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Isolation and characterization of biliprotein aggregates from *Acaryochloris marina*, a *Prochloron*-like prokaryote containing mainly chlorophyll *d*

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Abstract Phycobiliprotein aggregates were isolated from the prokaryote *Acaryochloris marina*, containing chlorophyll *d* as major pigment. In the electron microscope the biliprotein aggregates appear as rod-shaped structures of 26.0×11.3 nm, composed of four ring-shaped subunits 5.8 nm thick and 11.7 nm in diameter. Spectral data indicate that the aggregates contain two types of biliproteins: phycocyanin and an allophycocyanin-type pigment, with very efficient energy transfer from the phycocyanin- to allophycocyanin-type constituent. The chromophore-binding polypeptides of the pigments have apparent molecular masses of 16.2 and 17.4 kDa. They crossreact with antibodies against phycocyanin and allophycocyanin from a red alga.

Key words: Phycobiliprotein; Allophycocyanin; Phycocyanin; Light harvesting antenna; Chlorophyll *d*; Prochlorophyte; (*Acaryochloris marina*)

1. Introduction

Photosynthetic organisms contain very effective antenna complexes to optimize photosynthesis. Most of these antenna complexes contain bacteriochlorophyll or chlorophyll (Chl), usually Chl *a*, Chl *b* and/or a Chl *c*-type pigment. The recently discovered photosynthetic prokaryote *Acaryochloris marina* [1] is the only organism yet known which contains, in addition to small amounts of Chl *a* and a Chl *c*-type pigment, Chl *d* as a major pigment. Chlorophyll *d* was reported as a minor constituent from some red algae [2], but its in vivo occurrence in this algal group still remains doubtful.

In addition to Chl proteins, cyanobacteria, red algae and cryptophytes possess phycobiliprotein aggregates with light-harvesting function. In cyanobacteria and red algae, the phycobiliproteins are organized as phycobilisomes (PBS), which are attached to the cytoplasmic or stromal side of photosystem II. They are normally constructed of three-cylindrical core units from which several peripheral rods radiate forming hemi-discoidal or hemi-ellipsoidal arrays. The central core cylinders contain allophycocyanin (AP) while the peripheral rods are composed of phycocyanin (PC), either alone or with phycoerythrin (PE) or phycoerythrocyanin. The basic unit of the biliproteins is a heterodimer composed of an α- and β-subunit with molecular masses between 17 and 22 kDa. They are aggregated in ring-shaped trimers of the structure $(\alpha\beta)_3$ which in turn form hexamers by a tight face-to-face association. In vivo, the phycobiliprotein aggregates and PBS are assembled and organized by specific linker polypeptides. For reviews on PBS structure, see [3–5]. In contrast to cyanobacteria and red algae, cryptophytes contain no PBS. Here the biliproteins are located within the thylakoid lumen. They are probably organized as rod-shaped [6,7] or hemispherical structures [8], consisting either of PE or PC alone. Recently, Hess and co-workers [9] found evidence for the occurrence of PE-type biliproteins in a *Prochloron*-like cyanobacterium, the marine nanoplancter *Prochlorococcus marinus*. However, the concentration of PE was too low for isolation, and thus the in vivo structure and function of the biliproteins remains so far unclear.

In this communication we report the isolation and partial characterization of biliprotein aggregates from *Acaryochloris marina*, another *Prochloron*-type cyanobacterium. Its thylakoid membranes show a remarkably high degree of stacking and no PBS are evident [1]. The in vivo organization of the biliprotein pigments is unknown in these organisms. Therefore, the biliprotein complexes isolated from *Acaryochloris* might serve as a model for the organisation of biliproteins here and in other *Prochloron*-like organisms.

2. Material and methods

2.1. Culture conditions

Acaryochloris marina cells were grown in flat flasks in seawater-based K+ESM medium [10] with gentle air bubbling at a temperature of 28°C. The cultures were illuminated with white fluorescent light of 80 μmol photons m^{-2} s^{-1} with a 12-h light/dark cycle. The red alga *Galdieria sulphuraria*, applied for comparison, was cultivated in Allen's medium [11] at 35°C. The cultures were bubbled with air enriched with 2% CO_2 and illuminated with white fluorescent light of 25 μmol photons m^{-2} s^{-1} with a 14-h light/10-h dark cycle.

