Charles University in Prague, Faculty of Science

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Polyphasic approaches in the taxonomy of green aerophytic algae

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Contents

This thesis is based on the following five papers, referred to in the text as Papers 1-5:

1. **Variation and taxonomic significance of some morphological features in European strains of *Klebsormidium* (Klebsormidiophyceae, Streptophyta).**
   Pavel Škaloud
   Nova Hedwigia (2006), 83(3-4): 533-550.

2. **Comparative study of chloroplast morphology and ontogeny in *Asterochloris* (Trebouxiophyceae, Chlorophyta).**
   Pavel Škaloud & Ondřej Peksa
   Biologia (2008), in press.

3. **Phylogeny, morphology, reproduction and taxonomic revision of symbiotic alga *Asterochloris* (Trebouxiophyceae, Chlorophyta)**
   Pavel Škaloud & Ondřej Peksa
   Submitted manuscript.

4. **Confocal microscopy of chloroplast morphology and ontogeny in three strains of *Dictyochloropsis* (Trebouxiophyceae, Chlorophyta).**
   Pavel Škaloud, Jiří Neustupa, Barbora Radochová & Lucie Kubínová
   Phycologia (2005), 44: 261-269.

5. **Morphology, molecular phylogeny and taxonomy of green algal genera *Aerosphaera* and *Dictyochloropsis* (Trebouxiophyceae, Chlorophyta) with description of four new species.**
   Pavel Škaloud, Thomas Friedl & Jiří Neustupa
   Manuscript.

I hereby declare that I made this thesis independently, using the listed references, or in the co-operation with other authors of the papers. I did submit neither the thesis nor its any part to acquire any other academic title.

I was in general responsible for morphological investigations, confocal microscopy and writing papers. I was also responsible for molecular analyses in papers 2, 3. O. Peksa collected lichen samples, isolated and cultivated algal strains of *Asterochloris* and helped with DNA isolation and following PCR reactions (papers 2, 3). Jiří Neustupa helped with morphological investigation of *Dictyochloropsis* strains (papers 4, 5). In paper 5, he also helped with forming the leading idea and final writing of the manuscript. Barbora Radochová and Lucie Kubínová helped with the techniques of confocal microscopy (paper 4). Thomas Friedl performed molecular analyses of *Dictyochloropsis* strains (paper 5).

All co-authors helped to polish the manuscript text.

On behalf of all co-authors, we declare the keynote participation of Pavel Škaloud in acquiring the results and writing the papers, as described above.

Ondřej Peksa

Jiří Neustupa

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1 General introduction

At the generic and species levels, green microalgae are traditionally classified according to morphological characters of vegetative stages in their life cycle (Ettl & Gärtner 1995). However, since the 1990s, the application of phylogenetic analyses of molecular markers has demonstrated that this morphological concept is artificial for most of the green algal genera and needs to be revised (Chapman & Buchheim 1991; Huss et al. 1999; Krienitz et al. 2001; Pröschold et al. 2001). For example, the species of morphologically defined aero-terrestrial genus *Chlorella* were revealed to be dispersed over two classes of chlorophytes, the Chlorophyceae and the Trebouxiophyceae (Huss et al. 1999). It was demonstrated that only four species could be considered as true *Chlorella* taxa, whereas all other *Chlorella* species should be transferred into separate genera (Fig. 1). According to the present knowledge, the traditionally conceived genus *Chlorella* forms 9 particular lineages corresponding to different genera (*Chlorella*, *Mychonastes*, *"Heterochlorella", "Glaphyrella", Parachlorella, Auxenochlorella, "C. ellipsoidea clade", Scenedesmus* and *Desmodesmus*; An et al. 1999; Huss et al. 1999; Kalina unpubl.; Krienitz et al. 2004). Similarly, the rDNA data suggest the splitting of the well-known, widely-distributed freshwater genus *Pediastrum* into five particular genera: *Pediastrum*, *Monactinus*, *Parapediastrum*, *Pseudopediastrum* and *Stauridium* (Buchheim et al. 2005). The same results were also reported for marine green algae. Yamamoto et al. (2003) investigated the genus *Nannochloris* using 18S rDNA and actin genes and determined non-monophyly of these small planktonic, coccoid forms.

As demonstrated above, most of the green algal genera are polyphyletic and their status as well as species number needs a revision. Differentiation of genera and species based on single characters (e.g. morphology of vegetative cells, ultrastructural features) becomes to be insufficient and often leads to ambiguous classification (Pröschold & Leliaert 2007). Therefore, the usage of polyphasic approaches (i.e. combining morphological, ultrastructural, molecular, ecological or biochemical data) could reveal the real biodiversity and taxa relationships among the green algae. Polyphasic approaches can clearly distinguish and delimit species and genera, which predominantly lead to revealing more biological species (e.g. Fabry et al. 1999; Neustupa et al. 2007), but can also result to a reduction of described species (e.g. Pröschold et al. 2001). Following chapters briefly introduce the application, benefits and disadvantages of particular approaches to study green microalgal species.

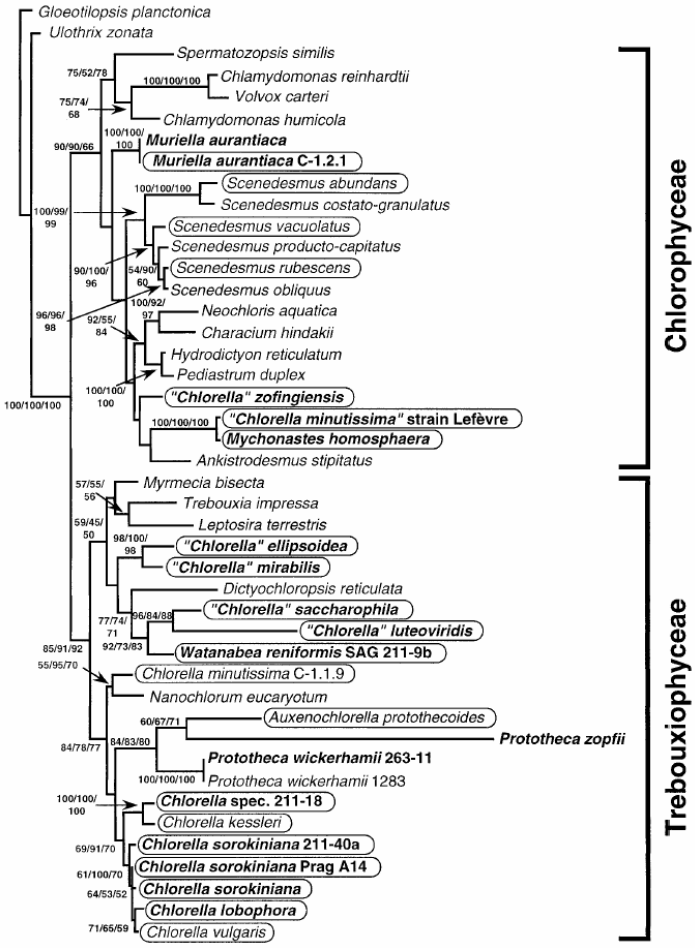


Fig. 1. Phylogenetic tree inferred from 18S rRNA gene sequences showing the polyphyly of the genus *Chlorella* within the Trebouxiophyceae/Chlorophyceae. Taxa that were traditionally assigned to *Chlorella* are circled (Huss et al. 1999).

1.1 Traditional morphology

Traditionally, green algae were classified according to the morphological species concept based on the organisation level of the vegetative state (Brunnthaler 1915; Ettl & Gärtner 1988; Hindák 1977, 1980, 1984; Komárek & Fott 1983). The main morphological and structural characteristics used as taxonomic criteria in green microalgae were: form and dimensions of cells, cell wall characteristics, number of nuclei, chloroplast morphology, presence or absence of pyrenoid, number and size of autospores, and possibility to form zoospores (Ettl & Gärtner 1995; Komárek & Fott 1983). Morphological observations and species descriptions made by a number of phycologists considerably increased our knowledge of the biodiversity, morphological variability and systematics of green algae. Morphological studies of several prominent cytologists are still much accounted for their quality of observations, being sometimes at the limits of the laws of optics (Fott & Nováková 1969[1]; Gärtner 1985; Tschermak-Woess 1989).

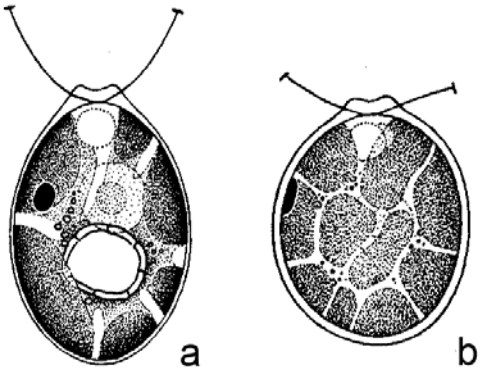


Fig. 2. Morphological distinction between genera *Chlamydomonas* (a) and *Chloromonas* (b) (modified after Ettl 1983).

However, recent molecular and physiological studies have demonstrated high plasticity of several morphological characters used for species delimitation and they evoked need for critical evaluation of observed morphological differences among the species. For example, presence of pyrenoid has been widely used to distinguish particular green algal species and genera, e.g. to differentiate flagellate species *Chlamydomonas* and *Chloromonas* (Ettl 1983, Fig. 2). However, phylogenetic analyses revealed that strains of both traditional genera could belong to the same clade and, in the case of *Chloromonas reticulata*, even to the same species (Buchheim et al. 1997, Pröschold et al. 2001). Nozaki et al. (1998) demonstrated that presence or absence of pyrenoids in *Chlorogonium* depends primarily on culture conditions (autotrophics vs. heterotrophics), instead on evolutionary differentiation. Similarly, production of mucilaginous envelopes around the cells, considered as discriminative character of green algal family Radiococcaceae (Komárek & Fott 1983), occurs in various unrelated algal lineages (Wolf et al. 2003). Moreover, it now appears that mucilaginous production often depends rather on the environmental conditions (Buzzelli et al. 1997, Reynolds 2007) than on phylogenetic position. Another example includes the genera *Chlorella* and *Micractinium* (Fig. 3), traditionally classified into different families of Chlorococcales (Chlorellaceae and Micractiniaceae). The 18S rDNA and ITS data revealed close relationship of these genera (Krienitz et al. 2004). Moreover, formation of colonies and cell wall bris-

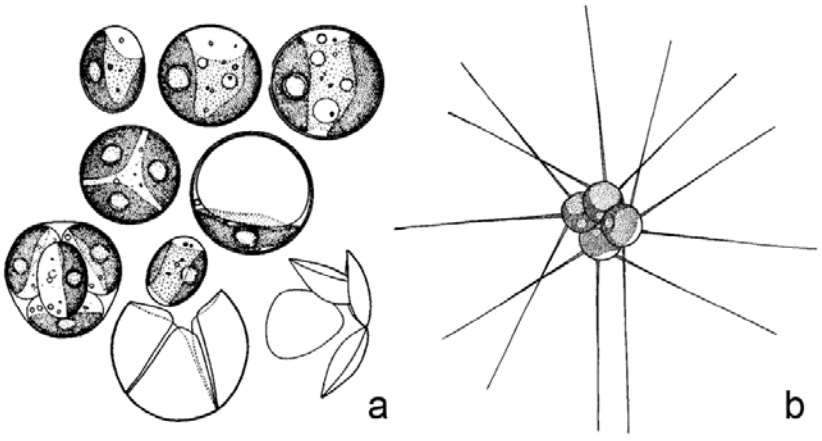


Fig. 3. Morphology of *Chlorella vulgaris* (a) and *Micractinium pusillum* (b) (modified after Komárek & Fott 1983).

[1] cited paper has presently 114 citations in WOS (ISI Web of Science, http://portal.isiknowledge.com/portal.cgi)

tles that characterize the genus *Monoraphidium* was proven to represent phenotypic adaptation against grazer pressure of *Brachionus calciflorus* (Luo et al. 2006).

Finally, morphology can be considerably influenced by the bacterial or fungal contamination. Common marine green alga *Ulva* is characterized by foliose morphology. However, by adding specific marine bacteria, strains of this genus are able to develop the *Enteromorpha*-like tubular thallus (Fig. 4, Provasoli & Pintner 1980).

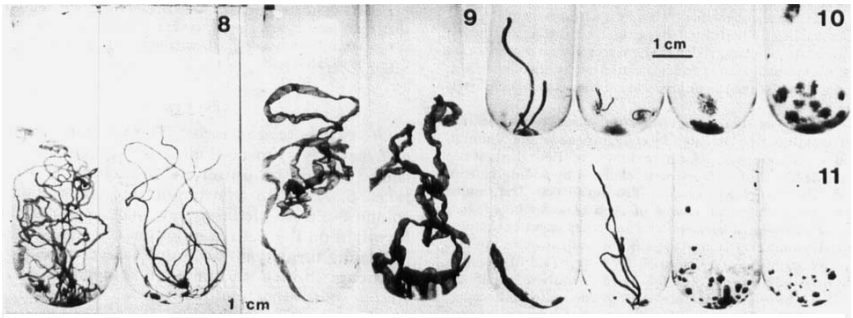


Fig. 4. *Enteromorpha*-like morphology of cultivated *Ulva lactuca*, induced by specific marine bacteria (after Provasoli & Pintner 1980).

The phylogenetic analyses of ITS rDNA sequences later proved that green seaweeds *Ulva* and *Enteromorpha* are not distinct genera, and therefore, *Enteromorpha* was included into the genus *Ulva* (Hayden et al. 2003). The same effect of considerable variation in morphology occurs in green microalgae. Morphology of "*Interfilum massjukiae*" seems to vary from filamentous to packet-like in dependence to degree of bacterial contamination (own observations, Mikhailyuk et al. 2007). Similarly, massive contamination of *Pseudococcomyxa simplex* by unidentified ascomycote fungus probably causes significant changes of cell width (Fig. 5, Nemjová 2007).

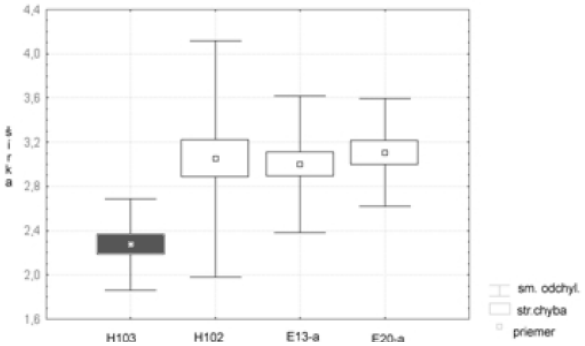


Fig. 5. Cell width variation in three *Pseudococcomyxa simplex* strains. Shaded box-plot indicates strain contamined by ascomycote fungus (modified after Nemjová 2007).

1.2 Confocal microscopy - an example of modern morphological approaches

In late 1980s, advent of a new procedure for optical sectioning of plant tissues using the confocal laser scanning microscope (CLSM) provided an exciting new tool for the morphological observation of living and intact cells. Although the principle of CLSM has been patented in 1957, the commercial application of this method in general started three decades later. The main advantages of CLSM consist in the increasing of micrograph contrast and the possibility to reconstruct three-dimensional images of investigated structures. The special confocal pinhole eliminates out-of-focus information, so that just the light within the focal plane can be detected (Fig. 6, Pawley 2006). Resulting image quality is much better than that of wide-field images obtained by conven-

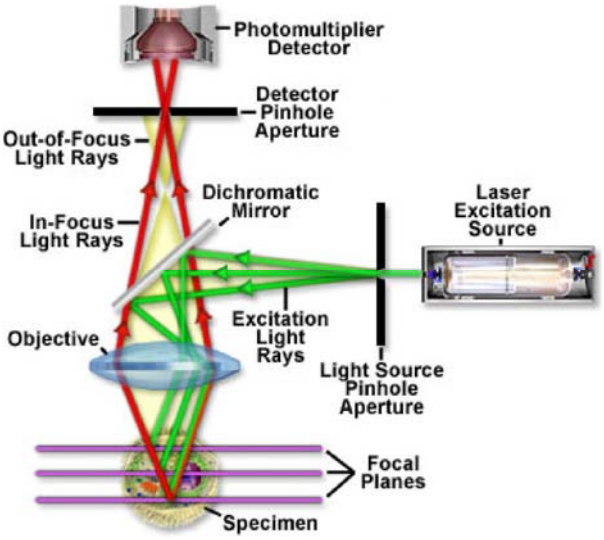


Fig. 6. The mechanics of confocal microscopy (from http://www.microscopyu.com/).

tional light or fluorescent microscopes. Moreover, three-dimensional objects displayed by CLSM can be virtually cut into several optical slices, which can be later used for computer analyses and shape reconstructions.

Above mentioned characteristics thus facilitate and greatly improve studies of plant chloroplasts, enabling their examination directly inside living cells using autofluorescence of the chlorophyll. Van Spronsen et al. (1989) presented some of the first observations of plant tissue using CLSM. To achieve their excellent images, they had simply placed whole pieces of leaf tissue between coverslips and then performed the confocal observations. Recently, CLSM has been repeatedly applied for the examination of chloroplast morphology and structural dynamics in higher plants (see Hepler & Gunning 1998; Wildman et al. 2004). However, it has only rarely been used in the investigations of algal chloroplasts, so far. The essential paper dealing with the algal chloroplast autofluorescence was published by Gunning & Schwartz (1999). These authors examined heterogeneity of chlorophyll fluorescence in chloroplasts of selected green algae and revealed differences in chloroplast ultrastructure between Chlorophyta and Streptophyta. Afterwards, CLSM was further used to investigate chloroplast morphology in *Euglena geniculata* (Zakrys et al. 2002), changes in the distribution of the fluorescence intensity within plastids of *Euglena gracilis* exposed to manganese excess (Ferroni et al. 2004) and plastid division in several *Mallomonas* species (Weatherill et al. 2007).

Despite its contemporary sporadic use, confocal microscopy and subsequent three-dimensional reconstructions can add useful information in studies of the phenotypic plasticity of algal chloroplasts and for detailed investigation of chloroplast ontogeny during cell cycle (Fig. 7). Therefore, CLSM represents an important tool to facilitate the morphological delimitation of particular species in taxonomic studies. Especially in green microalgae, the morphological investigation of often structurally complicated chloroplasts can be essential for the identification of even small morphological differences among particular species (Škaloud & Radochová 2004).

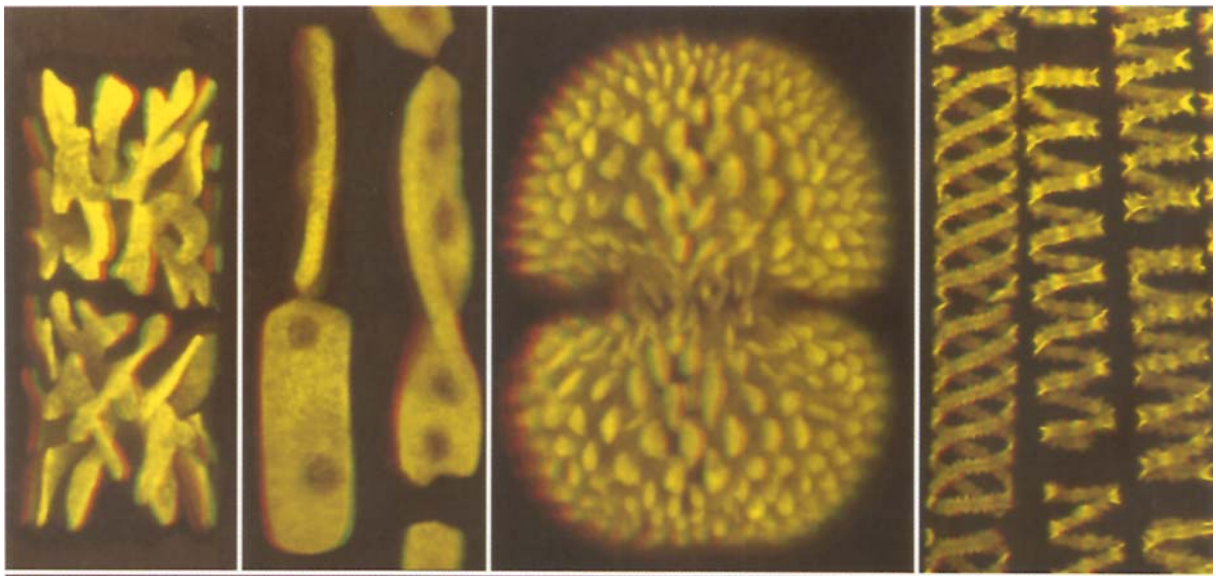


Fig. 7. Three-dimensional reconstructions of chloroplast morphology in several algae, as observed by confocal laser scanning microscope (from left to right: *Zygnema*, *Mougeotia*, *Cosmarium*, *Spirogyra* (after Hepler & Gunning 1998).

1.3 Molecular data

In the 1990s, application of phylogenetic analyses based on specific molecular markers was introduced into the systematics and taxonomy of algae. Molecular data now allow to formulate specific phylogenetic hypotheses and to trace the phylogenetic relationships among the taxa. Recent technological advantages allow rapid and accurate determination of the sequence of nucleotides in a nucleic acid molecule. Typical genetic markers sequenced in phycological studies are e.g. the nuclear ribosomal operon (SSU, 5.8S and LSU, including ITS1 and ITS2 regions), actin gene, several chloroplast genes (rbcL, atpB) and mitochondrial genes (coxI). Phylogenetic analyses of slowly evolving genes for small and large subunit ribosomal RNA (SSU and LSU rDNA) are often exploited in investigation of evolutionary divergences that led to the separation of major algal phyla and classes. However, SSU rDNA also contains some relatively variable regions, so that this marker can also be used at lower taxonomic levels, including microevolutionary investigations. Phylogenetic analyses of SSU rDNA provided solid support for existence of two main lineages among the green plants - Streptophyta and Chlorophyta (Friedl 1997), they revealed Trebouxiophyceae as a new green algal class (Friedl 1995), and demonstrated the polyphyly of many genera (e.g. Buchheim et al. 1997, 2005; Huss et al. 1999; Krienitz et al. 2004; Senousy et al. 2004).

For investigation of recent speciation events, the rapidly evolving sequences are preferred. The regions of the nuclear-encoded ribosomal RNA genes known as the internal transcribed spacers (ITS) have proved to be very useful in such studies (Fig. 8). In fact, ITS has now become the single most frequently utilized DNA region in plant studies (Hershkovitz et al. 1999). In taxonomy of green microalgae, ITS sequences were recently used e.g. in discovery of genetic variability among strains of *Chlorella* and *Micractinium* (Luo et al 2006), in investigation of relationships between *Paramecium* symbionts (Hoshina et al. 2005; Summerer et al. 2007) or in investigation of specificity and genetic variation in the genus *Asterochloris* (Nelsen & Gargas 2008; Yahr et al. 2006). Within the ITS region, the 5.8S gene sequence is highly conserved and it is useful for verifying identity of sequences. The ITS1 and ITS2 regions are much more variable in its primary sequence. Nevertheless, they exhibit a common secondary structure within all Viridiplantae (Mai & Coleman 1997). Although ITS represents the untranslated DNA region, the initial transcript of a nuclear ribosomal cistron is a long RNA that includes all three RNA genes plus the ITS. It promptly folds, and only then the ITS regions degraded (Venema & Tollervey 1999). Once secondary structure has been established for a group of related organisms, it can serve not only as a guide to alignment of all the nucleotide positions, but it may also contain information potentially useful for comparisons at family, order and even higher levels (Fig. 9, Coleman 2003). Nowadays, more than 550 ITS2 sequences along with the secondary structures are available for Chlorophyta (Schultz et al. 2006).

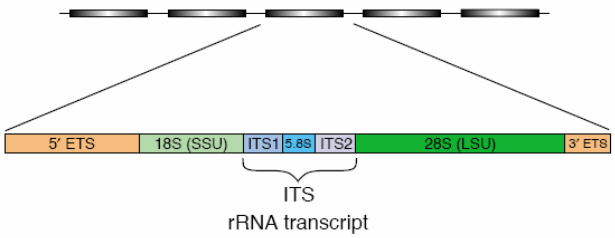


Fig. 8. Diagram illustrating the organization of the nuclear ribosomal cistrons (gray boxes) of a typical eukaryote and the position of ITS region in their primary RNA transcript (after Coleman 2003).

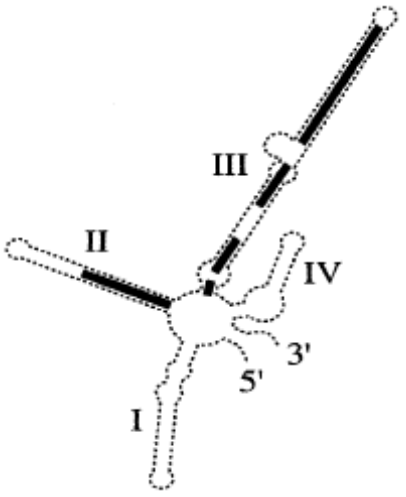


Fig. 9. ITS2 secondary structure of Volvocaceae. Relatively conserved nucleotide positions are in bold (after Coleman 2000).

More variable markers, such as the gene introns, provide high resolution in phylogenetic studies and they are useful in identifying and delimiting cryptic and phylogenetic species and for use in population genetic studies. One such variable marker, the actin type I intron, was reported by Liss et al. (1997), who observed high levels of its variation in *Chlamydomonas* and *Volvox* (Fig. 10). Afterwards, actin introns were used to investigate the cryptic diversity in lichen photobionts *Trebouxia* s. str. (Kroken & Taylor 2000) and *Asterochloris* (Nelsen & Gargas 2006, 2008).

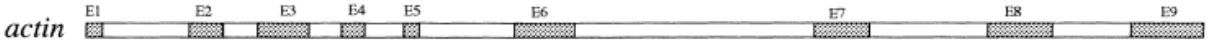


Fig. 10. Diagram illustrating the organisation of the actin genes of *Chlamydomonas reinhardtii* with respect to exons (shaded boxes) and introns (open boxes) (After Liss et al. 1997).

1.4 Species concepts

All the above-mentioned morphological and molecular markers are frequently applied for description and delimitation of particular species of coccal green algae. However, given two organisms, how can we distinguish, whether they belong to the same species or not? The answer may be straightforward for two divergent organisms, but it can be extremely difficult and laborious in closely related ones. That is why many different concepts of species have been and are held by biologists.

According to the **morphological species concept**, species are the smallest groups that can be repeatedly defined by structural characteristics that are relatively easy to distinguish (Fig. 11). However, some morphological species have been observed to undergo seasonal succession or distinct morphological variability depending on environmental conditions (Luo et al. 2006; Stoyneva et al. 2007; van Holthoon et al. 2003; Verschoor et al. 2004). Therefore, frequently observed intraspecific morphological variation avoids the descriptions of species based solely on the morphological differences. Moreover, traditional morphological species concept alone is not sufficient for species descriptions, since it does not recognize cryptic or sibling species (Behnke et al. 2004). However, due to its long tradition, previously published morphological investigations represent valuable information sources about morphological variability, uniqueness and distribution of particular green algal species.

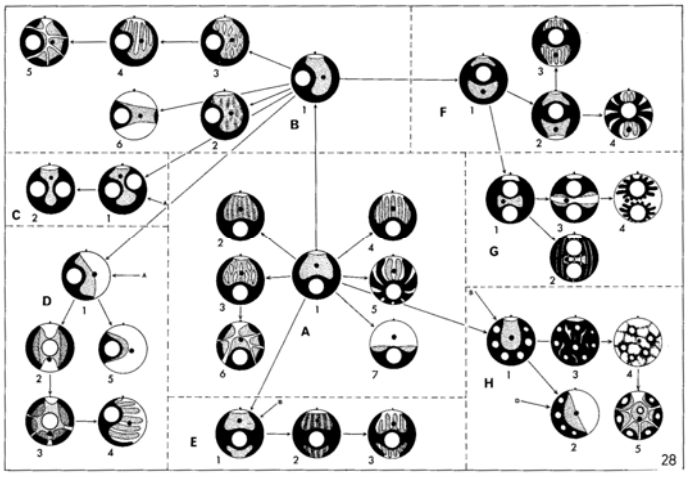


Fig. 11. Morphological species concept. Distinguishing *Chlamydomonas* species according to the chloroplast morphology (after Ettl 1983).

The **biological species concept**, the most widely accepted concept among the biologists, defines species in terms of interbreeding. According to this concept, species are groups of interbreeding natural populations that are reproductively isolated from other such groups (Mayr 1948). This is generally useful formulation for scientists working with living examples of the higher taxa like mammals, fish, and birds, but meaningless for organisms that do not reproduce sexually. In the majority of green microalgal genera, sexual reproduction has never been observed (Ettl & Gärtner 1995) and thus wide application of the biological concept is hard to imagine. However, indirect evidences on the interbreeding processes within the bio-

logical species could be obtained, e.g. by the examination of the recombination events between two gene alleles (Kroken & Taylor 2000).

Phycologists interested in algal evolution have advocated use of the **phylogenetic species concept**, in which a species is a smallest group of organisms that shares unique combination of character states (nucleotide states as well) as separate species (Nixon & Wheeler 1990). Ideally, a phylogenetic species is also monophyletic, i.e. includes an ancestor and all its descendants. In contrast to monophyletic groups, paraphyletic groups do not include all of the descendants of a common ancestor. Polyphyletic groups include some members that are more closely related to taxa outside the group. The phylogenetic species concept can be applied practically for all organisms. However, strict application of this species concept, regarding all evolutionary end-products (even a number of clonal asexual organisms) as unique species (Wheeler & Platnick 2000), could result in overestimating of real species number.

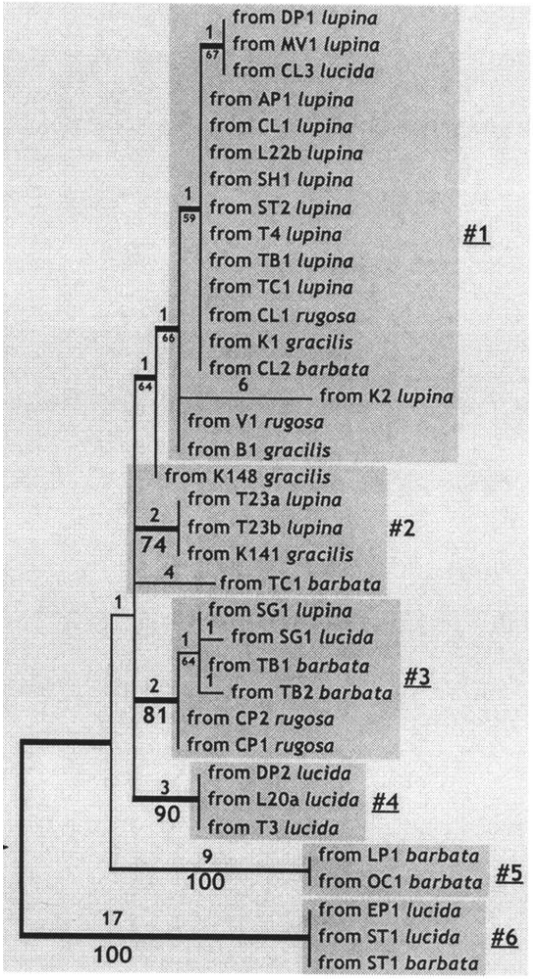


Fig. 12. Phylogenetic species concept. Recognizing cryptic species within *Trebouxia jamesii* species complex, based on actin I intron phylogeny (after Kroken & Taylor 2000).

According to the previously-mentioned characteristics, no single species concept seems to be the best for all groups of organisms. For species delimitation, the **polyphasic approaches** were suggested as the "gold standard" using a combination of morphology, sequence data, physiological characteristics and ecological data. This way, a combination of several species concepts applied for organisms studied will result in better delimitation of particular algal species. For example, the polyphasic approach was applied to recover cryptic species of *Gonium pectorale* by comparison of ITS sequences with a breeding data (Fabry et al. 1999; Coleman 2000) or to characterize the genera *Oogamochlamys* and *Lobochlamys* by means of combination of SSU rDNA data, morphological characters and sexual reproduction processes (Pröschold et al. 2001). In this thesis, I present several examples of polyphasic approach in investigation of taxonomy and phylogeny of some genera of green aerophytic algae.

2 Aims of the thesis

The general objective of the thesis was to taxonomically revise several genera of the green aerophytic algae, using both traditional morphological and modern molecular phylogenetic approaches. For the revision, I chose two coccal genera characterized by the complicated chloroplast structure (*Asterochloris* and *Dictyochloropsis*) and the ubiquitous filamentous genus *Klebsormidium*.

In particular, two principal aims can be summarized as follows:

1) **To test the stability and suitability of current and newly suggested morphological discriminating features for the distinction of the particular species** *(Papers 1, 2, 4)*.
2) **To revise the species concept of the genera, using the combination of selected morphological features and molecular markers** *(Papers 3, 5)*.

3 Outline of the thesis

The thesis comprehends the taxonomical investigation of three aerophytic green algal genera – *Klebsormidium*, *Asterochloris* and *Dictyochloropsis*. In *Klebsormidium*, the species concept differentiating in morphological view two closely related species was investigated by the comparison of several morphological characters in 40 isolates. In *Asterochloris* and *Dictyochloropsis*, the combination of detailed morphological study of chloroplast structure and the molecular phylogenetic analyses was performed to delimit particular species of the genera.

3.1 Species concept in *Klebsormidium*

The stability and suitability of several morphological characters was tested for the distinction between two narrowly related species *Klebsormidium flaccidum* and *K. nitens*. In forty isolated strains, the variability in cell dimensions, filament length, character of zoosporangia and zoospore germination was studied. Moreover, the habit of cell morphology in relation to the culture age was studied in six randomly chosen strains. In two selected strains, the effect of physico-chemical parameters (temperature, humidity, illumination and pH) on cell width was further studied. The study showed variability in taxonomically relevant morphological features during growing of the species in cultures and impossibility to clearly define the species by the combination of current morphological markers (***Paper 1***).

The comparison of ascertained morphological features with the molecular phylogenetic data was not performed, since the broad phylogenetic study of *Klebsormidium* is presently in progress, based on the comparison of rbcL sequences of forty isolated strains (Fabio Rindi, pers. comm.).

3.2 Taxonomy of *Asterochloris*

First, the confocal microscopy was utilized to compare the chloroplast morphology and ontogeny among five strains of the green alga *Asterochloris*. The examination revealed the existence of interspecific differences in the chloroplast ontogeny of *Asterochloris*, based upon the specific chloroplast structures observed in a single species (***Paper 2***).

Next, the broad phylogenetic study was performed on the 34 cultured strains, using nuclear-encoded ITS rDNA and actin intron sequences as the phylogenetic markers. The combination of molecular phylogenetic analyses and morphological examination resulted in the revision of the genus. 13 species were newly delimited, including the description of 7 species

new for science (*A. echinata*, *A. friedlii*, *A. gaertneri*, *A. leprariae*, *A. lobophora* and *A. woessiae*). All species were defined by both unique actin sequences and combination of selected morphological characters (chloroplast morphology, cell shape, etc.). Moreover, the isogamous sexual reproduction was observed in the genus, for the first time (***Paper 3***).

3.3 Taxonomy of *Dictyochloropsis*

Chloroplast morphology and ontogeny in three species of the genus *Dictyochloropsis* were investigated by using light and confocal microscopy. Four distinct morphological stages during the chloroplast ontogeny were revealed in all investigated strains. The stages were distinguished primarily by the number of differently structured chloroplast layers and by the inner structure of chloroplast lobes. The study detected significant differences in chloroplast morphology among the studied strains (***Paper 4***).

These differences were further supported by the 18S rDNA sequence analyses. The phylogenetic analyses revealed that various *Dictyochloropsis* strains form two distinct lineages within the Trebouxiophyceae. Based on detailed morphological investigation and comparing with literature data, the lineages were assigned two different genera, *Dictyochloropsis* and *Aerosphaera*. *Dictyochloropsis* comprises algae with a reticulate chloroplast, forming distinct parallelly-arranged lobes at some ontogenetic stages, and which reproduce only by means of autospores. *Aerosphaera* encompasses algae with evenly perforated chloroplast that can reproduce also by the formation of zoospores with typical separate insertion of the flagella.

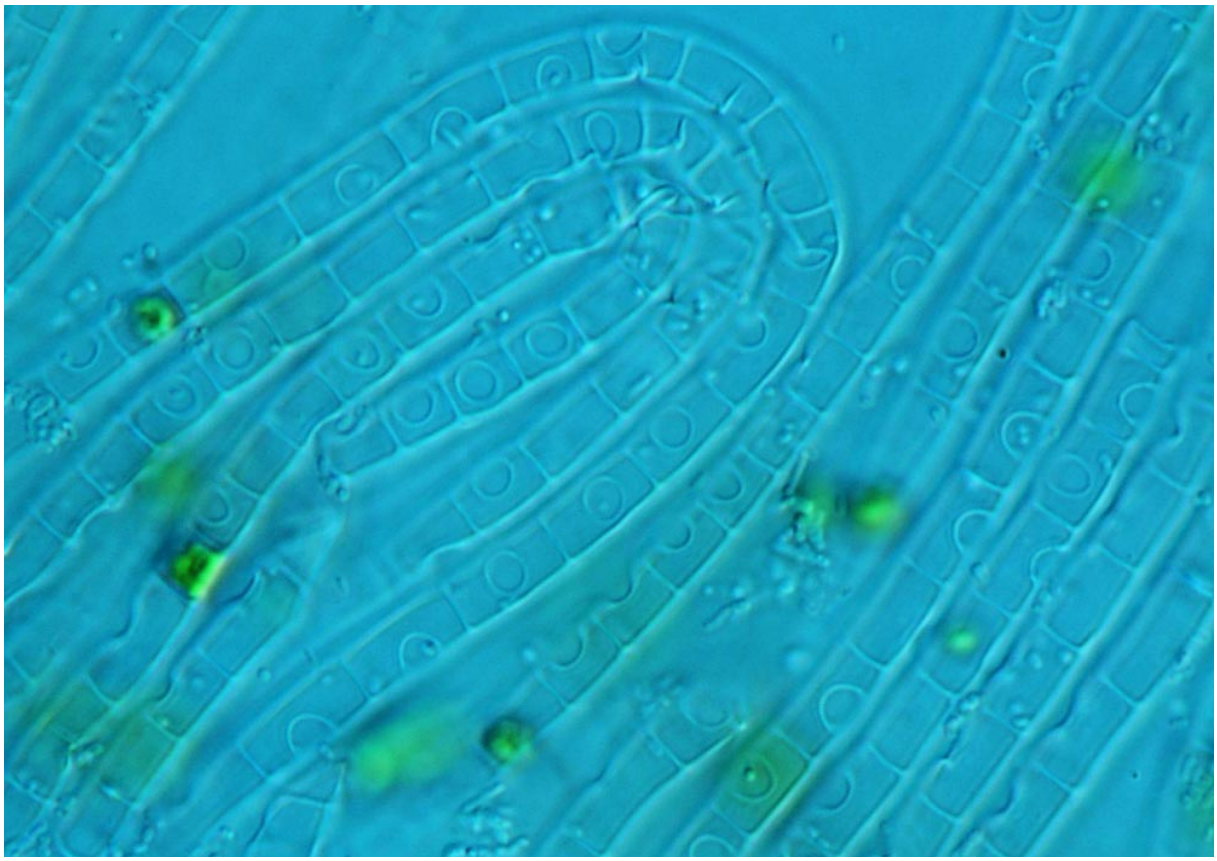
Based on congruencies found between morphological features and rDNA sequence analyses, four new combinations were proposed and four new species were described (*D. asterochloroides*, *A. handae*, *A. tropica* and *A. tschermakiae*) (***Paper 5***).

Paper 1

Variation and taxonomic significance of some morphological features in European strains of *Klebsormidium* (Klebsormidiophyceae, Streptophyta)

Pavel Škaloud

Nova Hedwigia (2006), 83(3-4): 533-550



Empty zoosporangia of *Klebsormidium nitens* with distinct apertures

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Variation and taxonomic significance of some morphological features in European strains of *Klebsormidium* (Klebsormidiophyceae, Streptophyta)

by

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With 18 figures and 5 tables

Škaloud, P. (2006): Variation and taxonomic significance of some morphological features in European strains of *Klebsormidium* (Klebsormidiophyceae, Streptophyta). - Nova Hedwigia 83: 533-550.

Abstract: Forty uni-algal strains of *Klebsormidium* Silva, Mattox & Blackwell (Klebsormidiophyceae, Streptophyta) were examined by light microscopy to test the stability of current discriminating features for the distinction of the narrowly related species *Klebsormidium flaccidum* (Kützing) Silva, Mattox & Blackwell and *K. nitens* (Meneghini in Kützing) Lokhorst. Cell dimensions, filament length, character of zoosporangia and zoospore germination were studied. The habit of cell morphology in relation to the culture age was studied in six randomly chosen strains. The effect of physico-chemical parameters (temperature, humidity, illumination and pH) on cell width was studied on two selected strains. Each strain was transferred to two extreme conditions of one physico-chemical parameter, whereas the other parameters remained the same. The study showed variability in taxonomically relevant morphological features during growing of the species in cultures. For example, there are strong indications that the type of aperture in empty zoosporangial cell walls, considered as a species-specific character, is dependent on environmental factors, in particular on habitat humidity.

In conclusion, it is possible to significantly divide the strains according to three attributes - cell width, character of zoosporangia and microbiotope of habitat. However, each character divides the strains into dissimilar groups. Therefore, it is impossible to decide which feature is the most suitable for proper differentiation of *Klebsormidium nitens* and *K. flaccidum*.

Introduction

The genus *Klebsormidium* contains unbranched filamentous green algae, common in aero-terrestrial habitats. It was originally proposed by Kützing (1843) under the

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name *Hormidium* to accommodate three filamentous green algae. However, this group of species was later found out to be heterogeneous (Lokhorst 1996). The generic name *Klebsormidium* was proposed by Silva et al. (1972) as a solution to the nomenclatural chaos emerged after Fott's discovery of the illegitimate use of the generic name *Hormidium* (Fott 1960). The European representatives of this genus were studied in detail by Lokhorst (1996), who presented a new classification of the genus. Form of growth in culture, cell diameter, shape of the chloroplast, length of filaments, presence of H-shaped pieces, the germination type of zoospores and the shape of emptied zoosporangia were used to distinguish the eight *Klebsormidium* species, reported for Europe.

Klebsormidium flaccidum and *K. nitens* are among the first described and the most investigated species of the genus. Both species were described by Kützing (1849) as representatives of genus *Ulothrix*, section *Hormidium*. These species were studied in detail by Klebs (1896), who distinguished them on the basis of a different cell width. Similar morphology of these species led several authors (Chodat 1902, Farooqui 1969) to consider *K. nitens* as a variety of *K. flaccidum*. Lokhorst (1996) regarded *K. flaccidum* and *K. nitens* as separate species, with different combination of zoosporangial types and zoospore germination.

These two taxa represent the most common species of the genus *Klebsormidium* (Lokhorst 1996, own observations), especially in aero-terrestrial habitats (Flechtner et al. 1998, Lukešová 2001, Neustupa 2001). In ecological and floristical studies, the species composition of terrestrial algal assemblages is often identified only by examination of algal colonies, grown up on agar plates (Ettl & Gärtner 1995). There are mainly two reasons for it: the need of studying the vegetative and reproductive features for correct identification and to trace secondarily in situ most of the species present. However, the correct delimitation of thin *Klebsormidium* species (such as *K. nitens* and *K. flaccidum*) in culture conditions seems to be still problematic, mainly due to similar morphologies and considerable variability in many features (for example cell dimensions and length of filaments). The main goal of the present investigation was therefore to test the usefulness of the current discriminating features (cell dimensions and reproductive characters) in culture conditions with emphasis on how environmental factors may affect the filament appearance during long-term laboratory cultivation. The implications of the results with regard to the species delimitation and taxonomy of genus *Klebsormidium* are discussed in detail.

Materials and methods

A total of 40 isolated strains of *Klebsormidium* were obtained from field algal samples collected from a variety of aero-terrestrial and (semi-)aquatic freshwater habitats, primarily from the Czech Republic. The samples were spread on the Petri dishes with agarized BBM medium (Bischoff & Bold 1963) at 25°C under a constant illumination of 50 - 200 μmol m^{-2} s^{-1} provided by 18W cool fluorescent tubes (Philips TLD 18W/33). Algal microcolonies grown up after 5-10 weeks were isolated into unialgal cultures and further cultivated under the same conditions. After several weeks, the strains were transferred to both agarized and liquid BBM culture tubes and then cultivated at 15°C under daylight conditions (the tubes were placed in a thermostat, placed beside a north facing window). One strain was obtained from the Collection of Algae at the Charles University of Prague, Czech Republic - CAUP (Škaloud & Neustupa 2005). Collection data and other characteristics of each strain are listed in Table 1.

The variation of cell morphology in relation to nutrient depletion was studied in six randomly chosen strains (K9, K13, K16, K29, K43 and K49). The individual strains were incubated on BBM agar plates containing approximately 42 ml of medium and cultivated at 25°C under the constant illumination of 200 μmol m^{-2} s^{-1}. Once a week, at the same time, a small part of the growing population was removed for determination of average cell width and length. The average values were counted from 50 randomly selected cells, measured to the nearest 0.5 μm.

The effect of several physico-chemical parameters (temperature, humidity, illumination and pH) on cell width was studied on two strains for which morphology and other characters (cell dimensions, germination of zoospores and type of zoosporangia) corresponded to the descriptions of *K. flaccidum* (CAUP J302) and *K. nitens* (K13) in Lokhorst (1996). Algal suspensions were prepared by adding a well-growing culture of each strain to distilled water (0.05 g of alga in 100 ml H_2O). In Erlenmeyer flasks sterile wet soil (5 g soil with 15 ml distilled water) was then inoculated by 2 ml of algal suspension. The flasks were well plugged to prevent soil drying.

To study the influence of temperature, two flasks of each strain were kept at 8°C and 26°C. The influence of soil humidity was studied by transferring each strain into the two flasks with soil wetted by 2.5 ml and 50 ml distilled water. To study the effect of illumination, two flasks of each strain were covered by aluminium foil to prevent the entrance of light; the remaining two flasks were kept under continuous illumination of about 200 mmol m^{-2} s^{-1}. The influence of pH was studied by transferring the strains to biphasic cultures (Pringsheim 1954). The pH in the cultures was maintained at 4.5 and 8.5 by adding drops of HCl or NaOH (approximately once per two weeks). During the experiments, only one of the physico-chemical parameters varied, while the others were kept constant (the standard parameters were as follows: temperature 25°C, humidity - 15 ml of sterile water in 5 g soil, illumination of about 200 μmol m^{-2} s^{-1}, pH 6.5). The strains were kept in the above-mentioned conditions for a period of four months; thereafter the cell width range was recorded.

Formation of zoospores was stimulated according to the following procedure: uni-algal cultures were transferred to agarized BBM medium and subsequently cultivated at 25°C under a constant illumination of about 200 μmol m^{-2} s^{-1}. After about 2 - 3 weeks, filaments were collected by sterile razor blades and transferred to tubes with both diluted BBM medium and fresh distilled water. After centrifuging, the tubes with diluted BBM medium were placed in darkness at a temperature of about 10°C. After zoospore formation (within one to two weeks), the tubes were placed in continuous lightness to induce their germination. Consequently, the germinating zoospores were carefully scraped off and observed in a solution of Indian ink.

