid occured more than once in the evolution of chlamydomonad species.

During my observations the pyrenoid was present in all but one chlorophyceae, in several strains more than one per cell (up to nine pyrenoids in *Sphaerocystis schroeteri* CCALA 483). In trebouxiphyceae the presence was either characteristic for the whole clade (e.g. the „*polydermatica*"-clade) or only for some members of the phylogenetic lineage (not observed in both *Neocystis* strains, hardly visible but present in *Sphaerocystis oleifera* isol. Elster 1998/26, present and surrounded by little starch grains in *S. bilobata* CCALA 482). These results advocate that the presence of pyrenoid should not be used as taxonomical criterion without the justification by molecular data.

What I find important is the form of pyrenoid. Among Trebouxiophyceae, the pyrenoid was always single, rather small and simple, without prominent margin, sometimes surrounded by little rounded starch grains, sometimes without. Among Chlorophyceae the pyrenoid was massive and bordered by prominent envelope.

4.2.7 number of chloroplasts

Usually, there is only one chloroplast per cell in Radiococcaceae. The only exceptions are *Planktosphaeria gelatinosa, Phacomyxa sphagnophila* and *Palmodictyon varium*. Among the 23 strains analyzed, numerous chloroplasts were observed only in adult cells of *Planktosphaeria gelatinosa* (CAUP H 1401). A similar feature was found also in *Coenochloris pyrenoidosa* (CCALA 324), but it is not sure yet whether the stage was an

adult cell or an early development of sporangium. Cells with numerous chloroplasts are unknown among Scenedesmaceae, where the strain belongs to (Komárek & Fott 1983).

4.2.8 shape of chloroplast

This marker was only occasionally used to separate few genera of distinct chloroplast shape, e.g. *Chondrosphaera* and *Pseudotetraspora* with stellate chloroplast in Fott (1974) and Bourrelly (1966), respectively. On the species level the shape of chloroplast was used by Komárek & Fott (1983) to separate between *Coenochloris mucosa* and *C. sphagnicola* and between *Palmodictyon* species.

Here very different appearance of chloroplast was found when comparing Chlorophyceae and Trebouxiophyceae. In the first class, the chloroplast was (~~almost~~) always massive and not smooth, filling the whole volume of cell except central area with nucleotide. In two strains it divided later into many small periferal chloroplast (see above). In the second class, the chloroplast was smooth and often comprised only part of the cell. Four distinct types of chloroplast were found among Trebouxiophyceae: cup-shaped, band-shaped, bilobed and through-like. Unfortunately, usually it was not possible to use a particular shape of chloroplast as a diacritical trait for whole clade. Through-shaped chloroplast, common among Oocystaceae, was found in *Coenochloris planoconvexa* (CAUP H 5502). Bilobed type was typical for *Schizochlamydella minutissima* (CAUP 57/1), *Coenochloris bilobata* (CAUP 176/1) and the *Neocystis*-clade, where, unfortunatedly, was not always apparent in *S. bilobata* (CCALA 482) and *S. oleifera* (isol. Elster 1998/26). Band shaped chloroplast was pronounced in some strains in the „*polydermatica*"-clade.

Recently, taxonomical stadies using polyphasic approach advocated use of chloroplast shape in taxonomy (Pröschold et al. 2001). In case of chlorophycean strains, the use of confocal microscopy could bear interesting results too.

4.2.9 number of autospores and mode of reproduction

The use of this autospore number on the generic level was given weight by Kostikov et al. (2002). In works of other authors (Koršikov 1953, Fott 1974, Hindák 1977, Komárek & Fott 1983) it was more a complementary criterion, or eventually was not used at all (Bourrelly 1966).

Kostikov distinguished two main groups of species: those producing „a small number, generally two" autospores and those producing generally four or eight. (The word „generally" means that exceptions were accepted). But for example in Hindák (1977), *Radiococcus* and *Coenochloris* differed in production of „always four" or „four or eight" autospores. According to Kostikov's concept, these two genera were in the same group.

Kostikov also admitted, that the number of autospores may depend on culture conditions (based on unpublished data) and „the stability of this feature needs further investigations" (Kostikov et al. 2002). This assumption was fully supported by my observations: the number of autospores proved to be the most variable morphological trait in different conditions of cultivation. In general, whilst some strains do not change at all (mostly Trebouxiophyceae), for others the number of autospores increases considerably when the strain is transferred from agar to liguid culture (mostly Chlorophyceae).

This could correspond with the finding of Hindák (1984) when the fresh material of *Planktosphaeria gelatinosa* produced commonly 16 or 32 autospores and the cultivated 4 or 8 autospores. It is possible that the aerated liguid culture simulates better the natural environment and so the number of autospores is closer to the state *in situ*.

Trebouxiophyte strains tend to be more stable, with the only exception of *Sphaerochlamydella minutissima* (CAUP 57/1) that produced much more eight-celled sporangia in liguid culture than on agar. Polysporic sporangia were not observed in any member of Trebouxiophyceae. On the other hand the number of autospores in Chlorophyceae was highly variable, spanning from two (on agar) to 32 (liquid culture for *Coenochloris hindakii* or any cultivation for the *Sphaerocystis*-clade) and was often limited by culture conditions.

The position of autospores was usually tetraedrical, only occasionaly parallel (*S. bilobata* isol. Elster 1998/26), but both types were observed in the same strain, so to this character no taxonomic weight is given, as was already done by Kostikov et al. (2002).

All chlorophyte strains were treated for zoospore production. The effort was succesful only in case of *Planktosphaeria gelatinosa* (CAUP H 1401), only supporting similar observation by Starr (1954) and Hindák (1984). The strain *Coenochloris hindakii* (CAUP H 5503) produced in the same conditions polysporic sporangia (aplanosporangia?). No other motile stages were observed except chlamydomonad strains *G. ampla* (UTEX LB 763) and *G. gigas* (UTEX LB 291).

4.3 taxonomical conclucions

Planktosphaeria gelatinosa CAUP H 1401

Under the name *Planktosphaeria gelatinosa* Smith (1915) a planctic organism showing no motile stages was described. The strain sequenced here was isolated by R. C. Starr in 1952 (deposited 1954). Based on his own isolates from soil, Starr reported the observation of zoospores in this species (Starr 1954). Some authors than understood the taxon as zoosporic, but others respected the original description and regarded Starr's zoosporic isolates as different organisms (Lukešová 1993, Ettl & Gärtner 1994, Kostikov et al. 2002).

According to A. Lukešová (unpubl.), zoosporic soil specimens commonly determined as *Planktosphaeria* probably all belong to the revised genus *Follicularia* MILLER 1924. This hypothesis needs to be verified by molecular data, however. In this work sequence of a freshwater and soil isolate cluster together. More confusingly, zoospore production was reported in both soil and planctic material by Hindák (1984).

Authentic strains of the type species of *Planktosphaeria* and *Follicularia* are not available, but authentic strains of other species of *Follicularia* are being analyzed by T. Pröschold (unpubl.). If *P. gelatinosa* sequenced here is related with *Follicularia* strains, than all the specimens should bear the name *Follicularia*. Or they could be all transferred to *Planktosphaeria*, with the emendation of the genus. If the two strains od *P. gelatinosa*

(CAUP H 1401 = UTEX B 124 and SAG 262-1b) lie in a distinct lineage, not with *Follicularia*, a new generic name should be applied.