2.2. Isolation of biliprotein aggregates

Cells were harvested by centrifugation, washed in 0.75 mol/l potassium phosphate buffer (pH 7.0), containing 10% sucrose, 2 mmol/l sodium ethylendiamine tetraacetic acid and 0.1 mmol/l phenylmethylsulfonyl fluoride, and broken by vigorous shaking with glass beads (1 mm diameter) in a cooled Vibrogen cell homogenizer (Bühler, Tübingen, Germany) for 15 min. The glass beads were removed by filtration, and the non-soluble cell fraction was sedimented by centrifugation at 47000×*g* for 1 h. The pellet was resuspended in potassium phosphate buffer containing 1.5% (final concentration) *N*,*N*-dimethyldodecylamine-*N*-oxide and stirred for 40 min at 17°C in the dark to solubilize the thylakoid membranes. Residual material was

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Abbreviations: AP, allophycocyanin; Chl, chlorophyll; PAGE, polyacrylamide gel electrophoresis; PBS, phycobilisome; PC, phycocyanin; PE, phycoerythrin; SDS, sodium dodecyl sulfate

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removed by centrifugation and the lysate was loaded on sucrose gradients (15–35% w/v) in potassium phosphate buffer. After 20 h of centrifugation at 100 000×*g* (17°C) the separated coloured bands were harvested and subjected to further investigation.

2.3. Polyacrylamide gel electrophoresis (PAGE) and immunochemical procedures

The fractions eluted from the gradient were diluted with phosphate buffer without sucrose and biliprotein aggregates were sedimented by centrifugation overnight at 200 000×*g*. The pellets were subjected to denaturing SDS-PAGE as described previously [12]. To visualize chromatophore-binding proteins, the gels were incubated for 30 min in 2 mol/l glycine containing 0.2 mol/l $ZnSO_4$ for 30 min and photographed on a UV transilluminator [13]. The gels were either Coomassie-stained with Serva Blue R-250 (Serva, Heidelberg, Germany) or blotted on Schleicher and Schuell (Dassel, Germany) BA 85 nitrocellulose membranes in a semi-dry system using a buffer given by Dunn [14]. The blots were immunodecorated as described before [15]. Antibodies against AP and C-PC from *Cyanidium caldarium* were a kind gift of Prof. R.F. Troxler (Dept. of Biochemistry, Boston University, School of Medicine, Boston, MA).

2.4. Spectroscopy

Fluorescence spectra were recorded at room temperature or at 77 K with the equipment described by Rhiel and co-workers [16]. The excitation wavelength was 570 nm. Absorbance spectra were taken at room temperature with a Hitachi U-3200 spectrophotometer.

2.5. Electron microscopy

Negative staining was performed according to Valentine and coworkers [17]. Isolated biliprotein aggregates were fixed with 0.1–0.3% glutaraldehyde (v/v) for 1 h and contrasted with 2% aqueous uranyl acetate. Electron microscopy was performed with a Philips 301 G electron microscope (Philips, Eindhoven, The Netherlands).