A correlation-based protocol was used to describe the relations among the characters used for strain differentiation (Table 4). Due to irregular data distribution, a nonparametric Spearman correlation matrix was used to examine individual relationships between variables (Sokal & Rohlf 1995). Similarities between isolated strains were estimated using the Gower distance measure for mixed data (Gower 1971). This coefficient is appropriate for comparisons based on quantitative and qualitative (binary) data. An UPGMA dendrogram tree was calculated from standardized data using the SYN-TAX 2000 program, module HIERCLUS (J.Podani, L.Eötvös University, Budapest, Hungary) (Fig. 16). A principal component analysis (PCA) using Canoco for Windows 4.5 (Ter Braak & Šmilauer 1998) was performed to illustrate the relationship among the characters used in analyses and to show the position of studied strains in the ordination space (Fig. 17). Using the same program, a redundancy analysis (RDA) was performed to test the influence of humidity to strain variability (Ter Braak & Šmilauer 1998). The results of ordination were summarized using the program CanoDraw for Windows 4.0 (Ter Braak & Šmilauer 2002). The differentiation of isolated strains in relation to the selected variables was tested via a two-group multivariate permutation test and canonical discriminant analysis (Table 5), performed in program PAST, version 1.29 (Hammer et al. 2001) and program NCSS (Hintze 2004), respectively. Finally, two-sample t-test in the program NCSS was performed to test the influence of humidity on the aperture type (Brown & Rothery 1993): The means of habitat humidity were compared between two groups, defined by aperture type. Only strains, for which the habitat humidity was measured, were used in the analysis. The habitat humidity was measured by a digital hygrometer Svelin TM977H. The soil humidity was determined following the method described in Neustupa (2001). If the strain was cultivated from water biotope, the humidity was accounted as 100 %.

Table 1. List of studied strains with their origin and other basic characteristics. The range of cell dimensions was ascertained from cultures grown on agar in culture tubes during whole life cycle. Annotation to habitat: Rock - mainly dry, only randomly wetted rock surface; Soil - occasionally wetted bare soil; Moss - the thallus of terrestrial moss; Water – continuously submerged environment (mainly plankton samples).

Strain	Location	Habitat	Habitat humidity (%)	Cell width (µm)	Cell length (µm)	Aperture in empty zoosporangial cell walls	Germination of zoospores
K1	České Středohoří Mts.	Rock	68	5(-6)	5-10	Distinct	Unipolar
K2	České Středohoří Mts.	Rock	68	(4.5-)5-6	5-15(-17)	Distinct	Unipolar
K3	České Středohoří Mts.	Rock	100	6.5-8	5.5-11	Inconspicuous	Unipolar
K4	České Středohoří Mts.	Soil	34	5-6	6-12	Distinct	Unipolar, bipolar
K5	České Středohoří Mts.	Rock	66	4.5(-5)	7-14(-18)	Distinct	Unipolar
K6	České Středohoří Mts.	Moss	66	6-6.5	5-14	Distinct	Unipolar, bipolar
K7	České Středohoří Mts.	Rock	78	4.5-6	5-17(-20)	Distinct	Unipolar
K8	České Středohoří Mts.	Rock	78	4.5-5	4-14	Distinct	Unipolar
K9	České Středohoří Mts.	Moss	-	6-6.5	5-18	Inconspicuous	Unipolar, bipolar
K10	Prague	Water	-	7	7-20	-	-
K11	České Středohoří Mts.	Soil	20	6-6.5(-7)	5-14	Distinct	Unipolar
K13	České Středohoří Mts.	Rock	67	4.5-5	5-15(-20)	Distinct	Unipolar
K14	České Středohoří Mts.	Moss	68	5.5-6	5-15	Inconspicuous	Unipolar, bipolar
K16	Šumava Mts.	Water surface	100	5-5.5	5-12	Distinct	Unipolar
K18	České Středohoří Mts.	Rock	76	4.5-5	5-14	Distinct	Unipolar
K19	Koleč. Central Bohemia	Moss	-	6-7	5-20(-26)	Distinct	Unipolar, bipolar
K20	Milská stráň. Central Bohemia	Soil	-	6-7.5	5-15	-	-
K22	NP České Švýcarsko	Water	100	5-6	8-18	Inconspicuous	Unipolar, bipolar
K25	České Středohoří Mts.	Rock	-	7.5-10(-11)	(4-)7-17(-26)	-	-
K26	České Středohoří Mts.	Rock	80	6-7	7-20	Distinct	Unipolar

K28	Krkonoše Mts.	Water	100	5-6	5-18	Inconspicuous	Unipolar
K29	Krkonoše Mts.	Water	-	(5-)5.5-6	5-24	-	-
K30	Krkonoše Mts.	Water	-	5.5-6	4-11.5	-	-
K31	Šumava Mts.	Soil	-	5-5.5	5-18	Inconspicuous	Unipolar
K32	Krkonoše Mts.	Water	100	5-6	6-20	Inconspicuous	Unipolar, bipolar
K33	Krkonoše Mts.	Water	100	5-6	7-20	Inconspicuous	Unipolar
K35	Drahanské údolí. Central Bohemia	Rock	-	6.5-8	7-25	Distinct	Unipolar
K36	Drahanské údolí. Central Bohemia	Rock	-	6.5-7	(4-)5-29	Distinct	Unipolar
K38	Klecany. Central Bohemia	Rock	-	6.5-7	7-25	-	-
K39	Budapest. Hungary	Rock	100	6-7	4-12	Distinct	Unipolar
K40	České Středohoří Mts.	Moss	66	7-7.5	8-15	Inconspicuous	Unipolar
K41	Prague	Wall	-	6-7	3-18	Inconspicuous	Unipolar, bipolar
K42	České Středohoří Mts.	Rock	-	6-7(-8)	5-19	Distinct	Unipolar
K43	Šumava Mts.	Water	100	5.5-6	4.5-15	Distinct	Unipolar
K44	Krkonoše Mts.	Water	100	6.5-7	4-11	Inconspicuous	Unipolar
K46	Drahanské údolí. Central Bohemia	Soil	-	8	6-20	Distinct	Unipolar
K47	Drahanské údolí. Central Bohemia	Rock	-	6.5-7	4-13	Distinct	Unipolar
K48	Drahanské údolí. Central Bohemia	Soil	-	7-7.5	7-20	Inconspicuous	Unipolar, bipolar
K49	Šumava Mts.	Water	100	8	6-16	Inconspicuous	Unipolar, bipolar
CAUP J302	Adršpach Mts.	Rock	-	6-7	6-18	Distinct	Unipolar

Results

Overall thallus morphology and life cycle

On agar plates, all strains show similar growth forms, composed of circular colonies several millimeters in diameter. The colonies differ only in the size and the degree to which they appeared filamentous at low magnification. In liquid cultures, most strains exhibit two different growth forms: free-floating tufts of filaments and a surface layer of water-repellent filaments, often organized in a parallel pattern. In some strains, the latter type was not observed.

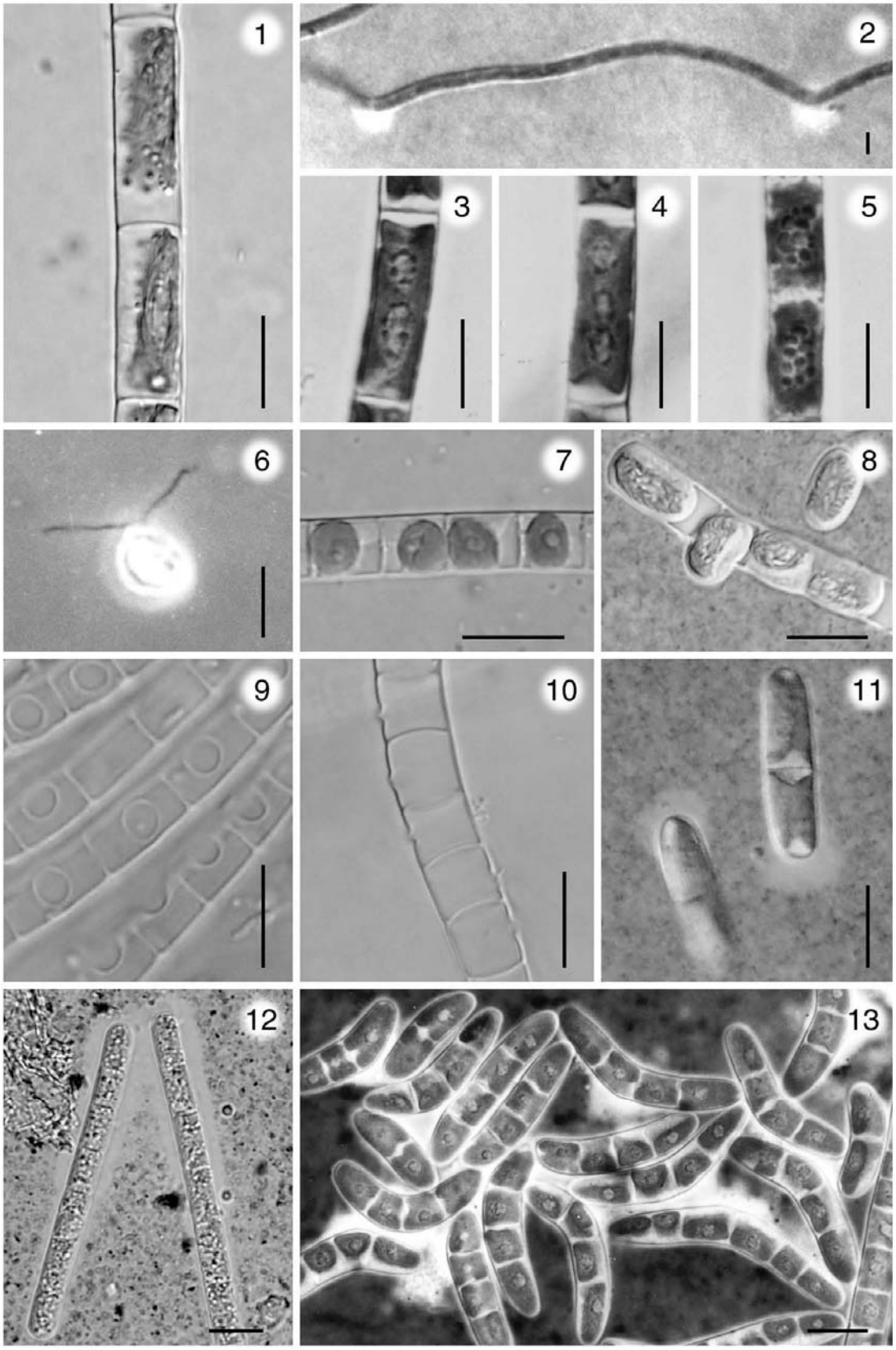
The general growth habit of filaments is identical in all strains. The filaments are uniseriate, unbranched, without morphologically differentiated cells or H-shaped pieces at the transverse cell walls (Fig. 1). The cell wall is smooth. In some strains, the production of mucilaginous discs was sometimes observed in liquid cultures. The discs are produced at regular intervals along the filament and apparently served as attachment (Fig. 2). Length of filaments varies from short, few-celled to very long (more than 1000 cells per one filament). In young cultures, the filaments are mostly short and straight, in old ones they are often bent and disintegrated easily into few-celled fragments. The cells are normally cylindrical; in older cultures they are sometimes slightly constricted at the transverse walls (doliiform). The length of the cells usually exceeds their width; the width/length ratio is quite variable through the life cycle. Cell dimensions of studied strains are given in Table 1. The cells contain one parietal chloroplast, covering about 40-70(-80)% of cell circumference. The chloroplast margin is usually straight, sometimes in older populations lobed along its longitudinal axis. Each chloroplast contains one, less frequently two (Fig. 3), rarely three (Fig. 4) pyrenoids, covered by a distinct layer of starch grains. With increasing age of culture, the starch grains become larger (Fig. 5).

The strains reproduce mostly by means of filament disintegration into few-celled fragments, produced at the ends of filaments. Less commonly, spontaneous production of biflagellate zoospores occurs in liquid cultures (Fig. 6). The zoospores are dorsiventral in structure, with flat ventral and rounded dorsal sides. They are produced in unspecialized cells of the filaments (Fig. 7). Very rarely, aplanospores are produced (Fig. 8).

Characteristics of zoosporangia and zoospore germination

From a total of 40 isolated strains, production of zoospores was induced in 34 strains. In all of these strains, the structure of empty zoosporangial cell wall including the release apertures and the zoospore germination were observed (see Table 1). Two

Fig. 1. Typical morphotype of studied strains. One chloroplast with one pyrenoid is visible in the cells. Fig. 2. Production of mucilaginous discs, serving filament attachment. Figs 3-4. Chloroplast morphology with distinct pyrenoids. Fig. 5. Ageing filament with large starch grains. Fig. 6. Zoospore. Fig. 7. Zoosporangia. Fig. 8. Aplanospore production. Fig. 9. Empty zoosporangia with distinct apertures. Fig. 10. Empty zoosporangia with inconspicuous margin of the apertures. Fig. 11. Unipolar germination of zoospores. Fig. 12. Specific unipolar germination of zoospores observed in strain K43. Fig. 13. Bipolar germination of zoospores. Scale bars = 10 μm.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types of the apertures were observed. In most strains the apertures were clearly visible by showing a distinctive margin (Fig. 9). In other strains the margin of the aperture was inconspicuous and the aperture was only distinct in side view (Fig. 10).

Two types of zoospore germination were observed. Unipolar germination was noticed in all reproductive strains (Fig. 11). A specific type of unipolar germination, characterized by mucilage production over the whole filament surface, was observed in all germinated filaments of strain K43 (Fig. 12). In 10 strains bipolar zoospore germination was observed (Fig. 13), always accompanied by unipolar germination of other zoospores in a culture.

Variability of cell morphology in relation to the nutrient depletion

A different cell width and length range was observed in the individual strains (Table 2). Whereas the average cell width fluctuated only in a range of several tenths of a μm, the average length of the cells often more strongly varied. The cell length variability of individual strains is demonstrated in Fig. 14. The average values of cell length differed both among all isolated strains and during ageing of the culture populations. Although the differences among the strains were obvious, the influence of nutrient depletion displayed identical features. During the first few weeks, a distinct reduction of cell length was observed, probably influenced by intensive cell dividing. However, at a certain stage the cells started to become longer. This phenomenon was observed in all strains, but they differed in timing at that particular stage (in strains K9 and K43 the shortest cells developed in the 3rd week, whilst in strain K49 those cells were not observed until the 5th week).

The cell length changes were notably linked with the length of filaments. The young cultures were typically quick in producing new cells per filament. Conversely, at maturity the filaments started to disintegrate into few-celled fragments. Around the sixth week after inoculation, fragments with symptoms of ageing (distinct starch grains covering the pyrenoid, disintegration of the chloroplast, production of abnormally-shaped cells) predominated the cultures. Interestingly, the start of filament disintegration corresponds with this time, when the shortest average cell length occurs (see Table 2).

The effect of physico-chemical parameters on the cell morphology variation

In the experiment, two selected strains were exposed to 8 different conditions of physico-chemical parameters. Differences in both cell length and width were observed between miscellaneous experimental populations of the same strain. The results of the experiment are listed in Table 3. In standard laboratory conditions (at 15°C under daylight conditions), the strains differed especially by cell width. The cell width of strain CAUP J302 (*K. flaccidum*) varied between 6-7 μm while the width of cells in strain K13 (*K. nitens*) fluctuated in the range of 4.5-5.0 μm (Table 1). By then, the cells of strain CAUP J302 were markedly wider. However, this distinctive cell width behavior was not hold in all experiments, as illustrated in Fig. 15.

At a lower pH, some widening of the cells of strain K13 was observed, whilst the cells of strain CAUP J302 remained unchanged. A high pH caused an increase of cell

Table 2. Variability of cell dimensions and number of cells per filament of six selected strains during population ageing. A - average, St.D. - standard deviation, St.E. - standard error of mean.

			1. week	2. week	3. week	4. week	5. week	6. week
K9	Cell length (µm)	**A**	**14**	**12.3**	**9.6**	**10.7**	**11.9**	**12.9**
		St.D.	3.63	3.23	2.26	2.12	2.65	2.64
		St.E.	0.51	0.46	0.32	0.3	0.37	0.37
	Cell width (µm)	**A**	**6.375**	**6.5**	**6.5**	**6.725**	**6.375**	**6.625**
		St.D.	0.30	0.32	0.39	0.28	0.27	0.28
		St.E.	0.04	0.04	0.06	0.04	0.04	0.04
	Cells per filament		**5-18**	**7-80**	**1-42**	**1-26**	**1-14**	**1-6**
K13	Cell length (µm)	**A**	**11**	**9.9**	**8.2**	**7.5**	**8.2**	**8.5**
		St.D.	2.76	2.02	2.14	1.62	2.14	1.63
		St.E.	0.39	0.29	0.30	0.23	0.3	0.23
	Cell width (µm)	**A**	**5.125**	**5**	**4.75**	**5.125**	**5.25**	**4.875**
		St.D.	0.13	0.19	0.20	0.26	0.22	0.13
		St.E.	0.02	0.03	0.03	0.04	0.03	0.02
	Cells per filament		**6-16(-30)**	**17-110**	**2-600**	**2-320**	**1-500**	**1-350**
K16	Cell length (µm)	**A**	**11.1**	**9.1**	**9**	**8.9**	**9.1**	**10**
		St.D.	2.46	2.34	2.44	1.67	2.08	1.83
		St.E.	0.35	0.33	0.35	0.24	0.29	0.26
	Cell width (µm)	**A**	**5.625**	**5.25**	**5.25**	**5.375**	**5.375**	**5.25**
		St.D.	0.31	0.18	0.18	0.13	0.13	0.20
		St.E.	0.04	0.03	0.03	0.02	0.02	0.03
	Cells per filament		**8-22**	**25-220**	**2-350**	**1-60**	**1-7**	**1-5**
K29	Cell length (µm)	**A**	**11.9**	**12.5**	**12.55**	**10.7**	**10.4**	**10.9**
		St.D.	2.97	3.03	3.39	2.60	2.22	3.08
		St.E.	0.42	0.43	0.48	0.37	0.31	0.44
	Cell width (µm)	**A**	**5.75**	**5.5**	**5.5**	**5.25**	**5.625**	**5.5**
		St.D.	0.21	0.20	0.19	0.20	0.29	0.18
		St.E.	0.03	0.03	0.03	0.03	0.04	0.03
	Cells per filament		**5-50**	**2-50**	**1-50**	**1-16**	**1-20**	**1-18**
K43	Cell length (µm)	**A**	**10.1**	**9.5**	**6.7**	**7.6**	**8.1**	**9**
		St.D.	2.49	2.24	1.71	1.66	1.86	1.80
		St.E.	0.35	0.32	0.24	0.24	0.26	0.25
	Cell width (µm)	**A**	**5.75**	**5.5**	**5.375**	**5.65**	**5.75**	**5.5**
		St.D.	0.21	0.20	0.13	0.13	0.19	0.00
		St.E.	0.03	0.03	0.02	0.02	0.03	0.00
	Cells per filament		**1-90**	**2-70**	**1-20**	**1-10**	**1-5**	**1-4**
K49	Cell length (µm)	**A**	**14**	**11.9**	**10.7**	**9**	**8**	**8.5**
		St.D.	5.37	2.98	2.34	1.97	2.29	2.23
		St.E.	0.76	0.42	0.33	0.28	0.32	0.32
	Cell width (µm)	**A**	**7.75**	**8**	**7.875**	**8**	**8.25**	**8**
		St.D.	0.21	0.21	0.13	0.21	0.19	0.22
		St.E.	0.03	0.03	0.02	0.02	0.02	0.03
	Cells per filament		**20-500**	**> 1000**	**> 1000**	**> 1000**	**> 1000**	**> 1000**

width range in both studied strains. Moreover, a high proportion of dead cells, disintegration of chloroplast and formation of abnormal-shaped cells (not included to the measured cell width) expressed the poor condition of both populations. No

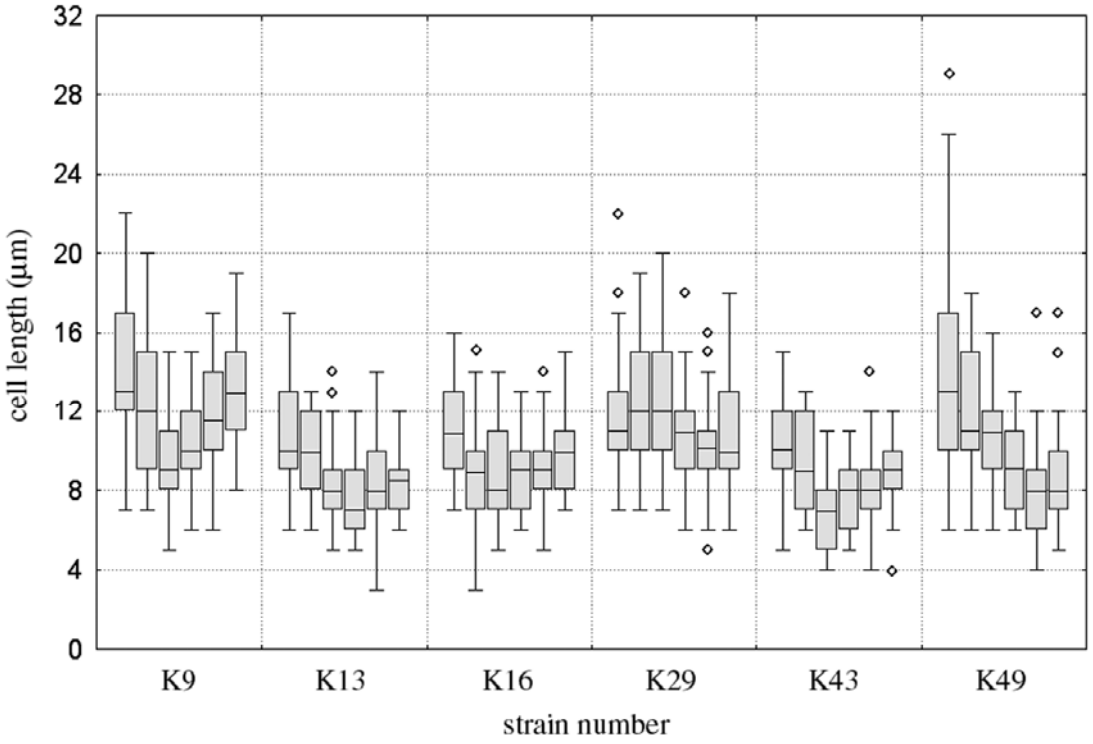

Fig. 14. Development of average cell length of six selected strains during six weeks of cultivation. For each strain, box plot on the left presents the variability of cell length in young cultures; the right box plot illustrates the cell length variability in six weeks old cultures.

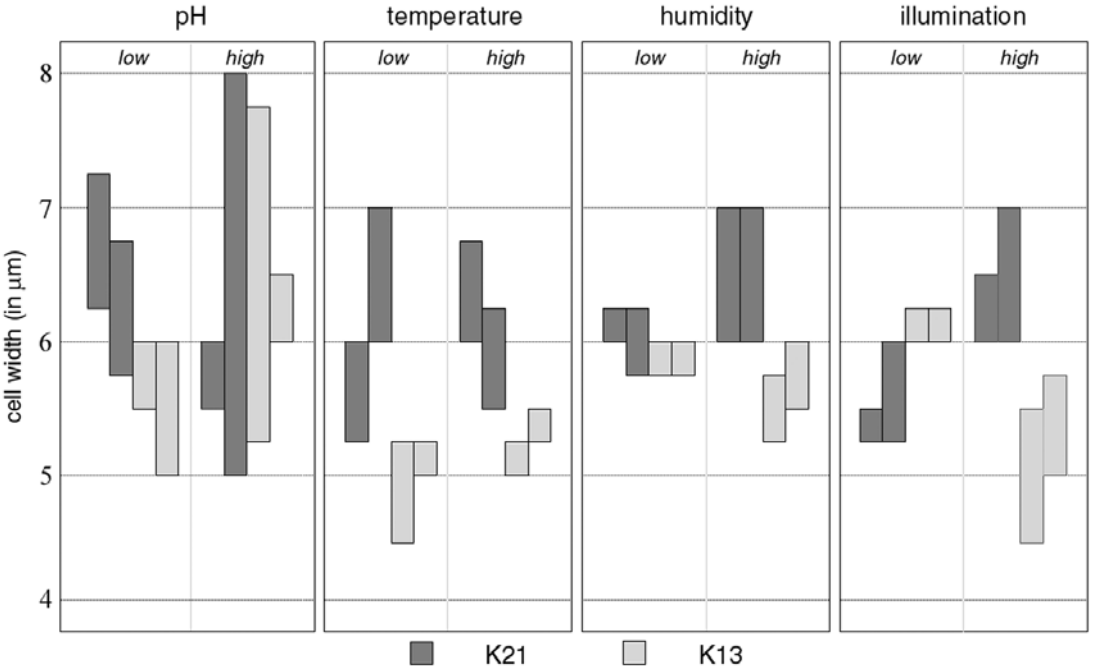

Fig. 15. Influence of four physical-chemical parameters on the cell width in strains CAUP J302 and K13. The values of two parallel measurements are shown.

Table 3. Cell dimensions of two selected strains (CAUP J302 - *Klebsormidium flaccidum* and K13 - *K. nitens*), cultivated in different environmental conditions. Results of both parallel measurements are listed in the table. During the experiments, only one of the physico-chemical parameters was varied while the others remained standard (temperature 25°C, humidity - 15 ml of sterile water in 5 g soil, illumination of about 200 µmol m^{-2} s^{-1}, pH 6.5).

Strain	Conditions		Cell width (µm)		Cell length (µm)	
CAUP J302	pH	Low (4.5)	6.25-7.25	5.75-6.75	5-14	6-20
		High (8.5)	5.5-6	5-8(-9)	5-22	5-17
	Temperature	Low (8°C)	5.25-6	6-7	5-18	5-17
		High (26°C)	6-6.75	5.5-6.25	5-15	5-18
	Humidity	Low (2.5 ml H_2O)	5.9-6.25	5.75-6.25	5-15	6-15
		High (50 ml H_2O)	6-7	6-7	3.5-14	5-15
	Illumination	No	5.25-5.5	5.25-6	7-16	6-15
		High (200 µmol m^{-2} s^{-1})	6-6.5	6-7	7-19	6-15
K13	pH	Low (4.5)	5.5-6	5-6	5-10	7-18
		High (8.5)	5.25-5.75(-8)	6-6.5	6-19	7-11
	Temperature	Low (8°C)	4.5-5.25	5-5.25	5.5-17	5-12
		High (26°C)	5-5.25	5.25-5.5	5-10	5-13
	Humidity	Low (2.5 ml H_2O)	5.75-6	5.75-6	6-15	4-16
		High (50 ml H_2O)	5.25-5.8	5.5-6	5-13	5-10
	Illumination	No	6-6.25	6-6.25	5-13	7-17
		High (200 µmol m^{-2} s^{-1})	4.5-5.5	5-5.75	5-15	6-15

influence of temperature on the cell width was noted. In both experimental conditions, cells of strain CAUP J302 were distinctly wider than those of strain K13. Another change in cell dimension was, however, observed in strains cultivated at high or low humidity. The effect of high humidity was similar to the effect of low pH - mild increase of the cell width of strain K13. Interestingly, at low humidity both strains showed a cell width of about 6 µm. Cell dimensions and overall morphology were alike in both populations. Under continuous high illumination, cell dimensions remained identical: the cells of strain CAUP J302 were distinctly wider in comparison to the cells of strain K13. A dissimilar behavior was noticed in strains which were cultivated in permanent darkness. Despite slow growth, the filaments were in good condition, without signs of ageing or degeneration. However, the cell width in both cultures changed considerably. The cells of strain CAUP J302 became thinner, whereas the cells of strain K13 became distinctly wider. Furthermore, the cells of K13 exceeded the cells of CAUP J302 in width, although in standard laboratory conditions they were clearly thinner.

The results of statistical analyses

The correlation matrix, shown in Table 4, describes the relationships among the characters used for strain description. No high correlation was detected. The highest correlation ($R = 0.71$) was noticed between the cell length/width ratio and length of the cells. Nevertheless, the l/w ratio was used in following analyses (e.g. in the cluster analysis or PCA, the use of this character gets the same weight to cell dimension variables and habitat variables). The characteristics of zoosporangia and zoospore

Table 4. Correlation matrix of the characters used for strain differentiation. The correlation between reproductive characters and habitat type is highlighted. Positive values of "Aperture" mean inconspicuous margins of apertures, positive values of "Germin" mean bipolar type of zoospore germination.

	Width	Length	Aperture	Germin	Rock	Soil	Moss	Water	L/w ratio
Width	1.00								
Length	0.25	1.00							
Aperture type	0.20	0.23	1.00						
Germination type	0.13	0.19	0.42	1.00					
Rock	-0.16	-0.15	**-0.46**	**-0.44**	1.00				
Soil	0.17	0.07	-0.05	0.04	-0.41	1.00			
Moss	0.14	0.07	0.19	**0.46**	-0.37	-0.19	1.00		
Water	-0.08	0.06	**0.42**	0.10	-0.49	-0.26	-0.23	1.00	
L/w ratio	-0.44	0.71	0.03	0.00	0.08	-0.13	-0.11	0.11	1.00

germination were only moderately correlated ($R = 0.42$). Interestingly, these characters were also influenced by environmental factors. Inconspicuous apertures in the empty zoosporangial cell walls occurred mainly in strains from aquatic microbiotopes ($R = 0.42$), whereas the distinct ones appeared in the strains from rock ($R = -0.46$). Similarly, the bipolar germination of the zoospores was correlated with moss microbiotope ($R = 0.46$), whilst the unipolar germination corresponded with microbiotope rock ($R = -0.44$).

No distinct large groups of strains were created in a cluster analysis of the 34 reproductive isolates, as illustrated in Fig. 16. Only several smaller groups were divided by means of cluster analysis. However, these groups correspond well with the microbiotope of the habitat. For example, all strains isolated from the rock surface formed one separate cluster. The strains with the same aperture type formed mostly in the separate sub-clusters within the microbiotope-defined clusters. The high occurrence of inconspicuous apertures in "water" strains and the low occurrence of this aperture type in "rock" strains are clearly visible in the dendrogram.

The ordination diagram in Fig. 17 shows the result of the principal component analysis. The first and second principal component axis explained 27.1 and 20.2% of the total variability, respectively. Rock microbiotope, type of aperture and the zoospore germination contributed to the first axis, whereas cell dimensions (especially length/width ratio) accounted for the second axis. The strains were evenly distributed in the ordination diagram, without formation of specific clusters. Moreover, none of the delimiting characters strictly separate the strains into two groups.

The distinction of strains by means of responded variables (character of zoosporangia, zoospore germination, type of microbiotope and the cell width) was tested via a two-group permutation test and canonical discriminant analysis (see Table 5). Three analyses significantly divided the strains into two separate groups: Analysis of aperture type (p-values 0.013 and 0.012) and the analyses of differences between the strains from microbiotopes "rock" and "moss" (p-values 0.013 and 0.009) and "rock" and "water" (p-values 0.035 and 0.042).

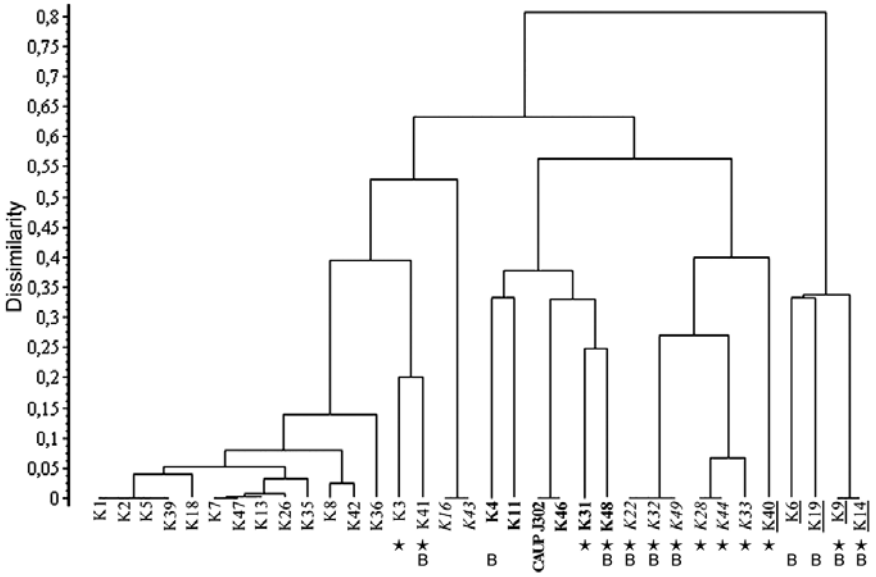

Fig. 16. Cluster analysis of 34 reproductive strains. The microbiotopes are distinguished by different font type (regular - rock, italic - water, bold - soil, underlined - moss). ★ - strains with inconspicuous aperture margins of empty zoosporangial cells. B - strains with bipolar zoospore germination.

The significant influence of habitat type led the author to interpret the variability in morphology of strains by means of habitat humidity (the values of humidity are listed in Table 1). This hypothesis was tested via redundancy analysis (RDA). The influence of humidity was significant both with aperture type (p-value 0.004) and the type of microbiotope (p-value 0.013) used as a covariable. In contrast, the test of apertures (with microbiotopes used as covariables) was not significant (p-value 0.358). Moreover, the effect of habitat humidity to the type of apertures in empty zoosporangial walls was significant in the two-sample t-test (p-value 0.022).

Table 5. Results of statistical analyses, tested the distinguishing of isolated strains by means of responded variables (listed in the left column). Separating of strains into two clusters by means of aperture types and microbiotopes rock vs. moss and rock vs. water was significant in both analyses (highlighted).

Tested pairs	Two-group permutation test	Discriminant analysis
Aperture type	**0.013**	**0.012**
Gernination type	0.067	0.068
Microbiotopes rock, soil	0.644	0.665
Microbiotopes rock, moss	**0.033**	**0.009**
Microbiotopes rock, water	**0.035**	**0.042**
Microbiotopes soil, moss	0.527	0.597
Microbiotopes soil, water	0.391	0.525
Microbiotopes moss, water	0.599	0.611
Cell width (boundary 6 µm)	0.471	0.492

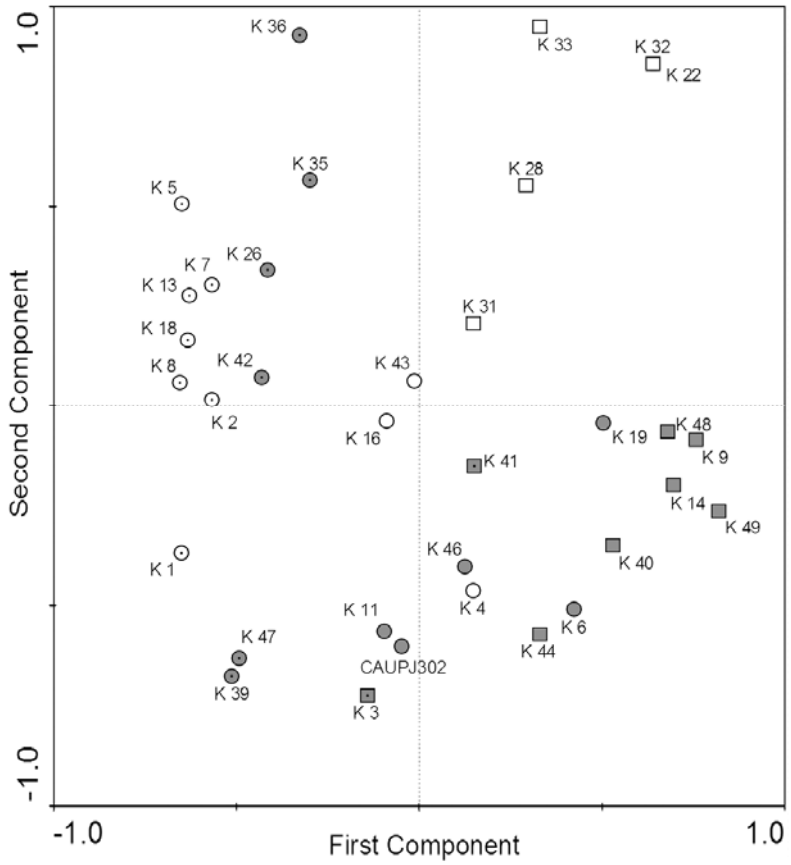

Fig. 17. PCA diagram showing the position of 34 isolated strains in the space of the first two ordination axes. The final shape of the symbols is characterized by the combination of three variables: CELL WIDTH - less than 6 µm (white colour), more than 6 µm (grey colour); APERTURE TYPE - distinctive aperture margins (circle), inconspicuous aperture margins (square) and MICROBIOTOPE - rock (symbols with dot), other microbiotopes (symbols without dot).

Discussion

For the morphological separation of thin *Klebsormidium* species, many authors mentioned a number of morphological features. Usually, the species have been differentiated on the basis of cell dimensions, especially cell width (Kützing 1843, Klebs 1896, Farooqui 1969, Lokhorst 1996). Lokhorst (1996) furthermore distinguished the species for the different habit of apertures in empty zoosporangial cell walls and occurrence of bipolar type of zoospore germination. However, the most of these morphological features seems to be taxonomically irrelevant when exposed to culture conditions.

The length and disintegration of filaments were significantly dependent on population age (in culture conditions, on nutrient content). The short cells occurred in well-

growing, mature populations, whereas the long ones were produced in very young or old cultures, where the nutrient depletion affects the disintegration of filaments into few-celled fragments. Despite the identical cultivation conditions, the onset of filament disintegration, equally as the rate of life cycle, was specific for each strain. In strain K49, several centimeters long filaments, containing more than 1000 cells, were observed during almost the whole life cycle (see Table 2). These long filaments were observed not only in strain K49 and not only in the culture conditions. For example, the longest filaments were noticed in strain K10, found in a fountain. There, *Klebsormidium* threads up to 2 meters long were found, consisting of several bundled filaments.

Contrary to cell length, the width of the cells varied only minimally during the life cycle. The cell width is considered to be one of the significant interspecific features in the genus *Klebsormidium* (Ettl & Gärtner 1995, Lokhorst 1996). However, the results of the above-mentioned experiment indicate the variability of cell width in dependence on some environmental conditions, especially humidity and intensity of illumination (Fig. 15). For example, cells cultivated in standard cultivation conditions differed from the cells of the same strain cultivated in darkness and low humidity. Similar results were presented by Poulíčková et al. (2001) who observed cell variability of a *Klebsormidium* strain obtained from a desert habitat cultivated under different culture conditions. They showed a high variation of cell width under different light, temperature and nutrient conditions.

On the other hand, the conditions used in present study are extreme and unnatural in biotopes where the *Klebsormidium* species are found (especially permanent darkness or pH 8.5). Under standard, non-extreme cultivation conditions the cell width observed was constant during the whole life cycle and therefore could be considered as a valuable strain (or species) feature (Table 2). Moreover, a boundary in cell width could be created to separate all 40 studied strains. During the whole life cycle, the cell width of 18 strains never exceeded 6 μm, whereas the cell width of the remaining 22 strains was always greater than 6 μm (Fig. 18). Even though a cell width of 6 μm was observed in some strains from both groups, no strain exhibited cell width fluctuation around this value (e.g. 5.5-6.5 μm).

Lokhorst (1996) pointed at the taxonomic value of zoospore germination and characteristics of apertures in the zoosporangial cell wall. In the present study, the unipolar germination was observed in all reproductive studied strains, whereas the bipolar type was present only in a few of them. Zoospore germination behavior characterized each strain, but not in a unique way. In addition, each reproductive *Klebsormidium* strain exhibited one of two types of apertures. In the majority of species the apertures were quite clearly visible, with distinct margins. In the rest of the studied strains the margin of aperture was inconspicuous. Following Lokhorst (1996), the type of zoospore germination and the aperture type are in coherence, i.e. the bipolar germination should be observed only in strains, where apertures with indistinctive margins are produced. Even though the present study indicated the correlation between these two characters (Fig. 17, Table 4), this link was not observed in all strains: in seven strains with bipolar germination the apertures were inconspicuous, in the remaining three strains they were obvious (Table 1). However,

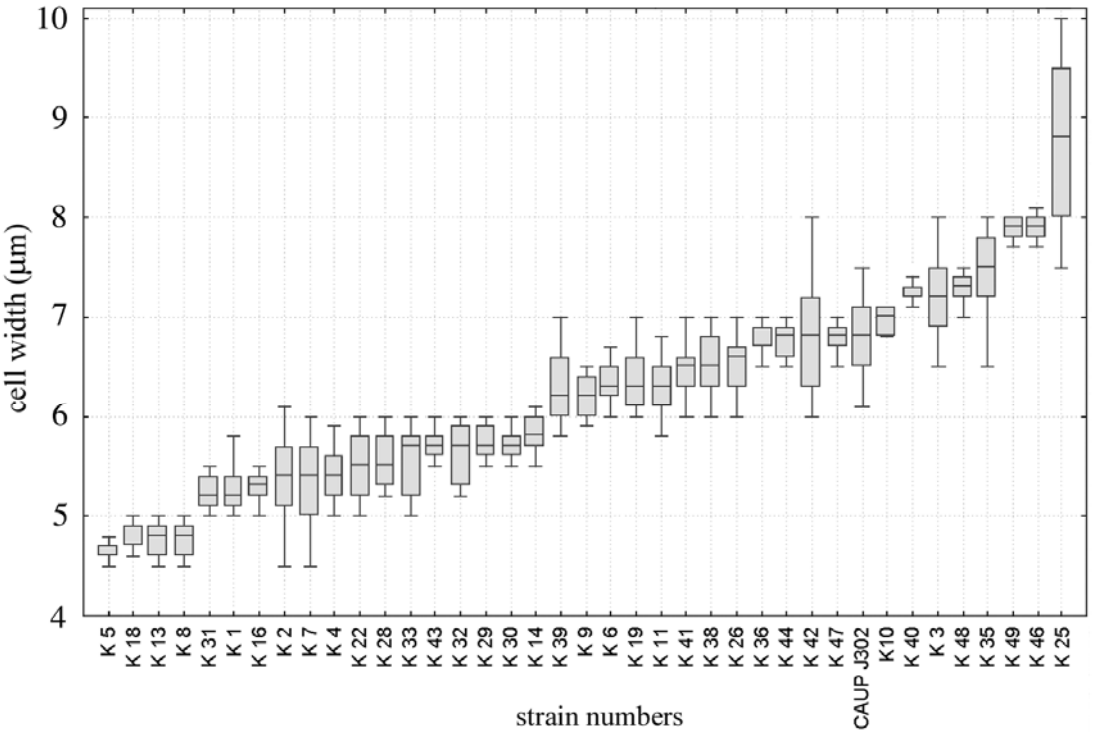

Fig. 18. Cell width range of all studied strains. The strains are ordered according to average cell width.

when only the aperture types were used as a delimiting character, the strains were successfully distinguished into two clusters (Table 5).

The most common species of the genus, *Klebsormidium nitens* and *K. flaccidum*, are traditionally differentiated by cell width - the cells of *K. nitens* should be thinner than the cells of *K. flaccidum* (Klebs 1896, Printz 1964, Starmach 1972, Lokhorst 1996). Considering the most studied strains as representatives of one of these two species, it is possible to divide the strains into two groups, using cell width of 6 mm as a boundary (Fig. 18). With the exception of three outlying cultures (K25, K46, K49, probably another species), the strains with cell width 6 and below 6 µm should represent *K. nitens*, whereas the other ones should belong to *K. flaccidum* (cell width 6 and above 6 µm). However, it is also possible to significantly separate the strains according to aperture type (but not germination behaviour). According to Lokhorst (1996), strains with distinctive margin of apertures should represent *K. nitens*, whereas strains with inconspicuous margin should characterize *K. flaccidum*. This delimitation is confirmed by the two-group permutation test and discriminant analysis (Table 5), equally as the strain separating by means of microbiotope of the habitat (Table 5, Fig. 16) or habitat humidity, respectively. In strains isolated from water biotopes, the inconspicuous apertures predominated. The type of habitat, and more precisely, habitat humidity seems to have a high impact on strain morphology.

Thus, it is possible to significantly divide the strains according to three attributes - cell width, character of zoosporangia and microbiotope of habitat. However, the

clusters of strains created on the basis of one delimiting character do not correspond with clusters constructed on account of other characters (Fig. 17). Although the cell width is suggested to be the main discriminant character, it is impossible to decide which feature is the most suitable for proper differentiation of *Klebsormidium nitens* and *K. flaccidum*. The present study, based on morphological features observed in culture conditions, indicates that the question of species concept in *Klebsormidium* can possibly be solved only by an appropriate combination of morphological and molecular data.

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Paper 2

Comparative study of chloroplast morphology and ontogeny in *Asterochloris* (Trebouxiophyceae, Chlorophyta)

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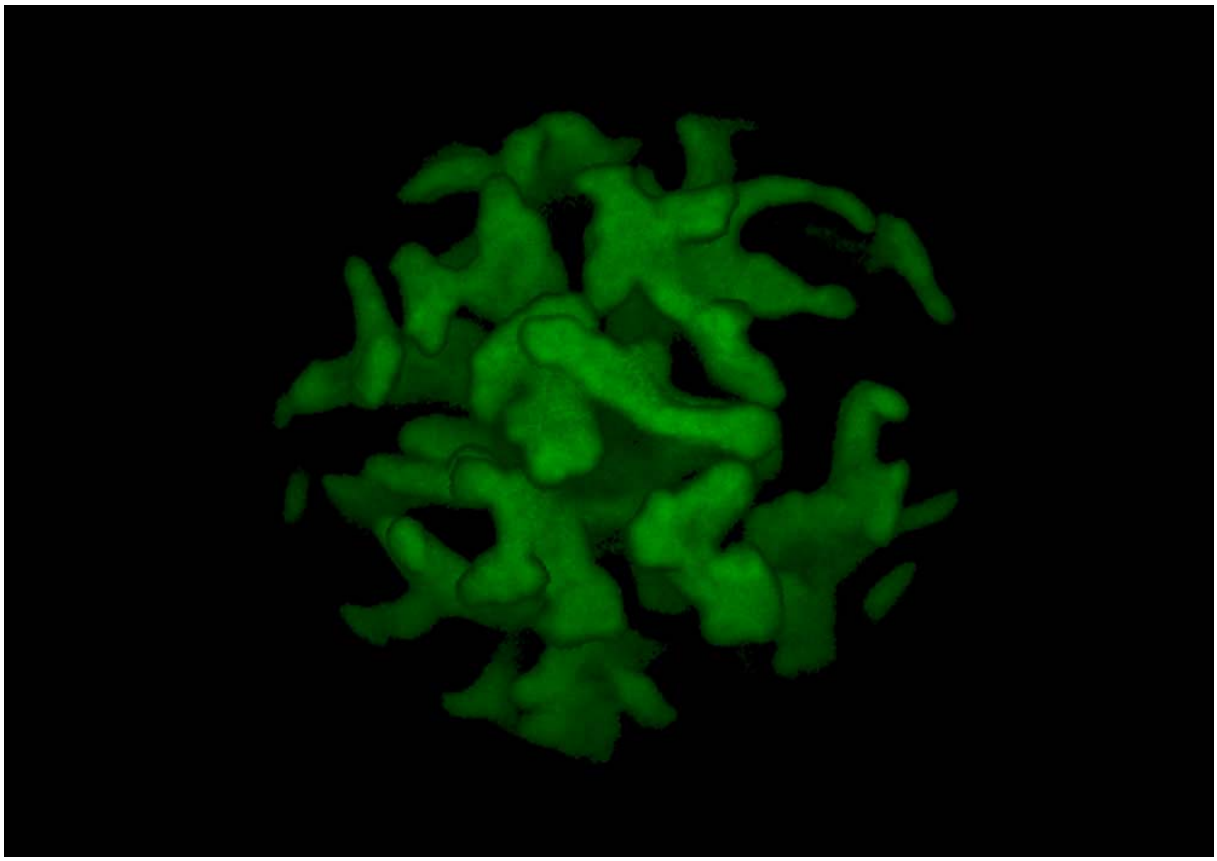

Confocal reconstruction of deeply lobed *Asterochloris* chloroplast

Comparative study of chloroplast morphology and ontogeny in *Asterochloris* (Trebouxiophyceae, Chlorophyta)

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Abstract: Confocal laser scanning microscopy was utilized to compare the chloroplast morphology and ontogeny among five strains of the green alga *Asterochloris*. Parsimony analysis inferred from the rDNA ITS sequences confirmed their placement in three distinct lineages: *Asterochloris phycobiontica*, *Trebouxia pyriformis* and *Asterochloris* sp. Examination by confocal microscopy revealed the existence of interspecific differences in the chloroplast ontogeny of *Asterochloris*; this was based upon either specific chloroplast structures observed in a single species, or on the differential timing of particular ontogenetic sequences. The occurrence of flat parietal chloroplasts prior to cell division, considered as a basic morphological discriminative character of *Asterochloris*, was clearly associated with the process of aplanosporogenesis. By contrast, chloroplast transformation prior to the formation of autospores proceeded simply by the multiple fission of the chloroplast matrix in the cell lumen.