Coenochloris pyrenoidosa SAG 217–1a, SAG 217–1b, SAG 217–1c and *Sphaerocystis schroeteri* CCALA 483

Although bearing different names, the strains SAG 217-1a, SAG 217-1b, SAG 217-1c and CCALA 483 show substantial similarity: relatively big sphaerical cells, massive chloroplast with prominent pyrenoid(s), thick gelatinizing layered cell walls, reproduction by numerous autospores. Being also phylogenetically related, these four strains should belong to a single genus.

The three SAG strains have different names in the collections of SAG and UTEX and according to Kostikov et al. (2002) and Wolf et al. (2003): *Coenochloris, Coelastrum* and *Radiococcus*. According to Koršikov (1953) the genus *Coenochloris* is characterized by oval cells in groups of four or eight (rarely 16) tightly accumulated in the centre of a structureless mucilage (the genus contained species either with or without pyrenoid). Athough it is not possible to observe the proper arrangement of cells in strains kept in agar cultures, I suppose that on the basis of cell size, number of pyrenoids, higher number of autospores and layered mucilaginizing cell wall, the name *Coenochloris* shold not be applied to the three strains discussed. The genus *Radiococcus* was firstly recognized by a radial structure in its mucilage, a feature that was discussed elsewhere (Fott 1974, Hindák 1984, above in this work), and after emendation (Fott 1974) by arrangement of cells in tetrads and reproduction by (~~strictly~~) four autospores (not (2–) 4–8 (–16) according to Kostikov et al. 2002). Therefore this name cannot be applied either. *Coelastrum* clearly has different morphology, here, for example, adult cells are not connected in coenobia, also sometimes more than one pyrenoid is present.

The four strains seem to fit the best within the delimitation of the genus *Sphaerocystis*: cells are spherical and tend to stay in groups after release of autospores, embedded within a substantial mucilage, with parietal chloroplast and one or more pyrenoids. Only the number of autospores commonly observed (usually 32 or 16) exceeds

the number presented in literature (4, 8 or 16) (Koršikov 1953) and the cell wall is rather thick. I suppose that in this situation, it is not reasonable to establish a new genus, omitting the traditionaly used, though poorly defined name. Because there is not an authentic culture of a type species, *Sphaerocystis schroeterii*, and because the genus was already emended, anyway, I suggest to establish the strain *Sphaerocystis schroeterii* as an epitype of the emended genus *Sphaerocystis* and to move the three strains of *Coenochloris polycocca* into this genus.

Coenochloris korsikovii CAUP H 5503

The authentic strain of *Coenochloris korsikovii* (CAUP H 5503) lies within the family Selenastraceae, most close to *Monoraphidium* spp., but not in close relationship to any particular isolate. The simple morphology is quite exceptional within the group, where species with elongated, sometimes curved or twisted cells are mostly present. The 18S rRNA data analysis itself was for Krienitz et al. (2004) a reason to establish a new taxon. Here the morphological as well as phylogenetic distinction of the strain *Coenochloris korsikovii* (CAUP H 5503) among Selenastraceae bears arguments enough to establish a new genus for the strain CAUP 5503. Because the alga did not commonly exhibit mucilaginous envelopes and fragments of broken mother cell wall and produced polysporic sporangia (probably aplanosporangia) under certain conditions, I do not find adequate to call it *Coenochloris*.

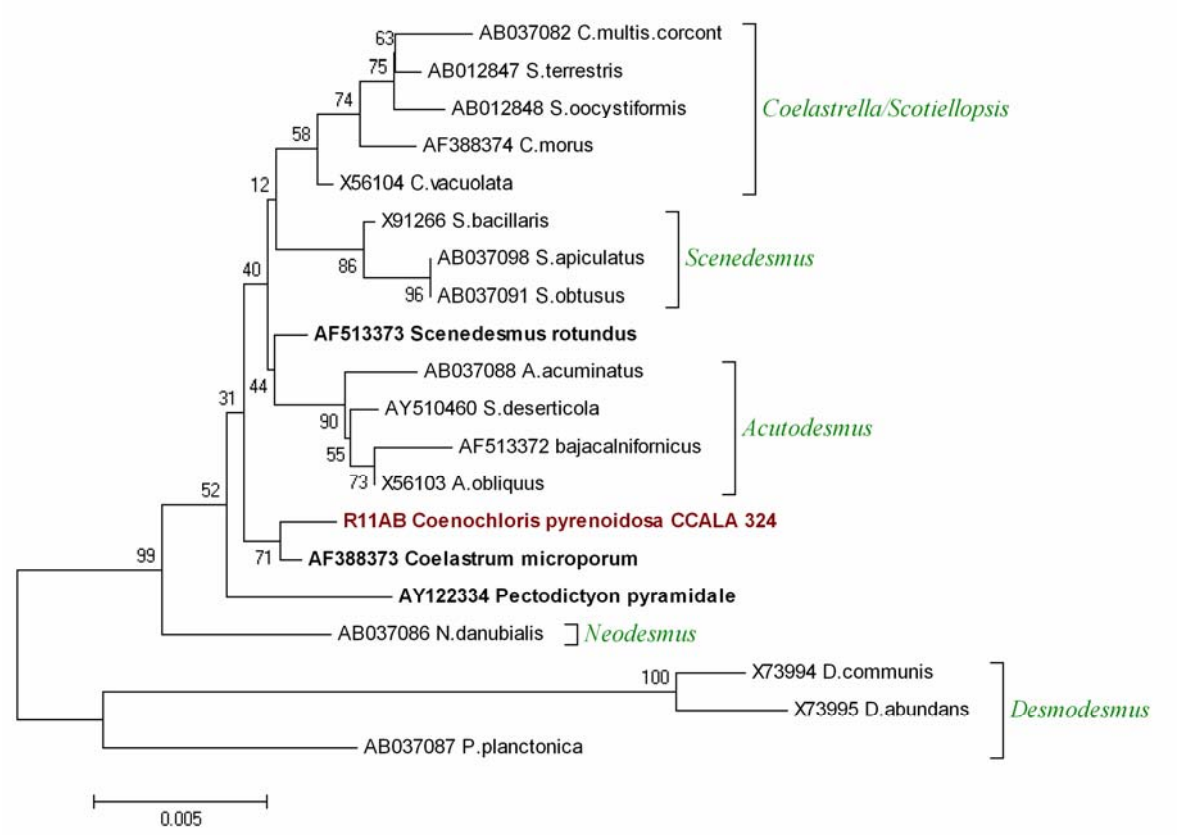
Coenochloris pyrenoidosa CCALA 324

The isolate from little pool named *Coenochloris pyrenoidosa* falls within the family Scenedesmaceae (its position in more detail is shown on fig. 3). It is probably a sister to *Coelastrum microporum*, isolate Kr1980/11 (Krienitz, unpublished). The sphaerical cell shape may be surprising within this family, but cell shape variability is not unknown among Scenedesmaceae. From the morphological point of view, two other specimens are interesting: *Pectodictyon pyramidale* was comprised in Radiococcaceae, subfam. *Dictyochlorelloideae* according to Komárek & Fott (1983). It is excluded from „true

Radiococcaceae“, because its cells are connected with mucilaginous strands (not free). This alga is more or less close to a specimen that almost does not produce mucilage at all. The second one is *Scenedesmus rotundus*, whose position within the clade is not clear. It is a recently described species of *Scenedesmus* (s.l.) from desert soil. It possesses very simple sphaerical cells and resembles the strain studied here.

To make final taxonomic decisions, thorough polyphasic study of the *Coenochloris pyrenoidosa* and *Coelastrum microporum* and possibly the two other strains together would be reasonable.

Fig. 3: Phylogeny of the family Scenedesmaceae, NJ, Tamura-Nei.