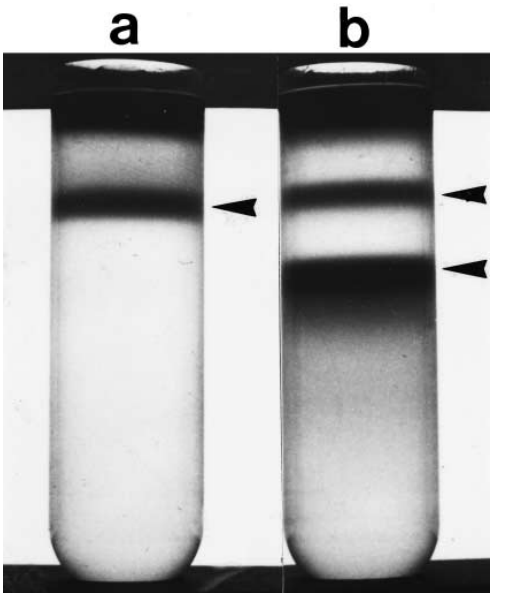

Fig. 1. Isolation of phycobiliprotein aggregates from *Acaryochloris marina* (a) and *Galdieria sulphuraria* (b) by sucrose density gradient centrifugation. *Galdieria* yielded a green band at the top of the gradient and two blue bands (arrowheads). The uppermost blue band contained phycobilisome fragments, the lowermost intact phycobilisomes. *Acaryochloris* yielded only one blue band (arrowhead) with a sedimentation behaviour similar to phycobilisome fragments.

3. Results and discussion

Membranes of *Acaryochloris marina* and *Galdieria sulphuraria* as a reference were solubilized with *N,N*-dimethyldodecylamine-*N*-oxide, and the lysates were fractionated by sucrose density gradient centrifugation. The banding patterns of these separations are shown in Fig. 1. The lysate from *Galdieria* separated into three coloured bands as shown in Fig. 1b. The uppermost band at the top of the gradient contained Chl and carotenoids, and the second band consisted of a mixture of PBS fragments, core particles, peripheral biliprotein rods and core-periphery fragments as determined by negative-staining electron microscopy and by absorption spectroscopy. Intact PBS were found in the lowermost band. The lysate from *Acaryochloris* (Fig. 1a) was separated into only two bands: a green band at the top and a blue band with a sedimentation behaviour similar to that of PBS fragments of *Galdieria*. However, the fraction corresponding to the PBS of the *Galdieria* preparation was missing.

The blue biliprotein fraction obtained from *Acaryochloris* was harvested and subjected to spectroscopic and biochemical characterization. The absorbance spectrum, shown in Fig. 2A (solid line), had a maximum at 616 nm and reassembled in general that of C-PC spectra of other cyanobacteria and of red algae (cf. [3,4]). These are characterized by an absorption maximum around 620 nm, depending on the aggregation state of the pigment–protein. The second derivative of the spectrum (Fig. 2A, broken line) showed a minimum at 618 nm, corresponding to the absorption maximum of C-PC, and, additionally, a remarkable shoulder at 641 nm, which is not present in purified PC preparations. This was the first indication of presence of an additional pigment type, namely AP.

The presence of two pigment types, PC and AP, was corroborated by fluorescence emission spectroscopy. The 273 K (room temperature) fluorescence spectrum of the sample had a maximum at 666 nm with a shoulder at 654 nm (Fig. 2B, solid line). The corresponding main minima in the second derivative of the spectrum (Fig. 2B, broken line) were at 646 and 667 nm. While the position of the short-wavelength component corresponds to the emission of C-PC with maxima at about 650 nm (see [3,4]), the fluorescence maximum must be attributed by its emission wavelength to an AP-type biliprotein. The fluorescence emission of native AP from cyanobacteria and red algae is at about 660 nm [3,4]. Thus, we infer that the separated complexes are composed of PC- and AP-type pigments. These pigments are energetically tightly coupled because most of the excitation energy is transferred from the short-wavelength to the long-wavelength component that obviously serves as terminal emitter within the aggregates. The position of the long-wavelength fluorescence maximum provides evidence that the biliprotein aggregates serve as photosynthetic antennae which transfer excitation energy to Chl *a*. The spectral characteristics of the two components do not perfectly match the values given for C-PC and AP from red algae and cyanobacteria. Thus, *Acaryochloris* might possess novel types of these phycobiliproteins. Cloning and sequencing of the genes encoding these proteins will be necessary to see how they are related to the other types of biliproteins.