Key words: *Asterochloris*, chloroplast morphology, confocal microscopy, ITS, molecular phylogeny, *Trebouxia*.

Introduction

The Swiss botanist Schwendener (1867) was the first to demonstrate that the microscopic green bodies in lichen thalli, the so-called gonidia, are in fact green or blue-green algae. Prior to that, lichenologists thought that the green bodies originated from the tips of colorless hyphae, even though their resemblance to algae was noticed. At present, an estimated 100 species in 40 genera of algae are reported as photobionts of various lichen species (Tschermak-Woess 1988; Friedl & Büdel 1996). In the majority of the associations, the phycobiont belongs to one of three genera, namely: *Trebouxia* Puymaly sensu lato, *Trentepohlia* Martius and *Nostoc* Vaucher ex Bornet et Flahault.

Among the leading researchers in lichen symbiosis was Elisabeth Tschermak-Woess (1917 – 2001) who greatly increased our knowledge of the morphology and systematics of many photobionts. Her extensive scientific work includes descriptions and morphological observations of some novel or rare photobiont species, e.g. those of the genera *Dictyochloropsis* Geitler, *Myrmecia* Printz, *Trebouxia* Puymaly and *Elliptochloris* Tschermak-Woess. In particular, she was recognized as an exceptional cytologist, sometimes working at the limits of the laws of optics (for more information see Hesse 2001). In 1980, she described a new algal genus and species, *Asterochloris phycobiontica* Tschermak-Woess, based on her observations of the phycobiont of lichen *Anzina carneonivea* (Anzi) Scheidegger (Tschermak-Woess 1980). She delimited the genus as having a mainly parietal, radially lobed cup-shaped

chloroplast ("sternförmig gegliederten Bechers") with a single large, or up to seven additional pyrenoids. Later however, she recognized the close relationship of *A. phycobiontica* with those species of *Trebouxia* that reproduce only by means of aplanospores. In accordance with these observations, she transferred *A. phycobiontica* into the genus *Trebouxia* subg. *Eleutherococcus* (Warén) Tschermak-Woess under the designation *Trebouxia phycobiontica* (Tschermak-Woess) Tschermak-Woess (Tschermak-Woess 1989). Additionally, Tschermak-Woess did not except the possible future elevation of the subgenera *Trebouxia* and *Eleutherococcus* as two separate genera; in that case, she suggested using the generic name *Asterochloris* for those species producing no autospores (Tschermak-Woess 1989).

Soon afterwards, ensuing molecular investigations revealed the polyphyly of the genus *Trebouxia* (DePriest 2004). Initially, Friedl & Zeltner (1994), Friedl (1995) and Friedl & Rokitta (1997) inferred from nrSSU and nrLSU rDNA sequence data that *Trebouxia magna* Archibald was more closely related to *Myrmecia biatorellae* Tschermak-Woess & Plessl than to *Trebouxia* s. str. In the light of this fact, Friedl (unpubl.) proposed a split of the genus *Trebouxia* into two genera, *Asterochloris* and *Trebouxia*, on the basis of congruencies found between morphology and DNA sequence analyses. In parallel, Rambold et al. (1998) referred to the lichen selectivity towards these two genera, assuming that all *Asterochloris* species would be the only compatible photobionts for the majority of the Cladoniaceae. Validity of *Asterochloris* was later supported by Piercey-Normore & DePriest (2001), who compared the nuclear internal transcribed spacer (ITS) sequences of many lichen photobionts and algal cultures. They revealed pairwise ITS sequence similarities among the *Asterochloris* taxa greater than 93%. Moreover, these sequences could not be aligned with those of *Trebouxia* s. str. Therefore, it appears that the *Asterochloris* algal symbionts are distinct from those of *Trebouxia* s. str. as proposed by Friedl (unpubl.).

Eight species are presently considered to be affiliated with the genus *Asterochloris*, based on ITS sequences and morphological characteristics (Piercey-Normore & DePriest 2001; Friedl & Gärtner 1988), including *Asterochloris phycobiontica* Tschermak-Woess, *Trebouxia erici* Ahmadjian, *T. excentrica* Archibald, *T. glomerata* (Warén) Ahmadjian, *T. italiana* Archibald, *T. irregularis* Hildreth et Ahmadjian, *T. magna* and *T. pyriformis* Archibald. In addition to considerably different ITS sequences, *Asterochloris* species can be recognized by their distinctive chloroplast ontogeny, as compared to *Trebouxia*. The chloroplasts of *Asterochloris* may flatten and assume a parietal position prior to cell division, while chloroplasts of *Trebouxia* species remain lobed and at a more central position during division (Ahmadjian 1960; Hildreth & Ahmadjian 1981; Friedl & Gärtner 1988).
In the present study, we observed the chloroplast morphology and ontogeny of selected *Asterochloris* species, using both type cultures and our own isolates from the lichen *Lepraria* Acharius. The main goals of this study were to investigate the process and function of chloroplast flattening prior to cell division, and to describe some additional patterns in chloroplast morphology that are typical for genus *Asterochloris*. A combination of conventional light microscopy and confocal microscopy was utilized to better observe the morphological variations of chloroplasts during cell ontogeny in detail.

Material and methods

Species sampling and algal cultures
Thallus fragments of three lichenized fungi, *Lepraria borealis*, *Lepraria neglecta* and *Lepraria* sp., were collected at various localities in Central Europe (Table 1). The algal symbionts were isolated into axenic culture according to the thallus fragmentation method of Ahmadjian (1993). Cultured strains of the isolated photobionts are maintained in the private culture collection of O. Peksa at the Department of Botany, Charles University in Prague. In addition, the type strains of *Asterochloris phycobiontica* and *Trebouxia pyriformis* were ob-

tained from 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), respectively (Table 1). Observations of the algal isolates were made on cultures grown on 2% agar slants of Bold´s Basal Medium (BBM) as modified by Bischoff & Bold (1963). All cultures were grown under standard conditions: at a temperature of 15 °C, under an illumination of 5-15 μmol m^{-2} s^{-1} in a Helkama C5G cool box.

Light and confocal microscopy

Observations using a conventional light microscope and a confocal microscope were made regularly at 7 day intervals on 2-11 week old cultures. The pure algal samples were examined by a Leica TCS SP2 confocal laser scanning microscope, equipped with an Argon-Krypton laser, using a 488 nm excitation line and an AOBS filter free system collecting emitted light between 498 and 700 nm. A Leica 63x/1.4 N.A. oil immersion objective fitted on a Leica DM IRE2 inverted microscope was used. A series of optical sections through chloroplasts were captured and used for 3-dimensional reconstruction of their morphology. The autofluorescence of the chlorophyll was exploited for the visualization of the chloroplast structure. For the final image processing we used Leica Confocal Software, version 2.61 (Leica Microsystems Heidelberg GmbH) and the Image J 1.34p program (Abramoff et al. 2004).

DNA extraction, PCR and DNA sequencing

Total genomic DNA was extracted from lyophilized algal cultures following the standard CTAB protocol (Doyle & Doyle 1987), with minor modifications. Algal DNA was resuspended in sterile dH2O and amplified by the polymerase chain reaction (PCR). The ITS1, ITS2, and 5.8S rDNA regions were amplified using the algal-specific primer nr-SSU-1780-5´ (5´-CTG CGG AAG GAT CAT TGA TTC-3´; Piercey-Normore & DePriest 2001) and a universal primer ITS4-3´ (5´-TCC TCC GCT TAT TGA TAT GC-3´; White et al. 1990). All PCR were performed in 20 μl reaction volumes (15.1 μl sterile Milli-Q Water, 2 μl 10´ PCR buffer (Sigma), 0.4 μl dNTP (10 μM), 0.25 μl of primers (25 pmol/ml), 0.5 μl Red Taq DNA Polymerase (Sigma) (1U/ml), 0.5 μl of $MgCl_2$, 1 μl of DNA (5 ng/ml)). After an initial denaturing step at 95 °C for 5 min, 35 cycles of denaturing at 95 °C for 1 min, annealing at 60 °C for 1 min and elongation at 72 °C for 1 min were performed, followed by a final extension at 72 °C for 7 min. The PCR products were quantified on 1% agarose gel stained with ethidium bromide and cleaned with GENOMED Jetquick kit. The purified amplification products were sequenced with a set of sequencing primers described above (nr-SSU-1780-5´ and ITS4-3´) using the protocol for the DNA sequencing kit (ABI Prism Big-Dye terminator cycle sequencing ready reaction, Applied BioSystems). Purified sequencing reactions were run on 3100-

Table 1. Species and strains of *Asterochloris* used in this study.

Phycobiont	Strain number [a]	Isolated from lichen	Locality	Collector	Year
Asterochloris phycobiontica	SAG 26.81	*Anzina carneonivea*	Italy, Trento, Madonna di Campiglio.	Tschermak-Woess E.	1976
Asterochloris phycobiontica	LEP 9	*Lepraria neglecta*	Ukraine, East Carpathians, Breskul Mt.	Slavíková Š.	2004
Asterochloris sp.	LEP 10	*Lepraria borealis*	Bulgaria, Stara planina Mts, Central Balkan NP.	Slavíková Š. & Slavík M.	2004
Asterochloris sp.	LEP 36	*Lepraria* sp.	Czech Republic, Máslovická stráň NR.	Peksa O. & Jindráková Z.	2006
Trebouxia pyriformis	UTEX 1712	*Cladonia squamosa*	USA, Massachusetts, Leverett	Hutchinson W.A.	1969

[a] SAG - culture collection of algae at the University of Göttingen (http://www.epsag.uni-goettingen.de/html/sag.html); UTEX - culture collection at the University of Austin, Texas (http://www.bio.utexas.edu/research/utex/); LEP – authors' strain designation.

Avant Genetic Analyzer (Applied BioSystems). Sequencing reads were assembled and edited using SeqAssem (SequentiX Software). Newly obtained sequences were deposited in the EMBL Nucleotide Sequence Database with following accession numbers: AM900490 (*Asterochloris phycobiontica*, SAG 26.81), AM900491 (*Asterochloris phycobiontica*, LEP 9), AM900492 (*Asterochloris* sp., LEP 10), AM900493 (*Asterochloris* sp., LEP 36).

Sequence alignment and phylogenetic analyses

After initial automatic alignment using ClustalX 1.83 (Thompson et al. 1997), the 18S rDNA sequences were manually aligned using MEGA 3.1 (Kumar et al. 2004) with the following reference sequences taken from GenBank: AF345382 (*Trebouxia glomerata* UTEX 895), AF345404 (*Trebouxia glomerata* UTEX 896), AF345405 (*Trebouxia glomerata* UTEX 897), AF345406 (*Trebouxia pyriformis* UTEX 1712), AF345407 (*Trebouxia pyriformis* UTEX 1713), AF345411 (*Trebouxia irregularis* UTEX 2236), AF345423 (*Trebouxia magna* UTEX 67), AF345433 (*Trebouxia excentrica* UTEX 1714), AF345439 (*Trebouxia erici* UTEX 910), AF345440 (*Trebouxia erici* UTEX 911), AF345441 (*Trebouxia erici* UTEX 912). Positions with deletions in most sequences were removed from the alignment, resulting in an alignment comprising 533 base positions. Alignment is available from EMBL-EBI (Accession No. ALIGN_001226). The phylogenetic tree was inferred from the aligned sequence data by the maximum parsimony (MP) method using the PAUP* 4.0b10 (Swofford 2003). Reliability of the resulting topology was tested using bootstrap analysis (10,000 replications). MP phylogenies were constructed using the branch-and-bound search option, with the simple addition of sequences and gap characters treated as a fifth base.

Results

The morphology of the isolated photobionts was compared with that of the type strains of *Asterochloris phycobiontica* and *Trebouxia pyriformis*. Comparisons under light microscopy revealed many shared morphological features, such as: the pyriform cell shape, chloroplast flattening prior to cell division, and frequent aplanosporogenesis. Further, the high similarity of photobiont ITS sequences with all available sequences from cultured strains of *Asterochloris* corroborated the assignment of the studied *Lepraria* photobionts to the genus *Asterochloris,* and revealed the close relationship among all studied strains. Parsimony analysis of the ITS data set recovered 14 most-parsimonious trees with a length of 33 steps. The resulting unrooted phylogeny of one of the most parsimonious trees is shown in Fig. 1. The tree topol-

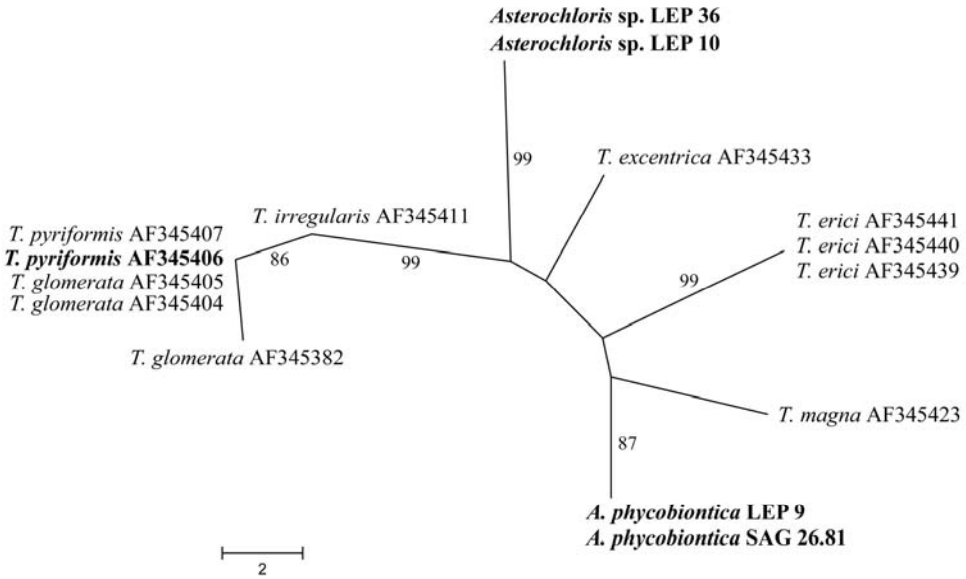
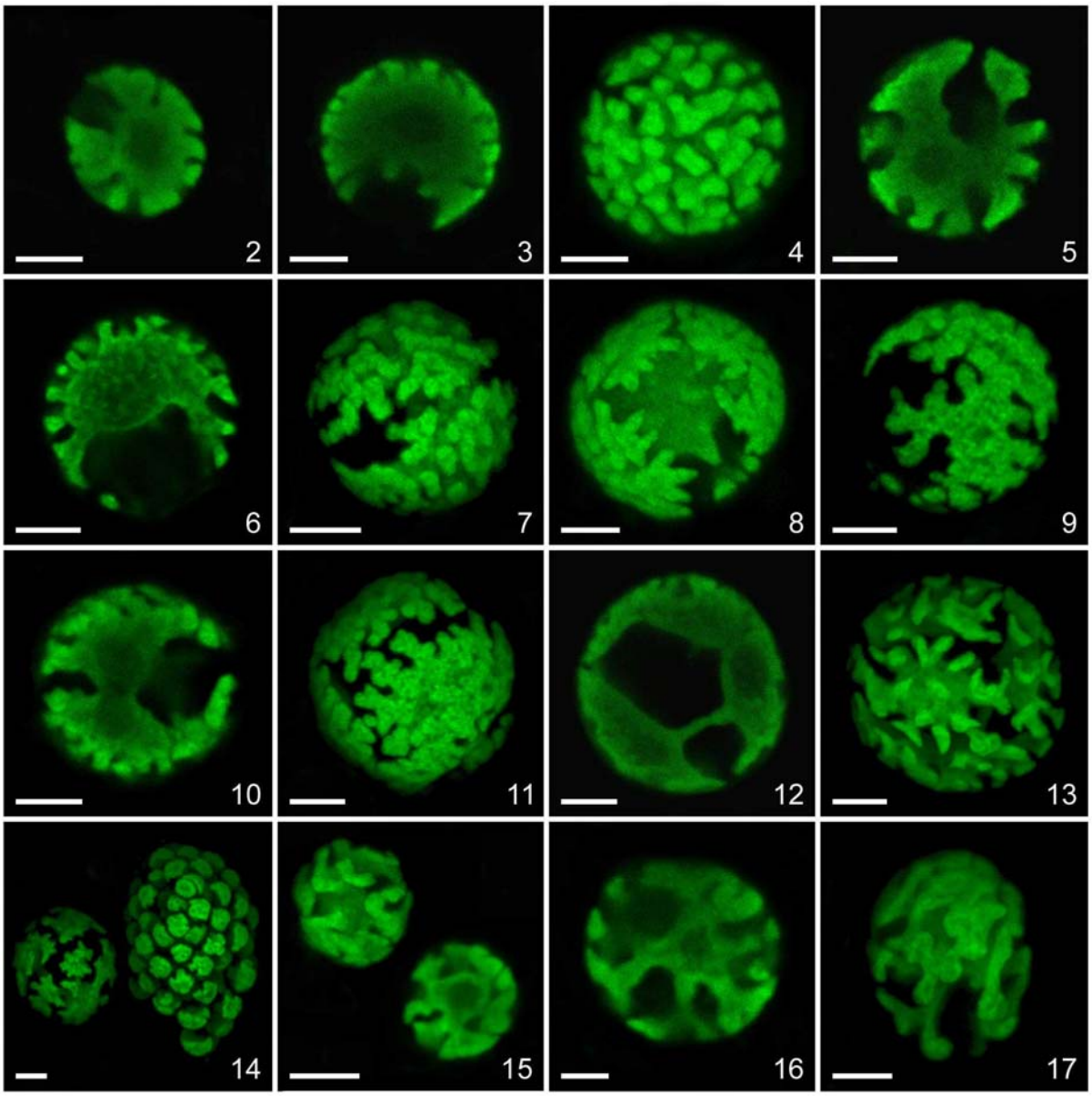


Fig. 1. Unrooted phylogeny of *Asterochloris* ITS rDNA sequences using the maximum parsimony method and a branch-and-bound search. Values at the nodes represent statistical support estimated by maximum parsimony bootstrapping. The scale indicates the distance due to two evolutionary steps. Investigated strains are indicated in **bold**.

ogy corresponded with the results of Piercey-Normore & DePriest (2001), distinguishing the species *Trebouxia glomerata*, *T. pyriformis* and *T. irregularis* (Clade I sensu Piercey-Normore & DePriest) from all other species (bootstrap support 99 %). The sequence of strain LEP 9 was identical with the type species of *Asterochloris phycobiontica* SAG 26.81. The ITS sequences of LEP 10 and LEP 36 were identical, thus, indicating that they formed a distinct branch separate from lineages representing other species (bootstrap value 99 %). To investigate chloroplast ontogeny in *Asterochloris*, algal strains from three different evolutionary lineages were chosen: *A. phycobiontica* (strains SAG 26.81 and LEP 9), *T. pyriformis* (strain UTEX 1712) and *Asterochloris* sp. (strains LEP 10 and LEP 36).



Figs 2-17. Confocal sections (CS) and maximum projections (MP) of chloroplast. 2-14: *Asterochloris phycobiontica*. 2 – simple chloroplast of young cell (CS); 3 – crenulate chloroplast (CS); 4 – crenulate chloroplast (MP); 5 – axial chloroplast with deep lobes (CS); 6 – parietal position of chloroplast (CS); 7 – chloroplast surface with many simple lobes (MP); 8 – parietal chloroplast with finger-like lobes (MP); 9 – smooth chloroplast surface with divided marginal lobes (MP); 10 – pyrenoid multiplication (CS); 11 – dividing of smooth parietal chloroplast (MP); 12 – chloroplast division into two parts (CS); 13 – lobed surface of divided chloroplast parts (MP); 14 – aplanospore production (MP). 15-17: *Trebouxia pyriformis*. 15 – simple chloroplast of young cells (MP, CS); 16 – deeply lobed axial chloroplast (CS); 17 – chloroplast with branched lobes (MP). Scale bar: 5 μm.

Asterochloris phycobiontica **(**SAG 26.81, LEP 9)

Young cells had a central crenulate chloroplast with many simple lobes and a central pyrenoid (Fig. 2). During cell growth, the chloroplasts either retained a crenulate form with a central mass of chloroplast matrix (Figs 3, 4), or had several deep incisions that cut the outer chloroplast layer into several separate lobes (Fig. 5). Very early in the cell ontogeny, the central asteroid chloroplast assumed a parietal position (Fig. 6). However, despite the eccentric chloroplast position, the simple crenulate chloroplast lobes were evenly distributed under the cell wall (Fig. 7). In the fully parietal stage, the chloroplast margin extended into simple, finger-like lobes, that were frequently divided (Fig. 8). Simultaneous to the formation of these lobes, the chloroplast surface simplified, as the superficial lobes decreased in size. Finally, the chloroplast assumed a parietal position, with the margins extended into the finger-like lobes (Fig. 9).

In conjunction with the above-mentioned processes, the chloroplast structure underwent distinct changes prior to aplanosporogenesis. Initially, the single pyrenoid divided equally (Fig. 10) giving rise to 2 – 4 pyrenoids within the chloroplast. These pyrenoids assumed opposite positions in the cell and became the centres of the new daughter chloroplasts. The chloroplast matrix usually occupied the area around the pyrenoids leading to the division of the chloroplast into several parts. The new chloroplasts had a smooth surface and simple undulated margins (Figs 11, 12). Further chloroplast multiplication was signalled by further pyrenoid divisions, and by increased complexity of the chloroplast surface. The chloroplasts migrated towards the cell centre and their surface was divided into the characteristic elongated lobes (Fig. 13). Finally, at the end of aplanosporogenesis, the chloroplast was separated into more than one hundred simple parts, entirely filling the cell lumen (Fig. 14).

Trebouxia pyriformis (UTEX 1712)

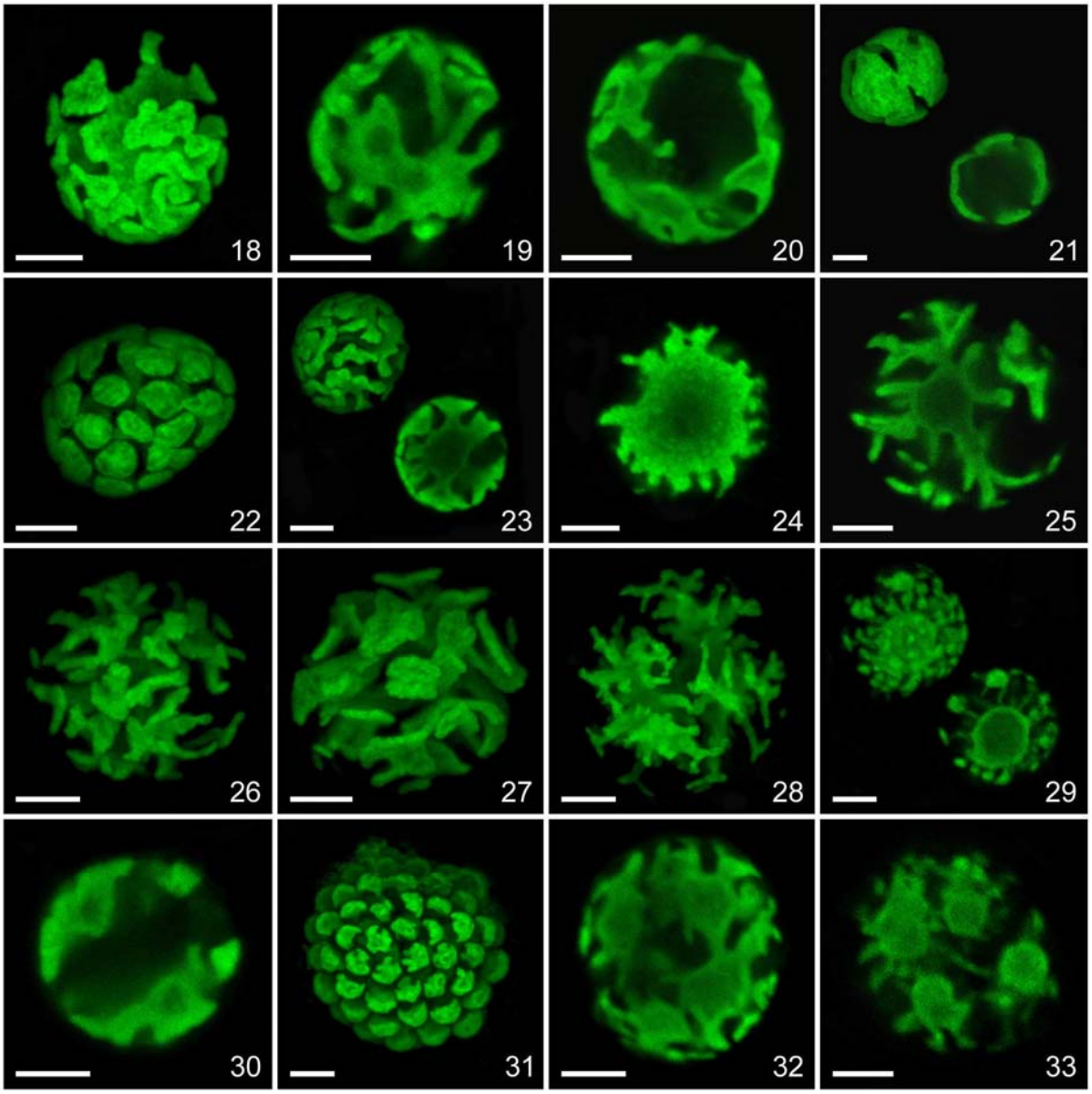
In young cells, the chloroplast assumed a central position with several lobes radiating towards the cell's periphery. The lobes were clearly extended longitudinally at their ends, leading to an elongate appearance in surface view. Terminal expansion was characterized by a T-shaped profile of the lobes as viewed in confocal optical sections (Fig. 15). Mature cells exhibited central chloroplasts with an asteroid or crenulate shape. Asteroid chloroplasts were characterized by deep lobes, emerging directly from the thin chloroplast layer spreading around the pyrenoid (Fig. 16). During cell growth, the chloroplast lobes branched both inside the cell and at the cell periphery (Fig. 17). The lobes then started to appear flattened over their entire length, with flat terminal portions of variable shape (Fig. 18). Concurrently with the above-mentioned increase of chloroplast complexity, the pyrenoids multiplied within the chloroplast matrix (Fig. 19). Before aplanosporogenesis, the chloroplast assumed a parietal position and began to divide (Fig. 20). The resulting smooth chloroplasts assumed an extremely flat shape, with no pyrenoids observed inside (Fig. 21). Finally, further chloroplast multiplication led to the formation of many simple chloroplast parts, entirely filling the cell lumen (Fig. 22).

Asterochloris **sp.** (LEP 10, LEP 36)

As in *T. pyriformis*, the chloroplasts of young cells assumed a central position with several lobes spreading to the cell periphery. Elongate ends of the lobes were characterized by a T-shaped profile, as viewed in confocal optical sections (Fig. 23). Rarely, a centrally positioned crenulate chloroplast with many simple lobes was present (Fig. 24). Larger cells displayed a typical asteroid chloroplast with deep lobes that emerged directly from the thin chloroplast layer spreading around the pyrenoid (Figs 25, 26). Mature cell chloroplasts exhibited several ontogenetic stages, alternating during the cell's ontogeny. Ordinary chloroplast lobes could

change into the flattened ones, with flat peripheral endings of variable shapes (Fig. 27). Alternatively, the chloroplast's surface was sometimes cut into many tubular branched lobes (Fig. 28). Finally, some cells were characterized by having a sun-like shaped chloroplast, formed by many thin radial lobes that emerged from the deep chloroplast layer (Fig. 29).

All the above-mentioned chloroplast stages could change as a consequence of the processes associated with asexual reproduction. Initial stages of aplanosporogenesis were signalled by multiplication of the pyrenoid, followed by the displacement of the chloroplast to a parietal position (Fig. 30). Then, the chloroplast matrix occupied the area around the pyrenoids, leading to the division of the chloroplast. The resulting smooth surfaced chloroplasts



Figs 18-33. Confocal sections (CS) and maximum projections (MP) of chloroplast. 18-22: *Trebouxia pyriformis*. 18 – flattened chloroplast lobes with flat terminal parts (MP); 19 –pyrenoid multiplication (CS); 20 – parietal position of chloroplast (CS); 21 – smooth parietal chloroplast during its division (MP, CS); 22 – aplanospore production (MP). 23-33: *Asterochloris* sp. 23 – simple chloroplast of young cells (MP, CS); 24 – crenulate chloroplast (CS); 25 – deeply lobed chloroplast (CS); 26 – deeply lobed chloroplast (MP); 27 – flattened chloroplast lobes with flat terminal parts (MP); 28 – chloroplast surface consisted of tubular lobes (MP); 29 – sun-like chloroplast (MP, CS); 30 – flattened parietal chloroplasts (CS); 31 – aplanospore production (MP); 32, 33 – chloroplast division during the autosporogenesis (CS). Scale bar: 5 μm.

further broke up into a large number of simple parts, filling up the cell lumen (Fig. 31). By contrast, autospore production was characterized by the direct fission of a central asteroid chloroplast into several parts, without any migration to a parietal position (Figs 32, 33). During the subsequent separation the resulting chloroplast parts filled up the whole cell lumen.

Discussion

Molecular analysis of the ITS rDNA sequences clearly placed all investigated photobionts in three distinct lineages within *Asterochloris*. Strains LEP10 and LEP 36 formed a lineage that was separate from all described species as well as from published *Asterochloris* sequences. Therefore, they very probably represent a new species of *Asterochloris* (Fig. 1). *Trebouxia italiana*, the last described species of *Asterochloris*, with no published sequence, has a very different chloroplast morphology and cell dimensions compared to both investigated strains (Gärtner 1985). These results indicate the presence of obvious cryptic species diversity in *Asterochloris*, as has been recently shown in other trebouxiophycean clades (Kroken & Taylor 2000; Neustupa et al. 2007).

Although it is undeniable that molecular characteristics play a leading role in the taxonomy of *Trebouxia* s.l., chloroplast morphology is still regarded as an important criterion in species delimitation (Beck et al. 1998; Friedl & Rokitta 1997). Despite the existance of different taxonomic concepts in *Trebouxia* s.l., the heterogeneity of *Trebouxia* was often demonstrated by the conspicuous differences in chloroplast structure. One of the main disparities, also applied recently for *Trebouxia* and *Asterochloris* separation, was the occurrence of flat parietal chloroplasts prior to cell division (Hildreth & Ahmadjian 1981; Friedl & Gärtner 1988). Our observations confirm the validity of this distinction, as the ontogenetic stage with flat parietal chloroplasts was noticed in all studied strains of *Asterochloris* (Figs 12, 21, 30). Moreover, further examination of mature cells revealed the specific occurrence of parietal chloroplasts only in the initial stages of aplano- or zoosporogenesis. On the other hand, chloroplast transformation prior to the formation of autospores occurred without chloroplast flattening, and simply involved the multiple fission of the chloroplast matrix in the cell lumen (Figs 32, 33). These observations appear to suggest the existence of two distinct ontogenetic pathways leading to chloroplast splitting prior to cell division. In the case of aplano- and zoosporogenesis, a large number (up to 128) of daughter cells is created compared to the production of autospores (Fig. 31). The requirement for a chloroplast to split into a large number of equal parts can lead to the necessity of chloroplast simplification prior to this process. The importance of this simplification can be demonstrated by *A. phycobiontica*: although the divided chloroplasts have a complicated structure with a lobed surface (Figs 13, 14), the splitting of the chloroplast precedes the formation of flat parietal chloroplasts with undulate margins (Figs 11, 12). Interestingly, these parietal chloroplasts have also been observed in *T. erici*, *T. glomerata*, *T. irregularis* and *T. pyriformis* (Friedl & Gärtner 1988), thus, in the majority of *Asterochloris* species. However, the specific stage of cell ontogeny demonstrating parietal chloroplasts has never been observed in *Trebouxia*, despite the evident prevalence of zoospores in this genus (Gärtner 1985). It would be interesting to compare the chloroplast ontogeny in *Asterochloris* and *Trebouxia* in greater detail which could clarify whether the parietal stage occurs in *Trebouxia,* or whether morphological transformation of chloroplasts during the process of aplano- and zoosporogenesis proceeds via a different ontogenetic pathway.

In addition to the above-mentioned stage characterized by flat parietal chloroplasts, there are some further morphologically identical stages between either all three species studied or at least two of them. These include a central axial chloroplast with elongate lobes that are T-shaped in profile (Figs 15, 23), a massive crenulate chloroplast with many simple lobes (Figs 3, 4, 24), a central chloroplast with deep long lobes (Figs 5, 16, 25, 26), and an axial

chloroplast with flattened lobes terminated by flat peripheral endings of variable shape (Figs 18, 27). Although these stages are shared amongst the species of *Asterochloris*, specific differences primarily concern the different timing of the particular stages in chloroplast ontogeny. However, since we did not study synchronized cultures, we were not able to precisely time the occurrence and duration of particular stage within cell ontogeny. The results presented here correspond with the observations of Škaloud et al. (2005), who identified several distinct ontogenetic stages shared by species of coccal green alga *Dictyochloropsis* Geitler. However, we found that certain specific chloroplast developmental stages occurred in one species only, for example, the simple lobed chloroplast margin of mature cells and the parietal position of chloroplasts in *A. phycobiontica*.

Although the results of molecular investigations demonstrated the polyphyly of *Trebouxia* and clearly segregated the genus *Asterochloris* (Piercey-Normore & DePriest 2001; DePriest 2004), the morphological diagnostic criteria of individual species remain vague. We hope that the distinctive differences in chloroplast ontogeny as demonstrated in this study will form a useful contribution towards future combined structural/molecular taxonomic investigations that are aimed at developing a clear species and genus concept in *Trebouxia* and *Asterochloris*.

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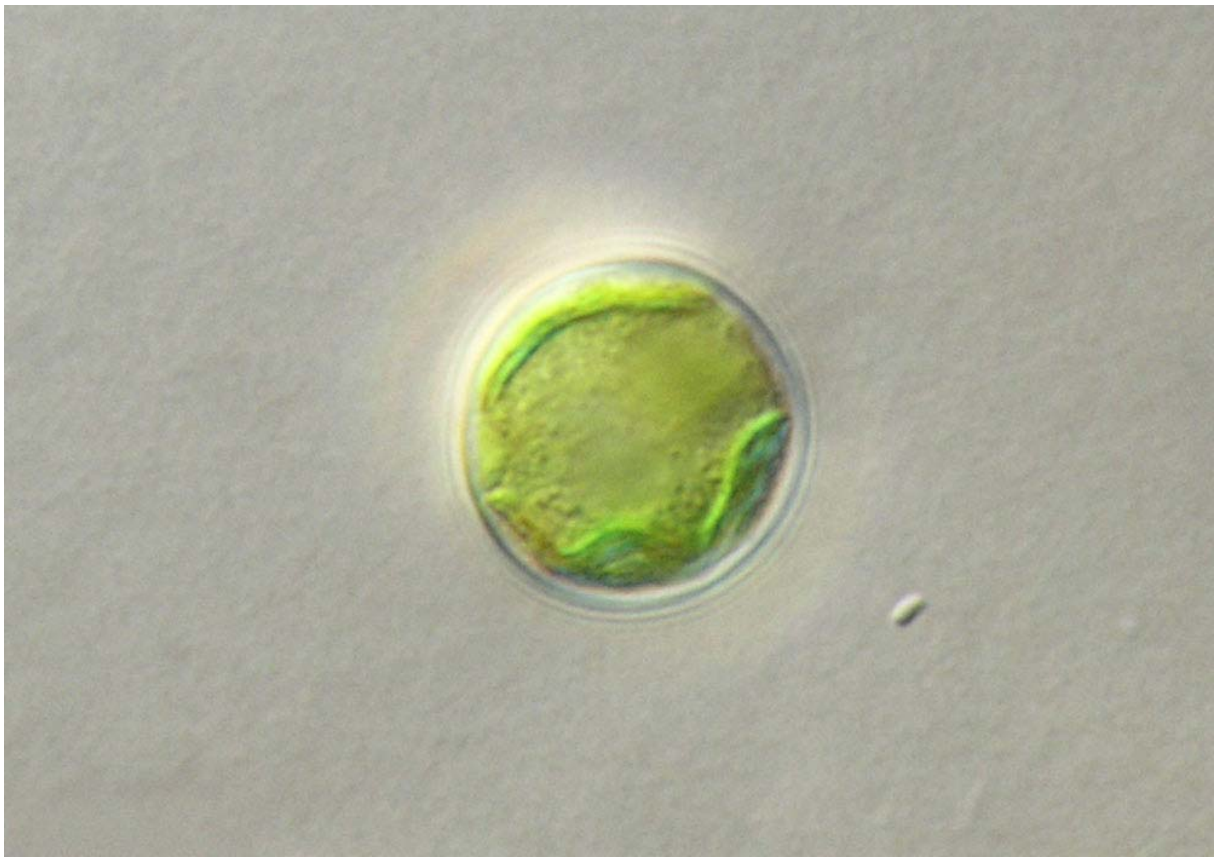
Paper 3

Phylogeny, morphology, reproduction and taxonomic revision of symbiotic alga *Asterochloris* (Trebouxiophyceae, Chlorophyta)

Pavel Škaloud & Ondřej Peksa

Submitted manuscript



Specific flattened parietal chloroplast, appearing in all *Asterochloris* species prior to zoospore production

Phylogeny, morphology, reproduction and taxonomic revision of the symbiotic alga *Asterochloris* (Trebouxiophyceae, Chlorophyta)

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Abstract

The genus *Asterochloris* is one of the most numerous lichen photobionts. We present a revision of the genus, based on the comparative morphological and molecular investigation of 34 cultured strains. For all strains, we determined nuclear-encoded ITS rDNA and partial actin I sequences. In addition, we studied morphology and reproduction by conventional light and confocal microscopy. Morphological comparisons reveal seven distinct chloroplast types appearing during cell ontogeny. We recognize and designate 13 monophyletic lineages, 7 of which represent currently described species (*Asterochloris phycobiontica*, *Trebouxia erici*, *T. excentrica*, *T. glomerata*, *T. irregularis*, *T. italiana* and *T. magna*). Concurrently with proposing new combinations for former *Trebouxia* species, we describe 6 species new to science (*A. echinata*, *A. friedlii*, *A. gaertneri*, *A. leprariae*, *A. lobophora* and *A. woessiae*); furthermore, *T. pyriformis* is reduced to a synonym of *A. glomerata*. All species are defined by both unique actin sequences and a combination of selected morphological characters (chloroplast morphology, cell shape, etc.). Variation in some of these characters correlates with genetic distances, and therefore, could be used for definition of particular clades. Asexual reproduction takes place by autospores, aplanospores and zoospores, which often bear special posterior extensions. Isogamous sexual reproduction was scarcely observed.

Keywords
Asterochloris, lichen photobionts, molecular phylogeny, morphology, reproduction, taxonomy

Introduction

Lichens are prime examples of symbiotic associations composed of a fungal (mycobiont) and a photosynthetic (photobiont) partner that may be either a green alga or cyanobacterium. The Swiss botanist Schwendener (1867) was the first to demonstrate that the microscopic green bodies in lichen thalli, the so-called gonidia, were in fact green or blue-green algae. Prior to that, lichenologists thought that the green bodies originated from the tips of colorless hyphae, even though their resemblance to algae was noticed. At present, an estimated 100 species in 40 genera of algae are reported as photobionts of various lichen taxa (Friedl and Büdel 1996; Tschermak-Woess 1988). The most common phycobiont genus, *Trebouxia* de Puymaly, is present in approximately 20% of all lichen species (DePriest 2004; Tschermak-Woess 1988).

Since the beginning of investigations of *Trebouxia* algae, some researchers found a disunity in this genus, and recognized the existence of two species groups. Initially, Warén (1920) established two subgenera based on differences in asexual reproduction. Those algae which divided vegetatively he designated as *Eucystococcus*, and those not exhibiting vegetative division but producing large numbers of autospores, he assigned to *Eleuterococcus*. Ahmadjian (1959b, 1960) divided *Trebouxia* into two main groups depending on the position of the chloroplast prior to sporogenesis as well as on cell shape. Group I was characterized by a parietal position of the chloroplast prior to cell division and rather oval cells, while group II was defined by a central position of dividing chloroplasts and rather spherical cells. Moreover, chloroplasts of group I algae were deeply lobed, with lobes reaching the cell periphery, as compared to group II algae containing a rather massive central chloroplast with a smoother surface. This differentiation was further confirmed by Jacobs and Ahmadjian (1968) and Peveling (1968) based on ultrastructural comparison of pyrenoid structure. The differences consisted of rather arcuate (group I) or swollen (group II) thylakoids penetrating the pyrenoid matrix and on a highly vesiculate pyrenoid in group II. Later, Archibald (1975) distinguished the genera *Pseudotrebouxia* Archibald and *Trebouxia* de Puymaly emend. Archibald, based on the occurrences of desmoschisis (*Trebouxia*) and eleutheroschisis (*Pseudotrebouxia*) in the process of asexual reproduction (Groover and Bold 1969). However, Gärtner (1985a, b) rejected establishment of *Pseudotrebouxia* because he exclusively observed protoplast division in all *Trebouxia* strains.

In 1980, Tschermak-Woess described a new genus and species, *Asterochloris phycobiontica* Tschermak-Woess, based on her observations of the phycobiont of a lichen *Anzina carneonivea* (Anzi) Scheidegger (Tschermak-Woess 1980, as *Varicellaria carneonivea*). Although some morphological features were similar to *Trebouxia* species, she delimited the genus as having a mainly parietal, radially lobed, cup-shaped chloroplast with a single large, or up to seven additional, pyrenoids. Later, when revising the taxonomy of *Trebouxia*, she split the genus into two subgenera, *Trebouxia* Tschermak-Woess and *Eleutherococcus* (Warén) Tschermak-Woess (Tschermak-Woess 1989), following the genus division given by Warén (1920). In her opinion, *Eleutherococcus* was defined by the strict absence of autospores, which occurred in subg. *Trebouxia*. Moreover, she recognized the close relationship of *A. phycobiontica* with those species producing no autospores. In accordance with these observations, she transferred *A. phycobiontica* into the genus *Trebouxia* subg. *Eleutherococcus* under the designation *Trebouxia phycobiontica* (Tschermak-Woess) Tschermak-Woess. Additionally, Tschermak-Woess did not exclude the possible of future reclassification of *Trebouxia* subgenera (*Trebouxia* and *Eleutherococcus*) into two separate genera; in that case, she suggested using the generic name *Asterochloris* for those species producing no autospores (Tschermak-Woess 1989).

Soon afterwards, molecular investigations revealed the polyphyly of genus *Trebouxia*. Initially, Friedl and Zeltner (1994), Friedl (1995) and Friedl and Rokitta (1997) inferred from nrSSU and nrLSU sequence data that *Trebouxia magna* Archibald and *Trebouxia erici* Ahmadjian were more closely related to *Myrmecia biatorellae* Tschermak-Woess and Plessl than to *Trebouxia* s. str. In light of this, the genus *Trebouxia* was split into two genera, *Asterochloris* and *Trebouxia*, was proposed (Friedl unpublished observations, in Rambold et al.

1998; Helms et al. 2001) based on the suggestion made by Tschermak-Woess (1989). Rambold et al. (1998) discussed the lichen selectivity towards these two genera and assumed that *Asterochloris* species would be the only compatible photobionts for the majority of the Cladoniaceae. Validity of *Asterochloris* was later supported by Piercey-Normore and DePriest (2001), who compared the nuclear internal transcribed spacer (ITS) sequences of many lichen photobionts and algal cultures. They revealed pairwise ITS sequence similarities among the *Asterochloris* taxa greater than 93%. Moreover, these sequences could not be aligned with those of *Trebouxia* s. str.

Comparing the ITS sequences of all species that cluster with *Asterochloris phycobiontica*, Piercey-Normore and DePriest (2001) reveal many discernable features to define the genus, as has been proposed by several authors in the past. They are as follows: parietal position of chloroplast prior to cell division (Ahmadjian 1960; Friedl and Gärtner 1988; Hildreth and Ahmadjian 1981), typical ultrastructure of pyrenoid matrix (Friedl 1989a; Jacobs and Ahmadjian 1968; Peveling 1968), deeply lobed chloroplast (Ahmadjian 1959b, 1960), rather oviform, elliptical and pyriform cell shape (Ahmadjian 1960; Hildreth and Ahmadjian 1981), high proportion or strict presence of aplanospores (Friedl 1993; Tschermak-Woess 1989; Warén 1920) and photobiont selection toward the Cladoniaceae (Ahmadjian and Jacobs 1981; Rambold et al. 1998). The above-mentioned morphological and molecular features clearly differentiate *Trebouxia* and *Asterochloris*. Additionally, several recent studies have illustrated that a wide range of *Asterochloris* taxa occur as photobionts of various lichen species (e.g. Beiggi and Piercey-Normore 2007; Cordeiro et al. 2005; Nelsen and Gargas 2008; Piercey-Normore 2004; Piercey-Normore and DePriest 2001; Yahr et al. 2004, 2006). Despite this, formal delimitation of *Asterochloris* as well as assignment of the species to that genus is still pending.

In the present study, we propose the revision of genus *Asterochloris* based on the comparative investigation of nine authentic strains in addition to twenty-five our own isolates from various lichen species. The polyphasic taxonomical approach was adopted to clearly define the species concept within the genus. Hence, a combination of conventional light and confocal microscopy was utilized in conjunction with the DNA sequencing of ITS rDNA and an intron-containing portion of the actin type I gene.

Results

Taxonomic revisions

Asterochloris Tschermak-Woess 1980; Pl. Syst. Evol. 135, pp. 291, 292 emend. Škaloud et Peksa

Type species: *Asterochloris phycobiontica* Tschermak-Woess emend. Škaloud et Peksa.

Emended diagnosis: Single asteroid chloroplast of lobed, crenulate or echinate form. Prior to aplano- and zoosporogenesis, the chloroplast flattens and assumes a parietal position. Asexual reproduction by (16-32-)64-128(-256) aplanospores and zoospores, occasionally by 2-4-8 autospores. Zoospores naked, dorsiventrally flattened, 4-10 μm long x 1.5-4 μm wide, with two apical flagella and one posterior extension; stigma present or absent. Sexual reproduction by biflagellated isogamous gametes. Zygotes motile, quadriflagellate. Photobionts of many lichens (genera *Anzina*, *Cladia*, *Cladonia*, *Diploschistes*, *Lepraria*, *Pilophorus*, *Pycnothelia*, *Stereocaulon*, etc.). Widely distributed, cosmopolitan.