Gloeocystis vesiculosa CCAP 31/3 and isol. Kašpárková 2004/3, *Gloeocystis ampla* UTEX LB 763, *Gloeocystis gigas* UTEX LB 291

Four strains of *Gloeocystis* spp. are members of the CW group of Chlorophyceae (*Chlamydomonas*-clade in broad sense). Their position within the clade, each strain lying within its distinct lineage, impairs seriously the validity of the *Gloeocystis* concept. It also shows evidence that the ability to form mucilaginous palmelloid stages is common among chlamydomonad algae.

The description of *Gloeocystis*, type spec. *Gloeocystis vesiculosa* Nägeli (1849), gives not sufficient information on the genus that would allow unambiguous interpretation. Although Fott & Nováková (1971) rejected this genus, Hindák (1978) was of different opinion and many authors kept his concept more or less similar till today (Komárek & Fott 1983, Ettl & Gärtner 1994, Kostikov et al. 2002). The name *Gloeocystis* should be applied (according to these works) to autosporic algae of either spherical (*G. vesiculosa*) or ellipsoidal (*G. polydermatica*) cells lying within concentric layers of mucilage.

Although Nägeli (1849) observed no motile stages, I am convinced that the specimen *originaly* described as *Gloeocystis vesiculosa* was in fact a palmelloid stage of some chlamydomonad alga. It could be a chlamydomonas-like organism who lost the ability to produce motile stages or where immotile form is the prevailing one. There are several points to support this assumption: (1) cells in the original drawing by Nägeli are quite similar to those in drawing of *Tetraspora* and *Palmella* by the same author; (2) the appearance of mucilaginous envelopes is very close to that observed on chlamydomonad strains in this work, and they are slightly different from the mucilage around *Gloeocystis polydermatica* that surely is not a chlamydomonad organism; (3) all spherical *Gloeocystis* spp. strains sequenced in this work are chlamydomonad organisms; (4) the „colourless area“ (or lumen in chloroplast) adjacent to sister-cell, as described by Nägeli for genus *Gloeocystis* but also for *Palmella* (1849), is a common feature in chlamydomonads (s.l.) and it was also observed in chlamydomonad strains during this study. On the other hand, this feature is not present in trebouxiophyte *Gloeocystis polydermatica*.

Basically, there are three ways to solve the problem of *Gloeocystis:*

a) to conclude that the genus was not appropriately described and the type species referres to a stage in life-cycle of another organism and the name should be rejected.

b) to isolate more representants of *Gloeocystis vesiculosa* with corresponding morphology and ecology (the strains analyzed here were of different origin, often freshwater, whilst the original material was aerophytic, growing on a stone) and subject these strains to further phylogenetic analysis. If the aerophytic algae form a uniform cluster within chlamydomonadales and none of them produces motile stages in any observable condition, than the original Nägeli's name can be applied to such organisms, aerophytic chlamydomonads (in broad sense) that lost the ability to form motile stages.

c) to prove that the type *Gloeocystis vesiculosa* was not appropriately delimited and all data about this species in literature were based on mistaken observations (all organisms determined as *G. vesiculosa* were stages of chlamydomonads). Because the name is traditionally used for aerophytic green algae with layered mucilage, *Gloeocystis polydermatica* might be conserved as a new type for the (trebouxiophyte) genus *Gloeocystis*.

Coenochloris planoconvexa CAUP H 5502

The new species *Coenochloris planoconvexa* was established mainly because its assymetrical, planoconvex cell shape (Hindák 1977), eventhough this feature was not present in all isolates of the same specimen (Hindák 1980, 1984). By the morphology itself, mainly based on the cell shape and presence of polar thickenings of the cell wall, the strain can be assigned to Oocystaceae. The same result was obtained here by analysis of 18S rRNA. The genus *Oocystis*, however, is not monophyletic (Hepperle et al. 2000). Without a revision of the genus, and when the strain CAUP H 5502 is not closely related to any of three sequenced *Oocystis* species, we can hardly assign *Coenochloris planoconvexa* to *Oocystis*.

The isolate CAUP 5502 exhibits an extraordinary shape of mucilaginous envelope, that could possibly serve as a morphological criterion to delineate a new genus, with the awareness that this particular trait may not be pronounced in some cultivations. Similar

feature was described earlier in the genus *Echinocoleum* JAO et LEE 1947. For comparison, strain *Echinocoleum elegans* SAG 37.93 was studied. The shape of mucilaginous envelope was essentially different, with only three or four massive mucilaginous arms comparing to many little rays in *Coenochloris planoconvexa*. Nor the 18S rRNA data support a close affinity of these two strains: *Echinocoleum elegans* is a sister to *Amphikrikos* sp. and *Schizochlamydella capsulata* within Oocystaceae.

Schizochlamydella minutissima CCAP 57/1

The strain *Schizochamydella minutissima* CCAP 57/1 constitutes a well supported lineage with few unknown *Nannochloris* sequences. It is placed in the neighbourhood with true *Nannochloris* species and the *Koliella spiculiformis*-clade. So it lies in the group where other algae with very small cell size are present, but suprisingly, this new clade appears closer to the *Koliella* clade than to the small coccoid forms.

S. minutissima was established as a type species of new genus *Sphaerochlamydella* by Kostikov et al. (2002). Because the strain sequenced here is a type strain isolated by Broady (1982), its phylogenetic position assigns a position of the genus *Sphaerochlamydella*. Other sequnces included in the clade represent undetermined organisms. It is probable that for the small size of these organisms, their particular morphology was simply omitted. This is not the case of the type strain, however, which apart its significantly small size exhibits distinct morphology, with deeply bilobed chloroplast without pyrenoid and usually a relatively big vacuole between the lobes.

I suppose that this particular characteristics also justify its removement from the genus *Schizochlamydella*, that should encompass species with (a) cup-shaped chloroplast, (b) two autospores (here commonly also 4 and 8), (c) persisting mother cell wall and (d) no particular arrangement in the mass of mucilage.

Into the new genus *Sphaerochlamydella* also *Catenococcus minutus* was moved by Kostikov et al. (2002). This organism should display a different arrangement of cells, with cell size even smaller (1,2 – 2,8 μm) than *S. minutissima* (Komárek & Fott 1983). Without

sequence to compare, it is impossible to assume about its position within the contemporary system.

Neocystis sp. CAUP D 801, *Neocystis brevis* CAUP D 802, *Sphaerocystis bilobata* CCALA 482, *Sphaerocystis oleifera* isol. Elster 1998/26

A new clade of trebouxiphytes is here constituted. All species originate from soil biotopes from different parts of the world. They have mostly oval or sometimes spherical cells, do not produce a lot of mucilage and have prevailingly bilobed chloroplast (this was not verified for *S. bilobata*, suprisingly, but the strain was determined and assigned to the „bilobata" species by an acknowledged algologist).

The old generic name *Sphaerocystis* is apparently not appropriate for two of the species (see discussion on the chlorophycean *Sphaerocystis* clade above). Nor would be *Coenochloris* (defined by the arrangement of cells) or *Radiococcus*, in which these species were moved by Kostikov et al. (2002). The name *Neocystis* may be considered. This genus was established by Hindák to accomodate non-spherical cells without pyrenoid and Koršikov's *Coenochloris ovalis* was established as a type species. Because the authentic strain is not availabe, here we must rely on the original description and drawing, which, unfortunately, does not show much. The usually periferal chloroplast could be cup-shaped, but surely is not bilobate. Because of the deeply bilobed chloroplast and the presence of pyrenoid in two of the strains, this clade should not bear the name *Neocystis* and rather be described with a new generic name.

