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

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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.

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: H 8601, H 8602 and H 8603.

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.

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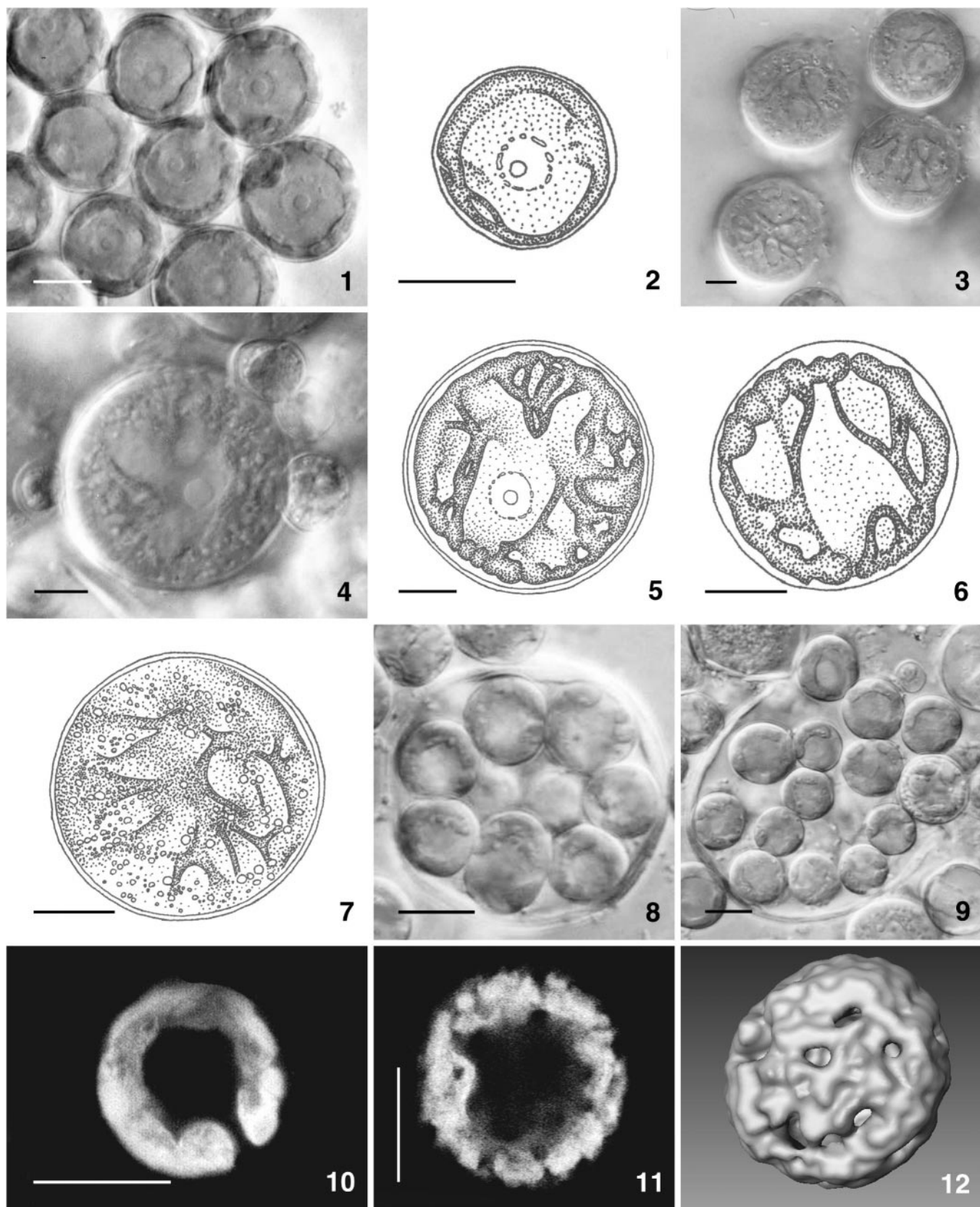
Figs 1–12. *Dictyochloropsis splendida* var. *splendida.* Scale bars = 10 μm.
  Figs 1, 2. Young cell with distinct nucleus.
  Figs 3–6. Vegetative cells with multilayered net-shaped chloroplast.
  Fig. 7. Vegetative cell with poorly visible chloroplast structure.
  Figs 8, 9. Globular autosporangia.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

A 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, Minneapolis, 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* (H 8601)

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 (H 8602)

CONVENTIONAL LIGHT MICROSCOPY: The investigated strain of this alga had uninuclear globular, or rarely ellipsoidal, cells.