The low-temperature (77 K) fluorescence emission spectrum (Fig. 2C, solid line) showed only a single maximum at 670 nm, confirming the excellent energy transfer to the final emitter. The fluorescence excitation spectrum of the sample recorded at 670 nm (not shown) had a maximum at 616 nm with a shoulder at 642 nm. When the biliprotein fraction was transferred to a buffer of lower ionic strength (0.075 mol/l), how-

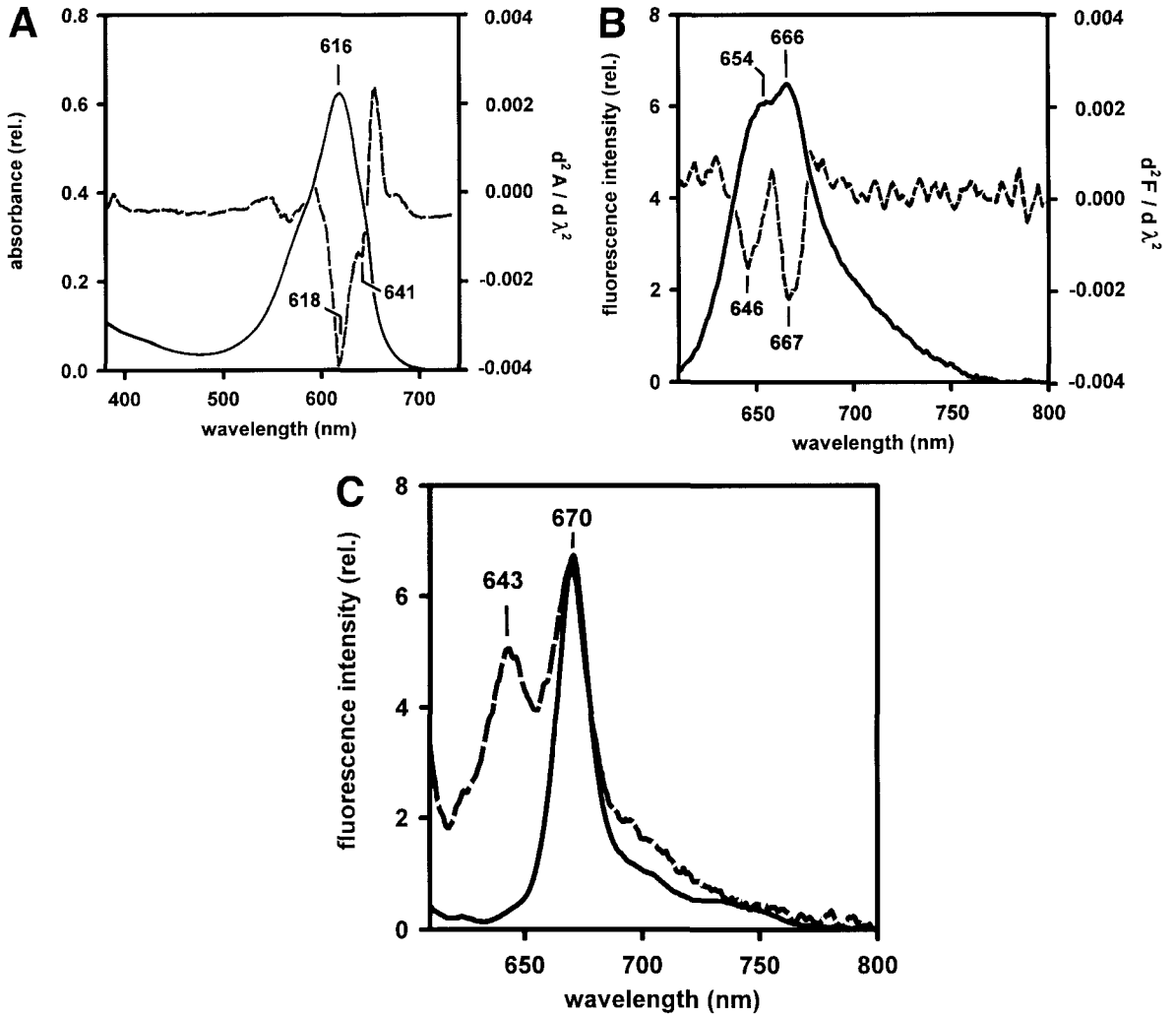

Fig. 2. a: Room temperature absorption spectrum (solid line) and second derivative (broken line). b: Room temperature fluorescence spectrum and second derivative (broken line). c: Low-temperature fluorescence spectrum in high-ionic-strength buffer (solid line) and low-ionic-strength buffer (broken line) of the phycobiliprotein aggregates of *Acaryochloris marina*. The excitation wavelength for the fluorescence spectra was 570 nm.