Key to the species

1a. Chloroplast without pyrenoid ***A. magna***
1b. Chloroplast with single or several pyrenoids 2
2a. Cells predominantly contain structurally complex, deeply lobed chloroplast 3
2b. Deeply lobed chloroplast occurs only occasionally or it is never produced 7
3a. Young cells often pyriform 4
3b. Young cells spherical 5
4a. Several smaller pyrenoids occur around the large central pyrenoid, zoospore length 4-4.5 μm ***A. glomerata***
4b. Pyrenoids of equal size, zoospore length 5-7 μm ***A. irregularis***
5a. Cells up to 17(-18) μm in diameter, never with a crenulate chloroplast ***A. excentrica***
5b. Cells up to 20(-24) μm in diameter, crenulate chloroplast can be produced 6
6a. Single pyrenoid per chloroplast ***A. friedlii***
6b. Several pyrenoids per chloroplast ***A. woessiae***
7a. Cells commonly exhibit parietal lobed chloroplast 8
7b. Parietal lobed chloroplast rare or absent 9
8a. Single pyrenoid per cell, elongated lobe terminations produced ***A. lobophora***
8b. Several pyrenoids per cell, elongated lobe terminations absent ***A. phycobiontica***
9a. Mature cells often exhibit globular chloroplasts ***A. echinata***
9b. Globular chloroplast never produced 10
10a. Cells up to 20 μm in diameter 11
10b. Cells larger 12
11a. Indistinct pyrenoid, spherical or slightly oval cells ***A. erici***
11b. Distinct, delimited pyrenoid, cells occasionally pyriform or cylindrical ***A. italiana***
12a. Large cells, up to 27(-33) μm in diameter, several smaller pyrenoids occur around the large central pyrenoid ***A. leprariae***
12b. Smaller cells, up to 23(-25) μm in diameter, pyrenoids of equal size ***A. gaertneri***

Asterochloris phycobiontica Tschermak-Woess 1980; Pl. Syst. Evol. 135, p. 292 emend. Škaloud et Peksa

Authentic strain: SAG 26.81

Emended diagnosis: Vegetative cells usually spherical, occasionally oviform to pyriform, up to 24(-25) μm in diameter (Fig. 1A). Cell wall thin, mature cells occasionally with expressive local thickenings (up to 5 μm wide) or rarely with wholly thickened cell wall. In young cells chloroplast is crenulate with many simple lobes and central pyrenoid. In mature cells, the chloroplasts either retain a crenulate form (Fig. 1B), or, more commonly, assume a parietal position with the margin extended into divided finger-like lobes (Fig. 1C). The chloroplast lobes are either simply terminated, or terminated by finger-like extensions. The chloroplast contains from one to many distinct pyrenoids having well-defined globular structures or indistinct striations (Fig. 1D). Besides the typical, centrally located pyrenoid, up to seven smaller ones may be present in its vicinity. Starch grains are embedded in a layer around the pyrenoid. Asexual reproduction by 64-128 aplanospores, or zoospores, produced in large spherical sporangia. Zoospores 4-4.5 μm long x 2.5 μm wide with posterior extensions.

Asterochloris glomerata (Warén) Škaloud et Peksa comb. nov.

Basionym: *Cystococcus glomeratus* Warén 1920; Reinkulturen von Flechtengonidien, pp. 56-60, Taf. I., Fig. 6.

Synonyms: *Trebouxia glomerata* (Warén) Ahmadjian 1960; Am. J. Bot. 47(8), p. 679, Figs 9, 10, 15. *Trebouxia pyriformis* Archibald 1975; Phycologia 14(3), pp. 130, 131, Fig. 11.

Emended diagnosis: Young cells usually spherical, mature vegetative cells pyriform, oviform, ellipsoidal, spherical or irregular, up to 20(-21) μm long x 15 μm wide (Fig. 1E). Cell wall thin, mature cells sometimes with local polar thickening, or rarely, with irregularly thickened cell wall. Chloroplast in young cells assumes the central position with several lobes spreading towards the cell's periphery. Mature cells exhibit central chloroplasts of either deeply lobed (Fig. 1F) or rarely shallowly lobed form (Fig. 1G). The chloroplast lobes are ex-

tended either longitudinally or finger-like at their ends. Occasionally, the chloroplast lobes start to appear flattened over their entire length exhibiting flat terminal portions of variable shape (Fig. 1H). Especially in older cells, the chloroplast can be asymmetrically positioned. The chloroplast contains from one to many distinct pyrenoids. Besides the typical, centrally located pyrenoid, up to four smaller ones are present in its vicinity (Fig. 1I). At times, indistinct striations or granulation can be visible inside pyrenoids. Starch grains are embedded in a layer around the pyrenoid. Asexual reproduction by 32-64-128 aplanospores or 64 zoospores that are produced in large ellipsoidal or spherical sporangia. Zoospores dorsiventrally flattened, 4-4.5 μm long x 3 μm wide.

Asterochloris irregularis (Hildreth et Ahmadjian) Škaloud et Peksa comb. nov.

Basionym: *Trebouxia irregularis* Hildreth et Ahmadjian 1981; Lichenologist 13(1), pp. 82, 83, Fig. 2C.

Emended diagnosis: Young cells usually spherical, mature vegetative cells pyriform, oviform, ellipsoidal, irregular or spherical, up to 20(-21) μm long x 11(-14) μm wide (Fig. 1J). Cell wall thin, in maturity often thickened, either unilaterally or along the entire surface. Chloroplast in young cells assumes the central position, with several lobes spreading towards the cell's periphery. Mature cells exhibit central chloroplasts of either deeply lobed (Fig. 1K) or shallowly lobed form (Fig. 1L). The chloroplast lobes generally are either extended longitudinally at their ends or terminated by finger-like extensions. Especially in older cells, the chloroplast is apparently asymmetrically positioned. It does not occupy the whole cell lumen, but forms a parietal mass with long lobes spreading towards the cell's periphery (Fig. 1M). In this case, the anterior part of the cell includes no chloroplast, containing a nucleus and cytoplasm. The chloroplast generally contains one conspicuous pyrenoid that can be indistinctly or distinctly striated (Fig. 1N). Sometimes, the pyrenoid is divided by invading thylakoids into several neighbouring parts. Asexual reproduction by 16-32-64-128 aplanospores or 16-32 zoospores produced in sporangia of variable, mainly pyriform shape. Zoospores 5-7 μm long x 1.5-2.5 μm wide with posterior extensions.

Asterochloris erici (Ahmadjian) Škaloud et Peksa comb. nov.

Basionym: *Trebouxia erici* Ahmadjian 1960; Am. J. Bot. 47(8), pp. 680, 681, Figs 6, 10, 15.

Authentic strain: UTEX 912

Emended diagnosis: Vegetative cells usually spherical, occasionally ellipsoidal, up to 16 μm in diameter (Fig. 1O). Cell wall thin, mature cells occasionally with local thickenings. Chloroplast in young cells simply asteroid or band-shaped, assuming the central position within the cell. Mature cells exhibit central chloroplasts of shallowly lobed form (Fig. 1P). The chloroplast lobes are distinctively or slightly extended longitudinally at their ends. The chloroplast contains one indistinct pyrenoid without delimited margin (Fig. 1Q). Sometimes, invading thylakoids are visible under confocal microscopy. Starch grains are embedded evenly throughout the chloroplast matrix. Asexual reproduction by 16 aplanospores or zoospores produced in spherical or ellipsoidal sporangia with locally thickened cell wall. Zoospores dorsiventrally flattened, 4.5-7.5 μm long x 2.5-3 μm wide with posterior extensions.

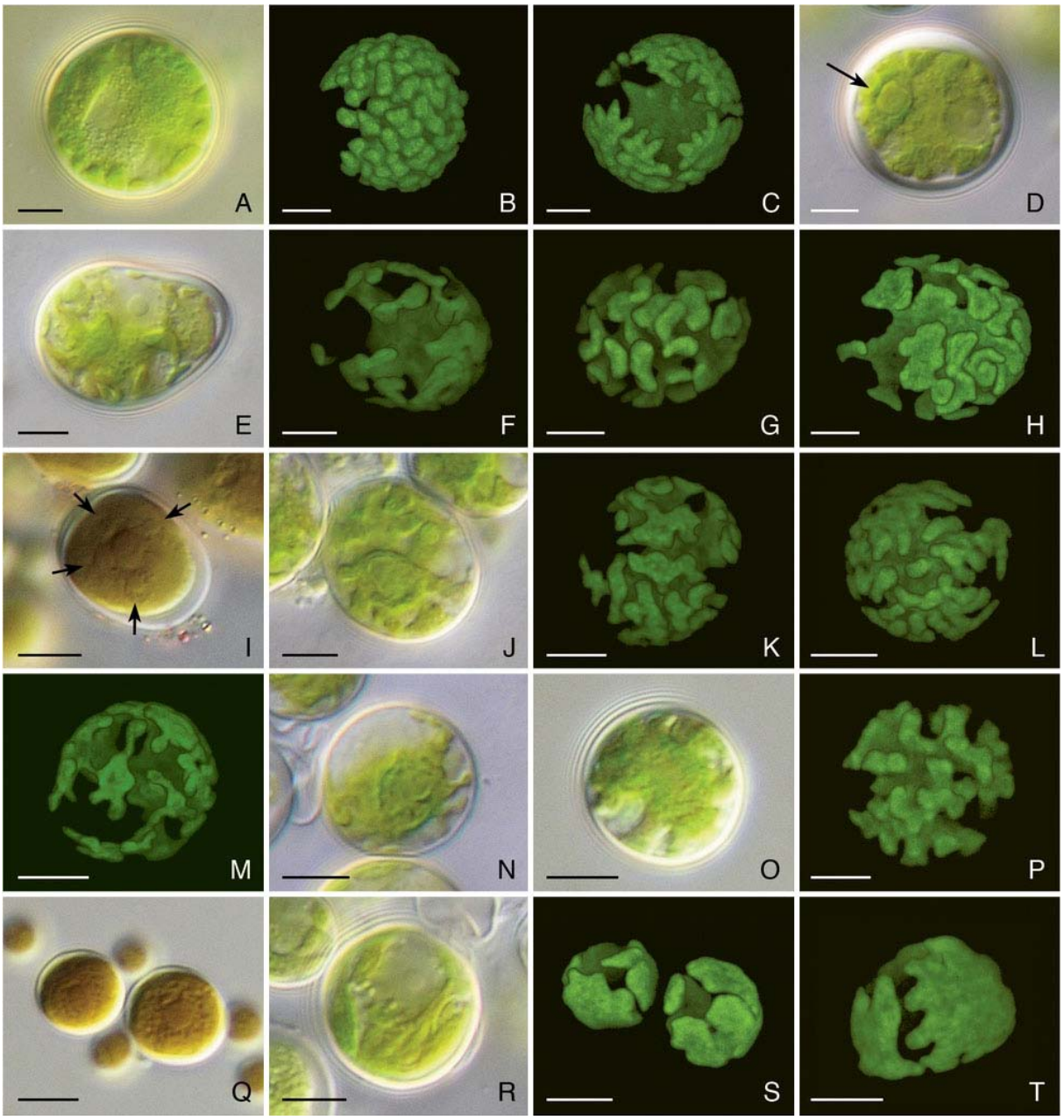

Figure 1. Light micrographs and confocal reconstructions of chloroplast structures in vegetative cells of *Asterochloris*. **A-D.** ***A. phycobiontica***. Characteristic morphology **(A)** and the confocal reconstructions of crenulate **(B)** and lobed parietal **(C)** chloroplasts. Globular structure inside the pyrenoid (arrow) is visible in the optical section **(D)**. **E-I.** ***A. glomerata***. Frequently produced pyriform cell **(E)**. Confocal reconstructions of deeply lobed **(F)**, shallowly lobed **(G)** and flat lobed **(H)** chloroplasts. Several pyrenoids (arrows) are visible in the vicinity of the larger, centrally located pyrenoid **(I)**. **J-N.** ***A. irregularis***. Characteristic morphology **(J)** and the confocal reconstructions of deeply lobed **(K)**, shallowly lobed **(L)** and parietal, asymmetrically positioned **(M)** chloroplasts. Single pyrenoid containing distinct elongate structures **(N)**. **O-Q.** ***A. erici***. Light micrograph **(O)** and confocal reconstruction **(P)** of simple, shallowly lobed chloroplast. Pyrenoid visualization **(Q)**. **R-T.** ***A. magna***. Characteristic morphology **(R)** and the confocal reconstructions of simple **(S)** and undulated **(T)** parietal chloroplasts. Cells in Figs **I**, **Q** stained by chloriodine solution. Scale bar – 5 μm.

Asterochloris magna (Archibald) Škaloud et Peksa comb. nov.

Basionym: *Trebouxia magna* Archibald 1975; Phycologia 14(3), p. 130, Fig. 10.

Synonym: *Trebouxia lambii* nomen nudum Ahmadjian 1959a: 55-57.

Lectotype: Ahmadjian 1959a; The taxonomy and physiology of lichen algae and problems of lichen synthesis. Ph.D. Dissertation, Harvard University. Illustration "Pilophorus acicularis" A-H inserted between pages 58 and 59 (as *T. lambii* sp. nov.). Botany Farlow Library, 22 Divinity Avenue, Cambridge, MA 02138, USA (*hic designatus*).

Authentic strain: UTEX 902

Emended diagnosis: Vegetative cells spherical or ellipsoidal, occasionally oval or pyriform, up to 19(-21) μm in diameter (Fig. 1R). Cell wall thin, mature cells sometimes with local thickenings. Chloroplast in young cells simply parietal. Mature cells exhibit smooth parietal chloroplasts that very quickly divide into several parts, equally distributed at the cell periphery (Fig. 1S). Larger cells can occasionally contain one parietal chloroplast with simply undulated margins (Fig. 1T). Generally, the chloroplast has a smooth surface and does not produce any extensions towards the cell's periphery. The chloroplast does not contain any pyrenoids. Asexual reproduction by 64-128 aplanospores produced in large spherical sporangia. Occasionally autosporangia are formed, containing 4-8 spherical autospores. Zoospores dorsiventrally flattened, 4.5-6.5 μm long x 2-2.5 μm wide with posterior extensions.

Asterochloris excentrica (Archibald) Škaloud et Peksa comb. nov.

Basionym: *Trebouxia excentrica* Archibald 1975; Phycologia 14(3), pp. 128, 130, Fig. 7.

Authentic strain: UTEX 1714

Emended diagnosis: Young cells usually spherical, mature vegetative cells spherical, occasionally ellipsoidal or oviform, up to 17(-18) μm in diameter (Fig. 2A). Cell wall thin, mature cells sometimes with local polar thickenings. Chloroplast in young cells assumes the central position with several lobes spreading towards the cell's periphery. Mature cells predominantly exhibit central deeply lobed chloroplasts (Fig. 2B). Occasionally shallowly lobed chloroplasts are formed (Fig. 2C). The chloroplast can be slightly asymmetrically positioned in mature cells. The chloroplast lobes are generally terminated by flat extensions (Fig. 2D). Infrequently, they also can be extended longitudinally at their ends or terminated by finger-like extensions. The chloroplast contains one distinct pyrenoid. Indistinct granulation or parallel striation can be visible inside the pyrenoid (Fig. 2E). Starch grains are embedded in a layer around the pyrenoid in the form of large granules. Asexual reproduction by 4-8 autospores, 16-32-64 aplanospores or 16 zoospores. Zoospores dorsiventrally flattened, 4-7 μm long x 2-3.8 μm wide; with posterior extensions. Autospores are produced relatively frequently, as compared to another species of the genus.

Asterochloris italiana (Archibald) Škaloud et Peksa comb. nov.

Basionym: *Trebouxia italiana* Archibald 1975; Phycologia 14(3), p. 130, Fig. 9.

Authentic strain: CCAP 219/5B

Emended diagnosis: Vegetative cells usually ellipsoidal, occasionally spherical, cylindrical and pyriform, up to 20 μm in length and 13 μm in width (Fig. 2F). Cell wall thin, seldom a flat local thickening of the cell wall can be made out. Chloroplast in young cells simply asteroid, assuming the central position within the cell. Mature cells exhibit central chloroplasts of either shallowly lobed (Fig. 2G) or, less commonly, crenulate form (Fig. 2H). Sometimes, the terminal lobes have broad bases and tapered ends, so the chloroplast itself looks like a many-pointed star. The chloroplast lobes are generally extended longitudinally at their ends, but occasionally they are only simply terminated. Pyrenoid generally one and distinct (Fig. 2I), occasionally up to four smaller ones occur in its vicinity. Sometimes, an indistinct granulation can be visible inside the pyrenoid. Starch grains are embedded centrally around the pyrenoid. Asexual reproduction by 32-64 aplanospores or zoospores produced in spherical, ellipsoidal or long cylindrical sporangia. Occasionally, 4-8 autospores are also produced. Zoospores dorsiventrally flattened, 3- μm long x 2-2.5 μm wide with posterior extensions.

Asterochloris woessiae Škaloud et Peksa sp. nov.

Latin Diagnosis: Cellulae vegetativae sphericae, interdum ellipticae et piriformes, ad 21(-24) μm magnae. Parietes cellularum raro localiter incrassati. Chloroplastus unicus, saepe lobularis, interdum echinatus, crenatus aut parietalis, pyrenoide unica vel aliquibus praeditus. Pyrenoides granis amylaceis dispersis circumcinctae. Nucleus unus, parietalis. Propagatio asexualis per zoosporas, aplanosporas et autosporas. Aplanosporae et zoosporae 128 per sporangio. Zoosporae complanatae, 4.5-7.5 μm longae et 2.5-4.5 μm latae, cum caudae posteriorum. Autosporae 2 vel 4 per sporangio. Propagatio sexualis per coniunctionem isogametarum, forma zoosporis similum. Specimina typica phycobionte *Leprariae borealis* Loht. & Tønsberg.

Holotype: Freeze-dried material obtained from strain LEP 10 (culture collection of O. Peksa) isolated from *Lepraria borealis* and deposited at the CAUP (Culture Collection of algae of the Charles University in Prague, Department of Botany, Benátská 2, 12801, Praha 2, Czech Republic) under designation LYO-H 1009 (*hic designatus*). The living cultures (exholotypes) have been deposited as CAUP H 1009 ibidem.

Type locality: Bulgaria, Stara planina Mts, Central Balkan National Park, on sunlit slate rock and over moss in fissures of rock, alt. 618 m – sample of *Lepraria borealis*, 1.7.2004 collected Š. Bayerová & M. Slavík, deposited in PRA No. 3401.

Etymology: The species epithet is in honour of the work of Dr. Elisabeth Tschermak-Woess, who described the genus *Asterochloris.*

Diagnosis: Vegetative cells usually spherical, occasionally oviform and pyriform, up to 21(-24) μm in diameter (Fig. 2J). Cell wall thin, seldom a flat local thickening of the cell wall can be made out. Very rarely the cell wall is slightly thickened along its entire surface. Chloroplast in young cells assumes the central position with several lobes spreading towards the cell's periphery. Mature cells display a structurally complicated, central, deeply lobed chloroplast with branched lobes that emerge directly from the thin layer spreading around the pyrenoid (Fig. 2K). Mature cell chloroplasts can further exhibit several other ontogenetic stages, alternating during the cell's ontogeny. The chloroplast lobes can appear flattened over their entire length (Fig. 2L), or the chloroplast can be formed by many thin radial lobes giving it a bristly appearance (Fig. 2M). Rarely, the crenulate and parietal chloroplast can be formed as well. Generally, the chloroplast lobes are terminated in three modes: they can be extended longitudinally at their ends, flat (Fig. 2N), or simply terminated. The chloroplast contains 1-3 distinctively delimited pyrenoids (Fig. 2O). Sometimes, an indistinct granulation can be visible inside the pyrenoids. Starch grains are embedded in a layer around the pyrenoid. Asexual reproduction by 128 aplanospores or zoospores produced in large spherical or ellipsoidal sporangia. Occasionally, 2-4 autospores are also produced. Zoospores dorsiventrally flattened, 4.5-7.5 μm long x 2.5-4.5 μm wide with posterior extensions. Sexual reproduction by biflagellated isogamous gametes.

Asterochloris leprariae Škaloud et Peksa sp. nov.

Latin Diagnosis: Cellulae vegetativae sphericae, interdum ellipticae, oviformes et piriformes, ad 27(-33) μm magnae. Parietes cellularum raro localiter incrassati. Chloroplastus unicus, crenulatus aut lobularis, interdum parietalis, pyrenoide unica vel aliquibus praeditus. Pyrenoides granis amylaceis dispersis circumcinctae. Nucleus unus, parietalis. Propagatio asexualis per 64-128 aplanosporas et 64 zoosporas, interdum per 2-4 autosporas. Zoosporae complanatae et recurvatae, 6-10 μm longae et 2.8-4 μm latae, cum caudae posteriorum. Specimina typica phycobionte *Leprariae neglectae* (Nyl.) Erichsen.

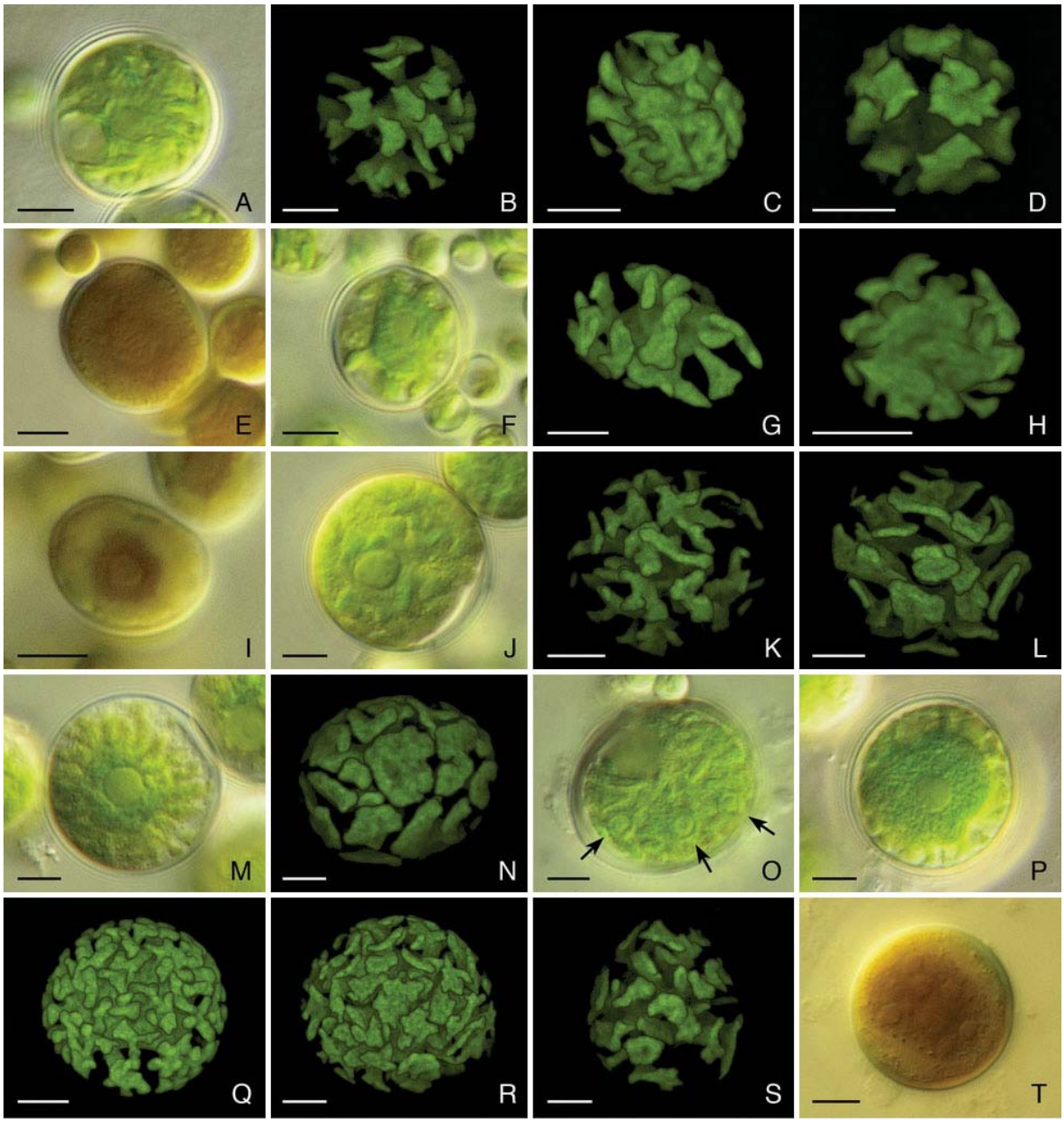

Figure 2. Light micrographs and confocal reconstructions of chloroplast structures in vegetative cells of *Asterochloris*. **A-E.** ***A. excentrica***. Characteristic morphology **(A)** and the confocal reconstructions of deeply lobed **(B)** and shallowly lobed **(C)** chloroplasts. Chloroplast lobes often terminated by flat extensions **(D)**. Parallelly striated pyrenoid **(E)**. **F-I.** ***A. italiana***. Characteristic morphology **(F)** and confocal reconstructions of shallowly lobed **(G)** and crenulate **(H)** chloroplasts. Single pyrenoid **(I)**. **J-O.** ***A. woessiae***. Characteristic morphology **(J)** and the confocal reconstructions of deeply lobed **(K)** and flat lobed **(L)** chloroplasts. Echinate chloroplast **(M)**. Flat terminations of chloroplast lobes **(N)**. Several pyrenoids (arrows) are formed within the chloroplast **(O)**. **P-T.** ***A. leprariae***. Light micrograph **(P)** and confocal reconstruction **(Q)** of crenulate chloroplast. Confocal reconstructions of shallowly lobed **(R)** and deeply lobed **(S)** chloroplasts. Several pyrenoids occur around the large central pyrenoid **(T)**. Cells in Figs **E**, **I**, **T** stained by chloriodine solution. Scale bar – 5 μm.

Holotype: Freeze-dried material obtained from strain LEP 16 (culture collection of O. Peksa) isolated from *Lepraria neglecta* and deposited at the CAUP (Culture Collection of algae of the Charles University in Prague, Department of Botany, Benátská 2, 12801, Praha 2, Czech Republic) under designation LYO-H 1010 (*hic designatus*). The living cultures (ex-holotypes) have been deposited as CAUP H 1010 ibidem.

Type locality: Czech Republic, Šumava Mts, Modrava, Rybárna, on siliceous rock, alt. 1000 m – sample of *Lepraria neglecta*, 23.5.2005 collected O. Peksa et al., deposited in herbarium of O. Peksa in PL, No. 183.

Etymology: The species is named in reference to the mycobiont genus *Lepraria* Ach.

Diagnosis: Vegetative cells usually spherical, occasionally ellipsoidal, oviform and pyriform, up to 27(-33) μm in diameter (Fig. 2P). Cell wall thin, seldom a flat local thickening of the cell wall can be distinguished . Very rarely, the cell wall is slightly thickened along its entire surface. Chloroplast in young cells assumes the central position with several lobes spreading towards the cell's periphery. Mature cells exhibit central chloroplasts of either crenulate (Fig. 2Q) or shallowly lobed form (Fig. 2R). Rarely, the deeply lobed (Fig. 2S) and the lobed parietal chloroplast is observed as well. Generally, the chloroplast lobes are simply terminated or extended longitudinally at their ends. Occasionally, the lobe ends are flat. The chloroplast contains from one to many distinct or indistinct pyrenoids. Besides the typical, centrally located pyrenoid, up to seven smaller ones may be present in its vicinity (Fig. 2T). Sometimes, an indistinct granulation or striation can be visible inside pyrenoids. Starch grains are embedded either in a layer around the pyrenoid or distributed evenly throughout the chloroplast. Asexual reproduction by 64-128 aplanospores or 64 zoospores produced in large spherical or ellipsoidal sporangia. Occasionally, 2-4 autospores are also produced. Zoospores dorsiventrally flattened, drop-shaped, arcuate in lateral view, 6-10 μm long x 2.8-4 μm wide with posterior extensions.

Asterochloris friedlii Škaloud et Peksa sp. nov.

Latin Diagnosis: Cellulae vegetativae sphericae et aliquantum ellipticae, ad 20(-22) μm magnae. Parietes cellularum raro localiter incrassati. Chloroplastus unicus, plerumque lobularis, interdum crenatus aut parietalis, pyrenoide unica praeditus. Pyrenoides granis amylaceis dispersis circumcinctae. Nucleus unus, parietalis. Propagatio asexualis per aplanosporas et zoosporas, 64-128 pro sporangio. Zoosporae complanatae, 4.5-7 μm longae et 3-3.5 μm latae, cum caudae posteriorum. Specimina typica phycobionte *Leprariae caesioalbae* (de Lesd.) J. R. Laundon.

Holotype: Freeze-dried material obtained from strain LEP 5 (culture collection of O. Peksa) isolated from *Lepraria caesioalba* and deposited at the CAUP (Culture Collection of algae of the Charles University in Prague, Department of Botany, Benátská 2, 12801, Praha 2, Czech Republic) under designation LYO-H 1011 (*hic designatus*). The living cultures (ex-holotypes) have been deposited as CAUP H 1011 ibidem.

Type locality: Slovakia, Slovenské Rudohorie Mts, Klenovský Vepor Mt, on bryophytes on siliceous rock, alt. 1330 m – sample of *Lepraria caesioalba*, 12.7.2004 collected O. Peksa & Z.Jindráková, deposited in herbarium of O. Peksa in PL, No. 235.

Etymology: The species epithet is in honour of the work of Dr. Thomas Friedl, who published several reports on *Trebouxia* s.l., and first proposed the polyphyly of the genus based on molecular data.

Diagnosis: Vegetative cells spherical or slightly ellipsoidal, up to 20(-22) μm in diameter (Fig. 3A). Cell wall thin, seldom a flat local thickening of the cell wall can be detected. Chloroplast in young cells assumes the central position with several lobes spreading towards the cell's periphery. Mature cells generally display a structurally complicated, central, deeply lobed chloroplast with branched lobes that emerge directly from the thin chloroplast layer spreading around the pyrenoid (Fig. 3B). Sometimes, the crenulate (Fig. 3C) or lobed parietal chloroplast (Fig. 3D) can be formed as well. In mature cells, the chloroplast can be slightly asymmetrically positioned. The chloroplast lobes are extended longitudinally at their ends, terminated by finger-like extensions, or simply terminated. The chloroplast generally contains a single distinct, granulated pyrenoid. Especially in older cells, the pyrenoid buds at its sur-

face and give rise to several smaller ones in its vicinity (Fig. 3E). Starch grains are embedded in a layer around the pyrenoid in the form of large granules. Asexual reproduction by 64-128 aplanospores or zoospores produced in large spherical, ellipsoidal or irregular sporangia. Zoospores dorsiventrally flattened, 4.5-7 μm long x 3-3.5 μm wide with posterior extensions.

Asterochloris echinata Škaloud et Peksa sp. nov.

Latin Diagnosis: Cellulae vegetativae plerumque sphericae, interdum ellipticae, ad 22(-24) μm magnae. Parietes cellularum vix incrassati. Chloroplastus unicus, lobularis et echinatus, interdum sphaericus, pyrenoide unica vel aliquibus praeditus. Pyrenoides granis amylaceis dispersis circumcinctae. Nucleus unus, parietalis. Propagatio asexualis per aplanosporas et zoosporas, 64-128 pro sporangio. Zoosporae raro formatae, complanatae, 6 μm longae et 4 μm latae. Specimina typica phycobionte *Leprariae* sp.

Holotype: Freeze-dried material obtained from strain LEP 31 (culture collection of O. Peksa) isolated from *Lepraria* sp. and deposited at the CAUP (Culture Collection of algae of the Charles University in Prague, Department of Botany, Benátská 2, 12801, Praha 2, Czech Republic) under designation LYO-H 1012 (*hic designatus*). The living cultures (ex-holotypes) have been deposited as CAUP H 1012 ibidem.

Type locality: Czech Republic, Lužické hory Mts, Klíč Mt., on bryophytes on siliceous rock, alt. 710 m – sample of *Lepraria* sp., 18.9.2004 collected O. Peksa & J. P. Halda, deposited in herbarium of O. Peksa in PL, No.186.

Etymology: The species epithet is named in reference to the echinate shape of chloroplast, which appeared in certain ontogenetic stages.

Diagnosis: Vegetative cells usually spherical, occasionally ellipsoidal, up to 22(-24) μm in diameter (Fig. 3F). Cell wall thin, without any local thickenings. Young and mature cells predominantly exhibit central crenulate chloroplasts (Fig. 3G). At times, the chloroplast can transform into the echinate form characterized by many thin radial lobes giving it a bristly appearance (Fig. 3H). The crenulate chloroplast of old cells frequently transform into a highly specific form that is characterized by a simple globular shape without any lobes (Fig. 3I). This form is typified by distinctive chloroplast ultrastructure in its central and marginal regions. In the centre, the starch accumulation causes the decrease of thylakoid numbers, and subsequent modification of the chloroplast's texture. The chloroplast lobes are either simply terminated, or very slightly extended longitudinally at their ends. The chloroplast contains from one to many distinct or indistinct pyrenoids. In the later case, up to eight smaller pyrenoids are present in the vicinity of the central one (Fig. 3J). Sometimes, an indistinct granulation can be visible inside pyrenoids. Starch grains are embedded evenly throughout the chloroplast. Asexual reproduction by 64-128 aplanospores produced in large spherical or ellipsoidal sporangia. Zoospores very rare, dorsiventrally flattened, 6 μm long x 4 μm wide.

Asterochloris gaertneri Škaloud et Peksa sp. nov.

Latin Diagnosis: Cellulae vegetativae plerumque sphericae et aliquantum ellipticae, interdum oviformes et piriformes, ad 23(-25) μm magnae. Parietes cellularum raro localiter incrassati. Chloroplastus unicus, plerumque lobularis, interdum crenatus, echinatus aut parietalis, pyrenoide unica vel aliquibus praeditus. Pyrenoides granis amylaceis dispersis circumcinctae. Nucleus unus, parietalis. Propagatio asexualis per 64-128-256 aplanosporas et 128 zoosporas, interdum per 4-8 autosporas. Zoosporae complanatae, 6-7.5 μm longae et 2.5-4 μm latae. Specimina typica phycobionte *Leprariae rigidulae* (de Lesd.) Tønsberg.

Holotype: Freeze-dried material obtained from strain LEP 6 (culture collection of O. Peksa) isolated from *Lepraria rigidula* and deposited at the CAUP (Culture Collection of algae of the Charles University in Prague, Department of Botany, Benátská 2, 12801, Praha 2, Czech Republic) under designation LYO-H 1013 (*hic designatus*). The living cultures (ex-holotypes) have been deposited as CAUP H 1013 ibidem.

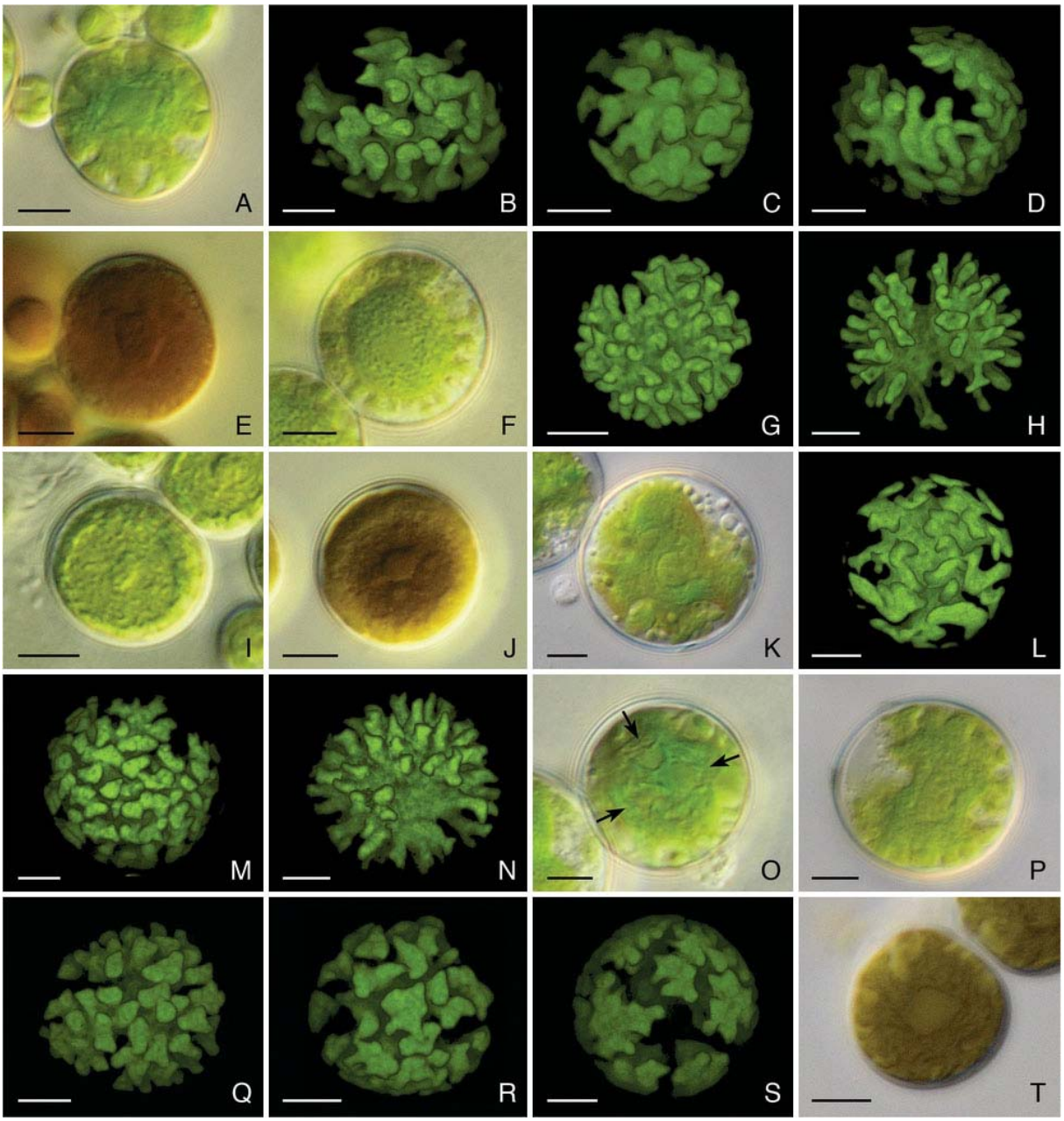

Figure 3. Light micrographs and confocal reconstructions of chloroplast structures in vegetative cells of *Asterochloris*. **A-E.** ***A. friedlii***. Characteristic morphology **(A)** and the confocal reconstructions of deeply lobed **(B)**, shallowly lobed **(C)** and lobed parietal **(D)** chloroplasts. Single budding pyrenoid **(E)**. **F-J.** ***A. echinata***. Light micrograph **(F)** and confocal reconstruction **(G)** of crenulate chloroplast. Confocal reconstruction of echinate chloroplast **(H)**. Globular chloroplast with single distinct pyrenoid **(I)**. Several pyrenoids occur around the large central pyrenoid **(J)**. **K-O.** ***A. gaertneri***. Characteristic morphology **(K)** and confocal reconstructions of shallowly lobed **(L)**, crenulate **(M)** and echinate **(N)** chloroplasts. Several pyrenoids of equal size (arrows) occur in the chloroplast's centre **(O)**. **P-T.** ***A. lobophora***. Characteristic morphology **(P)** and confocal reconstructions of crenulate **(Q)**, shallowly lobed **(R)** and parietal lobed **(S)** chloroplasts. A single pyrenoid is situated in the chloroplast lumen **(T)**. Cells in Figs **E**, **J**, **T** stained by chloriodine solution. Scale bar – 5 μm.

Type locality: Czech Republic, Králický Sněžník Mts, under Stříbrnická Mt., on bark of *Acer pseudoplatanus*, alt. 1030 m – sample of *Lepraria rigidula*, 10.2.2005 collected O. Peksa, deposited in herbarium of O. Peksa in PL, No. 236.

Etymology: The species epithet is in honour of the work of Dr. Georg Gärtner, who published several reports on *Trebouxia* s.l. and revised the genus according to the morphological characters.

Diagnosis: Vegetative cells spherical or slightly ellipsoidal, occasionally oviform to pyriform, up to 23(-25) μm in diameter (Fig. 3K). Cell wall thin, seldom a flat local thickening of the cell wall can be observed. Very rarely, the cell wall is slightly thickened along its entire surface. Chloroplast in young cells assumes the central position with several lobes spreading towards the cell's periphery. Mature cells predominantly exhibit shallowly lobed axial chloroplasts (Fig. 3L) characterized by a central irregular mass of chloroplast layer bearing simple or branched lobes terminated along the entire cell periphery. The chloroplasts can be further transformed into several other ontogenetic stages that alternate during the cell's ontogeny. The chloroplast can exhibit a crenulate shape (Fig. 3M), or it can be transformed into the echinate form with many thin radial lobes (Fig. 3N). Rarely, deeply lobed or lobed parietal chloroplasts occur as well. The chloroplast lobes are simply terminated, extended longitudinally at their ends, or terminated by finger-like extensions. The chloroplast contains from one to many distinct pyrenoids. If many, they usually jointly occupy the chloroplast's centre (Fig. 3O). Sometimes, an indistinct striation can be visible inside pyrenoids. Starch grains are embedded in a layer around the pyrenoid. Asexual reproduction by 64-128-256 aplanospores or 128 zoospores produced in large spherical or ellipsoidal sporangia. Occasionally, 4-8 autospores are also produced. Zoospores dorsiventrally flattened, 6-7.5 μm long x 2.5-4 μm wide.

Asterochloris lobophora Škaloud et Peksa sp. nov.

Latin Diagnosis: Cellulae vegetativae sphericae et ellipticae, ad 22(-25) μm magnae. Parietes cellularum raro localiter incrassati. Chloroplastus unicus, plerumque crenatus et lobularis, interdum parietalis, pyrenoide unica praeditus. Pyrenoides granis amylaceis dispersis circumcinctae. Nucleus unus, parietalis. Propagatio asexualis per 64-128 aplanosporas et 128-256 zoosporas. Zoosporae complanatae, 4-7 μm longae et 2.5-3.5 μm latae, cum caudae posteriorum. Specimina typica phycobionte *Leprariae caesioalbae* (de Lesd.) J. R. Laundon.

Holotype: Freeze-dried material obtained from strain LEP 13 (culture collection of O. Peksa) isolated from *Lepraria caesioalba* and deposited at the CAUP (Culture Collection of algae of the Charles University in Prague, Department of Botany, Benátská 2, 12801, Praha 2, Czech Republic) under designation LYO-H 1014 (*hic designatus*). The living cultures (exholotypes) have been deposited as CAUP H 1014 ibidem.

Type locality: Czech Republic, Šumava Mts, Kašperk Mt. (castle), on siliceous rock, alt. 900 m – sample of *Lepraria caesioalba*, 31.3.2005 collected O. Peksa, deposited in herbarium of O. Peksa in PL, No. 166.

Etymology: The species epithet is named in reference to the lobed chloroplast shape.

Diagnosis: Vegetative cells spherical or ellipsoidal, up to 22(-25) μm in diameter (Fig. 3P). Cell wall thin, seldom a flat local thickening of the cell wall can be distinguished. Very rarely, the cell wall is slightly thickened along its entire surface. Chloroplast in young cells assumes the central position with several lobes spreading towards the cell's periphery. Mature cells exhibit central crenulate (Fig. 3Q), or shallowly lobed chloroplasts (Fig. 3R) frequently transformed into the parietal lobed form (Fig. 3S). Even though the chloroplast can sometimes appears to be deeply lobed, the lobes never emerge directly from the pyrenoid surroundings. The chloroplast lobes are simply terminated, extended longitudinally at their ends, or terminated by finger-like extensions. The chloroplast contains single distinct granulated pyrenoid (Fig. 3T). Particularly prior to cell division, the pyrenoid sometimes divides into two parts.

Table 1. *Asterochloris* strains included in this study. Newly obtained sequences are in bold face.

Algal species	Strain	Mycobiont species	Accession numbers	
			ITS	Actin
Asterochloris echinata	CAUP H1012	*Lepraria* sp.	**AM905992**	**AM906017**
Asterochloris erici	UTEX 911	*Cladonia cristatella*	AF345440	**AM906018**
Asterochloris excentrica	UTEX 1714	*Stereocaulon dactylophyllum*	**AM905993**	**AM906019**
Asterochloris friedlii	LEP 4	*Lepraria* cf. *caesioalba*	**AM905994**	**AM906020**
Asterochloris friedlii	CAUP H1011	*Lepraria caesioalba*	**AM905995**	**AM906021**
Asterochloris friedlii	LEP 33	*Lepraria caesioalba*	**AM905996**	**AM906022**
Asterochloris friedlii	Nelsen 3974, 154	*Lepraria lobificans*	DQ229877	DQ229898
Asterochloris friedlii	Nelsen 3966, L36	*Lepraria caesioalba*	EU008664	EU008697
Asterochloris friedlii	Nelsen 3960, L12	*Lepraria lobificans*	EU008675	EU008704
Asterochloris friedlii	Nelsen 3973, 153	*Lepraria lobificans*	EU008678	EU008707
Asterochloris friedlii	Nelsen 2453, L59	*Lepraria* sp.	EU008691	EU008716
Asterochloris gaertneri	CAUP H1013	*Lepraria rigidula*	**AM905997**	**AM906023**
Asterochloris glomerata	UTEX 895	*Stereocaulon evolutoides*	AF345382	**AM906024**
Asterochloris glomerata	UTEX 1712	*Cladonia squamosa*	AF345406	**AM906025**
Asterochloris glomerata	DIP 2	*Diploschistes muscorum*	**AM905998**	**AM906026**
Asterochloris irregularis	UTEX 2236	*Stereocaulon* sp.	AF345411	**AM906027**
Asterochloris irregularis	STER 1	*Stereocaulon pileatum*	**AM905999**	**AM906028**
Asterochloris irregularis	B13	*Cladonia mitis*	**AM906000**	**AM906029**
Asterochloris irregularis	Talbot 153	*Stereocaulon botyosum*	DQ229880	DQ229889
Asterochloris irregularis	Talbot 167	*Stereocaulon subcoralloides*	DQ229881	DQ229890
Asterochloris italiana	CCAP 519/5B	*Xanthoria parietina*	**AM906001**	**AM906030**
Asterochloris italiana	UTEX 67	*Cladonia* sp.	AF345423	DQ229894
Asterochloris leprariae	CAUP H1010	*Lepraria neglecta*	**AM906002**	**AM906031**
Asterochloris leprariae	LEP 23	*Lepraria caesioalba* chemotype 1	**AM906003**	**AM906032**
Asterochloris leprariae	LEP 25	*Lepraria caesioalba* chemotype 1	**AM906004**	**AM906033**
Asterochloris leprariae	LEP 30	*Lepraria neglecta*	**AM906005**	**AM906034**
Asterochloris lobophora	LEP 1	*Lepraria caesioalba* chemotype 1	**AM906006**	**AM906035**
Asterochloris lobophora	LEP 2	*Lepraria* cf. *caesioalba*	**AM906007**	**AM906036**
Asterochloris lobophora	CAUP H1014	*Lepraria caesioalba* chemotype 1	**AM906008**	**AM906037**
Asterochloris lobophora	LEP 27	*Lepraria caesioalba* chemotype 1	**AM906009**	**AM906038**
Asterochloris lobophora	LEP 28	*Lepraria alpina*	**AM906010**	**AM906039**
Asterochloris lobophora	DIP 1	*Diploschistes muscorum*	**AM906011**	**AM906040**
Asterochloris lobophora	Nelsen 3950	*Cladonia cf. bacillaris*	DQ229878	DQ229892
Asterochloris magna	UTEX 902	*Pilophorus acicularis*	**AM906012**	**AM906041**
Asterochloris phycobiontica	SAG 26.81	*Anzina carneonivea*	AM900490	**AM906042**
Asterochloris phycobiontica	LEP 9	*Lepraria neglecta*	AM900491	**AM906043**
Asterochloris phycobiontica	LEP 7	*Lepraria neglecta*	**AM906013**	**AM906044**
Asterochloris sp.	Nelsen 2211a, L54	*Lepraria* sp.	EU008684	EU008711
Asterochloris sp.	Nelsen 2585, L60	*Lepraria* sp.	EU008690	EU008715
Asterochloris sp.	Nelsen 2233f	*Pilophorns* cf. *cereolus*	DQ229883	DQ229895
Asterochloris sp.	Talbot 101	*Stereocaulon paschale*	DQ229887	DQ229891
Asterochloris sp.	Talbot 400	*Stereocaulon tomentosum*	DQ229882	DQ229893
Asterochloris sp.	Talbot 281	*Stereocaulon vesuvianum*	DQ229885	DQ229888
Asterochloris sp.	Nelsen 2181b	*Stereocaulon* sp.	DQ229884	DQ229896
Asterochloris woessiae	CAUP H1009	*Lepraria borealis*	AM900492	**AM906045**
Asterochloris woessiae	LEP 36	*Lepraria nylanderiana*	AM900493	**AM906046**
Asterochloris woessiae	LEP 15	*Lepraria caesioalba* chemotype 1	**AM906014**	**AM906047**
Asterochloris woessiae	LEP 34	*Lepraria borealis*	**AM906015**	**AM906048**
Asterochloris woessiae	CLAD 1	*Cladonia foliacea*	**AM906016**	**AM906049**
Asterochloris woessiae	Nelsen 3637b, L55	*Lepraria nigrocincta*	EU008681	EU008710
Asterochloris woessiae	Nelsen 2166a, L18	*Lepraria* sp.	EU008687	EU008714
Asterochloris woessiae	Talbot KIS 187	*Stereocaulon saxatile*	DQ229886	DQ229897

Starch grains are embedded either in a layer around the pyrenoid or evenly throughout the chloroplast. Asexual reproduction by 64-128 aplanospores or 128-256 zoospores produced in large spherical or slightly ellipsoidal sporangia. Zoospores dorsiventrally flattened, 4-7 μm long x 2.5-3.5 μm wide with posterior extensions.