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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.
  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.
  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.
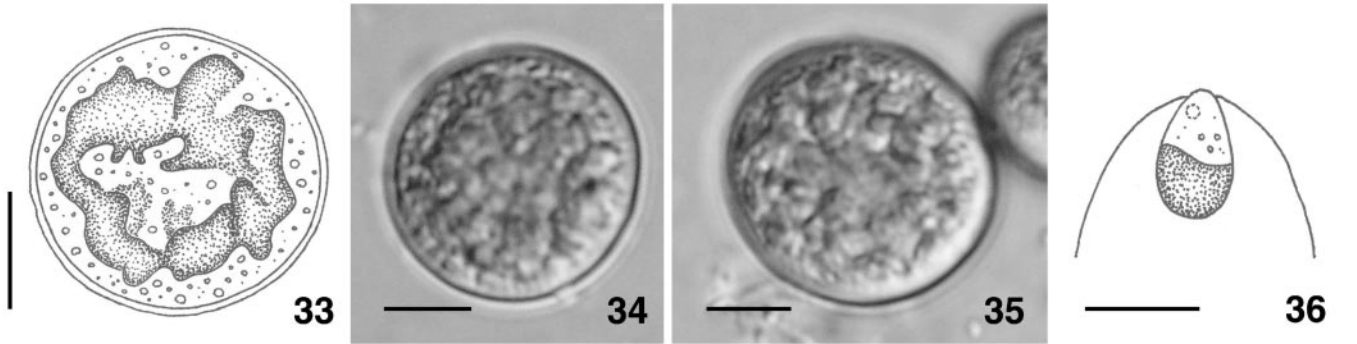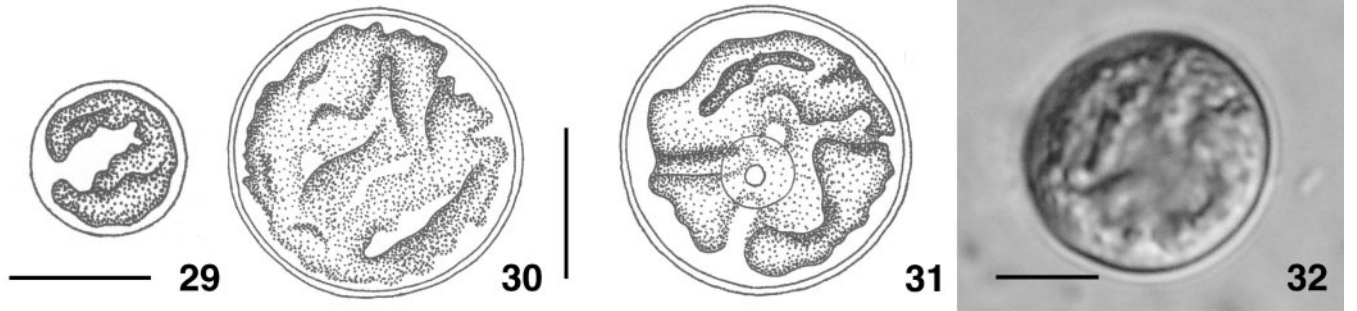 **Figs 41–43.