Gloeocystis polydermatica CAUP H 6701, CCAP 31/5, NP 20.02 and NP 20.04, *Eutetramorus* cf. *fottii* CAUP H 7701, *Coenocystis oleifera* CCAP 176/2, *Coenochloris signiensis* CCAP 176/3

Among this group algae with ellipsoidal, broadly ellipsoidal and spherical cells are present, with smooth parietal, often band-shaped chloroplast with a pyrenoid. Three autenthic strains are lying within this clade: *Gloeocystis polydermatica* Hindák *Coenocystis (Sphaerocystis) oleifera* and *C. signiensis* Broady. Since none of these strains

is a generic type, it is not appropriate to assign this clade to one of the genera, at least when a conservation under a new type species is not applied.

This clade does not exhibit any particular trait that would allow clear characterization of this new taxon in Trebouxiphyceae. Some of the specimens, but not arguably all of them, produced layered mucilaginous matrix in fresh state, eventhou this marker is hard to trace in cultures. So there is reason for choosing the name *Gloeocystis,* that was traditionally used for such an algae growing on rocks, wood etc. This question was already discussed above. Another name used *G. polydermatica*-like algae was *Dactylothece*. This genus was not thoroughly described either and resembles more the genus *Coccomyxa.* Both of the names are not adequate for species with often almost spherical cells, that sometimes tend to stay in groups of four or eight, have tetraedrically arranged autospores and discernible pyrenoid.

Sphaerochlamydella minutissima CAUP H 7501, *Coenochloris bilobata* CCAP 176/1

Sphaerochlamydella minutissima CAUP H 7501 and *Coenochloris bilobata* CCAP 176/1 are not relatives to any sequenced trebouxiphyte. Because they do not represent type species, new generic names shodl be proposed. Authentic strain of the type *Sphaerochlamydella minutissima* (under an older name *Schizochlamydella*) was sequenced in this work and discussed above. The authentic strain *Coenochloris* type species (*C. pyrenoidosa*) is not available, however the morphology of that species is apparently different and it probably belongs to Chlorophyceae.

The first specimen, *S. minutissima*, is spectacular when cultured on agar by its nice regular arrangement of cells. This arrangement is lost, however, in liquid medium. *Coenochloris micrococca*, described by Komárek, appears morphologically somewhat close, but it should have cells even smaller, posses (though hardly visible) pyrenoid and was found only in Cuba.

The culture of *Coenochloris bilobata* CCAP 176/1 was in very poor condition during all cultivation, unfortunately, so it is not possible to add more information to the original

description of *Broady* (1976) or choose a criterion to set up a new taxon except the sequence itself.

4.4 what to do with Radiococcaceae

As I discussed above, it is not appropriate to use common morphological characters without comparing their validity with molecular data. Regarding the latest system of Radiococcaceae, I suggest to abandon all new generic names, for which the authentic strain is not avaliable. When it is clear that the morphology itself needs not essentially reflect the real relationships of taxa, and more, that the characters in use are unstable, I don't find reasonable to confuse the more or less established, anyhow complicated system with even more complicated names. More strains should be isolated, sequenced and documented with thorough morphological data. Before new genera are defined, I would call for handling the original delimitations of genera more properly.

4.5 Conclusion:

With 23 new sequences, this work investigated the phylogenetic diversity of a the family Radiococcaceae and brought robust evidence that the group, as it was understood, is polyphyletic. Molecular analysis presented here revealed the diversity that woud not be apparent by morphological data themselves. Morphological data used as traditional taxonomical criteria were evaluated. A proposal for taxonomical changes was made.

5. References

Altschul, S. F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. (1990): Basic Local Alignment Search Tool. Journal of Molecular Biology 215: 403-410.

Bourrelly, P. (1966): Les algues d'eau douce. I. Les algues vertes. 511 pp., Boubée & Cie, Paris.

Broady, P. A. (1976): Six new species of terrestrial algae from Signy Island, South Orkney Islands, Antarctica. British Phycology 11: 387-405.

Broady, P. A. (1982): New Records of Chlorophycean Micro-Algae Cultured from Antarctic Terrestrial Habitats. Nova Hedwigia 36: 445-479.

Buchheim, M., Buchheim, J., Carlson, T., Braband, A., Hepperle, D., Krienitz, L., Wolf, M. & Hegewald, E. (2005): Phylogeny of the hydrodictyaceae (Chlorophyceae): Inferences from rDNA data. Journal of Phycology 41(5): 1039-1054.

Butcher, R. W. (1932): Notes on new and little-known algae from the beds of rivers. New Phytologist 31(5): 289-309.

Chodat, R. (1897): Études de biologie lacustre. Recherches sur les algues pélagiques de quelques lacs suisses et français. Bulletin Herb Boiss 5(5): 289-309,

Drouet, F. & Daily, W. A. (1956): Revision of the coccoid Myxophyceae. Butler Univ. bot. Stud. 12: 1-218.

Edgar, R. C. (2004): MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research 32(5): 1792-97.

Ettl, H. & Gärtner, G. (1995): Syllabus der Boden-, Luft- und Flechtenalgen. 721 pp., Gustav Fischer Verlag, Stuttgart.

Fott, B. (1956): Sinice a řasy. 520 pp.,Nakladatelství ČSAV, Praha.

Fott, B. (1959): Algenkunde. 482 pp., Gustav Fischer Verlag, Jena.

Fott, B. (1974): Taxonomie der palmelloiden *Chlorococcales* (Familie *Palmogloeaceae*). Preslia 46: 1-31.

Fott, B. & Nováková, M. (1969): A monograph of the genus *Chlorella*. The fresh-water species. – In: Fott, B. (ed.): Studies in Phycology. p. 50-74, Academia, Praha.

Fott, B. & Nováková, M. (1971): Taxonomy of the Palmelloid Genera *Gloeocystis* NÄGELI and *Palmogloea* KÜTZING (*Chlorophyceae*). Archiv für Protistenkunde 113: 322-333.

Guindon, S. & Gascuel, O. (2003): A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. – Systematic Biology 52(5): 696-704.

Hall, T. A. (1999): BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41: 95-98.

Hanagata, N. (1998): Phylogeny of the subfamily Scotiellocystoideae (Chlorophyceae, Chlorophyta) and related taxa inferred from 18S ribosomal RNA gene sequence data. Journal of Phycology 34(6): 1049-1054

Hanagata, N. & Chihara, M. (1999): Coenocystis inconstans, a New Species of Bark-Inhabiting Green Algae (Chlorophyceae, Chlorophyta). Journal of Japanese Botany 74(4): 204-211.

Hepperle, D. (2004): SeqAssem©. A sequence analysis tool, contig assembler and trace data visualization tool for molecular sequences. http://www.sequentix.de.

Hepperle, D., Hegewald, E., Krienitz, L. (2000): Phylogenetic position of the Oocystaceae (Chlorophyta). Journal of Phycology 36(3): 590-595.

Hindák, F. (1977): Studies on the chlorococcal algae (Chlorophyceae) I. Biologické práce. 195 pp., Veda, Bratislava.

Hindák, F. (1978): The genus *Gloeocystis* (*Chlorococcales*, *Chlorophyceae*). Preslia 50: 3-11.

Hindák, F. (1980): Studies on the chlorococcal algae (Chlorophyceae) II. Biologické práce. 196 pp., Veda, Bratislava.

Hindák, F. (1982): Systematic position of some genera of green algae characterized by the formation of mucilaginous or pseudofilamentous colonies. Preslia 54: 1-18.

