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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*; *Trebouxia*; chloroplast morphology; confocal microscopy; ITS; molecular phylogeny

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-Woessunder 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.) pro-

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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.

posed 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*, *L. 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 obtained 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

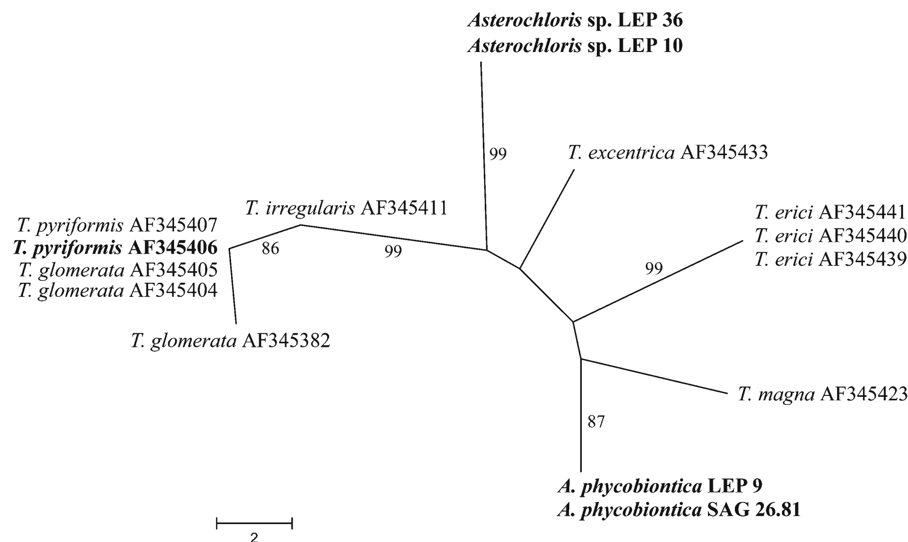


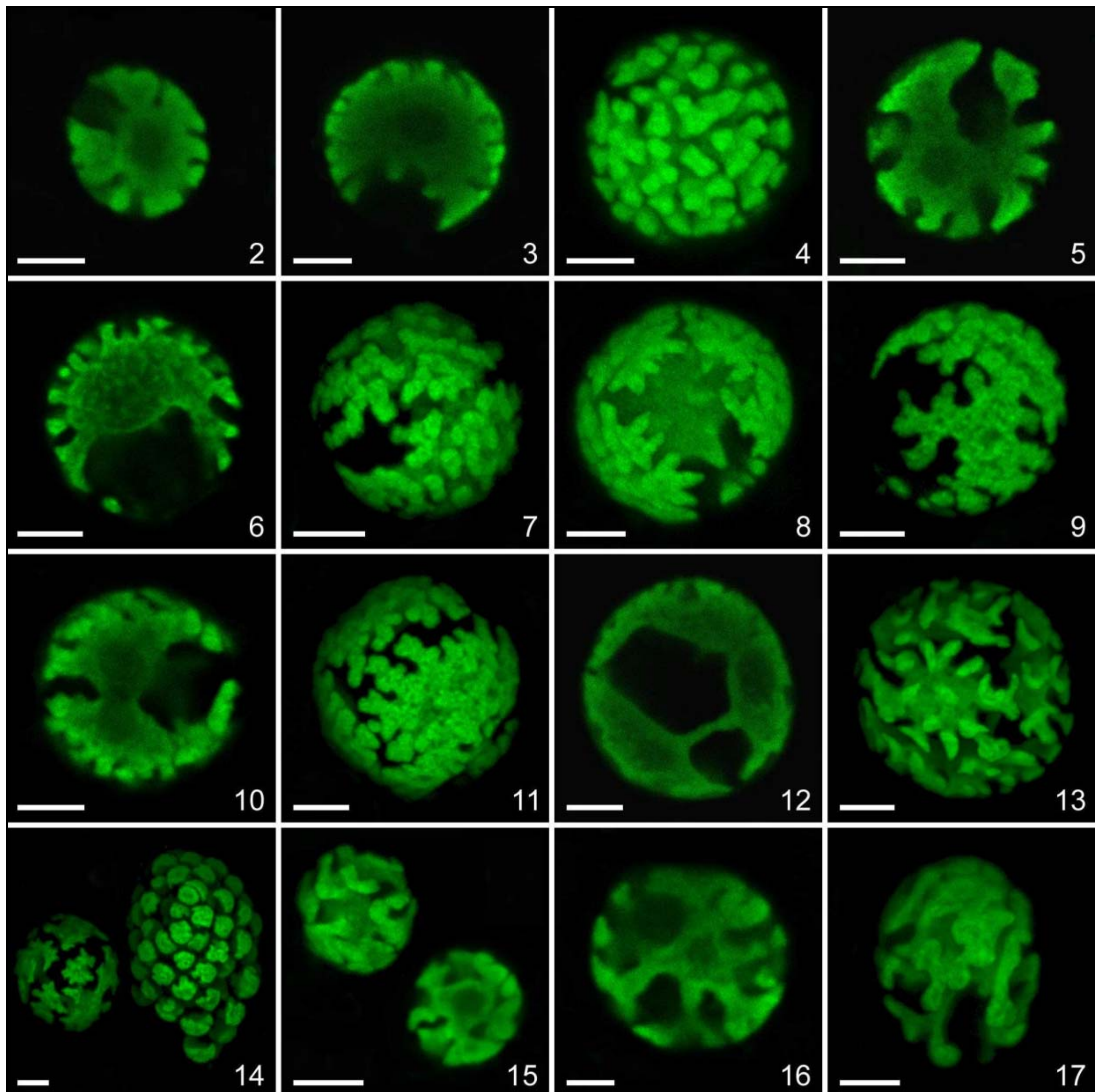
Fig. 1. Unrooted phylogeny of *Asterochloris* ITS rDNA sequences using the maximum parsimony method and a branch-and-bound search. Values at the nodes represent statistical support estimated by maximum parsimony bootstrapping. The scale bar indicates the distance due to two evolutionary steps. Investigated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