** Unilayered perforated chloroplast of young and vegetative cells.
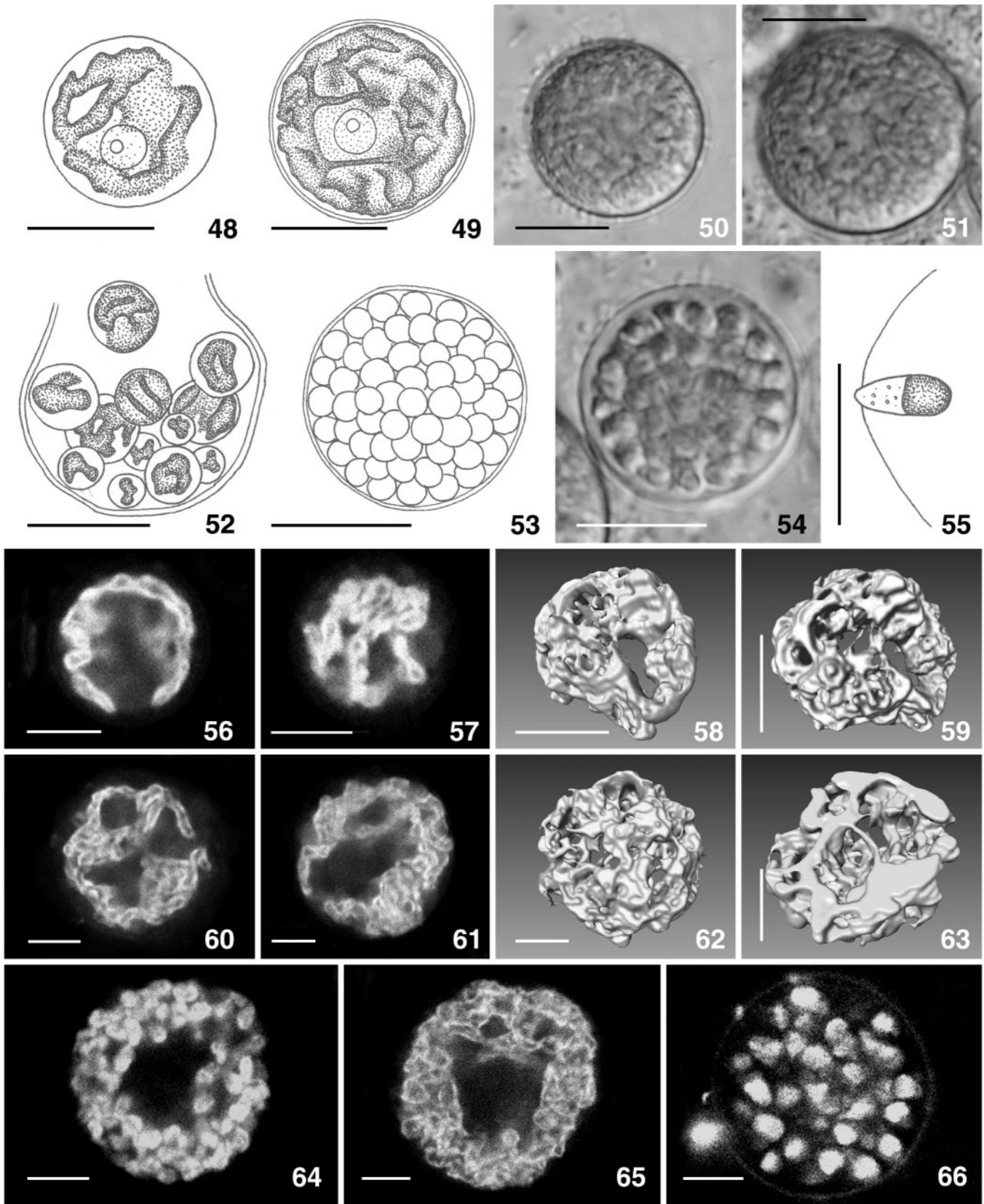 **Fig. 44.** Two-layered chloroplast of mature cells before cell division.
  Figs 45, 46. Multilayered chloroplast of interconnected chloroplast lobes.