Phylogenetic analyses

Sequences of the ITS region and the actin type I gene (2 introns and 1 complete exon) were determined for all strains. Information on the accession numbers of the sequences obtained is given in Table 1.

Specifications of the ITS, actin and concatenated datasets, as well as node resolution statistics obtained from bootstrap analyses and posterior probabilities are given in Table 2. Although both loci consisted of similar numbers of analyzed nucleotide pairs, they differ considerably in the amount of phylogenetic signal. The actin dataset contains a significantly higher amount of both variable and parsimony informative sites than ITS region that leads to higher average sequence divergence and better resolution of taxa relationships.

Table 2. Specification of data sets, model parameters obtained, and details on the ML, MP and BI analyses.

	ITS	actin	concatenated
Alignment length/analyzed	531/531	799/532	1330/1063
Variable sites/parsimony informative sites (in %)	70/41 (13.2/7.7)	319/238 (60.0/44.8)	389/279 (36.6/26.2)
Pairwise sequence divergence (max/average)	0.041/0.017	0.124/0.058	0.052/0.029
Empirical base frequencies (A/C/G/T)	0.21/0.30/0.26/0.24	0.19/0.26/0.32/0.23	0.20/0.28/0.29/0.23
Model estimated[a]	GTR + I + Γ	GTR + Γ	GTR + Γ
Node resolution[b]			
BI (GTR + I + Γ)	16/22/61/*42.6*	21/7/21/*62.8*	22/8/19/*63.8*
BI (GTR + Γ)	16/18/65/*42.4*	22/7/20/*62.8*	22/8/19/*63.6*
BI (GTR + I + Γ for ITS, GTR + Γ for Actin partition)	-	-	21/9/19/*63.3*
ML (GTR + I + Γ)	12/14/74/*28.6*	21/4/24/*51.5*	20/6/23/*53.0*
ML (GTR + Γ)	12/14/74/*27.9*	21/3/25/*52.1*	20/8/21/*53.4*
wMP	12/16/71/*31.7*	22/8/19/*59.7*	22/11/16/*61.1*
average resolution of all analyses	**14/17/69/*34.6***	**21/6/22/*57.8***	**21/8/20/*59.0***

[a]Estimated by the Akaike Information Criterion (AIC) with PAUP/MrModeltest 1.0b
[b]Percentage of all nodes receiving high (more than 94 % for BI; more than 79 % for ML and MP)/moderate (50-94 % for BI; 50-79 % for ML and MP)/low (less than 50 % for BI, ML and MP) bootstrap support/*average support of all nodes*

Phylograms inferred from ITS and actin sequences were constructed by the maximum-likelihood method. ITS phylogram (Fig. 4) contains a few well supported lineages corresponding to: species *A. friedlii*, *A. italiana* and *A. woessiae*; a pair of species *A. glomerata* and *A. irregularis*; and an undescribed lineage of *Asterochloris*. None of the other *Asterochloris* species, nor any internal branches, receive high bootstrap support. Distinct loss of phylogenetic signal occurs in a clade containing *A. phycobiontica*, with highly unresolved positions of four *Asterochloris* species and monophyly of *A. lobophora*. The actin phylogeny (Fig. 5) reveals support of all *Asterochloris* species represented by at least two analyzed representatives (i.e. *A. friedlii*, *A. glomerata*, *A. italiana*, *A. irregularis*, *A. leprariae*, *A. lobophora*, *A. phycobiontica* and *A. woessiae*). Results also indicate a sister-group relationship of *A. glomerata* to *A. irregularis*, *A. magna* to *A. erici* and *A. lobophora* to *A. phycobiontica*. Generally, *Asterochloris* is composed of three major, well-supported clades: (1) a lineage composed of *A. glomerata*, *A. irregularis*, *A. erici* and *A. magna*; (2) a lineage consisting of *A. excentrica*, *A. gaertneri* and *A. leprariae*; and (3) a large clade containing the remaining representatives of the genus, including a thus far undescribed, well-resolved lineage (clade 3A) consisting of *Lepraria* sp., *Stereocaulon* sp. and *Pilophorus* cf. *cereolus* photobionts isolated from lichens collected in North and Central America (Nelsen and Gargas 2006, 2008).

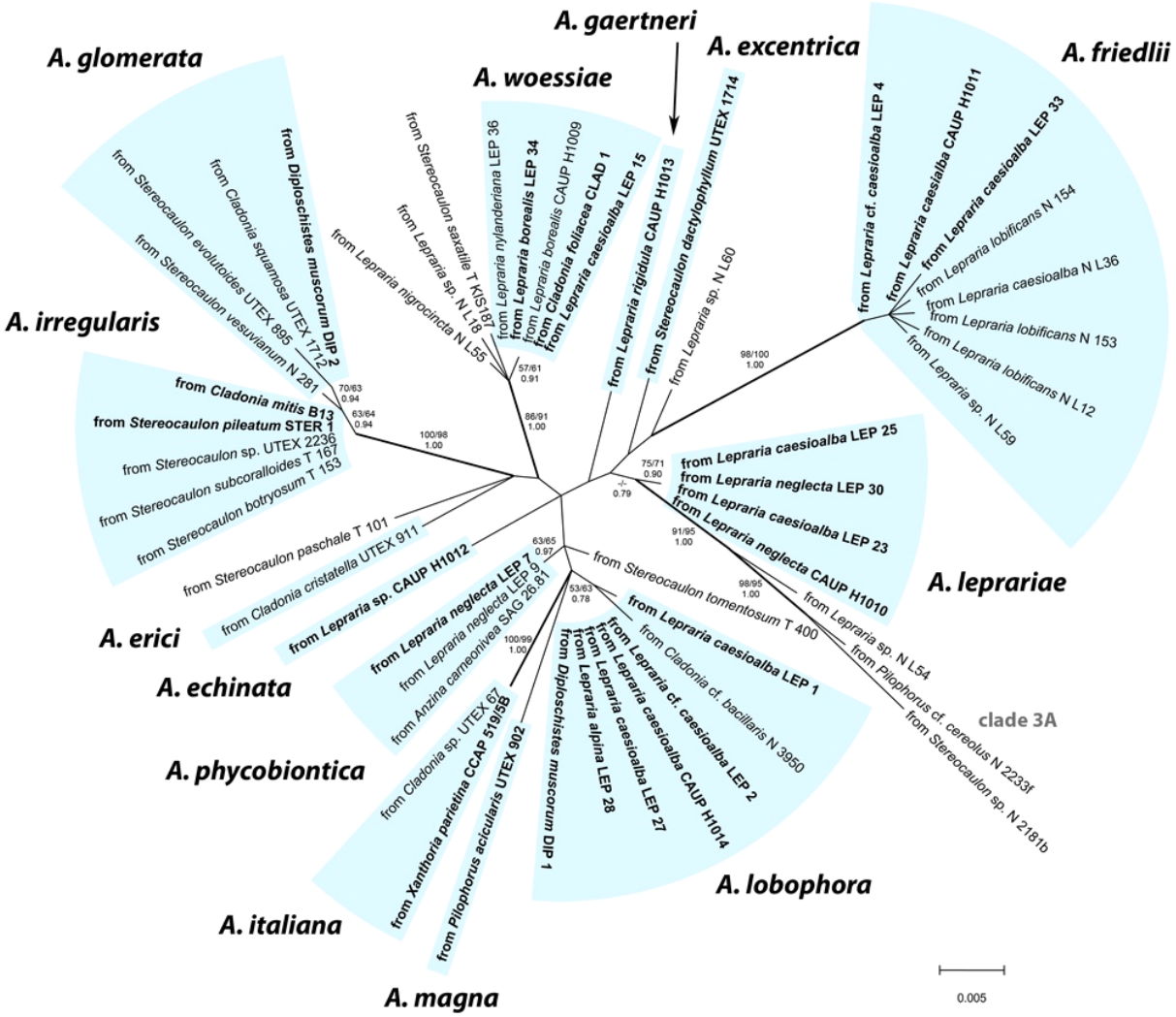

Figure 4. Unrooted maximum likelihood tree based on ITS sequences. Evolutionary model: GTR + G. Values at the nodes indicate statistical support estimated by three methods - maximum likelihood bootstrap (top left), maximum parsimony bootstrap (top right) and MrBayes posterior node probability (lower). Thick branches represent nodes receiving high statistical support in at least two bootstrap/posterior probability analyses. ITS sequences determined in this study are given in bold face. Scale bar – substitutions per site.

According to the partition homogeneity test (ILD test), the ITS and actin data sets were significantly incongruent (p = 0.001). Even if the ILD test was performed on parsimony informative sites only, the incogruence was still highly statistically significant (p = 0.001). Moreover, both Templeton (TT) and Kishino-Hasegawa (KHT) tests revealed significant incongruence between algal ITS and actin topologies (ITS data with actin topology: p = 0.0049 (TT), 0.0046 (KHT); actin data with ITS topology: p = 0.0001 (both TT and KHT)). The analyses of the concatenated data set led to slight increasing of average node resolutions. However, the proportion of nodes receiving high bootstrap support and posterior probabilities did not increase (Table 2). Regarding all above-mentioned results, we do not infer concatenated ITS+actin phylogeny; rather, we use actin sequences alone to define individual *Asterochloris* species and to reveal the relationships among taxa.

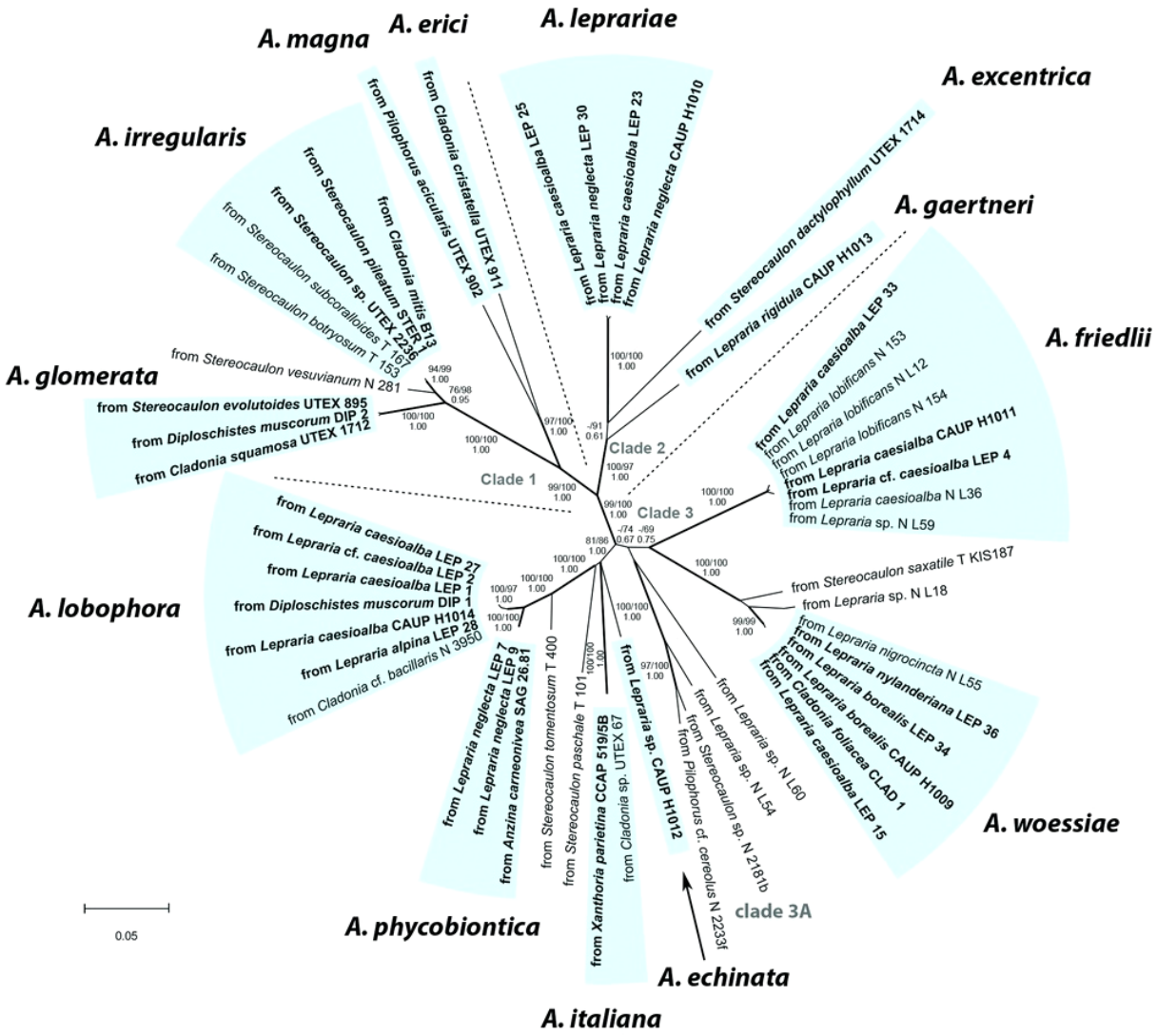

Figure 5. Unrooted maximum likelihood tree based on actin I locus sequences. Evolutionary model: GTR + I + G. Values at the nodes indicate statistical support estimated by three methods - maximum likelihood bootstrap (top left), maximum parsimony bootstrap (top right) and MrBayes posterior node probability (lower). Thick branches represent nodes receiving high statistical support in at least two bootstrap/posterior probability analyses. Actin sequences determined in this study are given in bold face. Strain affiliation to three particular clades is indicated. Scale bar – substitutions per site.

Comparative morphology

The major morphological characteristics used for species identification in *Asterochloris* are as follows: cell form, cell dimensions, cell wall, chloroplast morphology, pyrenoid number and structure, and the characteristics associated with asexual reproduction (Table 3). The cells' form and dimensions show sufficient variability, both among species and within strains belonging to a single species. The average dimensions of mature vegetative cells vary in the range of 13-24 μm. Cells are generally spherical, elliptical, or ovoid, and occasionally pyriform to irregular. Although the cell's form varies during cell ontogeny, differences in the prevailing shape of a species is maintained. For example, pyriform cells are frequently produced in *A. glomerata* and *A. irregularis*, although rare in *A. woessiae* and *A. phycobiontica*, and never observed in *A. erici* and *A. excentrica*. The cell wall is thin, and occasionally a flat local thickening of the wall can be detected in mature cells (except *A. echinata*). In old cultures the cell wall of some cells can be slightly thickened along their entire surface. A single nucleus with distinct nucleolus is situated parietally in the broad chloroplast infolding.

Table 3. Comparative table of morphological characters of *Asterochloris* species. Relative frequency of particular characters: *** prevailing, ** common, * occasional.

species	cell shape			maximum cell size	chloroplast types							lobe terminations				number of pyrenoids	satelite pyrenoids	zoospore dimensions
	pyrifrem	spherical	oval/ovoid		deeply-lobed	shallowly-lobed	crenulate	parietal lobed	flat-lobed	echinate	globular	excentrical	elongated	simple	tabular	fingerlike			
phycobiontica	*	***	**	24(-25)			**	***						***		*	many		4-4.5 x 2.5
glomerata	***	*	**	20(-21)	***	**			*			*	***		*	**	many	***	4-4.5 x 3
irregularis	***	*	**	20(-21)	***	**						**	***			**	many		5-7 x 1.5-2.5
erici		***	**	16		***							***				1		4.5-7.5 x 2.5-3
magna	*	***	**	19(-21)				***									0		4.5-6.5 x 2-2.5
excentrica		***	**	17(-18)	***	*						*	**		***	**	1		4-7 x 2-3.8
italiana	*	**	***	20		***	*						***	*			1	*	3-5 x 2-2.5
woessiae	*	***	**	21(-24)	***		*	*	**	**			***	*	**		1-3		4.5-7.5 x 2.5-4.5
leprariae	*	***	**	27(-33)	*	**	***	*					**	***	*		many	***	6-10 x 2.8-4
friedlii		***	**	20(-22)	***		**	*				*	***	**		**	1	**	4.5-7 x 3-3.5
echinata		***	*	22(-24)			***			**	**		*	***			many	***	6 x 4
gaertneri	*	***	**	23(-25)	*	***	**	*		**			**	***		**	many		6-7.5 x 2.5-4
lobophora		***	**	22(-25)		**	***	**					**	***		**	1		4-7 x 2.5-3.5

The chloroplast morphology in *Asterochloris* deserves special consideration, because its remarkable variability can be very useful in delimiting individual species. The chloroplast of young cells is not axial, but rather parietal or ribbon-shaped. Soon, it shifts to a central position and begins to develop into a lobed form. Thus, mature vegetative cells contain a central axial chloroplast with variously arranged lobes reaching the cell periphery. In the late ontogenetic stages, specifically prior to zoo- or aplanosporogenesis, the chloroplast transforms into the parietal type with smooth, never lobed, margins. After a short time, it begins to divide into numerous parts in preparation for asexual reproduction. The axial chloroplast of mature vegetative cells is characterized by a large morphological variability, enabling the distinction of seven specific chloroplast types:

Deeply lobed (Fig. 6A): axial chloroplast characterized by long, branched or unbranched lobes. These emerge directly from the thin chloroplast layer spreading around the pyrenoid ("Tieflappig Typ" sensu Gärtner 1985a).

Shallowly lobed (Fig. 6B): similar to the deeply lobed type, but chloroplast lobes emerge from the central mass of chloroplast layer encircling the pyrenoid. The lobes never emerge near the pyrenoid ("Normaltyp" sensu Gärtner 1985a).

Crenulate (Fig. 6C): central massive chloroplast with regularly nodulated surface ("Crenulater Typ" sensu Gärtner 1985b).

Parietal lobed (Fig. 6D): characterized by parietally positioned nodulated chloroplast with the margins extended into divided finger-like lobes.

Flat lobed (Fig. 6E): axial chloroplast with long lobes that appear flattened over their entire length.

Echinate (Fig. 6F): axial chloroplast with many thin radial lobes emerging uniformly from the central mass of chloroplast layer.

Globular (Fig. 6G): simple spherical chloroplast without, or with very shallow, lobes. Type observed only in *A. echinata*.

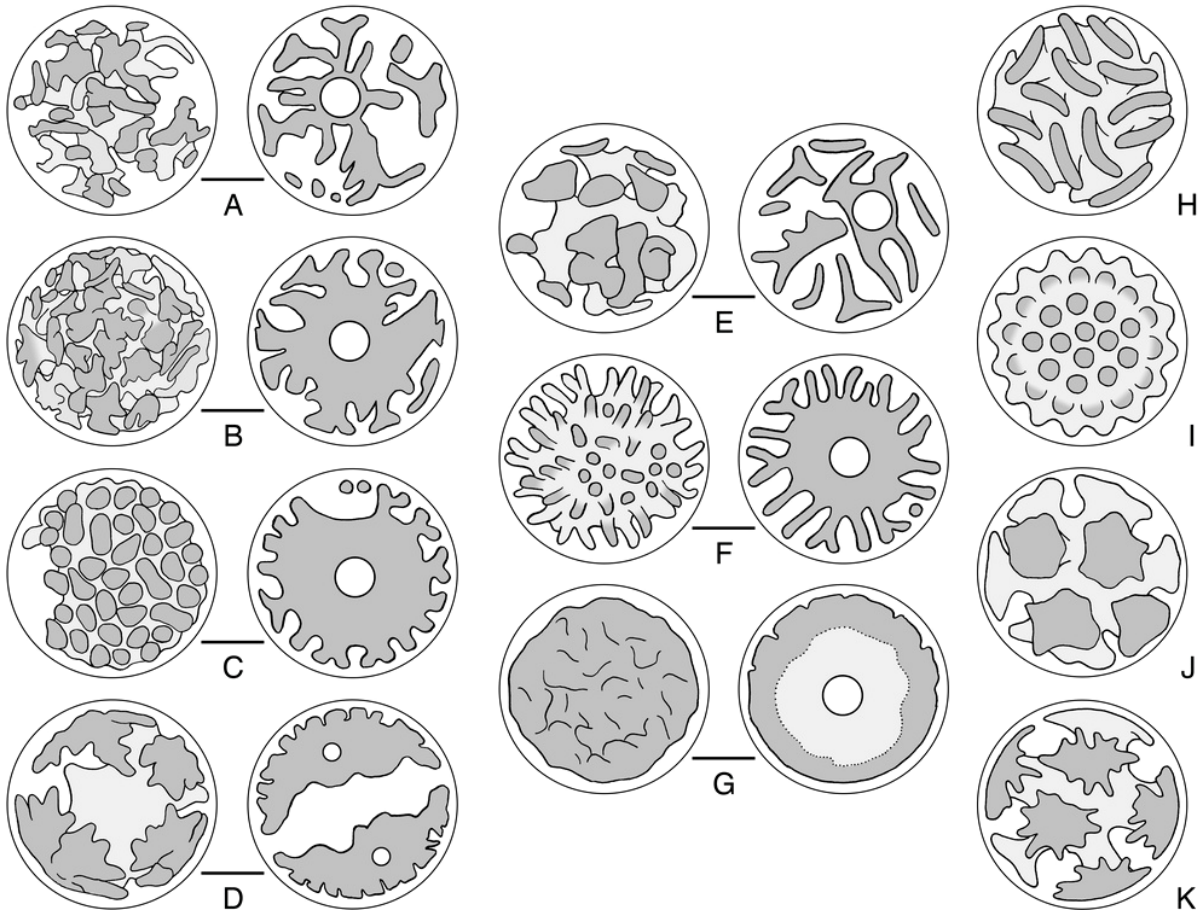

Figure 6. Schematic drawings of particular chloroplast and lobe termination types in *Asterochloris* algae. **A-G.** Chloroplast types (left – surface view, right – view in optical section). **A** deeply lobed, **B** shallowly lobed, **C** crenulate, **D** parietal lobed, **E** flat lobed, **F** echinate, **G** globular. **H-K.** Lobe termination types. **H** elongated, **I** simple, **J** flat, **K** finger-like.

In addition to these seven morphological chloroplast types, four types of lobe terminations can be distinguished:

Elongated termination (Fig. 6H): each chloroplast lobe is extended longitudinally at its end. Thus, giving the chloroplast a finny appearance in surface view ("Rippenförmig Typ" sensu Gärtner 1985a).

Simple termination (Fig. 6I): chloroplast lobes are simply terminated at their ends. This is specific to the crenulate, echinate, and generally also parietal lobed types of chloroplast.

Flat termination (Fig. 6J): chloroplast lobes are terminated by irregular plates, perpendicularly oriented with respect to the lobe axis.

Finger-like termination (Fig. 6K): chloroplast lobes branch at their ends to form several finger-like projections. These are perpendicularly oriented with respect to the lobe axis, spreading below the plasma membrane.

The pyrenoid is present in all *Asterochloris* species except *A. magna*. Beside *A. erici* and *A. lobophora* having single pyrenoid, the cells can contain from one to several pyrenoids, lying in the chloroplast centre. A characteristic arrangement of pyrenoids occurs in many species; one large centrally located pyrenoid is surrounded by several smaller satellite ones, created by budding. The pyrenoids are generally distinct, only in *A. erici* does the pyrenoid gradually change over to a chloroplast matrix without a distinct pyrenoid margin. Various structures can be sometimes seen in the pyrenoid matrix, both under conventional light and confocal microscopy. The pyrenoids can be granulated, striated or perforated. In *A. phycobiontica* the pyrenoid contains distinct rings of puzzling origin. The frequency and markedness

of subpyrenoidal structures significantly increase with cell age. Pyrenoids are usually surrounded by a conspicuous starch sheet (visualized by chloriodine solution in Figs 2I, 2T, 3J; dark area around pyrenoids).

Reproduction

Life cycle and the reproduction processes are schematically delineated in Fig. 7.

Asexual reproduction occurs by the formation of aplanospores, zoospores and autospores (sensu Tschermak-Woess 1989). Autospore production is relatively rare, and observed only in some species. It initiates by slight cell enlargement and subsequent chloroplast division. The chloroplast does not markedly flatten in the course of division, and fills up the entire lumen of the cell (Fig. 7B). In general, autospores are formed in relatively small numbers (mostly 4 or 8), as compared to aplano- and zoospores (Fig. 8A). In young autosporangia the daughter cells are tightly enclosed within the mother cell wall and are pressed against each other, which causes the slightly angular shape of autospores. During the growth of the autospores, the mother cell wall expands and becomes thin. The cells are liberated by either decomposition or rupturing of the mother cell wall, without producing any special openings (Fig. 7D).

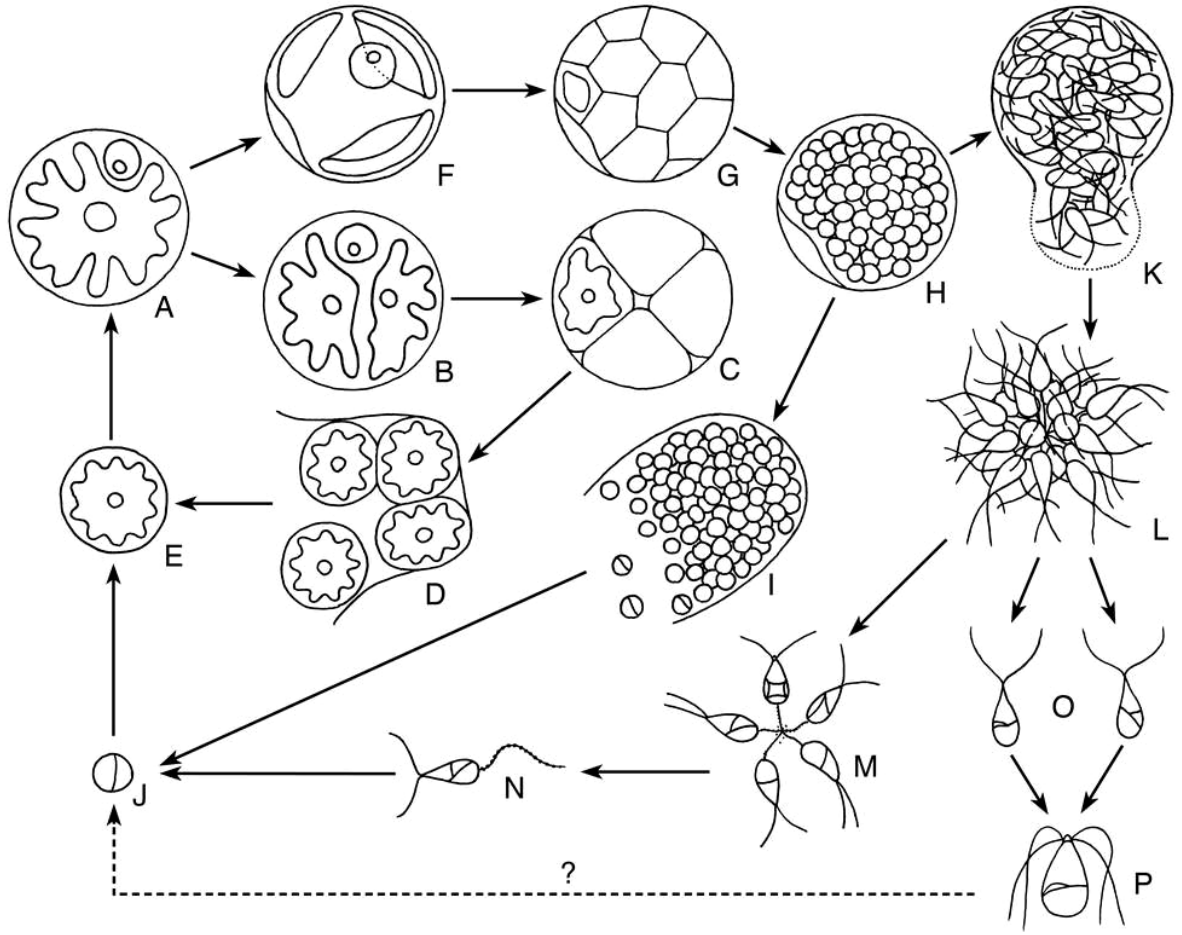

Figure 7. Schematic representation of the life cycle and reproduction processes in *Asterochloris*. **A** vegetative cell. **B-E.** Autosporogenesis. **B** chloroplast division, **C** young autosporangium, **D** release of autospores, **E** mature autospore. **F-N.** Aplano-, zoosporogenesis. **F** chloroplast flattening, note thickening of the cell wall, **G** young aplano/zoosporangium, **H** mature aplanosporangium, **I** release of aplanospores, **J** young aplanospore, **K** release of zoospores, note evagination of gelatinous vesicle, **L** zoospore packet, **M** releasing of zoospores from the packet, **N** single zoospore with posterior extension. **O-P.** Sexual reproduction. **O** gametes, **P** planozygote.

When compared to autospores, the formation of aplanospores and zoospores is frequent. Initial development of aplanospores and zoospores is identical, leading to distinct enlarging of the young sporangium and formation of a special thickening of the cell wall (Tschermak-Woess 2000). Prior to the first cleavage, the chloroplast flattens, assumes the parietal position, and starts to divide (Figs 7F, 8B). Finally, a high number of daughter cells (usually 64 or 128) is formed within the sporangium (Fig. 7H). Once all daughter cells are formed the distinction between aplanosporangia and zoosporangia is observable. In the case of aplanospores, the daughter cells round off and produce a cell wall of their own. Due to large sporangium size, pressure for space is not as drastic as in autosporangia, so they generally acquire a perfectly spherical form (Fig. 8C). Mature aplanospores are liberated by rupturing of the mother cell wall. During their liberation, they are often enclosed in a thin, gelatinous vesicle (Fig. 8D). Mature zoosporangia are discernible by the irregular shape of daughter cells (Fig. 8E). The zoospores are all liberated simultaneously with the rupturing of the mother cell wall. Similar to aplanospores, they are enclosed in a gelatinous vesicle that can slightly evaginate from the mother cell wall during the liberation process (Fig. 7K). The zoospores are dorsiventrally flattened with two equal anterior flagella, posterior chloroplast, median to posterior nucleus and indistinct stigma (Figs 8F, G). Following liberation from the sporangium they swim in a packet, remaining together by their posterior extensions (Figs 7L, 8H). Shortly afterwards, single zoospores start to detach from the packet and swim separately. Despite this, the majority of zoospores still bear posterior extensions even if observed several minutes after separation (Figs 7N, 8I).

Sexual reproduction is very scarce, and was observed only twice in *A. woessiae* (strain CAUP H1009). Biflagellated isogamous gametes are morphologically indistinguishable from zoospores. After their fusion, they give rise to large quadriflagellate planozygotes (Figs 7P, 8J).

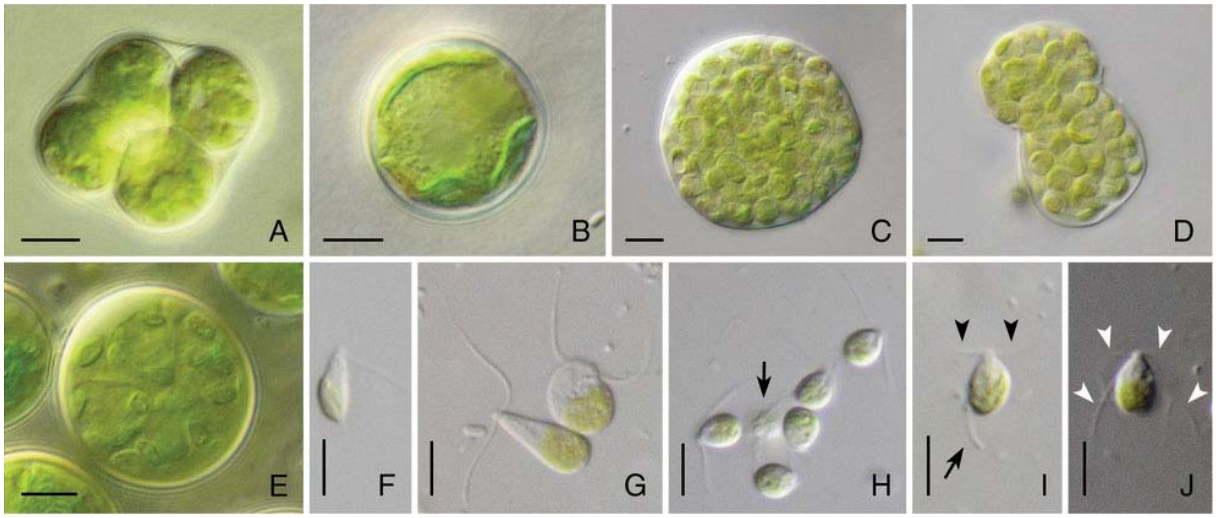

Figure 8. Light micrographs of reproductive structures in *Asterochloris*. **A** mature autosporangium, **B** remodelling of chloroplast prior to aplano-, zoospore production, **C** mature aplanosporangium, **D** release of autospores, note gelatinous vesicle containing liberated spores, **E** mature zoosporangium, **F, G** morphology of zoospores, **H** zoospore packet (arrow indicates association of posterior extensions), **I** single zoospore bearing the posterior extension (flagella are indicated by arrowheads, extension is marked by arrow), **J** planozygote (four flagella are indicated by arrowheads). Scale bar – 5 μm.

Analyses of morphological characters

Mantel tests were performed to determine which morphological characters congruently change with increasing genetic distances; i.e. which characters can be used to morphologically delimit the species clusters, and to trace the microevolution processes that led to the differentiation of the species. Differences in cell shape, overall chloroplast structure and the pattern of lobe termination significantly correlate with increasing genetic distance (Table 4). Specifically, the pyriform cell shape, the crenulate chloroplast type and the simple type of lobe terminations fit these correlations best. Therefore, closely related species share the same value for these characters (presence/absence). On the other hand, neither the number of pyrenoids nor the reproductive characters significantly change with increasing genetic distances. The complex test of all investigated characters was highly statistically significant, suggesting that unrelated species are clearly different in their morphology.

Table 4. Results of Mantel tests evaluating similarity of interspecific distances between actin sequence data and various morphological characters. Significant correlations are given in bold; * $p<0.05$; *** $p<0.01$.

Analyzed characters	*p* value	
All morphological characters	**0.0043**	***
Cell shape	**0.0388**	*
Pyriform	**0.0084**	***
Spherical	**0.0432**	*
Ellipsoidal/Oval	0.9238	
Chloroplast types - all	**0.0424**	*
Deeply lobed	**0.0304**	*
Shallowly lobed	0.6800	
Crenulate	**0.0026**	***
Parietal lobed	0.8554	
Flat lobed	0.3832	
Echinate	0.9208	
Excentrical	**0.0166**	*
Lobe terminations - all	**0.0189**	*
Elongated	0.7738	
Simple	**0.0034**	***
Tabular	0.0826	
Finger-like	0.4788	
Number of pyrenoids	0.2492	
All reproductive characters	0.3334	
Number of aplanospores per sporangium	0.5434	
Dimensions of zoospores	0.4766	
Production of autospores	0.0564	

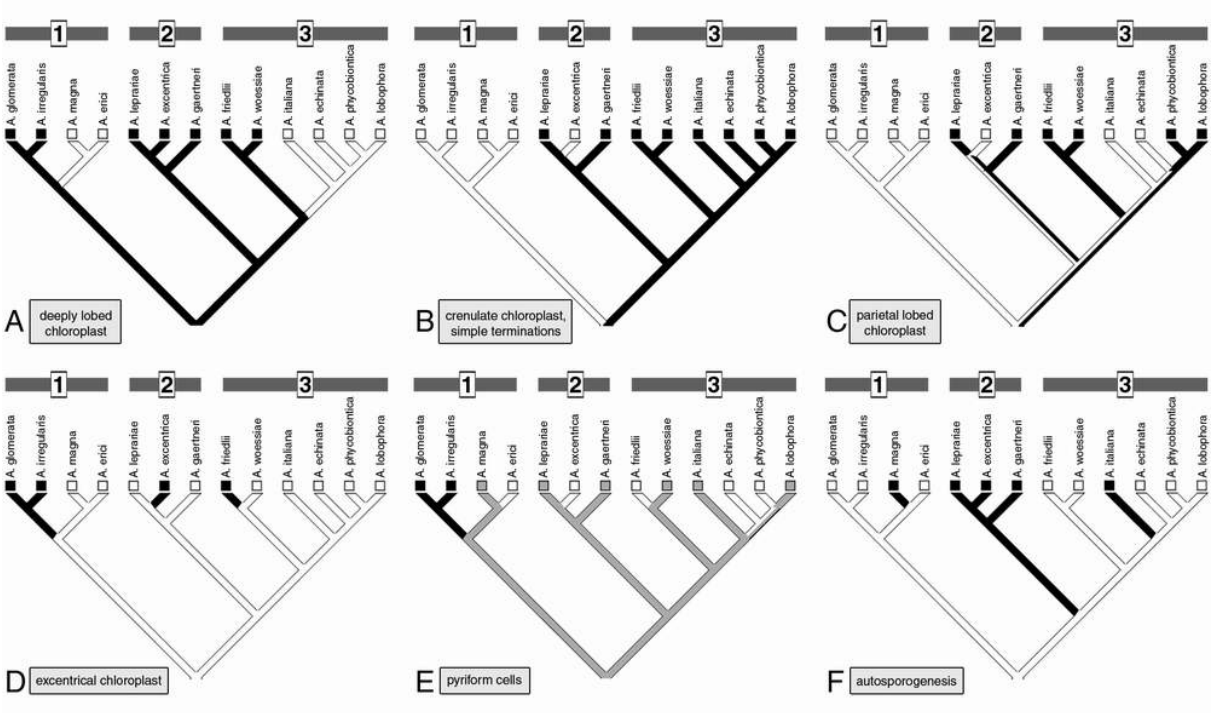

Figure 9. Evolution of morphological characters mapped onto the weighted maximum parsimony tree inferred for 13 *Asterochloris* species. Ancestral traits were reconstructed using maximum parsimony. Black color illustrates the presence of a displayed character. In Fig. **E** black color means frequent formation of pyriform cells, grey color their occasional presence.

The parsimony reconstruction of the evolution of a number of morphological characters (Fig. 9) shows that selected morphological characters are clearly correlated with the diversification of a genus. Clade (1) is defined by absence of crenulate and parietal lobed chloroplast types, and by the absence of simple lobe terminations (Figs 9B, C). Clade (2) is characterized by presence of deeply lobed chloroplast types, and autospore formation in all clade representatives (Figs 9A, F). Finally, all species belonging to clade (3) demonstrate crenulate chloroplasts with simple lobe terminations (Fig. 9B). Higher similarity in chloroplast morphology is present in subclade *A. italiana*, *A. echinata*, *A. phycobiontica* and *A. lobophora*. Together with crenulate chloroplasts, they also share the absence of deeply lobed and eccentrical chloroplasts (Figs 9A, D). The related species, *A. glomerata* and *A. irregularis*, share dominant presence of pyriform cells during the cell cycle (Fig. 9E). Generally, the morphological similarity of genetically related species can be well demonstrated in species pairs: *A. glomerata-A. irregularis*, *A. magna-A. erici* and *A. phycobiontica-A. lobophora*. These species pairs share all five characteristics of chloroplast morphology (Figs 9A-D).

Discussion

Taxonomical consequences

The affiliation of several *Trebouxia* species (*T. erici*, *T. excentrica*, *T. glomerata*, *T. irregularis*, *T. italiana*, *T. magna* and *T. pyriformis*) to the genus *Asterochloris* was repeatedly suggested by many authors (Friedl and Zeltner 1994; Friedl and Rokitta 1997; Helms et al. 2001; Nelsen and Gargas 2008; Normore and DePriest 2001; Rambold et al. 1998). Congruent to these suggestions, on the basis of presented comparative morphological and molecular investigations, we formally transfer all above-mentioned species into *Asterochloris,* along with the establishment of a new genus delimitation. The genus *Asterochloris* is characterized by a unique ITS and actin sequences, as well as by several morphological characteristics (chloroplast morphology, parietal position of chloroplast prior to cell division, frequent aplanospore production and morphology of zoospores). We further describe six new species of the genus, resulting in the existence of thirteen separate *Asterochloris* species well defined by both morphological and molecular data.

The investigation of authentic cultures of *Trebouxia glomerata* (UTEX 895) and *Trebouxia pyriformis* (UTEX 1712) revealed them to be highly similar, considering both morphological characteristics and molecular markers. The congruence in cell size and shape, chloroplast morphology and reproductive characters, as well as almost identical ITS and actin sequences (99.6% similarity in both loci), lead us to taxonomically join these two species and to establish *Trebouxia pyriformis* as a synonym of *Asterochloris glomerata*. Merging of these two species was already suggested by Friedl (1989b) and Piercey-Normore and DePriest (2001), based on their morphological similarity and RAPD amplification patterns, respectively. However, in contrast to another suggestion of Piercey-Normore and DePriest (2001), we retain the species rank of related *A. irregularis,* due to morphological differences in pyrenoid arrangement and zoospore dimensions, as well as dissimilar actin sequences.

Conversely, we revealed distinct differences in two authentic strains of *Trebouxia magna*, i.e. UTEX 902 and UTEX 67. Morphological variations between the strains were already diagnosed by Gärtner (1985b), proposing affiliation of the latter strain to *Trebouxia glomerata*. However, analyses of ITS and actin sequences revealed affiliation of strain UTEX 67 to *Asterochloris italiana* (Figs 4, 5). The strain UTEX 902 is, therefore, proposed to represent the only authentic strain of *Asterochloris magna*, given that its morphology well corresponds to the original species diagnosis made by Archibald (1975). Simultaneously, we established a new type of the species (i.e. lectotype), representing the first drawing of UTEX 902 (originally labelled under the invalid name *´Trebouxia lambii´*, Cult. Coll. 902) in the Ph.D. dissertation of Ahmadjian (1959a).

Morphological delineation of *Asterochloris* and its particular species

The investigated strains share several morphological features that allow the delimitation of the genus *Asterochloris*, as well as distinguish it from closely related genera (i.e. *Trebouxia* and *Myrmecia*). Along with the proposed morphological characters (for details see Introduction), we also consider the process of zoosporogenesis essential for *Asterochloris* delimitation. The characteristic simultaneous release of zoospores adhering together via posterior extensions seems to be unique within Trebouxiophyceae, as it has not been observed in any other class member so far. Interestingly, these unique posterior extensions are not mentioned in other works dealing with *Trebouxia* s.l. taxonomy (Archibald 1975; Gärtner 1985b; Tschermak-Woess 1989). We interpret this as being due either to the frequent application of iodine solution treatment, which leads to the disappearance/separation of extensions, or late zoospore observations. Nonetheless, Ahmadjian (1959b) mentioned frequent observations of "*Volvox*-looking forms" of zoospores. Although he considered these forms as zoospore malformations, we believe he simply observed the clusters of zoospores adhered by posterior extensions.

Several morphological characters have been determined to be highly plastic in culture conditions (e.g. cell size, thickness of cell walls and pyrenoid structure). Others exhibit large variability among strains of the same species (e.g. pyrenoid number in adult cells). All these characters are therefore inapplicable for species delimitation and can be used only for the additional circumscription of a particular species. On the other hand, chloroplast morphology, prevailing cell shape, number of aplanospores per sporangium and zoospore dimensions do appear to be suitable morphological characters for species delimitation in *Asterochloris*; regardless of the determination that the interspecific differences of the last two do not correlate with measured genetic distances (Table 4).

Chloroplast morphology in particular, exhibiting a high level of variability, could be considered the best morphological marker for species delineation (Škaloud and Peksa 2008). Generally, several specific chloroplast types occur during the species ontogeny (Figs 6A-K). Although these types are frequently shared by more than one species, the individual differences are primarily in the dominance and unique assemblage of the particular types (Table 3). For example, the parietal lobed chloroplast type prevails in *A. phycobiontica*, frequently occurs in *A. lobophora*, only occasionally appears in *A. gaertneri* and never develops in *A. glomerata*. Moreover, some chloroplast types occur in a small minority of species and could simply define them. For example, the combination of echinate and flat lobed chloroplast types occurs only in *A. woessiae*, while the presence of a globular type defines *A. echinata*.

Sexuality of lichen photobionts

According to contemporary symbiotic dogma, lichen symbiosis should lead to the loss of sexual reproduction in the algal symbiont as a result of highly evolved and integrated symbiotic association (Law and Lewis 1983). The absence of sexual reproduction in lichen photobionts (except the genus *Trentepohlia*) is in fact frequently mentioned in the literature (e.g. Ahmadjian 1987; Friedl and Büdel 1996; Gärtner 1992), and interpreted as preventing the production of novel genotypes that would be less suited to the mycobiont (Ahmadjian 1993). However, records confirming sexual reproduction of photobionts exist. The first exhaustive description of sexual reproduction in *Trebouxia* was published by Warén (1920). He observed frequent production of gametes and their subsequent fusion in photobionts of *Anaptychia ciliaris*, *Physconia distorta* (as *Physcia pulverulenta*) and *Xanthoria parietina*. Nine years later, Jaag (1929) reported sexual reproduction by iso- and anisogamy in a *´Cystococcus parmeliae´* photobiont isolated from *Flavoparmelia caperata*. Another observation was made by Ahmadjian (1959a, 1960), who described frequent sexual reproduction in *Trebouxia impressa* isolated from *Physcia stellaris*. In both cases, the isogamous sexual reproduction resulted in the formation of spherical smooth-walled zygotes. The additional, indirect evidence to support sexual reproduction in *Trebouxia* was presented by Kroken and Taylor (2000). The authors found a recombining population structure in photobionts of *Letharia* spp. by comparing ITS and actin sequences. Finally, we observed fusion of gametes and subsequent planozygote formation in *Asterochloris woessiae* (strain CAUP H1009), isolated from *Lepraria borealis*.

Although the existence of sexual reproduction was disregarded, e.g. by Gärtner (1985), we consider that the above-detailed direct observations, as well as indirect genetic data, definitely establish the presence of sexual reproduction in *Trebouxia and Asterochloris*. Up until now, sexual reproduction was reported only for *Trebouxia* s. str. photobionts (Beck et al. 1998; Dahlkild et al. 2001; Helms et al. 2001; Kroken and Taylor 2000; Nyati 2006). Therefore, the circumscribed gamete fusion in *A. woessiae* presents the first record of sexual reproduction being observed in *Asterochloris*. Considering the quantity and quality of published papers dealing with *Trebouxia* s.l. (see Gärtner 1985), and the scarcity of referred observations on gamete production, it could be simply concluded that sexual reproduction is a very rare, and a scarcely observed, event in the life of photobionts. However, both Ahmadjian (1959a, 1960) and Warén (1920) discussed frequent formation and fusion of gametes in their *Trebouxia* cultures isolated from lichen thalli. Surprisingly, this paradox can probably be explained by the prevalence of sexual reproduction in host fungi (i.e. *Anaptychia ciliaris*, *Physcia pulverulanta*, *Physcia stellaris* and *Xanthoria parietina*): the sexually reproducing mycobionts must associate with suitable algae to develop a new lichen thallus. This can be accomplished by germinating ascospores finding a free-living alga (Bubrick et al. 1984; Tschermak-Woess 1978). The free-living algae, not affected by symbiotic interaction, could preserve their ability to reproduce sexually. Therefore, if isolated soon after their uptake by the mycobiont, they could frequently exhibit sexual reproduction even in pure culture. However, sexual reproduction was also observed in lichens with predominantly vegetative reproduction, including *Lepraria borealis*. This species of *Lepraria* never develop ascomata or conidiomata (Ekman and Tønsberg 2002). The occurrence of sexually reproducing algae in the thallus of *Lepraria* can indicate the ability of a photobiont to retain its sexual reproductive mode during a long coexistence with a fungus, or the capability of the mycobiont to acquire free-living algae during its growth.