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 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-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 ITS 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 (10000 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 topology 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 sep-



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 5 μm.

arate 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).

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 pro-

cesses, 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).

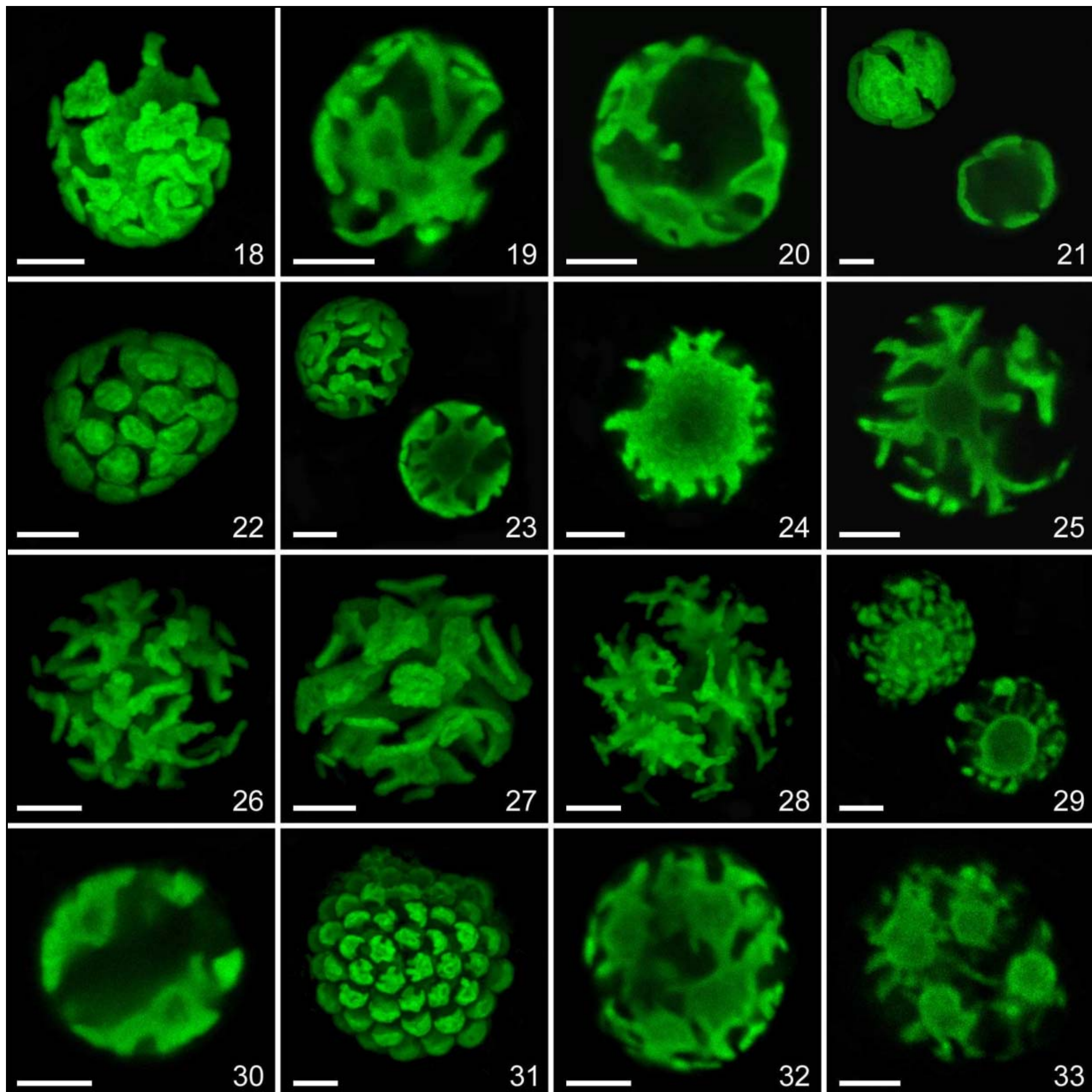
Asterochloris sp. (LEP 10, LEP 36)
As in *T. pyriformis*, the chloroplasts of young cells assumed a central position with several lobes spreading to the cell periphery. Elongate ends of the lobes were characterized by a T-shaped profile, as viewed in confocal optical sections (Fig. 23). Rarely, a centrally 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 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,



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 5 μm.

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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