  Fig. 47. Granular chloroplast of mature cells before the autospores production.
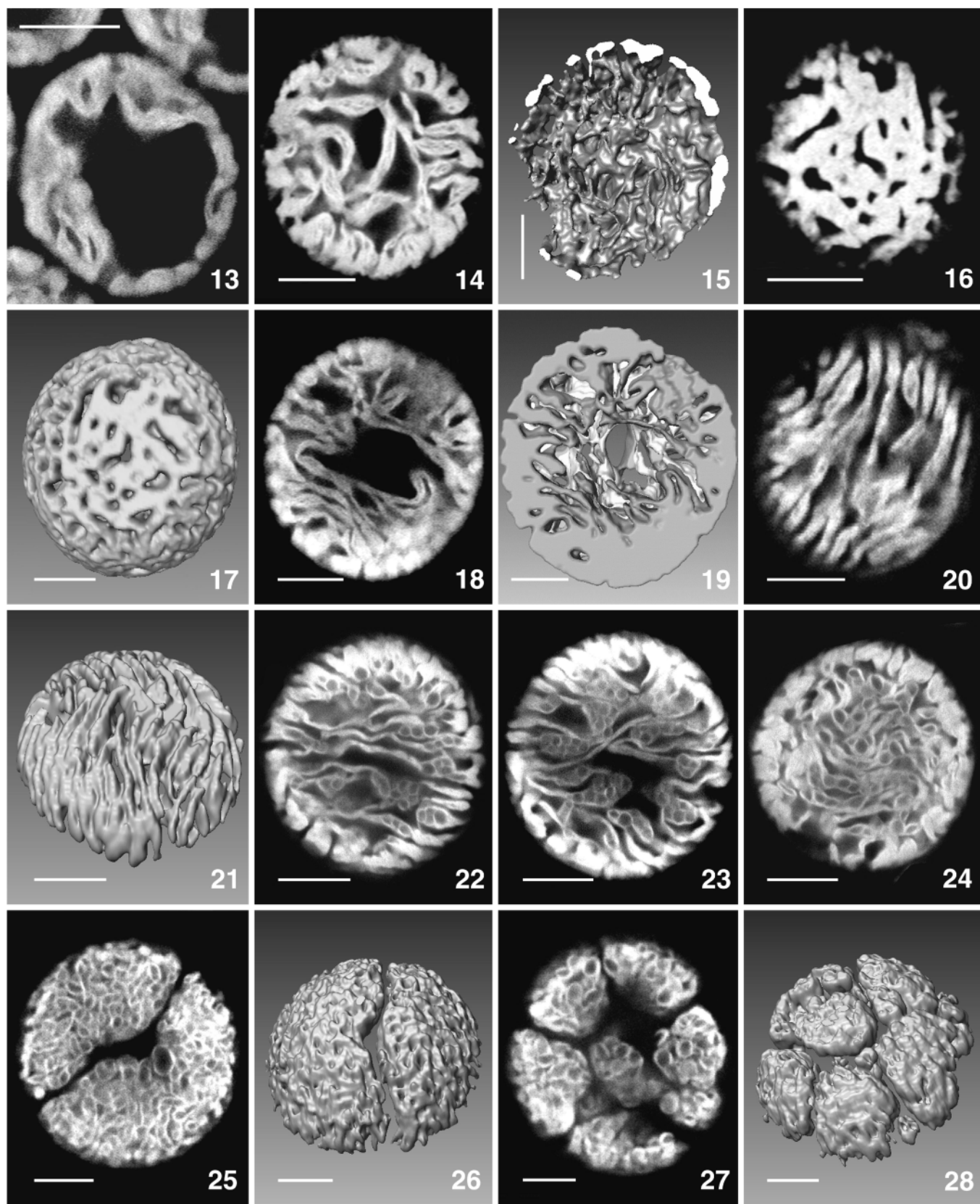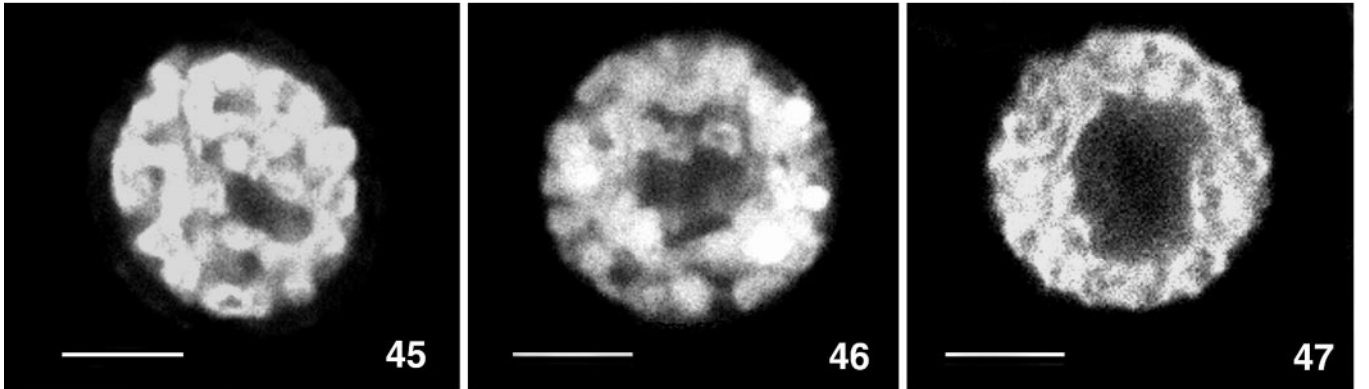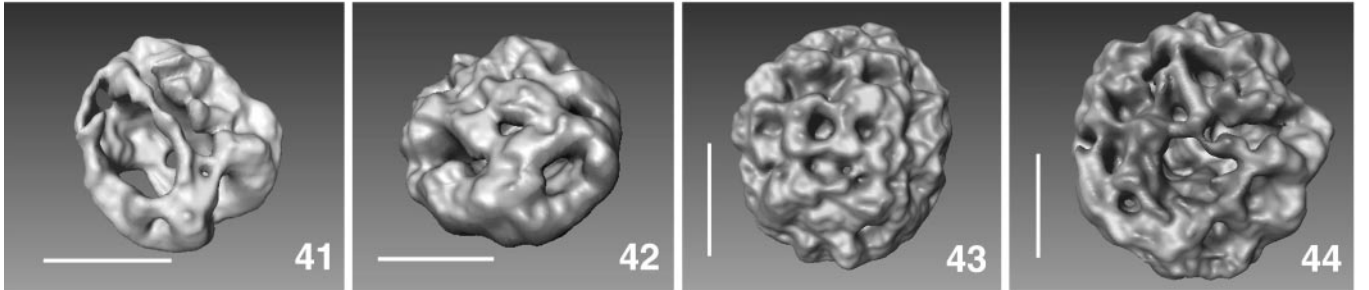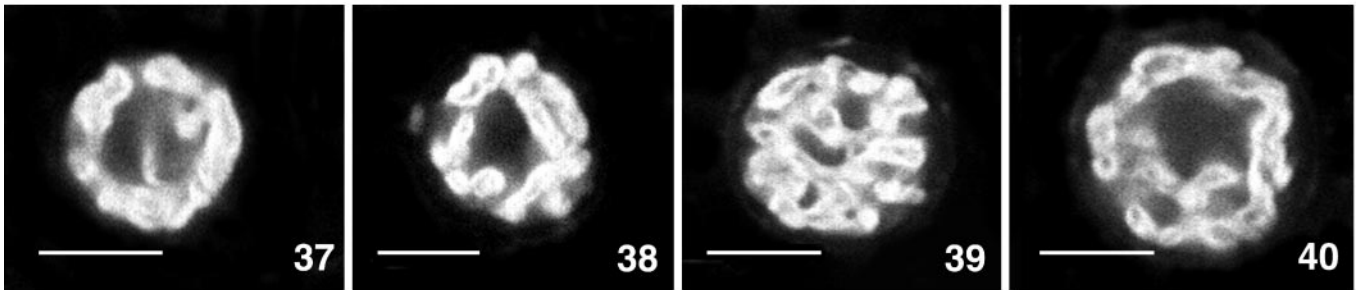
Figs 48–66. *Dictyochloropsis symbiontica.* Scale bars = 10 μm.
  Fig. 48. Young cell with single chloroplast layer.
  Figs 49–51. Vegetative cells with multilayered chloroplast and distinct nucleus.
  Fig. 52. Open sporangium with unequal autospores.
  Figs 53, 54. Globular aplanosporangia with a large number of aplanospores.