Specificity of *Asterochloris*

Many morphological and molecular investigations, as well as studies of resynthesis, revealed the specificity of *Asterochloris* algae to certain groups of lichen-forming fungi (e.g. Ahmadjian et al. 1980; Nelsen and Gargas 2008; Piercey-Normore and DePriest 2001; Yahr et al. 2004, 2006). The use of molecular methods, which provided an effective instrument for identification of photobionts, distinctly increased the number of investigated lichen taxa and identified photobionts (*Asterochloris* has recently been identified from approximately 104 lichen taxa – see Table 5).

Asterochloris algae are mentioned as photobionts of lichens (lichenized fungi) belonging mainly to the Lecanoromycetes, but also to Chaetothyriomycetes (*Verrucaria murina*, Chodat 1913). Within the class Lecanoromycetes, *Asterochloris* is in particular associated with the lichen-forming fungi from the order Lecanorales (Lecanoromycetideae), but references to the mycobionts from other algal groups (*Anzina*, *Diploschistes*) also exist. Within the Lecanorales, the major part of *Asterochloris* species (about 92% of those recorded) were found in Cladoniineae (Cladoniaceae, Squamarinaceae, Stereocaulaceae), with additional in Lecideaceae (Porpidiaceae) and Psoraceae. The most investigated lichen genera are *Cladonia* and *Lepraria*, where photobionts are known from 69 and 12 species, respectively.

Asterochloris has also been described as a photobiont of some lichens from family Parmeliaceae (Friedl 1989b; Meisch 1981). However, these observations are possibly mistaken, due to erroneous morphological determinations, and should be re-investigated. Similarly questionable, seem to be the records of *Asterochloris* from thalli of *Calicium*, *Chaenotheca* and *Cyphelium* (Raths 1938). Finally, the record of *Asterochloris italiana* from *Xanthoria parietina* (strain CCAP 219/5b) is almost certainly incorrect (Peršoh et al. 2004; Nyati 2006). In our view, this alga was either not really isolated from *X. parietina* or the culture was accidentally mistaken for another.

Table 5. List of taxa of lichen-forming fungi associated with *Asterochloris*. Number of species: number of particular lichen species whose photobiont identity is known. References (only primary sources of each known morphological and molecular records are included): 1 –

Ahmadjian (1960), 2 – Ahmadjian et al. (1980), 3 – Archibald (1975), 4 – Bačkor et al. (1998), 5 – Beck (2002), 6 – Beck et al. (2002), 7 – Beiggi and Piercey-Normore (2007), 8 – Chodat (1913), 9 – Cordeiro et al. (2005), 10 – Culberson (1963), 11 – Friedl (1987), 12 – Friedl and Gärtner (1988), 13 – Gärtner (1985b), 14 – Hildreth and Ahmadjian (1981), 15 – Meisch (1981), 16 – Nelsen and Gargas (2006), 17 – Nelsen and Gargas (2008), 18 – Piercey-Normore (2004), 19 – Piercey-Normore and DePriest (2001), 20 – Schaper and Ott (2003), 21 – Takeshita et al. (1991), 22 – Tschermak-Woess (1980), 23 – Warén (1920), 24 – Yahr et al. (2004), 25 – this study.

Genus	Classification	Number of species	References
Anzina	Ostropomycetidae inc. sed., Lecanoromycetes	1	19, 22
Cladia	Cladoniaceae, Lecanorales, Lecanoromycetes	1	17, 19, 21
Cladonia	Cladoniaceae, Lecanorales, Lecanoromycetes	69	1, 2, 3, 4, 7, 8, 9, 11, 13, 14, 15, 16, 18, 19, 23, 24, 25
Clauzadea	Lecideaceae, Lecanoromycetidae inc. sed., Lecanoromycetes	1	2
Diploschistes	Thelotremataceae, Ostropales, Lecanoromycetes	2	11, 12, 25
***Lecidea* s. l.**	Lecideaceae, Lecanoromycetidae inc. sed., Lecanoromycetes	1	20
Lepraria	Stereocaulaceae, Lecanorales, Lecanoromycetes	12	2, 5, 10, 16, 17, 25
Pilophorus	Cladoniaceae, Lecanorales, Lecanoromycetes	2	3, 16, 17
Porpidia	Lecideaceae, Lecanoromycetidae inc. sed., Lecanoromycetes	2	2, 14
Psora	Psoraceae, Lecanorales, Lecanoromycetes	1	20
Pycnothelia	Cladoniaceae, Lecanorales, Lecanoromycetes	1	19
Squamarina	Bacidiaceae, Lecanorales, Lecanoromycetes	2	6, 20
Stereocaulon	Stereocaulaceae, Lecanorales, Lecanoromycetes	8	1, 3, 14, 16, 19, 25
Verrucaria	Verrucariaceae, Verrucariales, Chaetothyriomycetes	1	8

Phylogenetic inference and species concepts in *Asterochloris*

ITS and partial actin sequence comparisons were used to analyze phylogenetic relationships between several *Asterochloris* members. The loci studied revealed considerable differences in their evolutionary dynamics as well as sequence variation (Table 2). According to previously published data (Kroken and Taylor 2000; Nelsen and Gargas 2006, 2008), the actin sequences show much greater variation, and the phylogenies yield strong resolution and support of many internal branches (compare Figs 4, 5). Moreover, the ITS locus cannot accurately resolve the closely related species *A. italiana*, *A. lobophora* and *A. magna*. According to the results of data and topological incongruence tests, the ITS and actin *Asterochloris* phylogenies were not congruent in their phylogenetic signal. This is in contradiction with previously published test results (Nelsen and Gargas 2006, 2008), which we explain by dissimilar taxon sampling and/or use of a different actin alignment. Contrary to these authors, the actin phylogenies were inferred using only the stabile alignment regions, assessed through SOAP comparisons (see Methods).

In general, both single-gene phylogenies show an absolute congruence in strain placement into the particular species. However, BP and PP support of species lineages was much lower in ITS tree. The most obvious discrepancy between the two single-gene trees relates to the ordering of internal branches and relative positions of certain species. Comparing tree topologies, the major contradictions are in the position of *A. echinata*, *A. friedlii*, *A. magna* and *A. woessiae*. Since the actin phylogeny receives an almost two-fold higher average support of all nodes, we recommend preferably using the actin locus for resolving phylogenies in *Asterochloris*. Howerver, ITS appears to remain applicable to determine the species membership of studied organisms. Although using statistical support for branches as the only criterion for choosing the better phylogeny is somewhat illegitimate (Gontcharov et al. 2004), the superiority of actin analysis is supported by several independent morphological characters. As an example, we cite the ultrastructural similarity of pyrenoid matrix in *A. erici* and *A. magna* (Friedl 1989a).

The actin phylogeny ascertained three highly supported clades (Fig. 5). These lineages share some morphological characteristics as shown in Fig. 9. For example, the ability to form morphologically well-defined crenulate chloroplasts distinguishes between the species of Clade 1 and 3; Clade 1 thus corresponds well with Clade I *sensu* Piercey-Normore and DePriest (2001), except for the additional inclusion of *A. erici* and *A. magna*. The close relationship of these two species, as well as the evident ingroup position of *A. erici* within *Asterochloris,* rule out use of *A. erici* as an outgroup, however frequently applied (Beiggi and Piercey-Normore 2007; Cordeiro et al. 2005; Nelsen and Gargas 2008; Piercey-Normore and DePriest 2001; Yahr et al. 2004, 2006). For the reason that incorrectly rooted trees may result in misleading phylogenetic and taxonomic inferences (Leliaert et al. 2007), we recommend the use of either unrooted phylogenies or those provisionally rooted by *A. glomerata* and *A. irregularis* clade, until the outgroup is detected.

Considering all previously mentioned morphological and molecular inferences, we believe the polyphasic approach that takes into account both methods is the best for defining particular species within *Asterochloris*. The traditional morphological species concept alone is not sufficient for species descriptions, as it does not recognize cryptic or sibling species (Behnke et al. 2004; Evans et al. 2007; Mayr 1948). However, due to its long tradition, previously published morphological obsevations provide valuable sources of information about morphological variability, uniqueness and distribution of particular *Asterochloris* species. On the other hand, the phylogenetic species concept, in the strict sense, regards the smallest aggregation of any lineages sharing a unique combination of character states (or nucleotide states) as separate species (Nixon and Wheeler 1990). A strict application of this species concept could result in overestimating the real numbers of species (e.g. three species within *A. friedlii*). In accordance with the polyphasic approach, we did not describe any new lineage as a new species without first considering the morphological data. For example, “clade 3A” remains undescribed, despite its high bootstrap support (Figs 4, 5). Similarly, we did not describe the photobiont from *Stereocaulon vesuvianum* N 281 (Clade 1; Nelsen and Gargas 2006) as a new species or assign it to *A. irregularis* without first completing morphological comparisons.

Finally, some *Asterochloris* species seem to exhibit strict ecological preferences. For example, *A. phycobiontica* was isolated from lichens collected only in the mountains (above alt. 950 m).Similarly, all *A. woessiae* strains were acquired from lichens growing on enriched silicate rocks (various types of schist, etc.), moreover, often dust-covered. Apart from the striking ecological preference, these data clarify the true nature of the *Asterochloris* species presented. In *Trebouxia*, similar ecological preferences were observed by Helms (2001), who revealed some photobiont genotypes to be preferentially isolated from lichens growing on specific substrata or in specific macroclimatic zones.

How many *Asterochloris* species are there?

By investigating both morphological and molecular data, thirteen species were identified as belonging to the genus *Asterochloris*, including six species new to science. However, this genus apparently still contains many undescribed species. The actin phylogram indicates 4-9 lineages that could represent other new species (Fig. 5), including a well-resolved lineage of purely American photobionts (clade 3A). However, actin data give us only partial insight into the recognized genetic variability of *Asterochloris*, as they have been used only twice to date (Nelsen and Gargas 2006, 2008). Conversely, ITS sequences have been frequently utilized in many studies concerning *Asterochloris* photobionts, and could aid us in calculating the actual species abundance. A lineage of North and Central American lichen photobionts (clade 3A) is described in a number of papers, demonstrating its wide distribution and endemism in the American Continent (Cordeiro et al. 2005 (Clade IIc); Nelsen and Gargas 2006 (photobionts from *Pilophorus* cf. *cereolus* and *Stereocaulon* sp.); Nelsen and Gargas 2008 (the uppermost lineage in Clade II); Piercey-Normore and DePriest 2001 (the uppermost lineage in Clade II); Yahr et al. 2004, 2006 (Clade IIa)).Furthermore, a number of lineages that could represent additional undescribed taxa was revealed by many authors; e.g. well resolved lineages of *Cladonia fimbriata* and *C. rangiformis* photobionts (Piercey-

Normore and DePriest 2001), Clade IIa *sensu* Cordeiro et al. (2005), Clades IIc and IId *sensu* Yahr et al. (2006) or a lineage of the *Cladonia poccilum*, *C. cornuta* and *C. pyxidata* photobionts (Beiggi and Piercey-Normore 2007). On the other hand, several originally unresolved lineages are described as a new *Asterochloris* species: e.g. *A. lobophora* - clade IIb *sensu* Yahr et al. (2004, 2006) and the lowest lineage in Clade II in Nelsen and Gargas (2008); or *A. friedlii* - clade IIL sensu Nelsen and Gargas (2008).

Our results reveal large hidden diversity in the genus *Asterochloris*. This study alone increases the total species number from seven to thirteen, i.e. almost double. Moreover, although previous studies investigated the photobionts from the same mycobiont genera and ecological groups (i.e. saxicolous, terricolous and epiphytic lichens), we uncovered three new, not yet reported lineages (*A. leprariae*, *A. echinata* and *A. gaertneri*). From twenty-five isolated photobionts considered in our study, only five of them could be assigned with certainty to previously described species (i.e. *A. glomerata*, *A. irregularis* and *A. phycobiontica*). In other words, 80% of our isolated strains are new to science. That amazing cryptic variability emphasizes the insufficiency of using morphological criteria only when making species determinations. Even if all previously described species are correct, more than three quarters of species determinations will be incorrect, due to the forced morphological affiliations with a few species described to date (Gärtner 1985b). However, considering the herein presented implications in *Asterochloris* species concepts, we believe in the possibility to morphologically and ecologically determine the majority of species correctly when additional species are described and delimited. Moreover, selected morphological characters (e.g. prevailing cell shape and chloroplast morphology) could be used to detect affiliation to previously described species, although new lineages will be uncovered.

Materials and Methods

Species sampling and algal cultures

Twenty-five thalli of lichen-forming genera *Lepraria*, *Cladonia*, *Stereocaulon* and *Diploschistes* were collected at various localities in Central and Eastern Europe to sample the wide spectrum of lichen symbionts. The algal symbionts were isolated into unialgal cultures according to the thallus fragmentation method of Ahmadjian (1993). In addition, eight authentic *Trebouxia* strains were obtained from the Culture Collection of Algae at the University of Texas at Austin, USA – UTEX (strains 67, 902, 911, 1712, 1714, 2236), the Culture Collection of Algae at the University of Göttingen, Germany – SAG (strain 26.81), and the Culture Collection of Algae and Protozoa at Argyll, United Kingdom – CCAP (strain 519/5B). Lichen specimens were maintained in the herbarium of O. Peksa in PL (Westbohemian Museum in Pilsen). Algal strains were deposited in the Culture Collection of algae of Charles University of Prague at Prague, Czech Republic (CAUP), either in the living form or cryo-preserved in a -70 °C freezer. Algal strains were cryo-preserved in a 10% dimethylsulfoxide (DMSO) using 5100 Cryo 1°C Freezing Container (Mr. Frosty, Nalgene). The 58 new sequences generated in this study were deposited in EMBL Nucleotide Sequence Database. All samples and sequences used in this study, with their accession numbers, are shown in Table 1.

Microscopic observations

Observations of the algal isolates were made from cultures grown on 2% agar slants of Bold's Basal Medium (BBM) as modified by Bischoff and Bold (1963). All cultures were maintained at a temperature of 15 °C, under an illumination of 5-15 μmol m^{-2} s^{-1} (cooling box Helkama C5G). Individual strains were regularly observed using a conventional light and a confocal microscope during three culture-to culture generations, from June 2006 until August 2007, to reveal morphological variability. Zoospore formation was induced by transferring the cultures to a 1% glucose solution (Hildreth and Ahmadjian 1981). Pyrenoid was visualized by staining with a chloriodine solution (an aqueous solution of 5 g I_2 and 10 g of 2,2,2-trichlor-1,1-ethandiol in 5 ml of distilled water).

The pure algal samples were examined under an Olympus BX51 light microscope with differential and phase contrasts, and Olympus Z5060 microphotographic equipment, as well

as by a Leica TCS SP2 laser scanning confocal microscope equipped with an Argon-Krypton laser. We used a 488 nm excitation line and an AOBS filter-free system collecting emitted light between 498 and 700 nm. A Leica 63x/1.4 N.A. oil immersion objective fitted on the Leica DM IRE2 inverted microscope was used. A series of optical sections through chloroplasts were captured and used for 3-dimentional reconstruction of their morphology. The autofluorescence of chlorophyll was exploited for visualization of the chloroplast structure. For the final processing of the confocal images and various chloroplast structure visualizations, Leica Confocal Software, version 2.61 (Leica Microsystems Heidelberg GmbH) and the ImageJ 1.34p Programme (Abramoff et al. 2004) were used.

DNA extraction, PCR and DNA sequencing

Total genomic DNA was extracted from lyophilized algal cultures following the standard CTAB protocol (Doyle and Doyle 1987), with minor modifications. Algal DNA was resuspended in sterile dH2O and amplified by polymerase chain reaction (PCR). The ITS1, ITS2, and 5.8S regions were amplified using the algal-specific primer nr-SSU-1780-5´ (5´-CTG CGG AAG GAT CAT TGA TTC-3´; Piercey-Normore and DePriest 2001) and a universal primer ITS4-3´ (5´-TCC TCC GCT TAT TGA TAT GC-3´; White et al., 1990). Actin type I locus (2 introns and 1 complete exon) was amplified using the algal specific primers a-nuact1-0645-5′ (GAC AGA GCG TGG KTA CAG) and a-nu-act1-0818-3′ (TGA ACA GCA CCT CAG GGC A; Nelsen and Gargas 2006). All PCR were performed in 20 μl reaction volumes (15.1 μl sterile Milli-Q Water, 2 μl 10´ PCR buffer (Sigma), 0.4 μl dNTP (10 μM), 0.25 μl of primers (25 pmol/ml), 0.5 μl Red Taq DNA Polymerase (Sigma) (1U/ml), 0.5 μl of $MgCl_2$, 1 μl of DNA (not quantified)).

PCR and cycle-sequencing reactions were performed in either a XP thermal cycler (Bioer) or a Touchgene gradient cycler (Techne). PCR amplification of the algal ITS began with an initial denaturation at 95 °C for 5 min, and was followed by 35 cycles of denaturing at 95 °C for 1 min, annealing at 54 °C for 1 min and elongation at 72 °C for 1 min, with a final extension at 72 °C for 7 min. Identical conditions were used for the amplification of the actin I locus, except that an annealing temperature of 60-62 °C was used. The PCR products were quantified on a 1% agarose gel stained with ethidium bromide and cleaned either with the JetQuick PCR Purification Kit (Genomed) or with QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's protocols. The purified amplification products were sequenced with the PCR primers using the protocol for the DNA sequencing kit (ABI Prism Big-Dye terminator cycle sequencing ready reaction, Applied BioSystems). Purification of sequencing reactions was carried out using an ethanol/sodium acetate precipitation provided with the sequencing kit. Products were run on an ABI 3100 Avant automated sequencer (Applied BioSystems). Sequencing reads were assembled and edited using SeqAssem programme (SequentiX Software).

Sequence alignment and phylogenetic analyses

Sequences were initially aligned using ClustalX 1.83 (Thompson et al. 1997) and MUSCLE (Edgar 2004). Only GenBank *Asterochloris* reference sequences acquired from organisms with both ITS and actin I locus sequences were used (Table 1). ITS sequences (comprised ITS1, 5.8S and ITS2 regions) were aligned on the basis of their rRNA secondary structure information with MEGA 3.1 (Kumar et al. 2004), using published ITS secondary structure of *Asterochloris* photobionts (Beiggi and Piercey-Normore 2007) as a model. Positions with deletions in a majority of sequences were removed from the alignment, resulting in an alignment comprising 531 base positions. The alignment of actin I locus sequences was more difficult than those of the ITS region. Although several successive MUSCLE alignments considerably improved alignment quality, a great deal of ambiguous positions remained. Due to an absence of published secondary structure of *Asterochloris* or a related genera, the stability of alignment has been assessed through comparison of ClustalW alignments produced under different gap opening/extension penalties using SOAP v.1.2 alpha 4 (Löytynoja and Milinkovitch 2001). Gap penalties were incrementally adjusted from 7 to 17 by steps of 2, and extension penalties were adjusted from 4 to 9 by steps of 1. Regions of instability were de-

leted by computing to a 90% consensus among the thirty-six different alignments, leaving an alignment of 532 positions. The robustness of an alignment was then tested by simply comparing NJ trees constructed in MEGA, from the resulting alignment, and those created by SOAP with the opening/extension penalty parameters varied either from 7/0.04 to 17/0.2 or from 14/4 to 16/9, respectively. The resulting tree topologies were consistent, with only bootstrap values slightly differing (trees not shown). Alignments are available from EMBL-EBI (Accession Nos. ALIGN_001221, ALIGN_001222 and ALIGN_001223 for the ITS, entire actin, and improved actin alignments, respectively).

The phylogenetic trees were inferred by maximum likelihood (ML) and weighted parsimony (wMP) criteria using PAUP*, version 4.0b10 (Swofford 2002), and by Bayesian inference (BI) using MrBayes version 3.1 (Ronquist and Huelsenbeck 2003). Three data sets were used consisting of 52 *Asterochloris* taxa: ITS (531 bp), actin (532 bp) and the concatenated set of ITS and actin sequences (1063 bp). A substitution model was estimated for each data set using the Akaike Information Criterion (AIC) with PAUP/MrModeltest 1.0b (Nylander 2004). For actin and the concatenated data set the best model was the GTR+G, whereas for ITS the GTR+G+I model was deemed best.

Maximum likelihood analyses consisted of heuristic searches with 1000 random sequence addition replicates and Tree Bisection Reconnection swapping. Reliability of the resulting topology was tested using bootstrap analysis (100 replications) consisting of heuristic searches with 10 random sequence addition replicates, Tree bisection reconnection swapping, and a rearrangement limit of 5000 for each replicate. The wMP bootstrapping was performed using heuristic searches with 100 random sequence addition replicates, Tree bisection reconnection swapping, random addition of sequences (the number limited to 10,000 for each replicate), and gap characters treated as a fifth character state. In BI analysis, two parallel MCMC runs were carried out for 2 million generations, each with one cold and three heated chains employing the above-stated evolutionary models for ITS and actin data sets. For the analysis of the concatenated alignment, the data set was partitioned into ITS and actin regions because of different substitution models estimated for the two partitions (Nylander et al. 2004). Trees and parameters were sampled every 100 generations. Convergence of the two cold chains was checked and burn-in was determined using the ´sump´ command. Bootstrap percentages and posterior probabilities were interpreted as weak (less than 50 %) moderate (50-94 % for BI; 50-79 % for ML and MP) or high (more than 94 % for BI; more than 79 % for ML and MP).

Congruence between ITS and actin data sets were tested using the incongruence length difference (ILD) test (Farris et al. 1995), as implemented by the partition homogeneity test in PAUP* (heuristic search, simple addition, TBR branching swapping, 1000 replicates). The ILD test was performed on both full concatenated data set and parsimony informative sites only, as suggested by Lee (2001). In view of several recent reports critizing the results of ILD tests (Dolphin et al. 2000; Ramirez 2006), we also explored whether resolution and support would be improved by increasing the amount of sequencing data, by direct comparison of bootstrap supports and posterior probabilities among analyses of all three data sets. To determine if topologies between data sets were congruent, wMP heuristic searches were performed for the ITS and actin sequences, using the same settings described above. For each data partition, a strict consensus tree was constructed from the most parsimonious trees from each search. The consensus topology was then enforced as a constraint on the other data set and a heuristic search was again performed. Trees obtained from the unconstrained and constrained searches were then compared by means of Templeton (Templeton 1983) and Kishino-Hasegawa (Kishino and Hasegawa 1989) tests.

To determine which morphological characters correlate well with genetic distances, the following procedure was used: the actin data set was used to compute the average between groups distances among all species in program MEGA 3.1 (Kimura 2-parameter distances). Manhattan distances were computed for single morphological characters as well as for some groups of them using program PAST v. 1.75b (Hammer et al. 2001). Similarity of both matrices was then tested by Mantel test in PAST v. 1.75b (10,000 permutations). To trace the evolution of morphological characters, the wMP tree was inferred for 13 taxa

representing individual *Asterochloris* species (heuristic searches with 1000 random sequence addition replicates, TBR swapping). The evolution of characters was traced along the tree using maximum parsimony in the Mesquite software package, v. 2.0 (Maddison and Maddison 2007). Clade 1 (*A. glomerata*, *A. irregularis*, *A. erici* and *A. magna*) was used to arbitrarily root the resulting tree.

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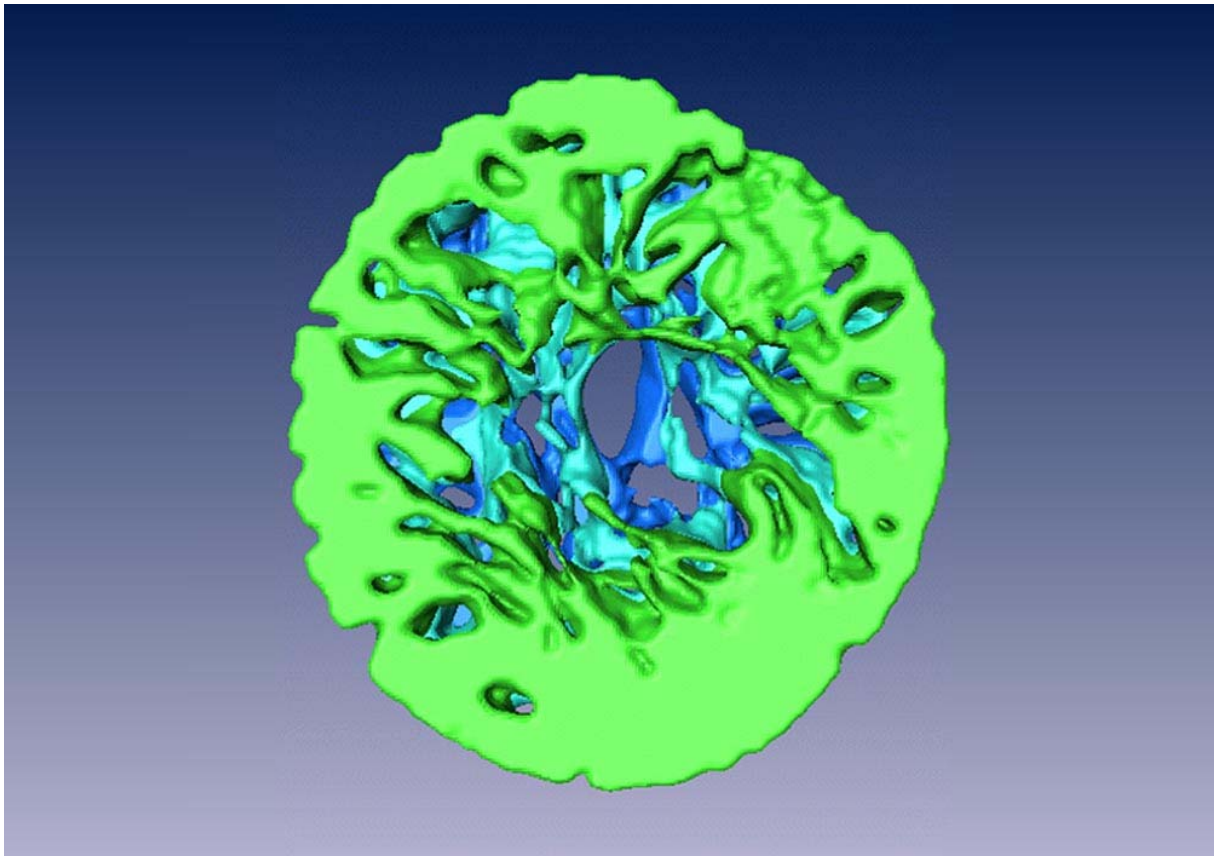
Paper 4

Confocal microscopy of chloroplast morphology and ontogeny in three strains of *Dictyochloropsis* (Trebouxiophyceae, Chlorophyta)

Pavel Škaloud, Jiří Neustupa, Barbora Kubínová & Lucie Kubínová

Submitted manuscript



Confocal reconstruction of the chloroplast structure in *Dictyochloropsis splendida*

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Confocal microscopy of chloroplast morphology and ontogeny in three strains of *Dictyochloropsis* (Trebouxiophyceae, Chlorophyta)

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P. ŠKALOUD, J. NEUSTUPA, B. RADOCHOVÁ AND L. KUBÍNOVÁ. 2005. Confocal microscopy of chloroplast morphology and ontogeny in three strains of *Dictyochloropsis* (Trebouxiophyceae, Chlorophyta). *Phycologia* 44: 000–000.

Chloroplast morphology and ontogeny in three species of the genus *Dictyochloropsis* – *D. splendida* var. *splendida, D. reticulata* and *D. symbiontica* – were investigated by using light and confocal microscopy. In a conventional light microscope, the complicated net-shaped chloroplast often appeared as a homogenous mass filling up most of the cell volume, while confocal microscopy enabled a detailed description of the chloroplast changes during its ontogeny. We identified four distinct morphological stages during the chloroplast ontogeny in all investigated strains. The stages are distinguished primarily by the number of differently structured chloroplast layers and by the inner structure of chloroplast lobes. The investigated *Dictyochloropsis* strains differed mainly in timing of these particular ontogenetic sequences. In the final stage of the chloroplast ontogeny, the transformation of the net-shaped chloroplast to a simple form allows the chloroplast division.

INTRODUCTION

The genus *Dictyochloropsis* was established by Geitler (1966), who described *D. splendida* as a type species of the genus from aeroterrestrial biotope. Later, Tschermak-Woess (1980, 1984) and Nakano & Isagi (1987) added several species isolated from subaerial biotopes and lichen thalli. At present the genus includes seven taxa: *D. splendida* Geitler var. *splendida, D. splendida* var. *gelatinosa* Tschermak-Woess, *D. symbiontica* Tschermak-Woess var. *symbiontica, D. symbiontica* var. *ellipsoidea* Tschermak-Woess, *D. symbiontica* var. *pauciautosporica* Tschermak-Woess, *D. reticulata* Tschermak-Woess and *D. irregularis* Nakano & Isagi.

The genus is characterized by single uninucleate cells and the asexual reproduction takes place by means of naked zoospores with typical separate insertion of flagella (Tschermak-Woess 1980, 1984). The individual species within the genus (Tschermak-Woess 1984, Ettl & Gärtner 1995) are distinguished mainly according to the chloroplast appearance under a conventional light microscope. *Dictyochloropsis* chloroplasts have a complicated structure formed by a reticulate net which spreads below the plasma membrane of adult cells. In some species the chloroplast lobes form multiple reticulate layers in the cytoplasm allowing their morphological and taxonomic delimitation. However, in some species it is impossible to investigate the chloroplast morphology, ontogeny and interspecific differences under a conventional light microscope, due to the complicated chloroplast structure and the small size of cells.

Recently, confocal microscopy has been repeatedly applied for the investigation of chloroplast morphology and structural dynamics in higher plants (Pyke & Page 1998; Sarafis 1998; Zheng *et al.* 2002). Confocal microscopy enables capture of sharp images of thin optical sections of living tissues and cells, however, it has been only rarely used in the investigations of algal chloroplasts so far (Kreimer *et al.* 1991; Gunning & Schwartz 1999; Zakrys *et al.* 2002).

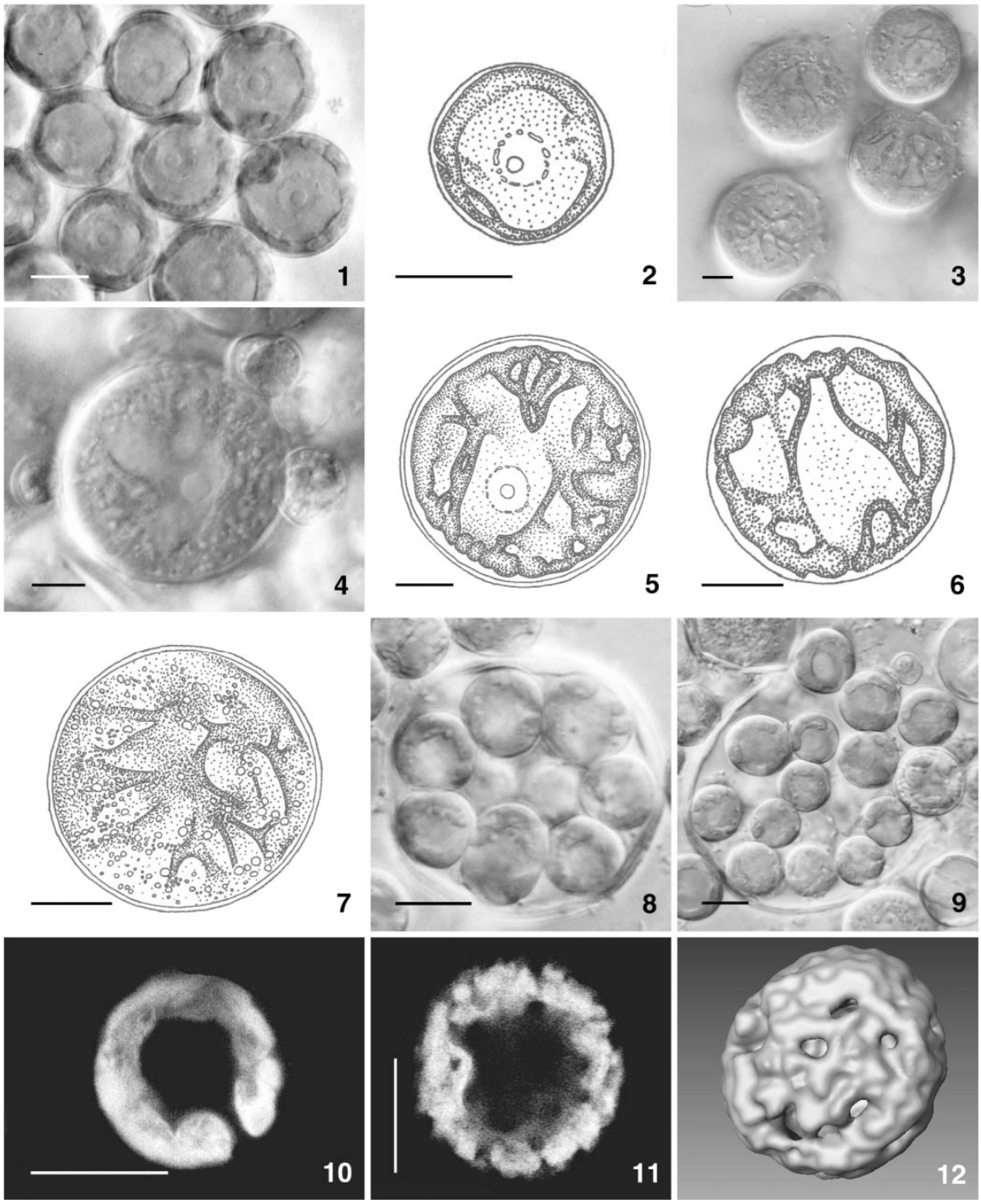
In the present paper, confocal microscopy, applied to chloroplasts in living cells of *Dictyochloropsis,* is used for a detailed description of morphological differences between particular strains and for the reconstruction of chloroplast ontogeny.

* Corresponding author (skaloud@natur.cuni.cz).

MATERIAL AND METHODS

Three *Dictyochloropsis* strains were investigated. The strain of *D. splendida* was isolated from a soil sample at the top of the Boreč hill in České Středohoří Mts., Czech Republic. The strain determined as *Dictyochloropsis reticulata* was isolated from a bark sample of an unidentified tree in the secondary tropical rain forest in the Kelantan province, Malaysia. The strain *D. symbiontica* was isolated from the bark sample of *Shorea* sp. in the primary tropical rain forest, Tioman Island, Malaysia. All investigated strains were deposited in the Culture Collection of Algae of Charles University in Prague (CAUP) and the following strain numbers were assigned to them:.

The strains were cultivated on agar-solidified BBM medium (Bischoff & Bold 1963) at a temperature of 25°C, under an illumination of about 200 μmol photons s^{-1} m^{-2} (light source: Philips TLD 18W/33, cool white). The production of zoospores was induced using several methods (Andreyeva 1998; Neustupa & Němcová 2001). It was most efficient to simply transfer vegetative cells from a growing culture into distilled water under a coverslip. The chloroplast structure was regularly examined under a confocal microscope during cell ontogeny. The algal samples were investigated by a laser scanning confocal microscope Bio-Rad MRC600 equipped with an argon–krypton laser using the 488-nm excitation line. A



Figs 1–12. *Dictyochloropsis splendida* var. *splendida*. Scale bars = 10 μm.
Figs 1, 2. Young cell with distinct nucleus.
Figs 3–6. Vegetative cells with multilayered net-shaped chloroplast.
Fig. 7. Vegetative cell with poorly visible chloroplast structure.
Figs 8, 9. Globular autosporangia.
Figs 10, 11. Unilayered chloroplast of young cells.
Fig. 12. Surface view of unilayered chloroplast with numerous perforations.

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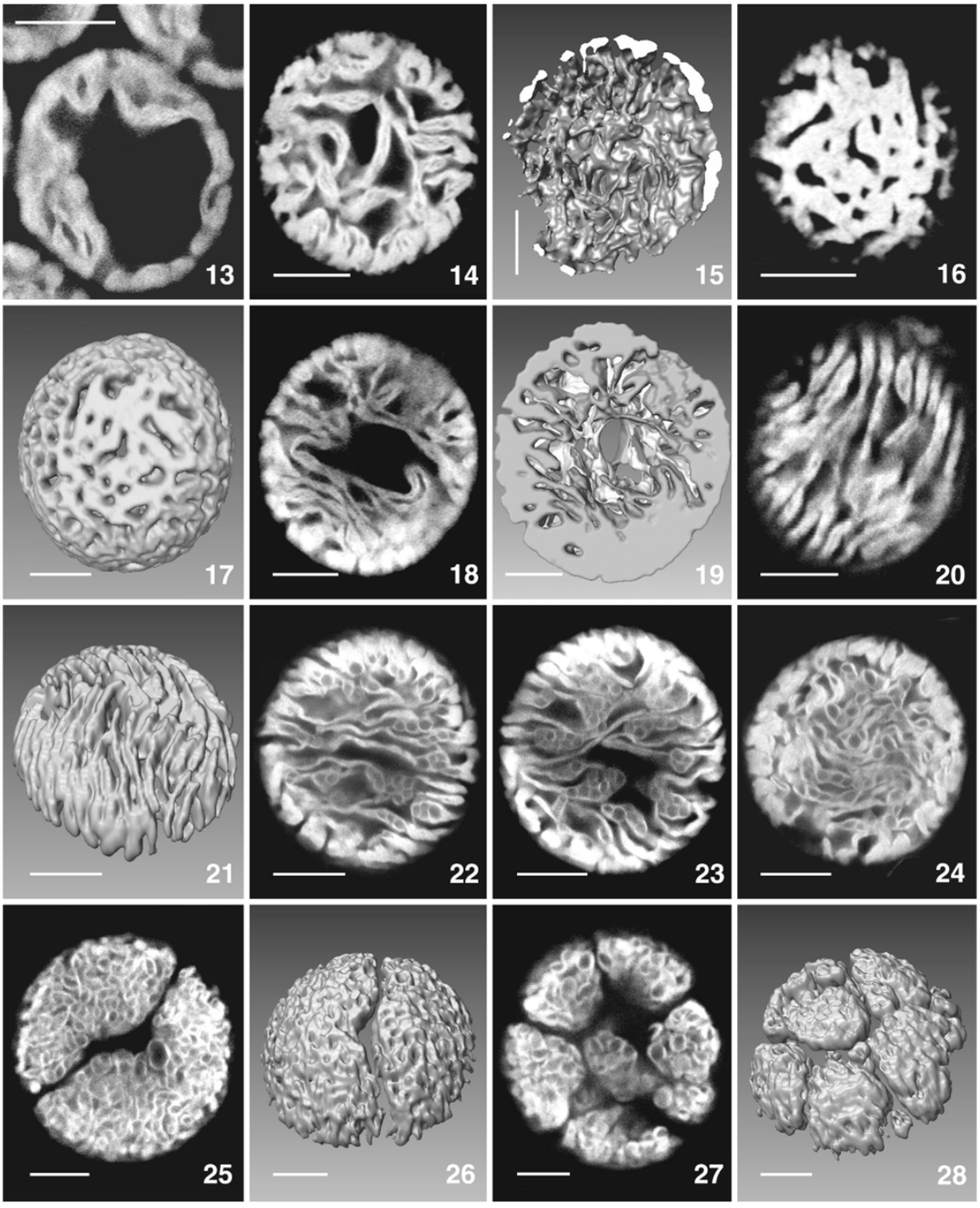

Figs 13–28. *Dictyochloropsis splendida* var. *splendida*. Scale bars = 10 μm.
Fig. 13. New chloroplast lobes production, forming the second layer of chloroplast.
Figs 14, 15. Net-shaped chloroplast of mature vegetative cells.
Figs 16, 17. Chloroplast structure of the first chloroplast layer (the layer below the plasma membrane) showing connected chloroplast with numerous perforations.

Nikon 100×/1.4 N.A. oil immersion objective fitted on the Nikon Diaphot inverted fluorescent microscope was used. Series of optical sections of chloroplasts, 0.5 μm apart, were captured and used for three-dimensional reconstruction of their morphology. The autofluorescence of chlorophyll was exploited for visualisation of the chloroplast structure. For the final processing of the confocal images, Confocal Assistant programme, version 4.02 (Todd Clark Brelje, University of Minnesota, , MN, USA) was used. The three-dimensional reconstruction images were created by Amira® 2.3 programme (Indeed – Visual Concepts GmbH, Berlin, Germany).

RESULTS

Dictyochloropsis splendida Geitler var. *splendida*

CONVENTIONAL LIGHT MICROSCOPY: The alga had globular uninuclear cells with diameters of 7–40(–50) μm. The chloroplast of young cells formed a single layer of lobes below the plasma membrane (Figs 1, 2). The chloroplasts of adult cells formed a complicated three-dimensional net of interconnected lobes (Figs 3–6). However, in most adult cells the chloroplast structure was not clearly visible under a conventional transmission light microscope and the chloroplasts appeared as a homogenous mass filling up the cell volume (Fig. 7). The reproduction took place by means of autospores (Figs 8, 9). Autospores were formed in autosporangia of globular shape. The autosporangium (having diameter of 45–48 μm) usually contained 8–16 autospores. No production of zoospores was observed during a long-term investigation.

CONFOCAL MICROSCOPY: It was clearly seen that in young cells the chloroplast spread below the plasma membrane as a single layer with numerous perforations (Figs 10–12). During the cell ontogeny, distinct chloroplast tubular lobes were producing further into the cell lumen (Fig. 13) and consequently formed a second chloroplast layer. Successively, further chloroplast lobes spread into the cell interior and formed more layers (Figs 14, 15). In external view, the perforated surface of the outer chloroplast layer was visible in this stage (Figs 16, 17). In cells having a diameter larger than 25 μm and at least three established chloroplast layers a new type of lobe production appeared. At this stage new lobes arose from original lobes by their longitudinal splitting (Figs 18, 19). In contrast to the original tubular lobes the new lobes were rather flat. These flat lobes further multiplied by subsequent longitudinal splitting. Finally, the whole chloroplast consisted of numerous parallel flat lobes (Fig. 20). Even the original outer chloroplast layer was modified at this later stage of chloroplast development (Fig. 21 – compare with Fig. 17 which shows the structure of the same chloroplast layer in the younger cells).

Before cell division occurred, the inner structure of chloroplast lobes changed. The lobes were widening and their structure was becoming more dense at the marginal regions (i.e. lighter due to higher emission of fluorescence light) and more loosened (i.e. darker) in the central part of the cell, as detected by confocal microscopy (Figs 22, 23), indicating the grouping of thylakoids within the chloroplast lumen. At the final stage the modified lobes fused into a single chloroplast with a granular structure where the regions with and without thylakoids could be distinguished (Fig. 24). Then the compact globular chloroplast encircling the nucleus divided into two equivalent parts (Figs 25, 26). Finally, the successive division produced several compact chloroplasts (Figs 27, 28).

Dictyochloropsis reticulata Tschermak-Woess

CONVENTIONAL LIGHT MICROSCOPY: The investigated strain of this alga had uninuclear globular, or rarely ellipsoidal, cells. The diameters of vegetative cells were in the range of (4.5–) 6–16(–18) μm. In a conventional light microscope the structure of the chloroplast could not be distinguished. In young cells and some adult cells the chloroplast formed a single layer below the plasma membrane (Fig. 29). In most of the adult cells, distinct chloroplast lobes were visible (Figs 30–32). However, under a conventional transmission light microscope, the chloroplasts of most adult cells appeared as a granular mass filling up the cell volume (Figs 33–35). Asexual reproduction took place by means of autospores and zoospores (Fig. 36). The number of autospores per autosporagium was 8–16 and they had a globular shape. The diameters of the autosporangia were 14–17 μm. The globular zoosporangia 16–23 μm in diameter contained 16–64 naked zoospores.

CONFOCAL MICROSCOPY: In young cells the chloroplast was unilayered with numerous perforations (Fig. 37). At this stage, chloroplasts of *D. reticulata* could not be distinguished from those of *D. splendida*. Later on, in some adult cells the isolated chloroplast lobes expanded into the central cell lumen (Figs 38, 39). The lobes were usually formed in one part of the cell (Fig. 40). However, in most cases the lobes did not form a continuous secondary layer. In adult cells, the structure of the original chloroplast layer was slightly changing. The perforations in the chloroplast became larger and the layer below the plasma membrane formed a net of connected tubular lobes (Figs 41–43).

Before cell division, the chloroplast structure was changing considerably to form a multilayered reticulate net (Fig. 44). At this very short ontogenetic stage, the tubular lobes changed to globular ones (Fig. 45). Immediately after the multilayered net was formed, the chloroplast lobes started to join into a single thick layer (Fig. 46). Afterwards, the thylakoids within the chloroplast lumen were grouped, appearing as lighter granular parts of the chloroplast (Fig. 47) (a similar stage is shown in Fig. 25 of *D. splendida*). Before the production of autos-

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Figs 18, 19. Parallel chloroplast lobes showing the longitudinal splitting of original chloroplast lobes.
Figs 20, 21. First chloroplast layer showing the parallel chloroplast lobes.
Figs 22, 23. Chloroplast of mature cells before cell division, showing the establishment of chloroplast compartments with granular structure.
Fig. 24. Granular chloroplast of mature cells.
Figs 25, 26. First division of granular chloroplast.
Figs 27, 28. Dividing of granular chloroplast before autospore production.

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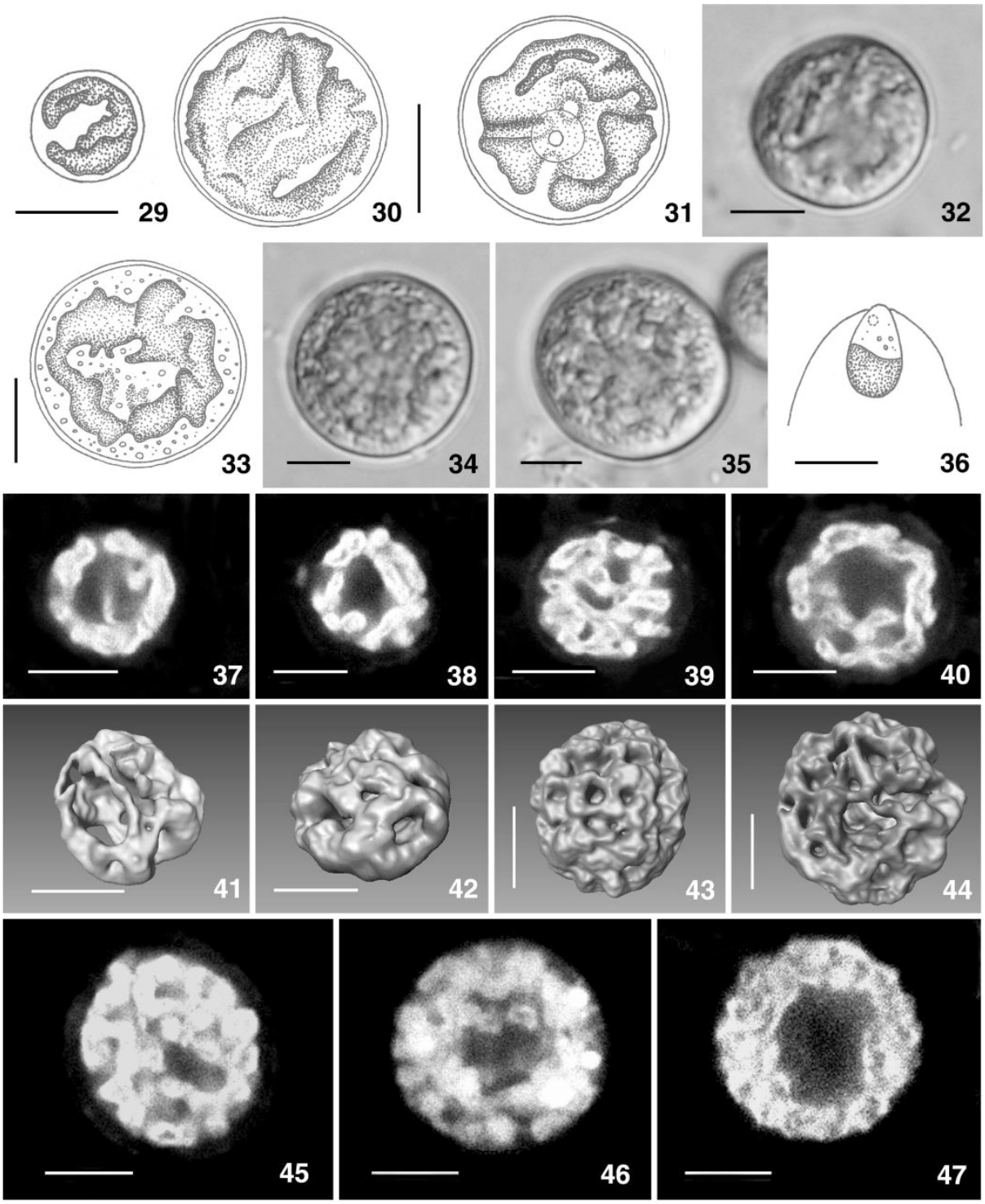

Figs 29–47. *Dictyochloropsis reticulata*. Scale bars = 5 μm.
Fig. 29. Young cell with single layer of chloroplast.
Figs 30–32. Vegetative cells with unilayered net-shaped chloroplast.
Figs 33–35. Barely visible chloroplast structure of most adult cells.
Fig. 36. Zoospore with separate insertion of flagella.

pores, the chloroplast was successively divided into several equivalent parts.