Hindák, F. (1984): Studies on the chlorococcal algae (Chlorophyceae) III. Biologické práce. 312 pp., Veda, Bratislava.

Hindák, F. (1988): Studies on the chlorococcal algae (Chlorophyceae) IV. Biologické práce. 294 pp., Veda, Bratislava.

Huelsenbeck J.P. & Ronquist F. (2001): MRBAYES: Bayesian inference of phylogenetic trees. – Bioinformatics Application Note 17(8): 754-755

Huss, V.A.R., Frank, C., Hartmann, E.C., Hirmer, M., Kloboucek, A., Seidel, B.M., Wenzeler, P. & Kessler, E. (1999): Biochemical taxonomy and molecular phylogeny of the genus *Chlorella* sensu lato (Chlorophyta). Journal of Phycology 35(3): 587-598.

Katana, A., Kwiatowski, J., Spalik, K., Zakrys, B., Szalacha, E. & Szymanska, H.(2001): Phylogenetic position of Koliella (chlorophyta) as inferred from nuclear and chloroplast small subunit rDNA. Journal of Phycology 37(3): 443-451

Krienitz, L., Hegewald, E.H., Hepperle, D., Huss, V.A.R., Rohrs, T. & Wolf, M. (2004): Phylogenetic relationship of Chlorella and Parachlorella gen. nov (Chlorophyta, Trebouxiophyceae). Phycologia 43(5): 529-542.

Komárek, J. (1979): Änderungen in der Taxonomie der Chlorokokkalgen. Algological Studies 24: 239-263.

Komárek, J. & Fott, B. (1983): Chlorococcales. – In Huber-Pestalozzi, G. (ed.) Das Phytoplankton des Süßwassers, Band 7. 1044 pp., Schweizerbart'sche Verlagsbuchhandlung, Stuttgart.

Koršikov, O. A. (1953): Pidklas Protokokovi (Protococcineae). In: Vyznachnik prisnovodnych vodorostej Ukrainskoj RSR. Vydavatelstvo Akademie Nauk Ukrainskoi RSR Kijev.

Kostikov, I. & Hoffmann, L. (2000): *Garhundacystis* gen. nov., a new genus of the Radiococcaceae (Chlorophyceae). Algological studies 96: 19-27.

Kostikov, I., Darienko, T., Lukešová, A. & Hoffmann, L. (2002): Revision of the classification system of Radiococcaceae Fott ex Komárek (except the subfamily Dictyochlorelloideae) (Chlorophyta). Algological studies 104: 23-58.

Krienitz, L., Hegewald, E.H., Hepperle, D., Huss, V.A.R., Rohrs, T. & Wolf, M. (2004): Phylogenetic relationship of *Chlorella* and *Parachlorella* gen. nov (Chlorophyta, Trebouxiophyceae). Phycologia 43(5) 529-542.

Kützing, F. T. (1843): Phycologia generalis oder Anatomie, Physiologie und Systemkunde der Tange. 458 pp., F.A. Brockhaus, Leipzig.

Kützing, F. T. (1845): Phycologia germanica, di Deutschlands Algen in bündigen Beschreibungen. 240 pp., Brockhaus,Nordhausen.

Kützing, F. T. (1849): Species algarum. 922 pp.,F.A. Brockhaus, Leipzig.

Lemmermann, E. (1915): Tetrasporales. In: Pascher, A. (ed.): Die Süsswasserflora Deutschlands, Österreichs und der Schweiz. Heft 5.

Lagerheim, G. (1883): Beitrag zur Algenflora von Schweden. Öfv. Kgl. Vetensk. Akad. Förh.1883 (3): 37-78.

Lanjouw, J., Baehni, C., Robyns, W., Rollins, R. C., Ross, R., Rousseau, J., Schulze, G. M., Smith, A. C., Vilmorin, R. de & Stafleu, F. A. (1956): International code of botanical nomenclature, adopted by the Eighth International Botanical Congress, Paris, July 1954. Regnum vegetabile 46.

Lewis, L. A. & Flechtner, V. R. (2004): Cryptic species of Scenedesmus (Chlorophyta) from desert soil communities of Western North America. Journal of Phycology 40(6): 1127-1137.

Lukešová, A. (1993): Revision of the genus *Follicularia* MILL. (Chlorophyceae). Archiv für Protistenkunde 143: 87-100.

Luo, W., Pflugmacher, S., Pröschold, T., Walz, N. & Krienitz, L. (2006): Genotype versus phenotype variability in *Chlorella* and *Micractinium* (Chlorophyta, Trebouxiophyceae). Protist 157(3): 315-333.

Lyngbye, H.C. (1819): Tentamen hydrophytologiae danicae continens omnia hydrophyta cryptogama Daniae, Holsatiae, Faeroae, Islandiae, Groenlandiae hucusque cognita, systematice disposita, descripta et iconibus illustrata, adjectis simul speciebus norvegicis. 248 pp., Hafniae, Kopenhagen

McNeill, J., Barrie, F.R., Burdet, H.M.,Demoulin, V, Hawksworth, D.L., Marhold, K., Nicolson, D.H, Prado, J., Silva, P.C., Skog, J.E., Wiersema J.H. & Turland, N.J. (2006): International Code of Botanical Nomenclature (Vienna Code). Regnum Vegetabile 146 A.R.G. Gantner Verlag KG.

Muller, J., Friedl, T., Hepperle, D., Lorenz, M. & Day, J.G. (2005): Distinction between multiple isolates of Chlorella vulgaris (Chlorophyta, Trebouxiophyceae) and testing for conspecificity using amplified fragment length polymorphism and its rDNA sequences. Journal of Phycology 41(6): 1236-1247.

Nägeli (1849): Gattungen einzelliger Algen. 139 pp., Friedrich Schulthess, Zürich.

N**ozaki, H., Ohta, N., Morita, E. & Watanabe M. M. (1998)**: Toward a natural system of species in *Chlorogonium* (Volvocales, Chlorophyta): A combined analysis of

morphological and rbcL gene sequence data. Journal of Phycology 34(6): 1024-1037.

Pascher, A. (1915): Einzellige Gattungen unsicherer Stellung. In: Pascher, A. (ed.): Die Süsswasserflora Deutschlands, Österreichs und der Schweiz. Heft 5. 250 pp., Gustav Fischer Verlag, Jena

Pascher, A. (1932): Drei neue Protococcalengattungen. Archiv für Protistenkunde 76: 409-419.

Posada, D. and Crandall, K. A. (1998): Modeltest: testing the model of DNA substitution. Bioinformatics 14 (9): 817-818.

Printz, H. (1914): Kristianiatraktens Protococcoider. Skrift. Videnskaps. Kristiania, Math.-naturvid. Kl. 1913 (6): 1-123.

Pröschold, T. & Leliaert, F. (2007): Systematics of the green algae: conflict of classic and modern approaches. – In. Broadie, J. & Lewis, J. M. (eds.) Unravelling the algae: the past, present, and future of algal systematics. p. 124 – 148, Taylor and Francis, Boca Raton.

Pröschold, T., Marin, B., Schlosser, U. G. & Melkonian, M. (2001): Molecular phylogeny and taxonomic revision of *Chlamydomonas* (Chlorophyta). I. Emendation of *Chlamydomonas* Ehrenberg and *Chloromonas* Gobi, and description of *Oogamochlamys* gen. nov. and *Lobochlamys* gen. nov. Protist 152(4): 265-300.

Přibyl, P. & Cepák, V. (2007): Evidence for sexual reproduction and zoospore formation in Botryosphaerella sudetica UTEX 2629, previously assigned to the genus Botryococcus (Chlorophyceae, Chlorophyta). Nova Hedwigia 85: 63-71.