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

Dictyochloropsis symbiontica Tschermak-Woess (H 8603)

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) 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

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Fig. 55. Naked zoospore.
Fig. 56. Unilayered chloroplast within the young cells.
Figs 57–59. Chloroplast structure of young cells with large perforations between the chloroplast lobes.
Figs 60, 61. Two-layered chloroplast with granular structure.
Fig. 62. Multilayered chloroplast of mature cells.
Fig. 63. Inner view of multilayered chloroplast showing the secondary chloroplast lobe spreading through the cell lumen.
Fig. 64. Multilayered chloroplast of interconnected globular lobes.
Fig. 65. Granular chloroplast of mature cells.
Fig. 66. Disintegrated chloroplast of early zoosporangium.

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 chloroplast divides. The development of the two-layered chloroplast stage, which was not observed in this species by Tschermak-Woess (1984), takes place shortly before cell division (Fig. 45). In *D. symbiontica* the stage of the 'granular' chloroplast composed of two-layered tubular lobes with grouped thylakoids predominates during the life cycle. The unilayered stage occurs only in young cells and the fused chloroplast mass with granular structure occurs in cells shortly before the cell division.

The most complicated chloroplast ontogeny occurs in *D. splendida.* In contrast to other investigated species a unique chloroplast structure develops during its ontogeny. Longitudinal division of primary chloroplast lobes produces flat lobes in all layers. Interestingly enough, these flat lobes are formed by parallel plates (Figs 20, 21). The lobes develop in such pattern in a vast majority of cells in populations of *D. splendida.* However, in some cells this stage does not occur.

Probably the most intriguing structural changes take place shortly before the cell divides. In all investigated species, the complex shape of chloroplast becomes simpler. In confocal images this process can be observed as a gradual formation of a granular region within the chloroplast (Figs 22–27). The chloroplast lobes are considerably enlarged, however, the number of traversing thylakoids remains identical with previous stages. Thylakoids, which are otherwise equally distributed in a chloroplast volume, aggregate into distinct fascicles. The dimensions of lobes are rapidly increasing and they fuse together. Subsequently, homogenous chloroplast regions with granular structure develop (Figs 22, 23). Gradually, the aggregation of these granular regions leads to a single massive homogenous chloroplast, filling up the whole cell volume (Fig. 24). This chloroplast stage has a considerably larger overall volume than previous stages with reticulated chloroplast. However, the enlargement of chloroplast matrix was not followed by additional production of new thylakoids, but the thylakoids were only regularly arranged in the matrix. Thus, the remodelled homogenous chloroplast is eventually prepared for the division in the course of autosporogenesis (Fig. 25).

CONCLUSION

The green algal genus *Dictyochloropsis* comprises several morphologically similar taxa. The confocal microscopy investigation revealed the existence of interspecific differences in the ontogeny of complex three-dimensional chloroplasts. The differences in *Dictyochloropsis* strains are based on a different timing of particular ontogenetic sequences rather than on the occurrence of entirely distinct and specific chloroplast structures. As the current infrageneric taxonomy of the genus and the discriminative criteria of individual species are rather vague, we assume that the features of chloroplast ontogeny could provide a useful platform for future complex combined structural/molecular taxonomic comparison involving numerous *Dictyochloropsis* isolates. As it concerns our investigated strains which we identified as three distinct species we consider the observed differences in chloroplast ontogeny as useful for species delimitation in *Dictyochloropsis.* However, the overall morphological variability of members of the genus *Dictyochloropsis* clearly does not fit the taxonomic criteria, on which the traditional taxonomy of the genus was based. Thus, potential species identifications should be made very cautiously for the time being.

Confocal microscopy and subsequent three-dimensional reconstructions can add useful information in studies of the phenotypic plasticity of algal chloroplasts, for detailed investigation of chloroplast ontogeny, and, in taxonomic studies as well, especially in those groups where chloroplast morphology is considered as one of the principle features in taxonomic evaluation.

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