***Dictyochloropsis symbiontica* Tschermak-Woess**

CONVENTIONAL LIGHT MICROSCOPY: The alga had globular uninuclear cells with diameter of 5–21(–26) μm. As in previous species, the chloroplast of young cells was unilayered with perforations (Fig. 48). In some of the young cells, the multilayered structure of the chloroplast was visible under a conventional light microscope (Figs 49, 50), however, in most cases the structure of the chloroplast could not be distinguished and the chloroplast appeared as a granular mass filling up the cell volume (Fig. 51). The reproduction took place by means of autospores (Fig. 52), aplanospores (Figs 53, 54) and zoospores (Fig. 55). The number of autospores per autosporagium was 12–16 and they had a globular shape. The diameters of autosporangia were 12–20 μm. The globular zoosporangia and the aplanosporangia contained 32–64 naked zoospores or aplanospores, respectively. The zoosporangia and aplanosporangia were 12–20 μm in diameter.

CONFOCAL MICROSCOPY: In young cells, the chloroplast exhibited a layer of pheripheral and interconnected tubular lobes (Fig. 56). The perforations in this layer were larger than in both previously investigated strains (Figs 57–59). During early ontogenetic stages the lobes became more dense in the marginal light regions and more loosened in the central dark regions, that indicated the grouping of thylakoids within the chloroplast lumen (Figs 60, 61), similarly as in the two previous species. At this stage, the second layer developed by the extension of individual chloroplast lobes into the cell lumen (Figs 62, 63).

Before cell division, globular lobes were formed (Fig. 64). The chloroplast lobes of the first and secondary layer fused into a single chloroplast mass with a granular structure (Fig. 65). Subsequently, the chloroplast was successively divided into a number of equivalent parts, which preceded the zoosporangial or autosporangial production (Fig. 66).

DISCUSSION

In general, light microscopic observations of three *Dictyochloropsis* strains correspond with most of the previous investigations (Geitler 1966; Tschermak-Woess 1980, 1984; Takeshita *et al.* 1991). Dimensions and morphology of the vegetative cells in the strain determined as *D. splendida* var. *splendida* correspond precisely both with Geitler's (1966) original description and the description given by Tschermak-Woess (1984). However, Tschermak-Woess (1984, 1995) did not observe the production of autospores in cultures of *D. splendida*. In contrast, Geitler (1966) observed the frequent production of autospores in that species, and our observations are in accordance with this. Thus, the absence of autospores in the life cycle cannot be considered as a principal discriminative character for the determination of *D. splendida* as stated by Tschermak-Woess (1984) which leaves the size of vegetative cells, which exceeds 30 μm in diameter, to be the only discriminative character for the light microscopic identification of *D. splendida*.

Morphological characteristics of the investigated strain of *D. reticulata* correspond with the original description (Tschermak-Woess 1984) in most aspects. However, Tschermak-Woess (1984) did not observe production of autospores in this species. The absence of autospore production was even stated as a discriminative character of *D. reticulata* in her identification key for the *Dictyochloropsis* species. However, Takeshita *et al.* (1991) observed the frequent production of autospores in *D. reticulata* isolated from the thallus of the lichen *Brigantiaea ferruginea*, and which is in accordance with our findings.

The observed morphological characters of *D. symbiontica* also correspond with those of the original description of this species in most cases. Tschermak-Woess (1980, 1984) described several varieties of *D. symbiontica* differing mainly in the dimension of vegetative cells and the frequency of autospore production. The dimensions of vegetative cells of our strain correspond with those of *D. symbiontica* var. *pauciautosporica* Tschermak-Woess (1984). This variety was characterized by the scarce production of autospores and by autosporangia with dimensions of 6–13 μm. However, we found autospores quite frequently in our strain and the autosporangia size varied from 12 to 20 μm in diameter. We decided not to assign our strain to a subspecific taxon in order to avoid confusion and because we believed that we observed only a small part of the overall variability of the species. The chloroplast morphology and ontogeny differs evidently between the three investigated species. However, chloroplast ontogeny of all strains comprises some morphologically identical stages: a single parietal layer of tubular interconnected chloroplast lobes (Figs 10, 37, 56); a two-layered chloroplast composed of a net of tubular lobes (Figs 13–16, 45, 60, 61); the 'granular' chloroplast stage of multilayered tubular lobes with grouped thylakoids (Figs 22, 23, 60, 61); and the stage of homogenous chloroplast mass with granular structure (Figs 24, 47, 65). The specific differences consist mainly in the different timing of the particular stage in the chloroplast ontogeny. In *D. splendida*, the individual stages are clearly established and evenly represented during the chloroplast ontogeny, whereas in *D. reticulata* the unilayered stage predominates during the life cycle. In the latter species further modifications of the chloroplast occur just a short time before the

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Fig. 37. Unilayered chloroplast of young cells.
Fig. 38. Unilayered chloroplast with isolated lobe, spreading into the cell lumen.
Fig. 39. Chloroplast surface with numerous perforations.
Fig. 40. Secondary chloroplast layer created in the half of cell.
Figs 41–43. Unilayered perforated chloroplast of young and vegetative cells.
Fig. 44. Two-layered chloroplast of mature cells before cell division.
Figs 45, 46. Multilayered chloroplast of interconnected chloroplast lobes.
Fig. 47. Granular chloroplast of mature cells before the autospores production.

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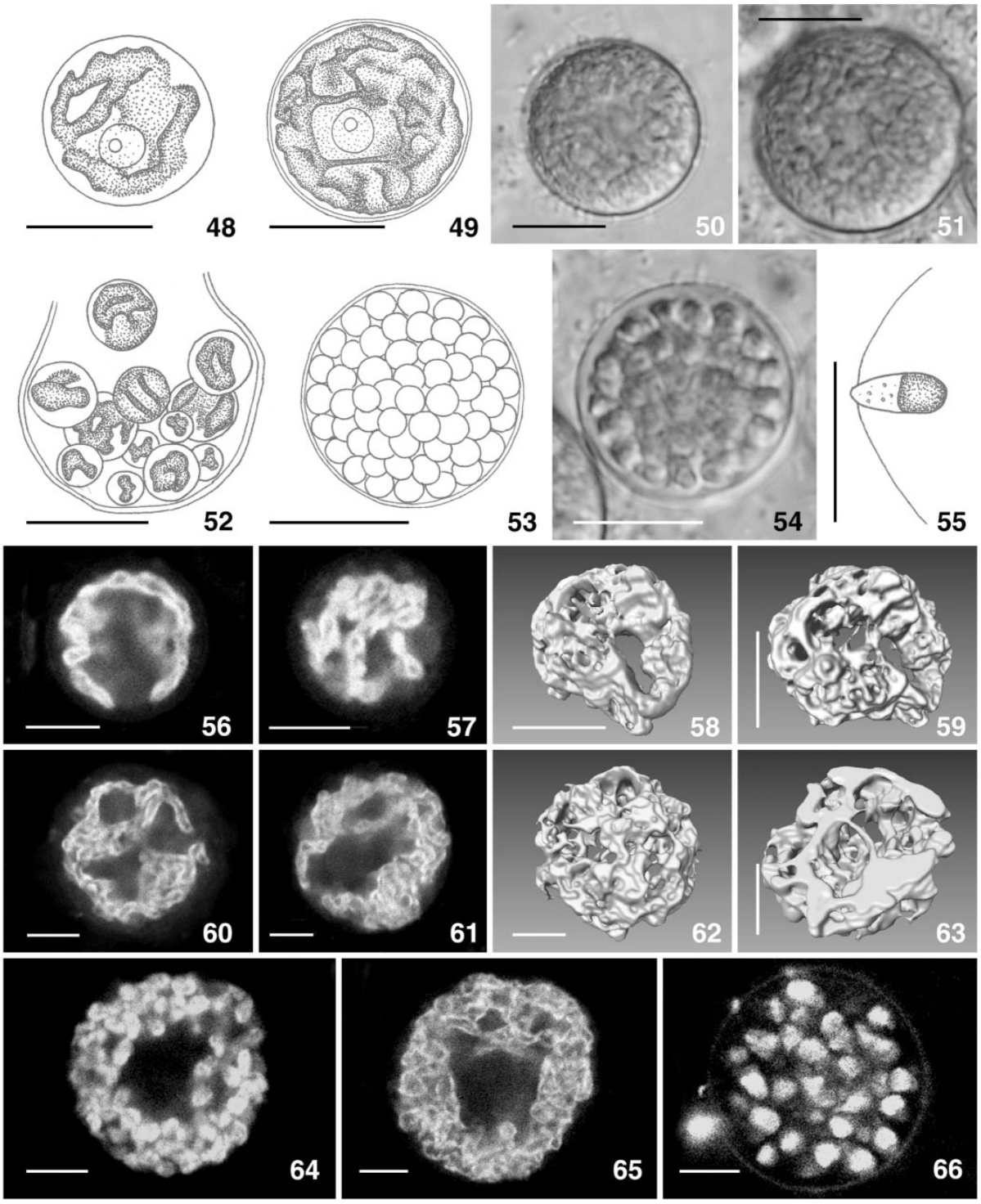

Figs 48–66. *Dictyochloropsis symbiontica*. Scale bars = 10 μm.
Fig. 48. Young cell with single chloroplast layer.
Figs 49–51. Vegetative cells with multilayered chloroplast and distinct nucleus.
Fig. 52. Open sporangium with unequal autospores.
Figs 53, 54. Globular aplanosporangia with a large number of aplanospores.

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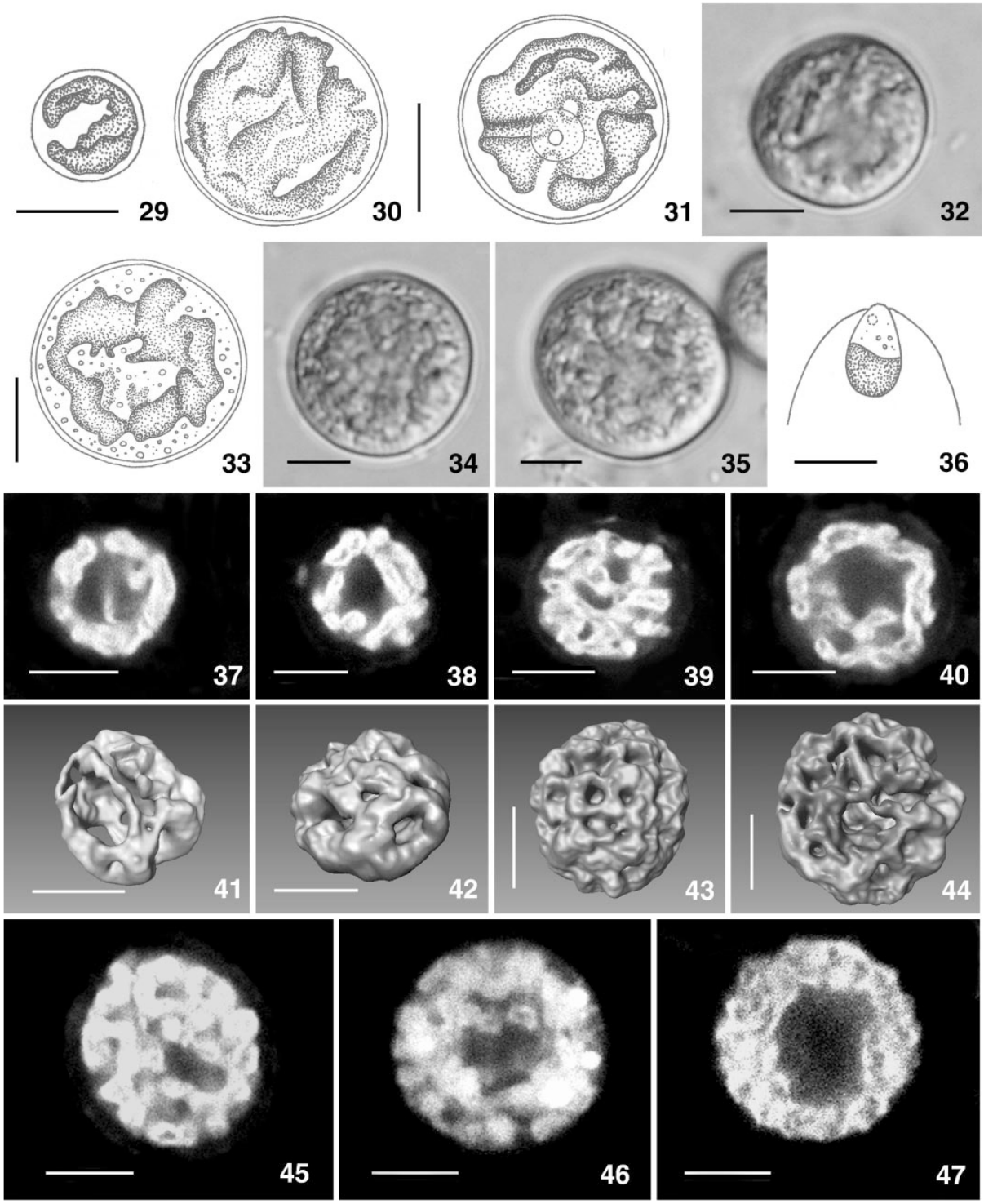

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***Dictyochloropsis symbiontica* Tschermak-Woess**

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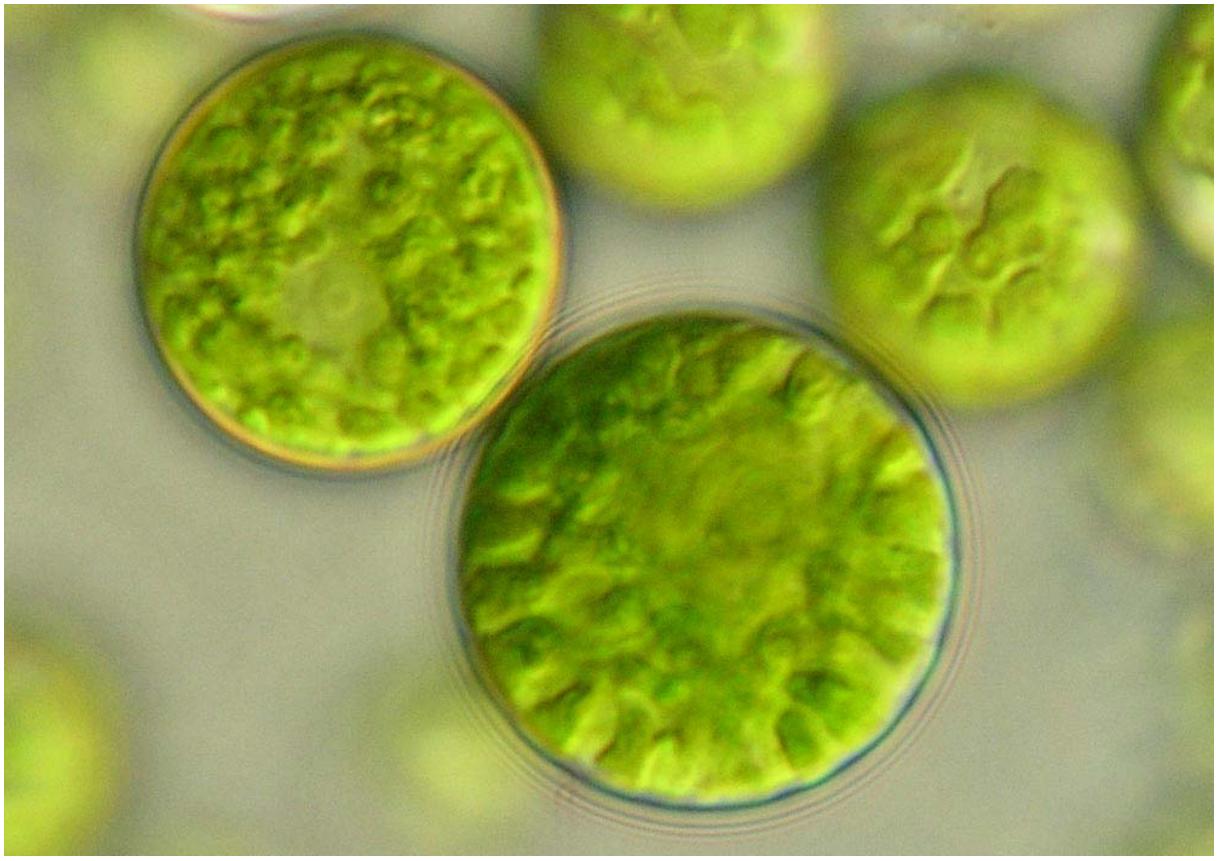
Fig. 47. Granular chloroplast of mature cells before the autospores production.

Paper 5

Morphology, molecular phylogeny and taxonomy of green algal genera *Aerosphaera* and *Dictyochloropsis* (Trebouxiophyceae, Chlorophyta) with description of four new species

Pavel Škaloud, Thomas Friedl & Jiří Neustupa

Manuscript



Complicated chloroplast structure of newly described *Dictyochloropsis asterochloroides*

Morphology, molecular phylogeny and taxonomy of green algal genera *Aerosphaera* and *Dictyochloropsis* (Trebouxiophyceae, Chlorophyta) with description of four new species

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ABSTRACT

Coccal green algae having complicated, three-dimensional, reticulate chloroplast without pyrenoid are traditionally classified into the genus *Dictyochloropsis* Geitler. In this study, the taxonomy of the genus was examined using comparative conventional light and confocal microscopy and 18S rDNA sequence analyses for 21 strains originating from geographically widely distributed locations. The phylogenetic analyses revealed two distinct lineages within the Trebouxiophyceae. Based on detailed morphological investigation and comparing with literature data, we have assigned these lineages to two different genera, *Dictyochloropsis* and *Aerosphaera* Gerneck. We have reconsidered the diacritic generic features as follows. *Dictyochloropsis* comprises algae with a reticulate chloroplast, forming distinct parallelly-arranged lobes at some ontogenetic stages, and which reproduce only by means of autospores. This is in congruence with Geitler's original definition of *Dictyochloropsis*, but in contrast to the later emendation of the genus by Tschermak-Woess. Consequently, the species of *Dictyochloropsis* sensu Tschermak-Woess are assigned to *Aerosphaera*, with new combinations proposed. *Aerosphaera* encompasses algae with evenly perforated chloroplast that can reproduce also by the formation of zoospores with typical separate insertion of the flagella. One new species of *Dictyochloropsis* (*D. asterochloroides*) and three new species of *Aerosphaera* (*A. handae*, *A. tropica* and *A. tschermakiae*) are described based on congruencies found between morphological features and rDNA sequence analyses.

INTRODUCTION

The coccoid green algal genus *Aerosphaera* was established by Gerneck (1907). He isolated species *Aerosphaera faginea* Gerneck from a moist trunk of a beech tree and studied its development in culture. The genus was characterized by large uninucleate cells, with single, complicated, net-forming chloroplast without pyrenoid. The alga reproduced only by means of autospores, no zoospores were observed. Soon afterwards, Wille (1910) transferred species *A. faginea* into genus *Chlorella* Beijerinck, section *Aerosphaera* Wille. Although many authors agreed with such taxonomical position (Brunnthaler 1915, Bourrelly 1966, etc.), including of *Aerosphaera faginea* in morphologically different genus *Chlorella* is presently not accepted (Komárek & Fott 1983, Hanagata et al. 1998). Since its description, *Aerosphaera faginea* was found in any later study.

In the second half of the 20th century, Geitler (1966) described genus *Dictyochloropsis* Geitler, with a *D. splendida* Geitler as a type species of the genus. The ecology and morphology of this species, isolated from a moist wooden roof, was very similar to *Aerosphaera fagi-nea* (large uninucleate cells with reticulate chloroplast, reproduced only by means of autospores). Be aware of this similarity, Geitler differentiated *Dictyochloropsis* by starch and oil accumulation within the cells. Later, Tschermak-Woess (1978) found *Dictyochloropsis splendida* to be a phycobiont of lichen *Chaenotheca brunneola* (Acharius) Müller Argoviensis and added several other taxa isolated from lichen thalli (Tschermak-Woess 1980, 1984): *D. splendida* var. *gelatinosa* Tschermak-Woess, *D. symbiontica* Tschermak-Woess var. *symbiontica*, *D. symbiontica* var. *ellipsoidea* Tschermak-Woess, *D. symbiontica* var. *pauciautosporica* Tschermak-Woess and *D. reticulata* Tschermak-Woess. The individual species within the genus were distinguished mainly according to the chloroplast structure, cell size and type of asexual reproduction (Tschermak-Woess 1984). In all studied species, Tschermak-Woess (1978, 1980, 1984) observed production of characteristic naked zoospores, with separate insertion of flagella. Since Geitler (1966) had not observed zoospores in *Dictyochloropsis splendida*, she emended Geitler's description of *Dictyochloropsis* (Tschermak-Woess 1984) and defined zoospore shape and separate flagella insertions as a typical feature of genus.

The last described species, *Dictyochloropsis irregularis* Nakano & Isagi, was isolated from bark of *Picea jezoenzis* Carrière in Hokkaido, Japan (Nakano & Isagi 1987) and later isolated from similar habitat in Europe (Tschermak-Woess 2000). This species is well characterized by polymorphic cell shape. Recently, Škaloud et al. (2005) observed differences in the morphology and ontogeny of chloroplast structure in three isolates of *Dictyochloropsis*, using confocal fluorescent microscopy.

Our recent results of extended morphological and molecular investigations based on more than twenty unialgal strains led us to reconsider the taxonomic position of genera *Dictyochloropsis* and *Aerosphaera*.

MATERIAL AND METHODS

Twenty-one strains were obtained from the following algal culture collections: the Culture Collection of Algae at the University of Göttingen, Germany (SAG); the Culture Collection of Algae of Charles University in Prague (CAUP); the Culture Collection of Algae at the University of Texas at Austin, USA (UTEX) and the private culture collection of prof. Shinji Handa, Japan. All strains used in this study, with their origin description and strain numbers, are shown in Table 1. Observations of the algal isolates were made from cultures grown on 2% agar slants of Bold's Basal Medium (BBM) as modified by Bischoff and Bold (1963). All cultures were cultivated at a temperature of 15 °C, under an illumination of 5-15 μmol m^{-2} s^{-1}. Individual strains were regularly observed using a conventional light and a confocal microscope to reveal well whole morphological variability. Zoospore formation was induced by transferring the cultures to 10 °C and dark. The pure algal samples were examined by Olympus BX51 light microscope with differential and phase contrasts and Olympus Z5060 microphotographic equipment as well as by a laser scanning confocal microscope Leica TCS SP2 equipped with an Argon-Krypton laser. We used a 488 nm excitation line and an AOBS filter free system collecting emitted light between 498 and 700 nm. A Leica 63x/1.4 N.A. oil immersion objective fitted on the Leica DM IRE2 inverted microscope was used. A series of optical sections through chloroplasts were captured and used for 3-dimentional reconstruction of their morphology. The autofluorescence of the chlorophyll was exploited for visualization of the chloroplast structure. For the final processing of the confocal images and various chloroplast structure visualizations, Leica Confocal Software, version 2.61 (Leica Microsystems Heidelberg GmbH) and the ImageJ 1.34p Programme (Abramoff et al. 2004) were used.

DNA extraction, PCR, DNA sequencing and phylogenetic analyses were performed as described in Helms et al. (2003).

Species	Strain	Origin; *isolator*
Aerosphaera handae	CCHU 5616	Japan; *Takeshita*
Aerosphaera irregularis	SAG 2036	Austria, tree bark; *Tschermak-Woess*
Aerosphaera irregularis	Handa 906d	Japan, leaf; *Handa*
Aerosphaera pauciautosporica	SAG 12.86	Spain, lichen phycobiont; *Tschermak-Woess*
Aerosphaera pauciautosporica	UTEX 2599	USA, lichen phycobiont; *Tschermak-Woess*
Aerosphaera pauciautosporica	UTEX 2612	USA, lichen phycobiont; *Tschermak-Woess*
Aerosphaera reticulata	SAG 53.87	Spain, lichen phycobiont; *Tschermak-Woess*
Aerosphaera symbiontica	SAG 27.81	Austria, lichen phycobiont; *Tschermak-Woess*
Aerosphaera symbiontica	SAG 2070	Japan, lichen surface; *Handa*
Aerosphaera symbiontica	SAG 2099	Japan, lichen surface; *Takeshita*
Aerosphaera tropica	CAUP H8602	Malaysia, tree bark; *Neustupa*
Aerosphaera tropica	CAUP H9603	Malaysia, tree bark; *Neustupa*
Aerosphaera woessii	SAG 46.85	New Zealand, lichen phycobiont; *Tschermak-Woess*
Aerosphaera sp.	SAG 244.80	Austria, lichen phycobiont; *Tschermak-Woess*
Aerosphaera sp.	SAG 2069	Japan, rock; *Handa*
Dictyochloropsis asterochloroides	SAG 2073	Japan, rock; *Handa*
Dictyochloropsis asterochloroides	SAG 2098	Japan, concrete; *Handa*
Dictyochloropsis splendida	CAUP H8601	Czech Republic, soil; *Škaloud*
Dictyochloropsis splendida	SAG 2071	Japan, rock; *Handa*
Dictyochloropsis splendida	SAG 2097	Japan, rock; *Handa*
Dictyochloropsis splendida	Handa 939d	Japan, tombstone; *Ohmura*

Table 1. List of strains investigated in this study.

RESULTS

According to molecular analyses, all studied strains were significantly separated into two distinct clades. This division is clearly supported by morphology, especially by the chloroplast structure of adult cells and type of asexual reproduction. The first group comprehends the algae with reticulate chloroplast, forming in some ontogenetic stages distinct parallelly-arranged lobes, and reproduced only by means of autospores. The second clade encompasses the strains with evenly perforated chloroplast, reproduced by means of autospores, aplanospores and zoospores. Comparing with literary data, we have assigned these clades to *Dictyochloropsis* and *Aerosphaera*, respectively. Detailed explanation of our taxonomical conclusions is given in Discussion.

Morphology and taxonomic revisions

Dictyochloropsis Geitler 1966, Österr. Bot. Z. 133, p. 162.

Synonym: non *Dictyochloropsis* Geitler emend. Tschermak-Woess 1984, Pl. Syst. Evol. 147, p. 317.

Type species: *Dictyochloropsis splendida* Geitler

Diagnosis: Cells are usually spherical, uninucleate, with smooth, thin cell wall. Chloroplast is without pyrenoid, net-forming, and complicated in structure. In adult cells, chloroplast fills up whole cell lumen except for the centre, where the nucleus is present. It consists of many interconnected lamellae, which often form parallel-arranged structure at chloroplast periphery. Asexual reproduction takes place only by means of spherical autospores, no zoo-

spores and aplanospores are produced. Autosporangia spherical, in maturity they assume the shape of inlying autospores. The daughter cells are released by rupturing of sporangium cell wall.

Dictyochloropsis splendida Geitler 1966, Österr. Bot. Z. 133, pp. 162, 163.

Investigated strains: CAUP H8601, Handa 939d, SAG 2071, SAG 2097.

Diagnosis: Young cells have one parietal chloroplast, forming a single layer with numerous perforations below the plasma membrane. One nucleus with nucleolus is clearly visible in the centre of the cells (Fig. 1a). The chloroplasts of adult cells form a complicated three-dimensional net of interconnected lobes (Fig. 1b). In most adult cells the chloroplast lobes are evenly distributed within the cell volume and the surface of the chloroplast has the form of one compact mass with many perforations (Fig. 1c). In specific stage, the flat lobes are produced within the chloroplast, changing considerably the chloroplast appearance. These flat lobes are parallel-oriented and less interconnected as compared to the younger cells. The chloroplast surface is therefore formed by parallel, sometimes bifurcating lobes (Figs 1d-f). The production of these specific lobes was observed in all studied strains of this species, however with different frequency. Cell diameters vary in range of 6–40(–50) μm. The reproduction takes place by means of autospores (Fig. 1g), formed in autosporangia of globular shape. The autosporangia (having diameter of 45–48 mm) usually contain 8–16 autospores, of size about 8-15 μm in diameter. They are liberated by rupturing of mother cell wall (Fig. 1h). The chloroplast of autospores has the shape of simple spherical, perforated chloroplast below the plasma membrane.

Dictyochloropsis asterochloroides Škaloud, Friedl et Neustupa sp. nov.

Latin Diagnosis: *Cellulae solitariae, globosae vel ellipsoideae. Chloroplastus pyrenoide nullo, in cellulis junioribus centrails, stellatus, in statu adolescente irregulariter globose-excavatus, particulatim reticulatus, peripheriam versus vario modo lobatus, intus trabeculis vel ligamentis praeditus. Nucleus unus, in cellulis junioribus parietalis, vetustioribus plus minusve centralis. Cellulae usque ad 22, rarius 24 μm diametro metientes. Reproductio asexualis per 4 vel 8 autosporas divisionibus succedaneis formatis. Autosporae per rupturam membrane sporangii liberatae.*

Holotype: Lyophilized material deposited in the Culture Collection of algae of the Charles University in Prague, Czech Republic (CAUP) as specimen No. LYO-H8604 (*hic designatus*). The living cultures (ex-holotypes) have been deposited as SAG 2098 in the Culture collection of algae at the University of Göttingen, Germany and as CAUP H8604 in the Culture Collection of algae of the Charles University in Prague, Czech Republic.

Type locality: Concrete, Yokogawa, Hiroshima-City, Japan. Sample was collected by Shinji Handa in May 2004.

Etymology: The species epithet refers to the axial shape of chloroplast in young cells.

Investigated strains: SAG 2073, SAG 2098

Diagnosis: Young cells are spherical or slightly ellipsoidal, with parietal or central positioned asteroid chloroplast (Fig. 1i). Very indistinct nucleus with nucleolus is present parietal, near the cell wall. In some portion of adult cells, the chloroplast is still central, with a few thick (Fig. 1j) or many thinner lobes spreading out the cell periphery (Fig. 1k). In the rest of adult cells, the chloroplast become to be more complicated, consisting of many interconnected chloroplast lobes, evenly distributed inside the cell. In this stage, the nucleus comes to occupy more central position. As well as in previous species, the flat, parallel-oriented chloroplast lobes are often produced, changing the chloroplast appearance in external view (Figs 1l,m). These lobes start to be produced in both cells with asteroid and reticular chloroplast. The species is also characteristic by sporadic occurrence of thin tubular apertures inside the chloroplast, well visible by confocal microscope (Fig. 1n). These apertures probably originate dur-

ing the chloroplast rebuilding from asteroid to reticular stage. Cell diameters vary in range of 6–22(–24) μm. The reproduction takes place by means of 4-8 autospores, formed in globular autosporangia (Fig. 1o). Mature autospores are globular or triangular, with diameter of about 6 μm. They are liberated by rupturing of mother cell wall (Fig. 1p). One daughter cell often remains inside the mother cell wall. The chloroplast of autospores is parietal, with a few perforations.

Aerosphaera Gerneck 1907, Beih. Bot. Centralbl. 21, pp. 251-253 emend. Škaloud, Friedl et Neustupa

Type species: *Aerosphaera faginea* Gerneck

Synonyms: *Dictyochloropsis* Geitler emend. Tschermak-Woess 1984, Pl. Syst. Evol. 147, p. 317, non *Dictyochloropsis* Geitler 1966, Osterr. Bot. Z. 133, p. 162.

Latin diagnosis: *Cellulae solitariae, globosae vel ellipsoideae. Paries tenuis, firmus, interdum. Chloroplastus pyrenoide nullo, in statu adolescente globoso-excavatus, reticulatus, trabeculis nonnisi peripheric is aut peripheric is et internis praeditus. Nucleus unus, plus minusve centralis. Propagatio aliquarum specierum zoo-, aplano- et autosporis, alternarum specierum tantum zoo- et aplanosporis. In sporangiis (16)32 vel 64(128) zoo- aut aplanosporae aut 4 vel 16(32) autosporae formatae. Zoosporae saepe parum complanatae, cum flagelis remote insertis, vacuola contractili una, sine stigmate.*

Diagnosis: Cells are spherical, ellipsoidal or irregularly shaped, with smooth, thin cell wall. One nucleus with a prominent nucleolus occupies the central portion of the cell. The chloroplast varies in shape with the age of the cell. In young cells, the chloroplast forms one peripheral layer of inter-connected lobes. Later, in more mature stages, the chloroplast can develop to the multilayered network of interconnected lobes, filling up the cell lumen. Pyrenoids are absent. Contrary to *Aerosphaera*, the lobes never form parallel-arranged structure, so the chloroplast has a regular, net-like appearance in the surface view. Asexual reproduction occurs by means of zoospores and two types of immobile daughter cells – aplanospores and autospores. Aplanospores occurs in all species. Young aplanosporangia are characterized by indistinct cell content, starch accumulation and yellowish or pale green colour. Mature aplanosporangia are spherical or ellipsoidal, containing high number (up to 128 cells) of spherical daughter cells. The aplanospores are mainly released by rupturing of sporangial cell wall, although in some species aplanosporangial cell wall rather dissolves. The chloroplast of aplanospores is cup-shaped, occupied about half of the periphery of the wall. Contrary to aplanospores, autospores are not produced in all studied species. Young autosporangia are much smaller than aplanosporangia; they are characterized by distinct, angular cell walls of new produced cells, no starch accumulation and clear green colour. Mature autosporangia assume the shape of inlying autospores. The shape of mature autospores is deformed by close contact of daughter cells inside the sporangium, so they are not strictly spherical when released. Commonly, 4-8-16 autospores are produced within the sporangium. The autospores are released either by rupturing or dissolving of sporangial cell wall. In the fact, autospores are always greater than aplanospores and possess more developed reticular chloroplast, similar to the single young cells.

The zoospores are produced in spherical or ellipsoidal zoosporangia, having the same characteristics as above-mentioned aplanosporangia. In early stages of zoosporangia development, the inner cell thickening appears for some time in one side of the cell. The zoospores are released by sporangial cell wall rupture, developed in the position of former thickening. All zoospores are simultaneously flow out from the sporangium, enveloped by shared mucus. During several seconds, the zoospores are liberated from the mucus and swim freely. The biflagellated zoospores of *Dictyochloropsis* are characterized by separate insertion of flagella. They are ellipsoidal or cylindrical, with one parietal chloroplast without an observable stigma. The zoospores lack cell walls, so when ceasing to move, they rapidly become spherical.

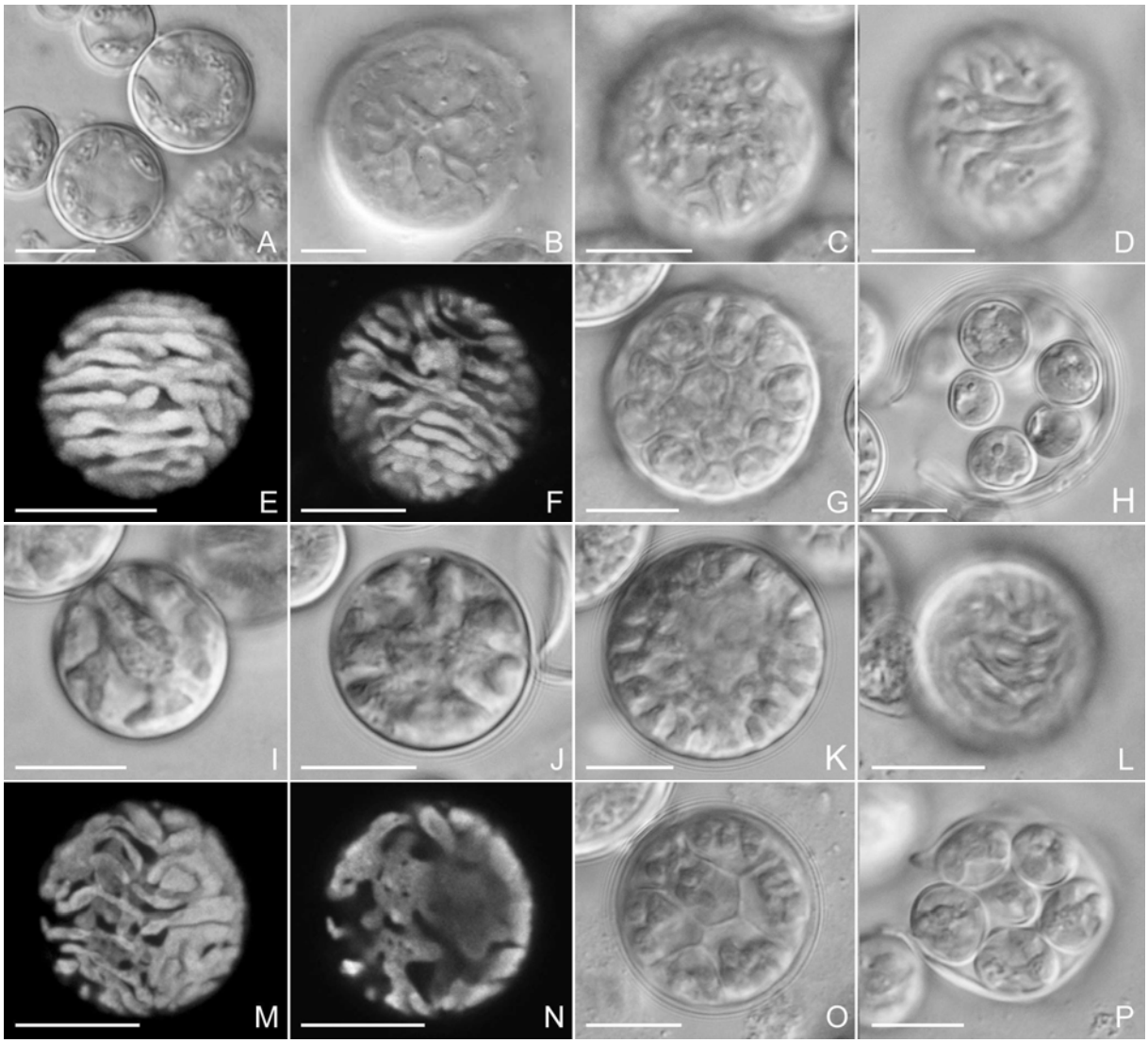

Fig. 1. Micrographs of *Dictyochloropsis*. (A-H) *Dictyochloropsis splendida*. (A) Young cells. (B) Mature cell in optical section. (C) Mature cell, surface view. (D) Chloroplast surface consisting of many parallel-arranged lobes. (E-F) Chloroplast structure of mature cell, maximum projection of multiple serial confocal sections. (G) Young autosporangium. (H) Opened mature autosporangium. (I-P) *Dictyochloropsis asterochloroides*. (I) Young cell with asteroid chloroplast. (J) Mature cell with a few thick chloroplast lobes. (K) Mature cell with many fine chloroplast lobes. (L) Parallel chloroplast lobes, surface view. (M) Chloroplast structure of mature cell, maximum projection of multiple serial confocal sections. (N) Chloroplast section showing thin tubular apertures, single median confocal section. (O) Young autosporangium. (P) Opened mature autosporangium. Scale bar = 10 μm.

Aerosphaera symbiontica (Tschermak-Woess) Škaloud, Friedl et Neustupa comb. nov.

Basionym: *Dictyochloropsis symbiontica* Tschermak-Woess 1980, Pl. Syst. Evol. 136, pp. 304-305, Figs 5-7.

Investigated strains: SAG 27.81 (authentic strain), SAG 2070, SAG 2099

Emended diagnosis: Cells are spherical or slightly ellipsoidal. In young and many adult cells, the chloroplast spreads along the whole inner surface of the cell wall as a single layer with many perforations. In the cross-section and surface view, this unilayered chloroplast is very characteristic by granulate structure, suggestive many small tightly-apressed spherical chloroplasts (Figs 2a, b). Many small vacuoles are often situated between chloroplast and central nucleus. In adult cells with diameter more than 12 μm, the chloroplast can develop into

multilayered stage of many interconnected lobes (Figs 2c, d). The diameters of vegetative cells are in the range of 4.5-16(-18) μm. The asexual reproduction takes place mainly by means of autospores (Fig. 2e). Relatively small autosporangia (up to 11 μm in diameter) contains 4, 8 or 16 polygonal, later spherical autospores. Mature autospores are released either by dissolving or rupturing of sporangial cell wall. When dissolving, the autospores remain attached each other and form cell packets (Fig. 2f). If the cell wall ruptures, the autospores are not released from sporangium, but remains attached to the mother cell wall. Even in the deeply opened sporangium, the autospores still remained adhered to the sporangial cell wall, forming characteristic chains of cells (Fig 2g). Rarely, the cells form colony-like packets, adhering each to other by sporangium cell wall remnants, located in the middle of the colony (Fig. 2h). The remnants of sporangial cell wall are often present on the surface of mature autospores and vegetative cells, resembling the scales (Fig. 2i).

Very rarely, globular zoosporangia and aplanosporangia appear in the culture (Fig. 2j). Usually, 32 zoospores and 16 or 32 aplanospores are produced within the sporangium (Fig. 2k). Mature aplanospores are released by the rupturing of sporangia, so the remnants of sporangial cell wall remain in the culture.

Aerosphaera tschermakiae Škaloud, Friedl et Neustupa sp. nov.

Latin Diagnosis: *Cellulae solitariae, globosae vel ellipsoideae, rarius pyriformes. Chloroplastus pyrenoide nullo, globoso-excavatus, reticulatus, trabeculis externis et internis praeditus. Nucleus unus, in statu adolescente plus minusve centralis. Cellulae vegetativae ad 16, rarius 19 μm diametro metientes. Reproductio asexualis per 4, 8 vel 16 autosporas, rarius per 32 aplanosporas divisionibus succedaneis formatis. Aplanosporae per rupturam, autosporae per rupturam vel solvoram membrane sporangii liberatae.*

Holotype: Lyophilized material deposited in the Culture Collection of algae of the Charles University in Prague, Czech Republic (CAUP) as specimen No. LYO-H8605 (*hic designatus*). The living cultures (ex-holotypes) have been deposited as SAG 46.85 in the Culture collection of algae at the University of Göttingen, Germany and as CAUP H8605 in the Culture Collection of algae of the Charles University in Prague, Czech Republic.

Type locality: Phycobiont of lichen *Pseudocyphellaria aurata*, North Auckland, North Island, New Zealand. Sample was collected by Elisabeth Tschermak-Woess in 1981.

Etymology: The species epithet is in honour of Dr. Elisabeth Tschermak-Woess, who has contributed much to our knowledge of *Dictyochloropsis*.

Investigated strain: SAG 46.85

Diagnosis: Cells are mainly spherical; slightly ellipsoidal or pyriform cells are produced only sporadically in the culture. Chloroplast of young cells forms a single reticular layer below plasma membrane (Fig. 2l). In adult cells, chloroplast develops into multilayered stage, formed by many fine interconnected chloroplast lobes (Fig. 2m). In early stages of chloroplast development, new lobes are produced in one side of the cell and central nucleus slightly moves contrariwise (Fig. 2n). Although the most of adult cells contains multilayered chloroplast filling up cell lumen, chloroplast of some adult cells can be unilayered and the high portion of cell lumen is filled up by cytoplasm and nucleus (Fig. 2o). In surface view, the chloroplast is evenly perforated by rather spherical distinct pores (Fig. 2p).

The diameters of vegetative cells vary in the range of 5-16(-19) μm. The asexual reproduction takes place by means of autospores, aplanospores and zoospores. Young autosporangia are well distinguished by many obvious cell walls, formed among young autospores (Fig. 2q). Mature sporangia grow up to 13 μm in diameter and contain 4, 8 or 16 autospores (Fig. 2r). Autospores are released by dissolving or disturbing of sporangial cell wall. They often remain attached each other forming cell packets, though the mother cell wall disappears. Sometimes, very thin remnants of sporangial cell walls can be found in the culture, rarely

containing one autospore. The globular aplanosporangia reach the diameters of 16-18 μm. When young, they are characteristic by production of inner thickening of the cell wall (Fig. 2s). The aplanosporangia usually contain 32 spores (Fig. 2t), released by the rupturing of sporangial cell wall. Despite extensive investigation, the production of zoospores was not observed.

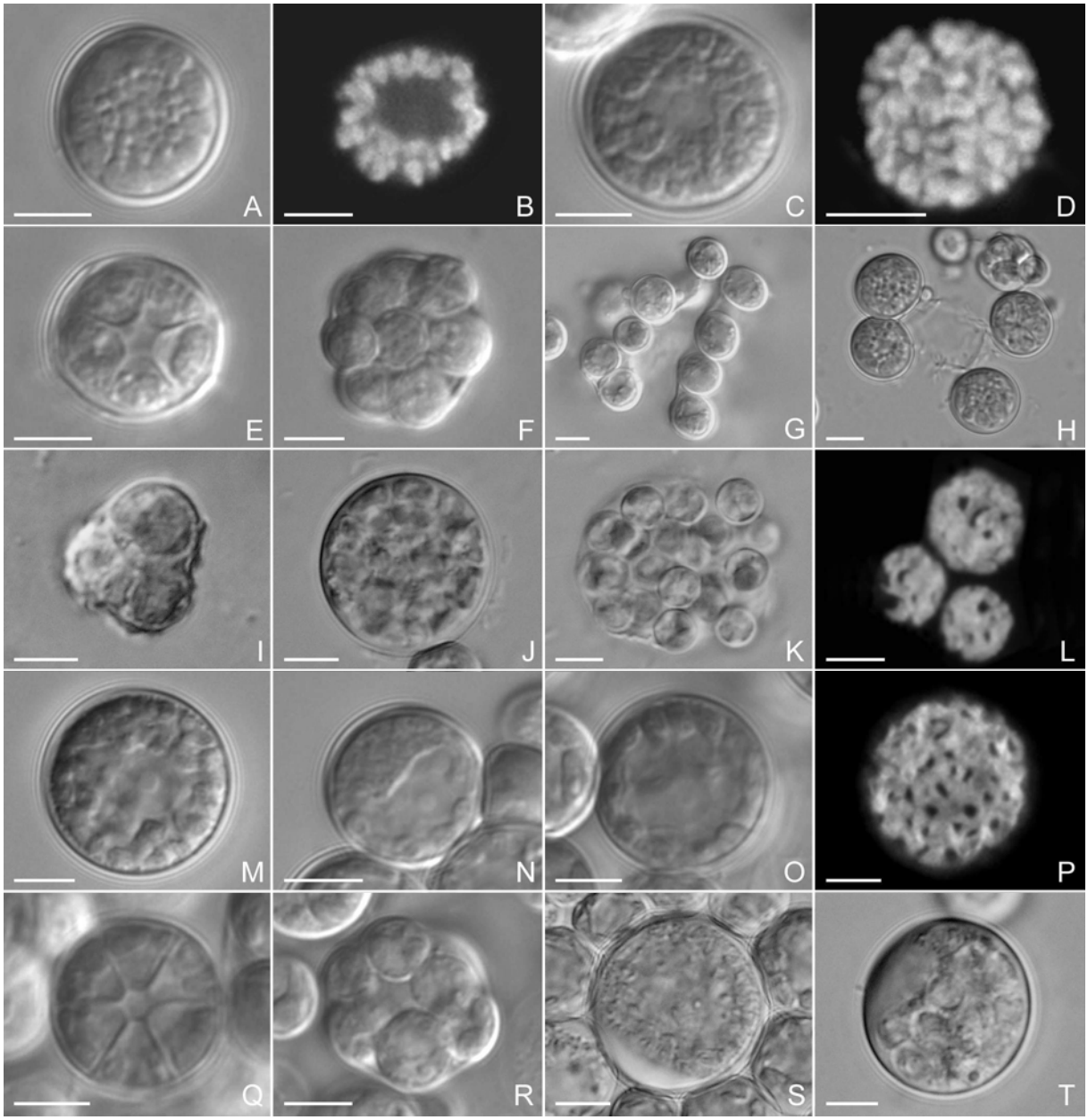

Fig. 2. Micrographs of *Aerosphaera*. (A-K) *Aerosphaera symbiontica*. (A) Young cell. (B) Young cell, single median confocal section. (C) Mature cell. (D) Chloroplast structure of mature cell, maximum projection of multiple serial confocal sections. (E) Young autosporangium. (F) Mature autosporangium. (G) Chain of autospores adhered to the mother cell wall. (H) Packet of four cells adhered each to other by mother sporangium cell wall remnants. (I) Mature autospores with scales stuck on the cell surfaces. (J). Young aplanosporangium. (K) Opened mature aplanosporangium. (L-T) *Aerosphaera tschermakiae*. (L) Chloroplast structure of young cells, maximum projection of multiple serial confocal sections. (M) Mature cell with multilayered chloroplast. (N) Young cell with new chloroplast lobe production. (O) Mature cell with unilayered chloroplast. (P) Chloroplast structure of mature cell, maximum projection of multiple serial confocal sections. (Q) Young autosporangium. (R) Mature autosporangium. (S) Young aplanosporangium showing inner thickening of the cell wall. (P) Mature autosporangium. Scale bar = 5 μm.