Schmidle, W. (1901): Über drei Algengenera. Ber. Deutsch. Bot. Gesel. 19: 10-24.

Schmidle, W. (1902): Über die Gattung Radiococcus Schmidle n. gen. Allgem. Bot. Zeitschr. 8: 41-42.

Senousy, H. H., Beakes, G. W., Hack, E. (2004): Phylogenetic placement of *Botryococcus braunii* (Trebouxiophyceae) and *Botryococcus sudeticus* isolate UTEX 2629 (Chlorophyceae). Journal of Phycology 40(2): 412-423.

Silva, P. C. (2007): Historical Review of Attempts to Decrease Subjectivity in Species Identification, with Particular Regard to Algae. Protist 159(1): 153-161.

Skuja, H. (1956): Taxonomische uber biologische Studien über das Phytoplankton schwedischer Binnengewässer. Nova Acta Reg. Soc. Sci. Uppsal. 4(16): 164.

Smith, G. M. (1918): A second list of algae found in Wisconsin Lakes. Trans Wiscons. Acad. Sci. Arts a. Lett 19: 614-654.

Smith, G. M. (1950): The fresh-water algae of the United States. Ed 2. 716 pp., McGraw-Hill Book Co., New York.

Starr, R. (1954): Reproduction by zoospores in *Planktosphaeria gelatinosa* G. M. Smith. Hydrobiologia 6: 392-397.

Stafleu, F. A., Demoulin, V., Greuter, W., Hiepko, P., Linczevski, I.A., McVaugh, R., Meikle, R. D., Rollins, R. C., Ross, R., Schopf, J. M. & Voss, E. G. (1978): International code of botanical nomenclature, adopted by the Twelfth International Botanical Congress, Leningrad, July 1975. Regnum vegetabile 97: I-XIV, 1-457.

Swofford, D.L. (2000): PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods), Version 4.0b10, Sinauer Associates, Sunderland, MA.

Škaloud, P. (2004): Aero-terestrické řasy vrcholových partií NPP Boreč. – Diploma thesis. Department of botany, Charles University, Prague.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007): MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24: 1596-1599.

Thompson,J.D., Gibson,T.J., Plewniak,F., Jeanmougin,F. and Higgins,D.G. (1997): The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research, 24:4876-4882.

Walton, L. B. (1918): *Eutetramorus globosus*, a new genus and species of algae belonging to the *Protococcoideae*. Ohio Journal of Sciences 1918: 126.

West (1904): A treatise of the British freshwater algae. Cambridge University Press, Cambridge.

Wille, N. (1906): Algologische Untersuchungen an der biologischen Station in Drontheim Kgl. Norske vid. selsk. Skr 1906(3): 5-39.

Wolf, M., Hepperle, D. & Krienitz, L. (2003): On the phylogeny of Radiococcus, Planktosphaeria and Schizochlamydella (Radiococcaceae, chlorophyta). Biologia 58 (4): 759-765.

Zachleder, V. & Šetlík, I. (1982): Effect of irradiance on the course of RNA synthesis in the cell cycle of Scenedesmus quadricauda. Biologia Plantarum 24: 341-353.

6. Appendices

Tab. 3: Strains sequenced and analyzed in this work. The strains names are put as they are labeled in the collections.

Strain code	Name	Origin	Isolated by	Authentic?
CCAP 176/1	***Coenochloris bilobata***	Wet moss and peat, Signy Island, South Orkney Islands, Antarctica	Broady 1972	**YES**
CAUP H 5503	***Coenochloris korsikovii***	Danube river, Bratislava-Železná Studienka, Slovakia	Hindák, 1982/6	**YES**
CAUP H 5502	***Coenochloris planoconvexa***	Morava river, Bratislava, Slovakia	Hindák, 1975/129	**YES**
SAG 217-1a	***Coenochloris polycocca***	Garden pond, Cambridge, UK	E.G. Pringsheim 1940	
SAG 217-1b	***Coenochloris polycocca***	Freshwater, Sweden	W. Rodhe	
SAG 217-1c	***Coenochloris polycocca***	Freshwater, Cambridge, UK	R. C. Starr 1951	
CCALA 324	***Coenochloris pyrenoidosa***	Fishpond, Prague - Břevnov, Czech Republic,	Řeháková 1960/13	
CCAP 176/3	***Coenochloris signiensis***	Soil and moss, Signy Island, South Orkney Islands, Antarctica	Broady 1972	**YES**
CCAP 176/2	***Coenocystis oleifera***	Soil and moss, Signy Island, South Orkney Islands, Antarctica	Broady 1972	**YES**
CAUP H 7701	***Eutetramorus* cf. *fottii***	Rock surface, ventarole, Boreč hill, Czech Republic	Škaloud, 2003	

Strain code	Name	Origin	Isolated by	Authentic?
LB 763	***Gloeocystis ampla***	?, Bloomington, Indiana, USA	A. Wilbois Coleman 1965	
LB 291	***Gloeocystis gigas***	Pool, Loch na Neil; Mull, Ebbudes, UK	R.A. Lewin, 1952(?)	
CAUP H 6701	***Gloeocystis polydermatica***	Wet sandstone rocks, Adršpach, Czech Republic	Nováková, 1968/2	**YES**
CCAP 31/5	***Gloeocystis polydermatica***	Domestic lawn, Windermere, England	De Ville 1993	
NP 20-02	***Gloeocystis polydermatica***	Wet sandstone rock, Bohemian Switzerland National Park, Czech Republic	Pažoutová 2006	
NP 20-04	***Gloeocystis polydermatica***	Wet sandstone rock, Bohemian Switzerland National Park, Czech Republic	Pažoutová 2006	
Kašpárková 2004/3	***Gloeocystis vesiculosa***	Stone, Dolomites Mts., Italy	Kašpárková 2004/3	
CCAP 31/3	***Gloeocystis vesiculosa***	Freshwater; Greely's Pond, Connecticut, USA	Lewin 1950	
CAUP D 801	***Neocystis* sp.**	Soil, Antarctic	Flint, 1964/3	

Strain code	Name	Origin	Isolated by	Authentic?
CAUP D 802	***Neocystis brevis***	Soil, Unterengadin, Switzerland	Vischer, 1941, No. 267	**YES**
CAUP H 1401	***Planktosphaeria gelatinosa***	Soil from garden, Woods Hole, USA	Starr, 1652	
CAUP H 7501	***Sphaerochlamydella minutissima***	Rock surface, ventarole, Boreč hill, Czech Republic	Škaloud, 2003	
CCALA 482	***Sphaerocystis bilobata***	Soil in field, Chelčice, Czech Republic.	Lukešová 1987/3	
CCAP 57/1	***Schizochlamydella minutissima***	Freshwater; moss clump, Vestfold Hills, Princess Elizabeth Land, Antarctica	Broady 1979	**YES**
Elster 1998/26	***Sphaerocystis oleifera***	Soil, Svalbard, Norway	Elster 1998/26	
CCALA 483	***Sphaerocystis schroeteri***	Pool on road in forest, Tapaste, Cuba	Komárek 1964/143	