Aerosphaera reticulata (Tschermak-Woess) Škaloud, Friedl et Neustupa comb. nov.

Basionym: *Myrmecia reticulata* Tschermak-Woess 1969, Österr. Bot. Z. 98, pp. 412-419.

Synonym: *Dictyochloropsis reticulata* (Tschermak-Woess) Tschermak-Woess 1984, Pl. Syst. Evol. 147, p. 317.

Investigated strain: SAG 53.87

Emended diagnosis: Cells are spherical or slightly ellipsoidal. In young and adult cells, the chloroplast spreads along the whole inner surface of the cell wall as a single layer with many perforations. As in *D. symbiontica*, chloroplast has a granulate structure in the cross-section, suggestive many small tightly-apressed spherical chloroplasts (Fig. 3a). Many vacuoles often occurred between chloroplast and nucleus. Although sporadic chloroplast lobes can be produced toward cell lumen, the chloroplast is strictly unilayered during whole its ontogeny (Fig. 3b). In surface view, the chloroplast is evenly perforated by large distinct pores (Fig. 3c). The diameters of vegetative cells are in the range of 4-13(-15) μm. The asexual reproduction takes place by means of autospores, aplanospores and zoospores. Autosporangia (up to 11 μm in diameter) contains 4, 8, seldom 16 autospores (Fig. 3d). Mature autospores are released either by dissolving or rupturing of sporangial cell wall. If the sporangial wall ruptures, one autospore often remains inside the mother cell wall (Fig. 3e). Rarely, the globular aplanosporangia are produced, measuring about 12-13 μm in diameter (Figs 3f, g). Usually, 16 or 32 aplanospores are produced within the sporangium. Mature aplanospores are mainly released by the rupturing of sporangial cell wall. Despite extensive investigation, the production of zoospores was not observed.

Aerosphaera irregularis (Nakano et Isagi) comb. nov.

Basionym: *Dictyochloropsis irregularis* Nakano et Isagi 1987, Phycologia 26, p. 224, Figs 1-16.

Investigated strains: SAG 2036, Handa 906d

Emended diagnosis: The very young cells are mainly spherical, containing one parietal chloroplast. However, the adult cells are very characteristic by their variable shape. The mature cells are mainly ellipsoidal, ovoid, pyriform, reniform or irregularly oblong, although small portion of adult cells remains spherical. Chloroplast of young and some adult cells forms a single reticular layer below plasma membrane (Fig. 3h). During the cell ontogeny, new chloroplast lobes are produced further into the cell lumen. These lobes are primarily formed in the cell poles of irregular cells, as the nucleus is situated in the cell centre. Finally, the chloroplast of mature cells develops into multilayered stage, formed by many interconnected lobes (Fig. 3i). In surface view, the chloroplast is evenly perforated by rather spherical distinct pores (Fig. 3j). The diameters of young or adult spherical cells vary in the range of 3.5-14 μm. The irregular cells reach a maximum of 25 μm in length and 18 μm in width. The asexual reproduction takes place by means of aplanospores and zoospores. No autospores were observed. Mature aplanosporangia of pyriform or reniform shape contain 32, 64 or 128 spherical aplanospores with cup-shaped parietal chloroplast, measuring 2.5-4 μm in diameter. The zoosporangia usually enclose 64 or 128 zoospores. The sporangia are characteristic by production of inner thickening of the cell wall, located at the broader pole of the cell (Fig. 3k). Mature aplanospores and zoospores are released by the rupturing of sporangia (Fig. 3l) so the remnants of sporangial cell wall remain in the culture.

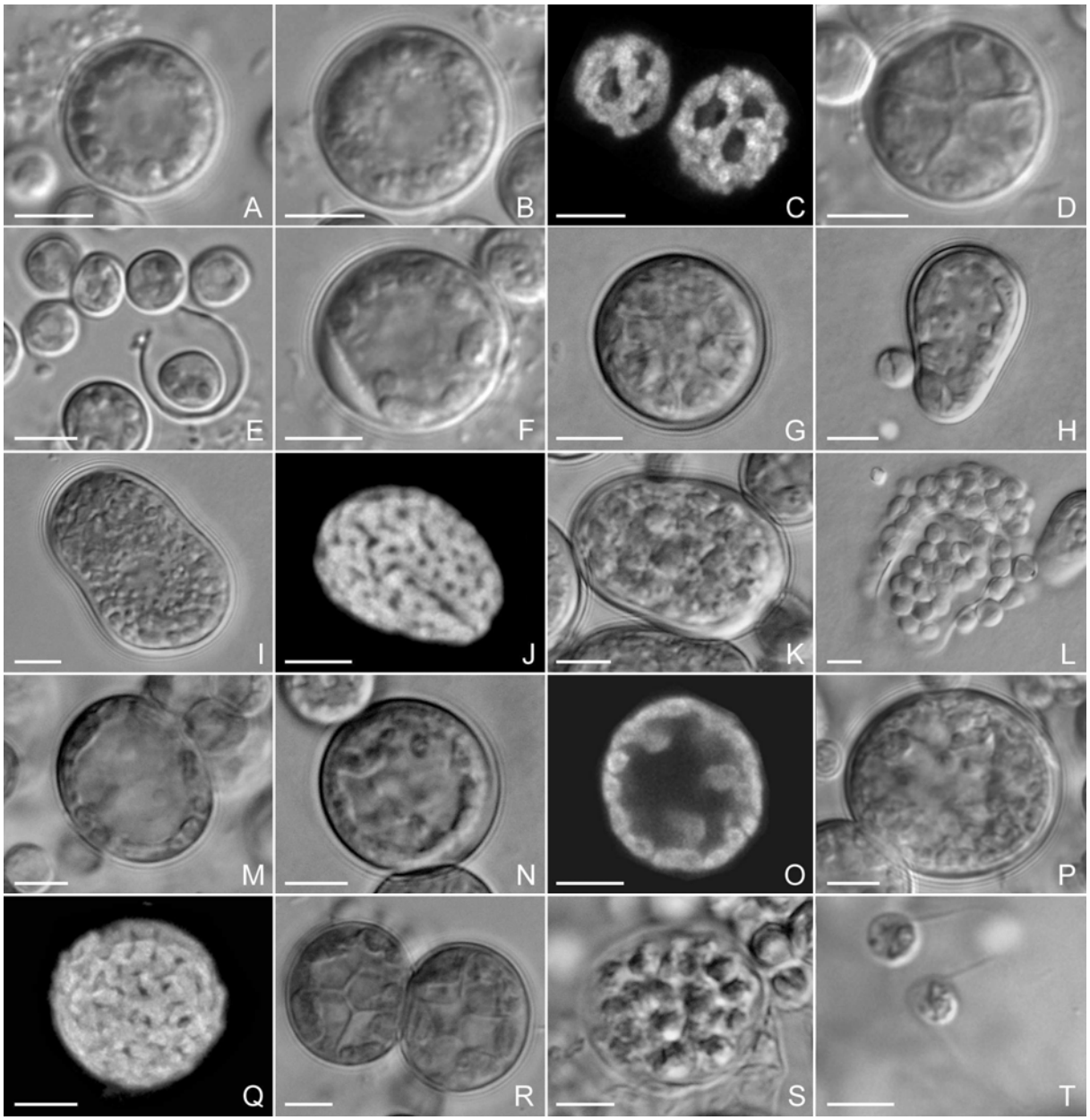

Fig. 3. Micrographs of *Aerosphaera*. (A-G) *Aerosphaera reticulata*. (A) Young cell. (B) Mature cell. (C) Chloroplast structure of mature cells, maximum projection of multiple serial confocal sections. (D) Young autosporangium. (E) Mature opened autosporangium with one arrested autospore. (F) Young aplanosporangium showing inner thickening of the cell wall. (G). Mature aplanosporangium. (H-L) *Aerosphaera irregularis*. (H) Young cell. (I) Mature cell with multilayered chloroplast. (J) Chloroplast structure of mature cell, maximum projection of multiple serial confocal sections. (K) Young aplanosporangium showing inner thickening of the cell wall. (L) Opened mature aplanosporangium. (M-T) *Aerosphaera handae*. (M) Young cell with unilayered chloroplast. (N) Mature cell with a few solitary lobes spreading the cell centre. (O) Chloroplast section showing solitary lobes spreading the cell centre, single median confocal section. (P) Mature cell with multilayered chloroplast. (Q) Chloroplast structure of mature cell, maximum projection of multiple serial confocal sections. (R) Young autosporangium. (S) Mature aplanosporangium. (T) Zoospores with separate flagella insertions. Scale bar = 5 μm.

Aerosphaera handae Škaloud, Friedl et Neustupa sp. nov.

Latin diagnosis: *Cellulae solitariae, globosae vel ellipsoideae. Chloroplastus pyrenoide nullo, in cellulis junioribus globoso-excavatus, vetustioribus reticulatus, trabeculis externis et internis praeditus. Nucleus unus, in statu adolescente plus minusve centralis. Cellulae vegetativae ad 26, rarius 30 μm diametro metientes. Reproductio asexualis per zoo-, aplano- et autosporas. In sporangiis 8, 16 vel 32 autosporae aut 64 vel 128 aplanosporiae aut zoosporae formatae. Autosporae per diluam, aplanosporae per diluam et rupturam membrane sporangii liberatae. Zoosporae ellipsoidae, duo flagella longitudinis aequae praebentes, cum flagellis remote insertis, sine stigmate.*

Holotype: Lyophilized material deposited in the Culture Collection of algae of the Charles University in Prague, Czech Republic (CAUP) as specimen No. LYO-H8606 (*hic designatus*). The living cultures (ex-holotypes) have been deposited as SAG 2150 in the Culture collection of algae at the University of Göttingen, Germany and as CAUP H8606 in the Culture Collection of algae of the Charles University in Prague, Czech Republic.

Type locality: Japan, Hiroshima?

Etymology: The species epithet is in honour of Dr. Shinji Handa, who has collected the most of the strains, investigated in this study.

Investigated strain: SAG 2150

Diagnosis: Cells are mainly spherical or ellipsoidal. Sometimes longer cylindrical cells can appear in the culture. Chloroplast of young cells forms a single reticular layer below plasma membrane. In many adult cells, the chloroplast is still unilayered, without any subsequent chloroplast lobes spreading towards the cell centre. Therefore, high portion of the cell lumen fills up the cytoplasm and one distinct nucleus (Fig. 3m). During the cell ontogeny, several new chloroplast lobes can arise from the parietal chloroplast layer. These lobes are oriented perpendicularly to the original layer, so in the light microscopic observation, a few solitary lobes spreading the cell centre are well visible in the cross section (Figs 3n, o). Depending to the culture conditions, all adult cells can posses only unilayered chloroplast with above-mentioned perpendicular lobes or in some of them the chloroplast can develop into the multilayered stage. Multilayered chloroplast is then formed by many interconnected lobes, reaching the nucleus in the middle of the cell (Fig. 3p). However, multilayered chloroplast of *D. handae* can be distinguished from other species of genus by having the net of interconnected lobes more sparse (compare Fig. 3p with Fig. 4c and Fig. 4j). Moreover, in surface view, the chloroplast is perforated by small linear fissures (Fig. 3q), rather than by distinct spherical pores. Cells of *Dictyochloropsis handae* reach one of the biggest dimensions among all studied strains. The diameters of vegetative cells vary in the range of 3.5-26(-30) μm. In very old culture, a few cells having diameters slightly over 30 μm were observed. The asexual reproduction takes place by means of autospores, aplanospores and zoospores. The autosporangia are mainly ellipsoidal, although spherical sporangia were sometimes observed. Both are well distinguished by many obvious cell walls formed among young autospores (Fig. 3r). Mature sporangia grow up to 22 μm in diameter and contain 8, 16 or 32 autospores, released by dissolving of sporangial cell wall. Mature autospores and vegetative cells often remain attached each other forming very large cell packets. Even the new autosporangia are formed inside the cell packets.

Zoospores and aplanospores are formed in globular or slightly ellipsoidal sporangia, reaching the diameters of 22 μm (Fig. 3s). Usually, 64 or 128 aplanospores and 64 zoospores are produced within the sporangium. Characteristic inner thickening of the cell wall is produced during young stage of sporogenesis. The zoospores are released by rupturing of sporangial cell wall, whereas the aplanosporangial cell wall first ruptures and then it is dissolved. The zoospore production is quite common. The zoospores are ellipsoidal to cylindrical, with-

out stigma, 5-8 μm in length and 3 μm in width. They have rounded anterior end with separate insertion of flagella (Fig. 3t).

Aerosphaera pauciautosporica (Tschermak-Woess) Škaloud, Friedl et Neustupa comb. nov.

Basionym: *Dictyochloropsis symbiontica* var. *pauciautosporica* Tschermak-Woess 1984, Pl. Syst. Evol. 147, p. 317, Figs 6, 7.

Investigated strains: SAG 12.86 (authentic strain), UTEX 2599, UTEX 2612

Emended diagnosis: Cells are spherical or slightly ellipsoidal. In young cells, the chloroplast spreads along the whole inner surface of the cell wall as a single layer with many perforations (Fig. 4a). In some adult cells, the chloroplast is also unilayered, without any chloroplast lobes spreading towards the cell centre. In these cells, high portion of the cell lumen fills up the cytoplasm and one distinct nucleus (Fig. 4b). However, in many adult cells, the complicated multilayered chloroplast is formed, reaching the nucleus in the middle of the cell (Figs 4c, d). The chloroplast lobes of *D. pauciautosporica* seem to be a little broader (about 2 μm) contrary to another species of the genus. In surface view, the chloroplast is evenly perforated by rather spherical distinct pores (Fig. 4e). Cells reach one of the biggest dimensions among all studied strains. The diameters of vegetative cells vary in the range of 3.5-26(-30) μm. The asexual reproduction takes place by means of aplanospores and zoospores. No autospores were observed. Dimensions of mature spherical or slightly ellipsoidal sporangia vary between 16-25 μm in diameter. Usually, 64 zoospores and 64 or 128 aplanospores are produced within the sporangium (Fig. 4f). Mature aplanospores and zoospores are released by the rupturing of sporangia (Fig. 4g) so the remnants of sporangial cell wall remain in the culture. The zoospores are produced only sporadically.

Aerosphaera tropica Škaloud, Friedl et Neustupa sp. nov.

Latin diagnosis: *Cellulae solitariae, globosae vel ellipsoideae. Chloroplastus pyrenoide nullo, globoso-excavatus, reticulatus, trabeculis externis et internis praeditus. Nucleus unus, in statu adolescente plus minusve centralis. Cellulae vegetativae ad 24, rarius 26 μm diametro metientes. Reproductio asexualis per 32 vel 64 aplanosporae atque zoosporae formatis, sine autosporis. Zoosporae et aplanosporae per rupturam membrane sporangii liberatae. Zoosporae ellipsoidae, duo flagella longitudinis aequae praebentes, cum flagellis remote insertis, sine stigmate.*

Holotype: Lyophilized material deposited in the Culture Collection of algae of the Charles University in Prague, Czech Republic (CAUP) as specimen No. LYO-H8604 (*hic designatus*). The living cultures (ex-holotypes) have been deposited as CAUP H8602 in the Culture Collection of algae of the Charles University in Prague, Czech Republic.

Type locality: Bark sample, unidentified tree in the secondary lowland rain forest, Hulu Kelantan, Malaysia. Sample was collected by Jiří Neustupa in 2000.

Etymology: The species epithet refers to the tropical origin of both isolated strains.

Investigated strains: CAUP H8602, CAUP H8603

Diagnosis: Cells are mainly spherical or ellipsoidal. Rarely, broad cylindrical or pyriform cells can appear in old culture (Fig. 4h). In young cells, the chloroplast exhibited a layer of peripheral interconnected lobes. However, in early ontogenetic stages the isolated lobes expand into the central cell lumen, evolving the complicated multilayered chloroplast reaching the nucleus (Figs 4i-k). Since the multilayered chloroplast is established so soon, no cells with high portion of the cell lumen filled by the cytoplasm occur (as in *D. pauciautosporica* or *D. handae*). The multilayered chloroplast of *D. tropica* has a unique structure, in comparison to all other aplanosporogenic species. The chloroplast does not posses a granulate structure (suggesting many small tightly-apressed spherical chloroplasts), but it is formed by many

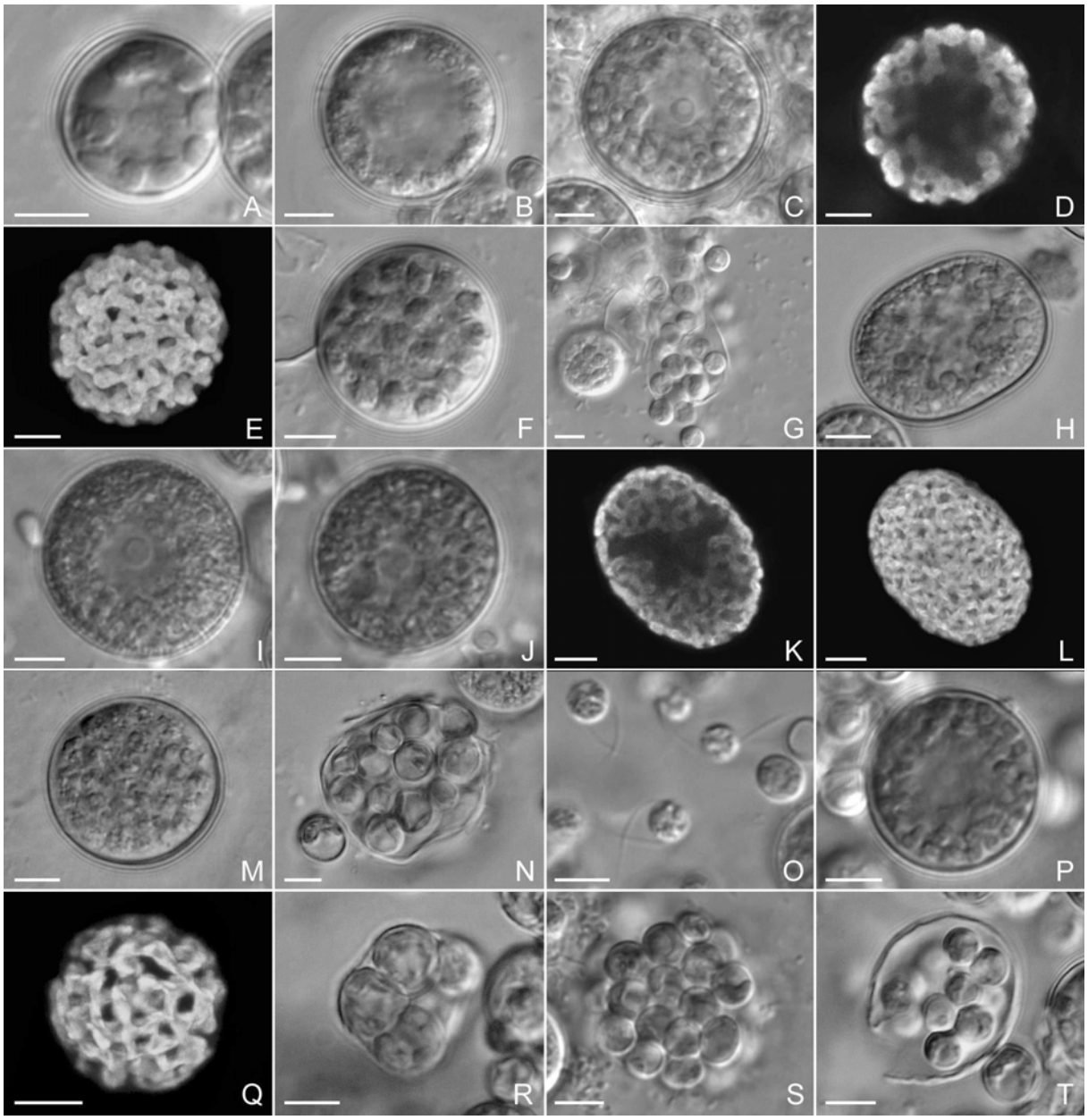

Fig. 4. Micrographs of *Aerosphaera*. (A-G) *Aerosphaera pauciautosporica*. (A) Young cell. (B) Mature cell with unilayered chloroplast. (C) Mature cell with multilayered chloroplast. (D) Chloroplast structure of mature cells, single median confocal section. (E) Chloroplast structure of mature cells, maximum projection of multiple serial confocal sections. (F) Young aplanosporangium. (G) Mature opened aplanosporangium. (H-O) *Aerosphaera tropica*. (H) Cylindrical shape of some cells. (I, J) Mature cells with multilayered chloroplast. (K) Chloroplast structure of mature cells, single median confocal section. (L) Chloroplast structure of mature cells, maximum projection of multiple serial confocal sections. (M) Young aplanosporangium. (N) Opened mature aplanosporangium. (O) Zoospores with separate flagella insertions. (P-T) *Aerosphaera* sp. (P) Mature cell with multilayered chloroplast. (Q) Chloroplast structure of mature cell, maximum projection of multiple serial confocal sections. (R) Mature autosporangium. (S) Mature aplanosporangium with dissolving sporangial cell wall. (T) Mature opened sporangium. Scale bar = 5 μm.

thin tubular lobes (compare Fig. 4i with Fig. 4c and Fig. 4k with Fig. 4d). In surface view, the chloroplast is evenly perforated by rather small pores (Fig. 4l).

The diameters of vegetative cells vary in the range of 3-24(-26) μm. The asexual reproduction takes place by means of aplanospores and zoospores. No autospores were observed. Aplanospores and zoospores are formed in globular or slightly ellipsoidal sporangia, reaching the diameters of 25 μm (Fig. 4m). Usually, 32 or 64 zoospores or aplanospores are produced within the sporangium. Mature aplanospores and zoospores are released by the rupturing of sporangia (Fig. 4n) so the remnants of sporangial cell wall remain in the culture. The zoospore production is very common. The zoospores are ellipsoidal, without stigma, 5.5-6 μm in length and 2-3 μm in width. They have rounded anterior end with separate insertion of flagella (Fig. 4o).

Aerosphaera sp.

Investigated strains: SAG 244.80, SAG 2069

Cells are mainly spherical or slightly ellipsoidal. In young cells, the chloroplast spreads along the whole inner surface of the cell wall as a single layer with many perforations. Later, the complicated multilayered chloroplast is formed inside adult cells, reaching the nucleus in the middle of the cell (Fig. 4p). Regardless, some adult cells contain only unilayered chloroplast, without any subsequent chloroplast lobes spreading towards the cell centre (analogous to *D. pauciautosporica* or *D. handae*). In surface view, the chloroplast is evenly perforated by rather spherical distinct pores (Fig. 4q). The diameters of vegetative cells vary in the range of 3.5-19(-20) μm (strain SAG 2069) and 3.5-20(-23) μm (strain SAG 244.80). The asexual reproduction takes place by means of aplanospores and zoospores. Rarely, autosporogenesis was observed in strain SAG 2069. Mature autosporangia grow up to 12 μm in diameter and contain 16 or 32 autospores (Fig. 4r), released by dissolving of sporangial cell wall.

Aplanospores and zoospores are formed in globular or slightly ellipsoidal sporangia. Usually, 64 zoospores and 64 or 128 aplanospores are produced within the sporangium. Mature aplanospores of strain SAG 244.80 are released mainly by the dissolving of sporangial cell wall (Fig. 4s), whereas the spores of strain SAG 2069 are released by the rupturing of sporangia, so the remnants of sporangial cell wall remain in the culture (Fig. 4t).

Key for the species determination **(Fig. 5):**

Note: We highly recommend to determine the species using fresh (one month old) cultures, cultivated at 15 °C.

1a. Chloroplast surface of some adult cells consists of parallel-arranged lobes (Fig.). Asexual reproduction only by autospores. ***Dictyochloropsis*** 2

1b. Chloroplast surface is evenly perforated, no parallel-arranged lobes are formed. Asexual reproduction by aplanospores, zoospores and/or autospores. The young zoo- and aplanosporangia possess the inner thickening of the cell wall. ***Aerosphaera*** 3

2a. Cells up to 40(-50) μm. Chloroplast of young cells parietal. ***Dictyochloropsis splendida***

2b. Cells up to 22(-24) μm. Chloroplast of young cells asteroid. ***Dictyochloropsis asterochloroides***

3a. Prevailing type of asexual reproduction autosporogenesis 4

3b. Prevailing type of asexual reproduction aplano- and zoosporogenesis 6

4a. Young autospores often remain attached to the sporangial cell wall, forming chains of cells or cell packets ***Aerosphaera symbiontica***

4b. Young autospores never form chains 5

5a. Multilayered chloroplast of adult cells ***Aerosphaera tschermakiae***

5b. Unilayered chloroplast of adult cells ***Aerosphaera reticulata***

6a. Adult cells spherical or ellipsoidal 7

6b. Adult cells variable in the shape ***Aerosphaera irregularis***

7a. Distinct perpendicularly oriented chloroplast lobes often arise from the parietal chloroplast layer. Autospore production frequent ***Aerosphaera handae***

7b. No perpendicularly oriented chloroplast lobes appear inside the cells. Autospores very rare or absent. 8

8a. Chloroplast of some adult cells only unilayered. In these cells, high portion of the cell lumen fills up the cytoplasm with nucleus ***Aerosphaera pauciautosporica***

8b. Chloroplast of adult cells strictly multilayered ***Aerosphaera tropica***

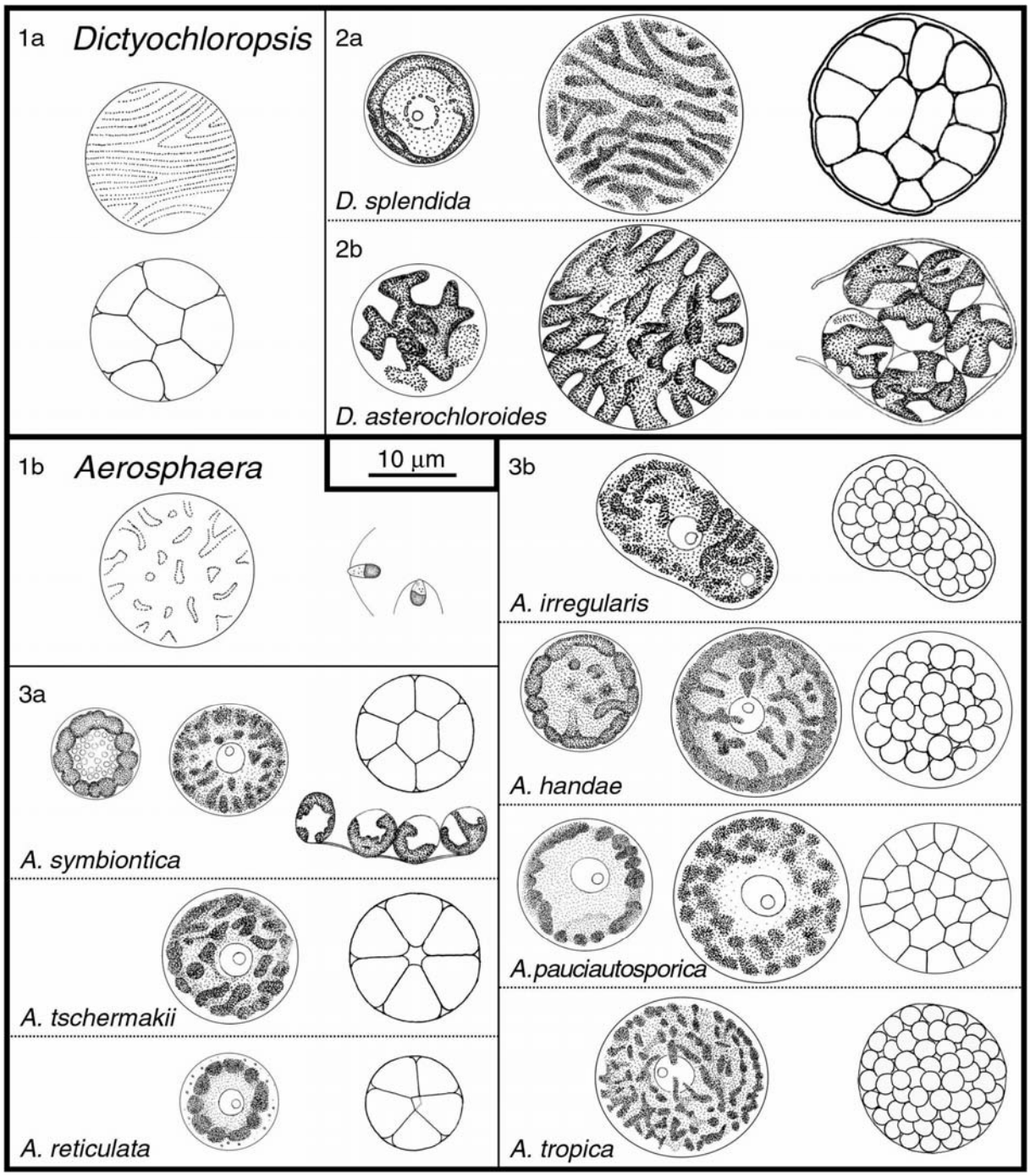

Fig. 5. Illustrated key for the species determination.

Phylogenetic analyses

Phylogram inferred from 18S rDNA sequences clearly separate the genera *Dictyochloropsis* and *Aerosphaera* (Fig. 6). Both genera are positioned within the class Trebouxiophyceae. *Aerosphaera* is a member of *Watanabea* clade, comprising the coccal autosporine algae *Viridiella*, *Watanabea* and *´Heterochlorella´* sensu Krienitz et al. (2004). By contrast, *Dictyochloropsis* forms a separate lineage within Trebouxiophyceae, with any closely related species.

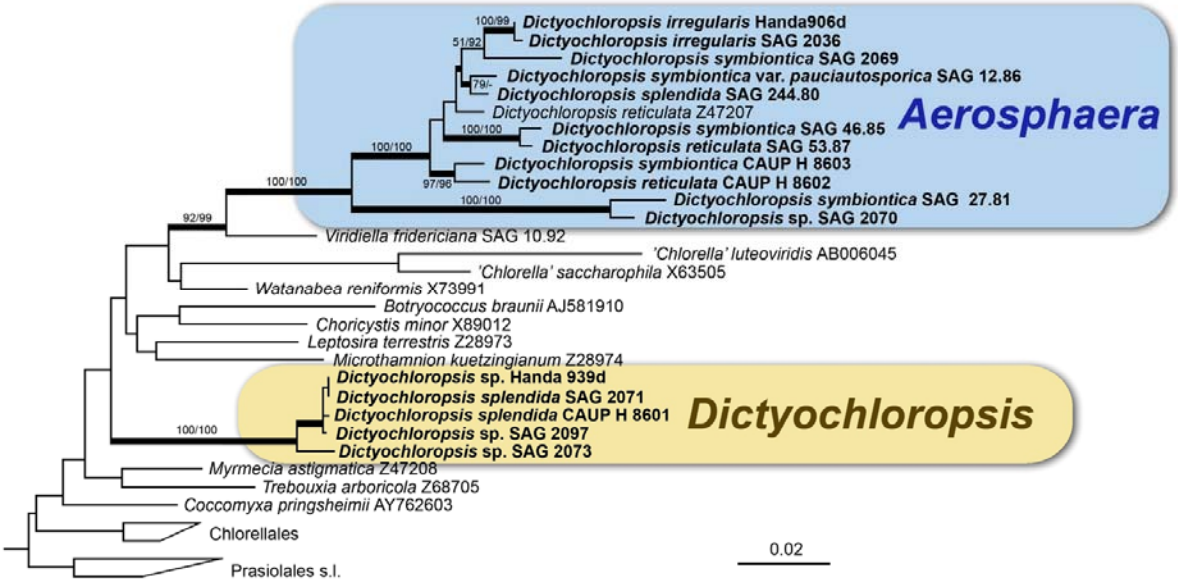

Fig. 6. Maximum Likelihood (ML) tree based on 18S rDNA sequences. A total of 1736 characters with 489/312 variable/parsimony informative characters were used. Numbers at internal branches are bootstrap values from 2000 replicates using neighbor-joining distance (HKY 85 model; left) and weighted Maximum Parsimony (1000 replicates; right).

ITS phylogram was inferred to show the intergeneric variability more precisely (Fig. 7). Generally, in *Aerosphaera* we detected seven lineages representing particular species, while in *Dictyochloropsis* only two species were recognized. Moreover, *Aerosphaera* is composed of two major, well-supported clades: the unispecific lineage of *A. symbiontica* isolates and a lineage composed of all other *Aerosphaera* species.

DISCUSSION

The production of characteristic naked zoospores with separate insertion of flagella is considered to be one of the main diagnostic characters of *Dictyochloropsis* (Tschermak-Woess 1980, 1984, Nakano & Isagi 1987). However, the production of such zoospores is not mentioned in the former genus description. Geitler (1966), who described *Dictyochloropsis* with a *D. splendida* as a type species of the genus, declared autosporogenesis as an exclusive type of asexual reproduction. The production of zoospores was mentioned as late as by Tschermak-Woess, who described several new species of *Dictyochloropsis* and emended Geitler´s description of the genus (Tschermak-Woess 1984). Since no authentic strain of type species *D. splendida* retained, she modified the genus description based on observation of own strain SAG 244.80 *D. splendida*, isolated from lichen *Chaenotheca brunneola* (Tschermak-Woess 1978). This strain was characteristic by frequent zoospore production. Later, Tschermak-Woess isolated many additional strains of *D. splendida* from the thalli of Euro-

pean lichen *Phlyctis argena* (Tschermak-Woess 1995). Two of these strains (UTEX 2599 and UTEX 2612) are considered in this study. Our observations on the morphology and the process of asexual reproduction of strains SAG 244.80, UTEX 2599 and UTEX 2612 do not agree with the original Geitler's (1966) description of *D. splendida*. Strict autosporogenesis and chloroplast shape (rather ribbon-like than granulated) of Geitler's *D. splendida* let us consider the addition of all other species into the genus *Dictyochloropsis* (Tschermak-Woess 1978, 1980, 1984; Nakano & Isagi 1987) as incorrect.

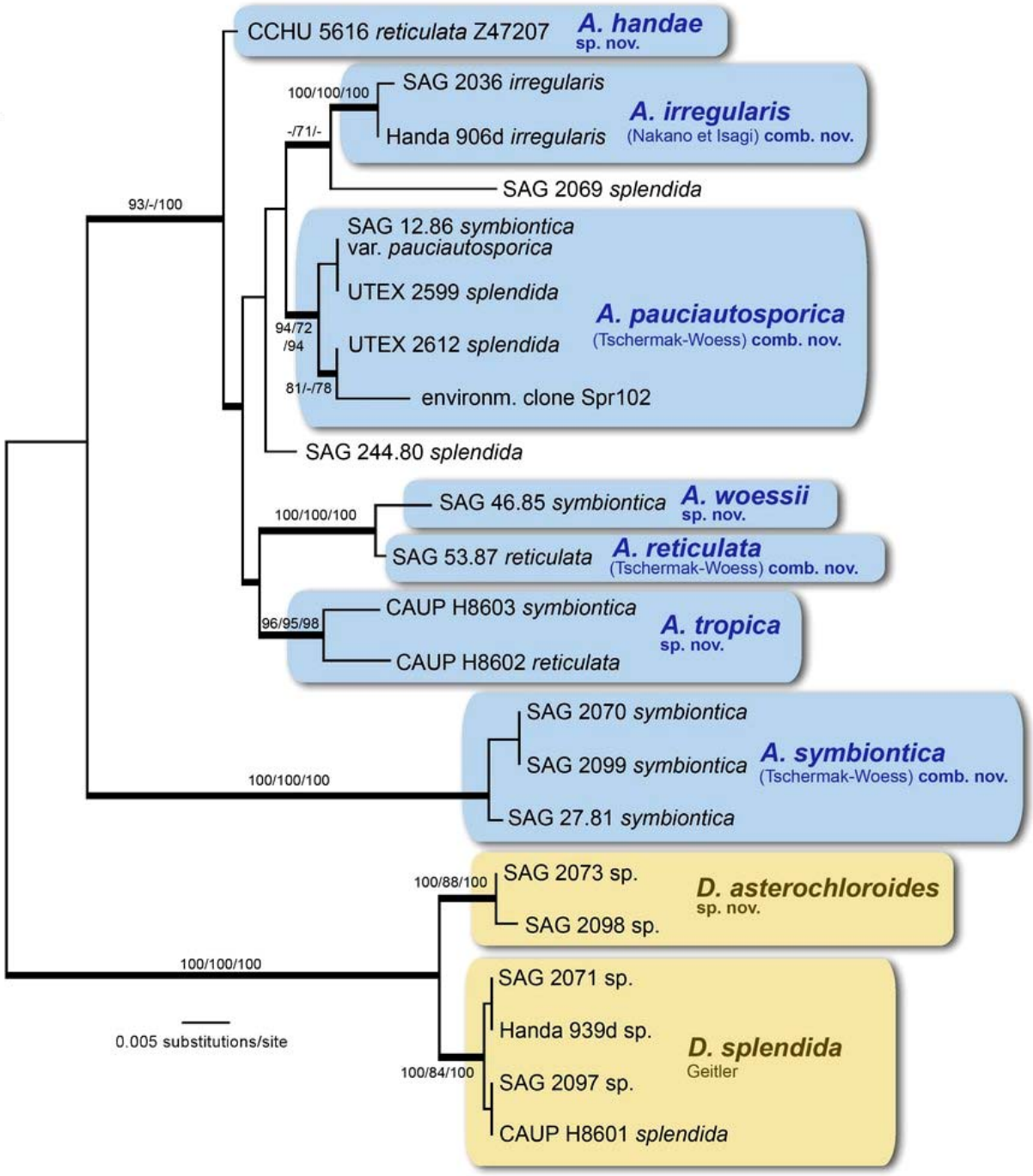

Fig. 7. Maximum Likelihood (ML) tree based on ITS-1,2 rDNA sequences. A total of 1486 characters with 229/200 variable/parsimony informative characters were used. Numbers at internal branches are bootstrap values from 2000 replicates using neighbor-joining distance (HKY 85 model; left), 1000 replicates using minimum-evolution distance (TrN+I+G model; in the middle) and weighted Maximum Parsimony (1000 replicates; right).

Aside from the strains isolated by Tschermak-Woess, we dealt with two additional strains, independently determined as *D. splendida* by two phycologists: CAUP H8601 (Škaloud et al. 2005) and SAG 2071 (Handa, unpublished). According to results of molecular analyses, both strains are recently placed to the clade, separated from all zoosporogenic species, described by Tschermak-Woess (1978, 1980, 1984) and Nakano & Isagi (1987).

Since all important morphological characteristics (cell size, shape of chloroplast, type of asexual reproduction) are in absolute congruence with Geitler´s definition of *Dictyochloropsis splendida*, we determined our strains as this species. Dealing with our results, *Dictyochloropsis* consists in fact of only two species – *D. splendida* and recently described *D. asterochloroides*. All other species should be transferred into another genus.

Instead of describing new genus we decided to transfer these species into the genus *Aerosphaera*. Despite poor genus description (missing Latin diagnosis, unmatched illustration with description given in the text), many morphological characteristics are very similar to *Dictyochloropsis* sensu Tschermak-Woess and the genus name *Aerosphaera* is closely connected with the morphology characteristic for that algae. Thus, we established the Latin diagnosis of *Aerosphaera* following the emendation made by Tschermak-Woess (1984) to clearly define the algae with reticular chloroplast of granular structure, asexually reproduced by means of zoospores with separate flagella insertions.

Concluding the taxonomical revision, the genera *Aerosphaera* and *Dictyochloropsis* differ in two main features – chloroplast structure of adult cells and type of asexual reproduction. In *Dictyochloropsis*, the chloroplast surface is in some ontogenetic stages formed by distinct parallelly-arranged lobes, whereas the reticulate chloroplast of *Aerosphaera* is always evenly perforated in the surface view. This difference has been already observed by Škaloud et al. (2005), who studied chloroplast ontogeny in three strains assigned to genus *Dictyochloropsis*. They described unique parallelly-arranged flat lobes in strain H 8601, presently recognized as a strain of *Dictyochloropsis splendida*. We have observed these lobes in all six studied strains of *Dictyochloropsis* and consider the production of parallel lobes as a very good discriminate character. The ability to form zoospores seems to comprise another good discriminate character for *Aerosphaera* and *Dictyochloropsis* recognition. Although the zoosporogenesis can be quite rare in some species, at least the aplanospores are produced during the cell cycle of all studied strains of *Aerosphaera*. By contrast, *Dictyochloropsis* never forms zoospores and aplanospores and the asexual reproduction take place only by means of autospores.

The species of *Aerosphaera* (=*Dictyochloropsis* sensu Tschermak-Woess) were traditionally determined by means of cell shape and chloroplast structure (Tschermak-Woess 1984, Ettl & Gärtner 1995). Although the chloroplast structure (especially number of layers) is essential for correct determination of some species, it seems to be very variable depending to the cultivation conditions. For example, in the temperature of about 15 °C all strains of *A. symbiontica* hold multilayered chloroplast comprising of many interconnected lobes. In slightly higher temperatures, however, we observed distinct simplification of chloroplast structure. When cultivating at 20 °C, the absolute majority of cells possess only single chloroplast layer below the plasma membrane. Moreover, if we increase the temperature over 20 °C, many *Aerosphaera* strains stop to grow and die. Conversely, all *Dictyochloropsis* strains are able to grow even in temperatures of about 30 °C, demonstrating clear difference between the genera.

The prevailing type of asexual reproduction has an important taxonomic value within the genus *Aerosphaera*. Several species are characteristic by frequent production of autospores, strictly differentiated from another type of daughter cells – aplanospores. Our observations are identical with the definition made by Tschermak-Woess (1989, 2000): Autospores are formed in relatively small cells and in low number (mostly 4-16, rarely 32); the daughter cells are tightly pressed against each other that cause slightly angular shape of mature autospores. Even in early stages of aplanosporogenesis, distinct cell walls can be visible inside the

sporangium. Aplanospores, by contrast, are formed in bigger cells and in a large number (mostly 64-128); pressure for space is not so drastic, so they generally acquire a perfectly spherical form. The inner cell walls are less distinct compared to autosporangia. In fact, aplanospores represent arrested zoospores, so they have the same appearance in early ontogenetic stages. In *Aerosphaera*, early stages of both aplanosporangia and zoosporangia are characteristic by production of inner thickenings of the cell wall (for more information see Tschermak-Woess 2000). The absence of inner thickenings in all *Dictyochloropsis* strains can be considered as a confirmation of zoospores absence within this genus.

Both *Aerosphaera* and *Dictyochloropsis* are positioned deeply within the Trebouxiophycean lineage together with autospore (eg. *Chlorella*, *Coccomyxa*) and zoospore (e.g. *Trebouxia*, *Myrmecia*) producing algae (Fig. 6). Thus, they probably evolved from the common ancestor, asexually reproduced by means of zoospores. Many clades within Trebouxiophyceae comprise by only autosporogenic species (e.g. *Chlorella*, *Coccomyxa*), so they probably lose the ability to form the zoospores in the past. The closest relatives of *Aerosphaera* represent typical autosporogenic algae (*Viridiella*, *Watanabea*, "*Chlorella" luteoviridis* clade), so we can suppose the loss of zoosporogenesis in the phylogenic history of this lineage. However, the crown position of *Aerosphaera* within Trebouxiophytes and the unique, highly specific morphology of zoospores predicate the secondary origin of zoosporogenesis in the early evolutionary history of *Aerosphaera*.

As compared to *Dictyochloropsis*, the genus *Aerosphaera* exhibits large species diversity. Very probably, the number of *Aerosphaera* species is not final and will grow in the future as new investigations will come. The splitting of presently delimited species is also possible. For example, species *A. tropica* represents the clade consisting of two strains isolated from tropical biotopes. Even if these two strains share many morphological features, some characteristics are not common for both strains. Thus, if new tropical isolates will be investigated, new data can support the splitting of the species.

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4 Conclusions

Traditional taxonomy often depends on single characters, used for distinguishing particular species. In this thesis, I illustrate the application of polyphasic approaches in the taxonomy of green aerophytic algae. These approaches are based on the combination of various characters (traditional morphology, modern morphological approaches, SSU, ITS rDNA and gene intron sequencing) for the species recognition in green algae.

The morphological characters alone are often not sufficient for species delimitation, though they can be used for species determination, e.g. in keys to the species. Modern morphological approaches like confocal microscopy allow detecting clear morphological differences among the studied species, even if at first sight morphologically very uniform algae are investigated. A number of morphological characters does not reflect the genetic relationships, rather they are influenced by various environmental or culture conditions. For example, cell dimensions in *Klebsormidium* can be distinctively influenced by pH, humidity, illumination and temperature conditions. However, with the aid of molecular markers we can detect proper morphological features useful for species differentiation. In *Asterochloris*, the test of correlation between genetic distances and differences in various morphological characters revealed the usefulness of particular chloroplast characters for the species delimitation.

The molecular phylogenetic analyses are also essential to reveal cryptic diversity within the green algal genera. Analyses of molecular sequences detected the existence of 10 new lineages within genera *Asterochloris* and *Dictyochloropsis*, consequently described as new species. However, their description was based not only on molecular characters, but also on distinct morphological differences in chloroplast morphology. Similarly to morphological characters, exclusive application of molecular data in species delimitation can lead to erroneous taxonomical conclusions, as well, caused by the fault in differentiation between real biological species and intraspecific variability.

Accordingly, the application of polyphasic approaches combining morphological and molecular data will result in better delimitation of particular algal species and improve the taxonomy of green aerophytic algae.

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Publications impacted

- **ŠKALOUD, P.** & PEKSA, O. (2008): Comparative study of chloroplast morphology and ontogeny in *Asterochloris* (Trebouxiophyceae, Chlorophyta). - Biologia (in press).
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