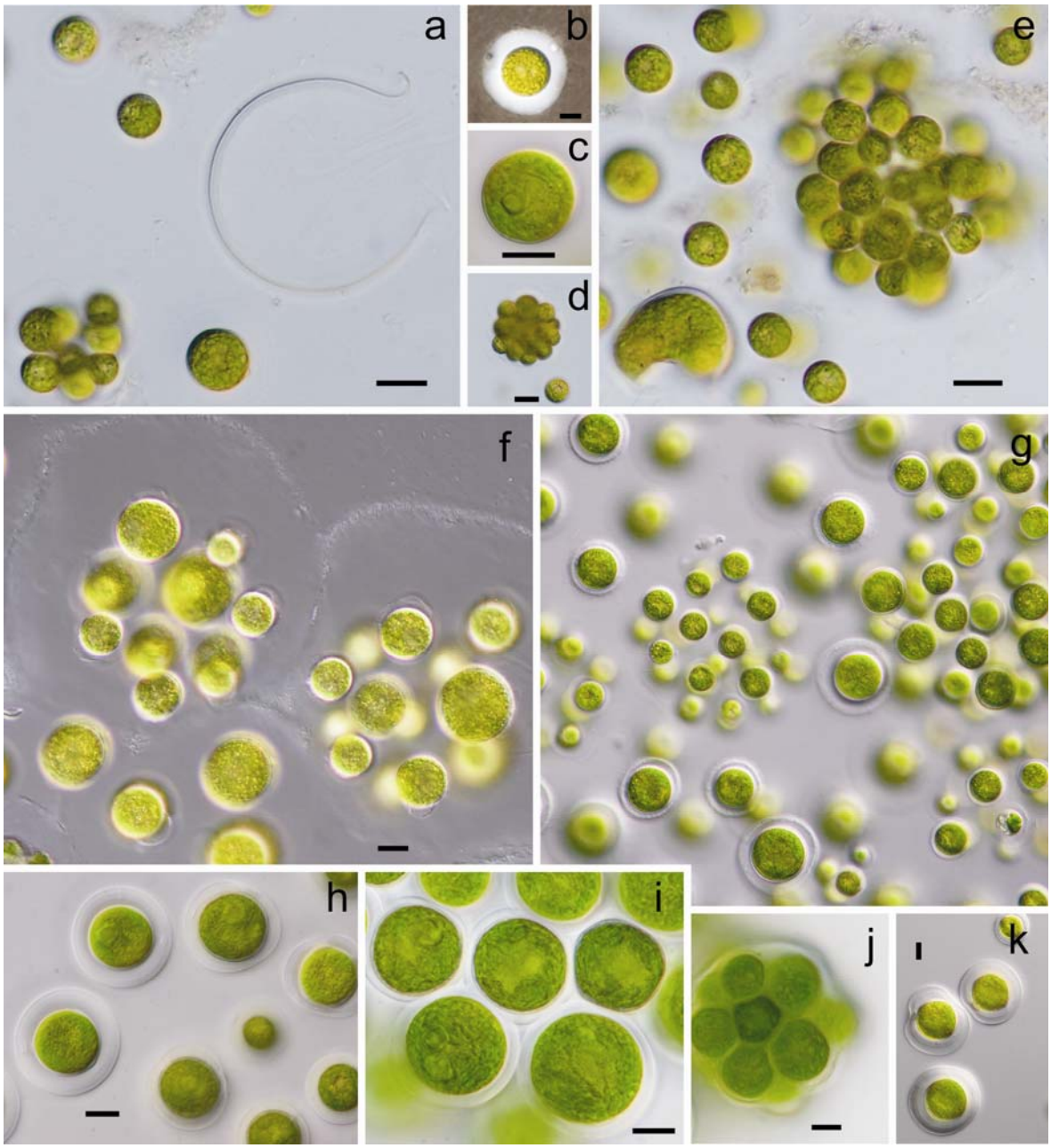


Plate 1: Chlorophyceae I.
a-e – *Sphaerocystis schroeterii* CCALA 483 (**a** – empty sporangium; **b** – mucilage in sample stained with Indian ink; **d** – sporangium; **e** – spore release); **f** – *Coenochloris polycocca* SAG 217-1b (form of colonies in common mucilage); **g-h** – *C. polycocca* SAG 217-1c; **i** – *C. polycocca* SAG 217-1b (cell with two pyrenoids); **j** – *C. polycocca* SAG 217-1a (*Coelastrum*-resembling sporangium); **k** – *C. polycocca* SAG 217-1c (shape of layered mucilage). Scale bar = 10µm.

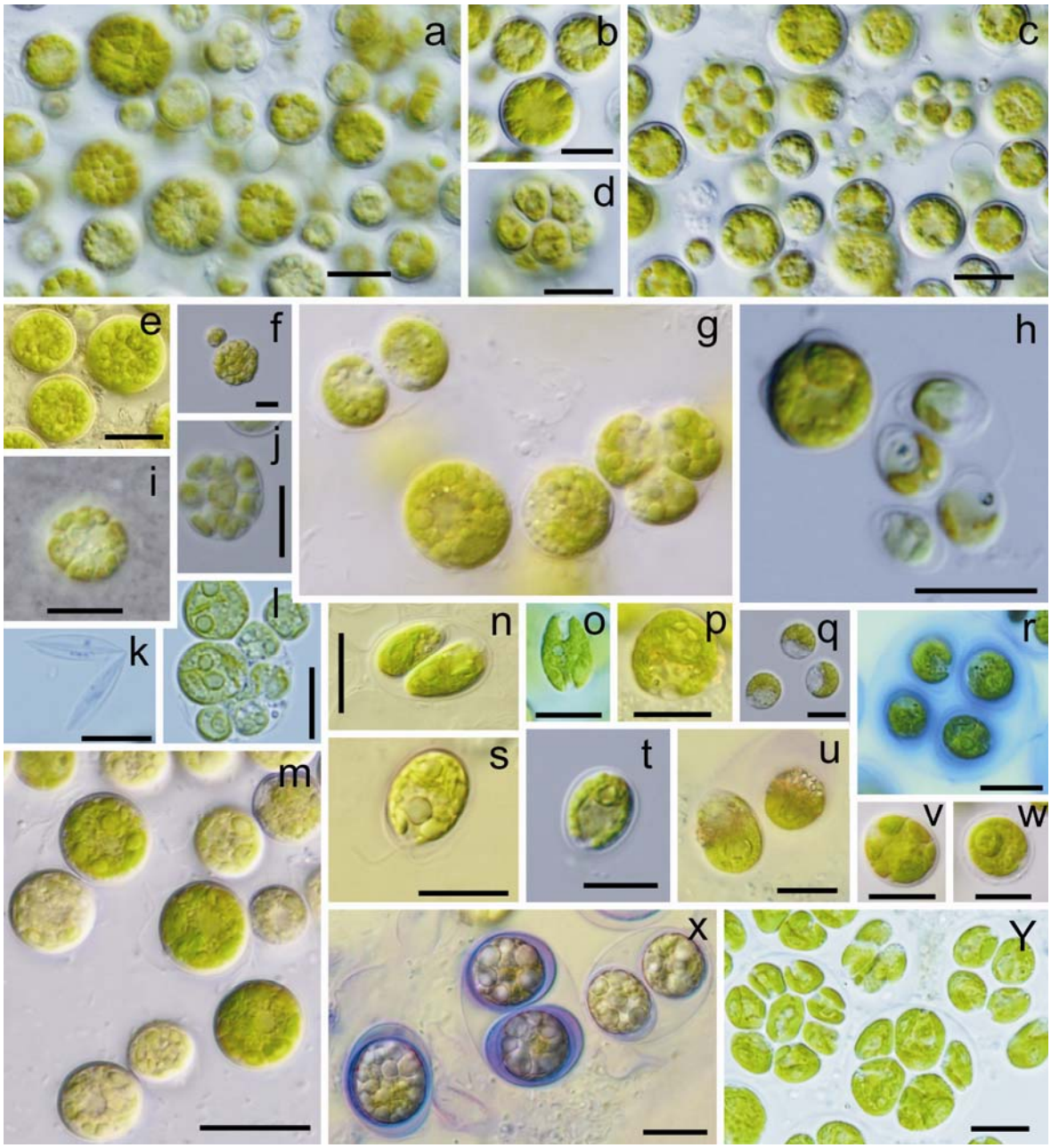


Plate 2: **Chlorophyceae II.**
a-e – *Planktosphaeria gelatinosa* CAUP H 1401 (**a** – surface view of the cells; **b** – chloroplast morphology, LM.; **c** – sporangia; **d** – surface view of sporangium; **e** – chloroplast morphology, agar); **f-h** – *Coenochloris korsikovii* CAUP H 5503 (**f** – sporangium, LM.; **g** – cells and sporangia, agar; **h** – cell and spores, LM.); **i-m –** *C. pyrenoidosa* CCALA 324 (**i** – young sporangium, LM.; **j** – sporangium, LM.; **k** – empty ruptured sporangium; **l** – cells with two pyrenoids in nutrient-poor LM.; **m** – cells with many chloroplasts?, agar); **n** – *Gloeocystis ampla* UTEX LB 763 (sporangium); **o** – *G. vesiculosa* CCAP 31/3 (chloroplast morphology); **p** – *G.gigas* UTEX LB 291 (cell with pyrenoid and apical papila); **q-r** – *G. vesiculosa* CCAP 31/3 (**q** – apical papila, LM.; **r** – mucilage stained with MB); **s-t** – *G. ampla* (**t** – chloroplast morphology); **u** – *G.gigas* UTEX LB 291; **v-w** *G. vesiculosa* isol. Kašpárková 2004/3 (**v** – lobate chloroplast); **x** – *G. ampla* (encysted cells with lot of oil droplets stained with MB); **y** – *G. vesiculosa* isol. Kašpárková (LM); LM = liquid medium; MB = methylene blue. Scale bar = 10µm.

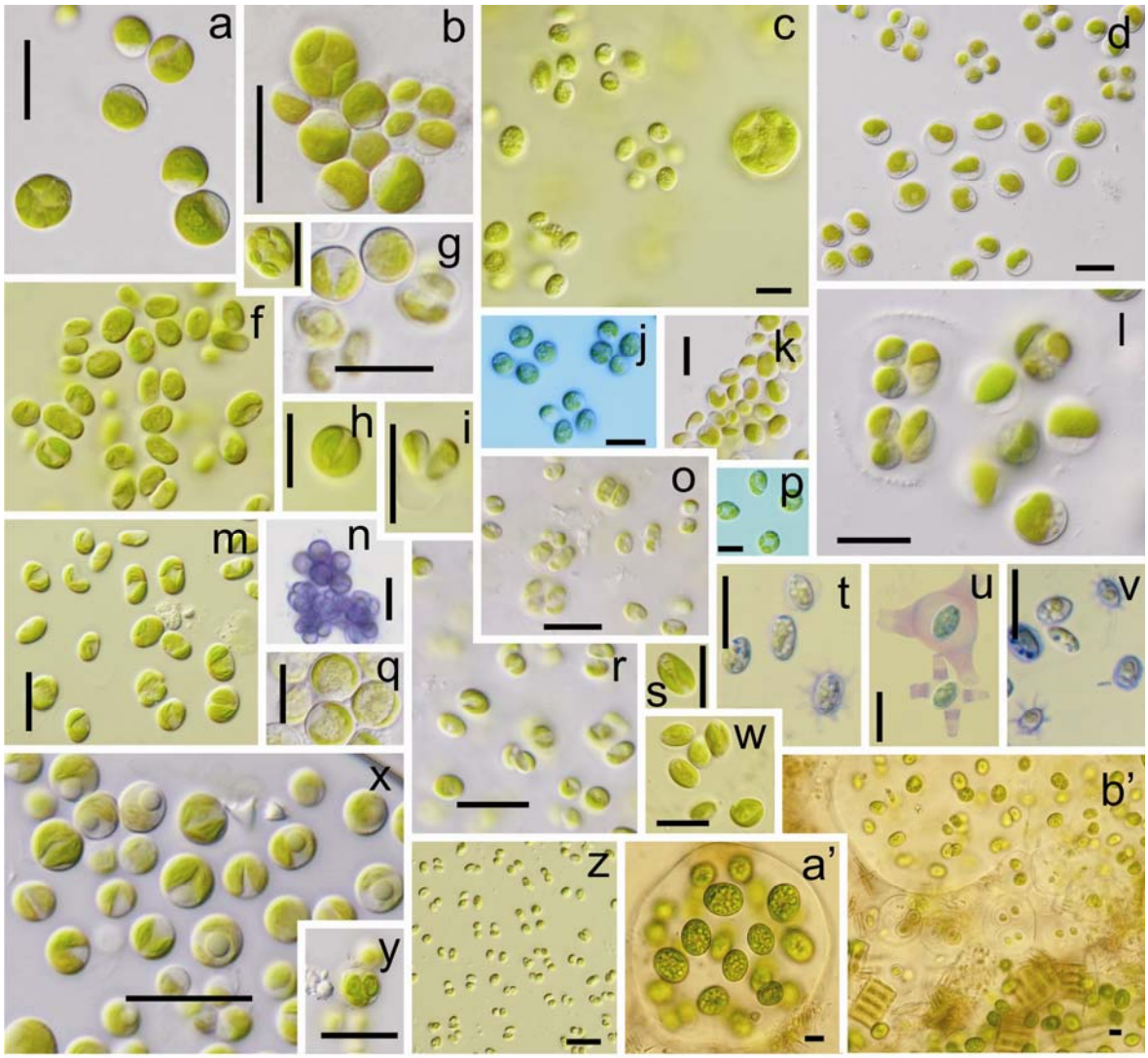


Plate 3: **Trebouxiophyceae.**
a-b – *Sphaerocystis bilobata* CCALA 482 (cells and sporangia); **c** – *G. polydermatica* NP 20-04 (groups of cells and sporangium); **d** - *Eutetramorus* cf. *fottii* CAUP H 7701; **e** (not marked) – *Neocystis brevis* CAUP D 802 (sporangium); **f** – *Neocystis* sp. CAUP D 801; **g** – *Sphaerocystis bilobata* CCALA 482; **h** – *N. brevis* (bilobate chloroplast); **i** – *Neocystis* sp. (autospore release); **j** – *G. polydermatica* CCAP 31/5 (mucilage stained with MB); **k** – *Coenocystis oleifera* CCAP 176/2; **l** – *Eutetramorus* cf. *fottii* (autospores); **m** – *N. brevis*; **n** – *Sphaerochlamydella minutissima* CAUP H 7501 (groups of cells in LM, stained with methylene blue); **q** – *Coenochloris bilobata* CCAP 176/1; **r** – *Sphaerochlamydella minutissima*; **s-t** – *Coenochloris planoconvexa* CAUP H 5502 (**s** – cell with trough shaped chloroplast; **t –** cells with ray-like mucilage, LM, stained with methylene blue); **u** – *Echinocoleum elegans* SAG 37.93 (massive ray-like mucilage, LM, stained with methyleme blue); **v-w** – *C. planoconvexa*; **x-y** – *Schizochlamydella minutissima* CCAP 57/1 (**x** – cells with bilobate chloroplast and single vacuole; **y** – sporangium); **z** – *Sphaerochlamydella minutissima* (arrangement of cells); **a'-b'** – specimens of „Radiococcaceae“ in fresh material from sandstone surface.