ever, the tight energy coupling was destroyed and a second fluorescence maximum at 643 nm was revealed as already shown in the room temperature spectrum. This can be explained by a partial dissociation of the PC-type complexes from the AP-type components resulting in the uncoupling of energy transfer. A similar behaviour has been described for PBS, which are stabilized in high-ionic-strength and dissociate in low-ionic-strength buffer and also change their fluorescence properties [18].

The biliprotein aggregates were separated by SDS-PAGE (Fig. 3a) to determine their subunit composition. Electrophoresis yielded two major bands with apparent molecular masses of 16.2 and 17.4 kDa and minor bands of 24.5, 26.3, 31.6, 51.3 and 57.5 kDa. The major bands carried bilin chromophores as indicated by an orange-coloured fluorescence when excited with ultraviolet light after treatment with $ZnSO_4$ (Fig. 3b). Their apparent molecular masses are similar to those of the α- and β-subunits of phycobiliproteins of red algae and cyanobacteria [18]. The true relation of these subunits to biliproteins was demonstrated by immunodecoration with antibodies against PC and AP from *Cyanidium caldarium*. The antiserum against PC does not crossreact with AP from *Cyanidium* or other sources, and vice versa. The antibodies, however, show crossreactivity with heterologous PC or AP, respectively, from other red algae and cyanobacteria [19]. The antiserum against PC gave rise to strong labelling of the 16.2 and 17.4 kDa bands (Fig. 3c). The antibody against AP showed a strong reaction with the 17.4 kDa band, while the labelling of the 16.2 kDa band was considerably weaker (Fig. 3d). The nature of the minor bands is still unsolved. They might be uncoloured linker polypeptides of phycobiliproteins (24.5–31.6 kDa), but it cannot be excluded that at least some of them (51.3–57.5 kDa) were only co-migrating with the biliprotein aggregates in the gradient.

The electron microscopic investigation of the negatively stained biliprotein aggregates showed a mainly homogenous fraction of rod-shaped structures (Fig. 4). The largest rods were 24.0 ± 1.0 nm long and 11.3 ± 1.1 nm in diameter (means of 50 particles ± SD). These rods were composed of four units of 5.8 ± 1.4 nm thickness, representing hexameric biliprotein units $(\alpha\beta)_6$, aggregated linearly with their flat faces. Crosslinking was not complete and smaller rods composed of three and two biliprotein hexamers are also observed. Circular structures of 11.7 ± 1.7 nm diameter, some with central holes,

represent face-on views of dissociated hexameric $(\alpha\beta)_6$ or dodecameric $(\alpha\beta)_6 \times (\alpha\beta)_6$ units making up the rod structures [20]. The biliprotein rods are made up of PC as the dominant component. The organization of the second pigment type, AP, within the rods remains unclear, because its concentration is rather low. We speculate, however, that one of the end-on biliprotein hexamers is heterogeneously composed of one PC and one AP-like trimer each.

The electron microscopic studies and the sedimentation experiments prove that the native biliprotein units of *Acaryochloris* are significantly smaller than intact PBS from cyanobacteria and red algae. They rather resemble the peripheral rods of these PBS. Nevertheless, we think that the rod-shaped aggregates represent the entire phycobiliprotein complexes of *Acaryochloris*, because (i) there is no indication for the existence of PBS structures in ultrathin sections of the cells [1], (ii) we could not isolate larger biliprotein structures under conditions that stabilize PBS of red algae and cyanobacteria, and (iii) within the isolated aggregates there is an efficient energy transfer from a PC-type biliprotein to a terminal AP-like emitter. Thus, *Acaryochloris* may contain a new type of biliprotein aggregate that has not been described previously in cyanobacteria. This structure can be regarded as a model for

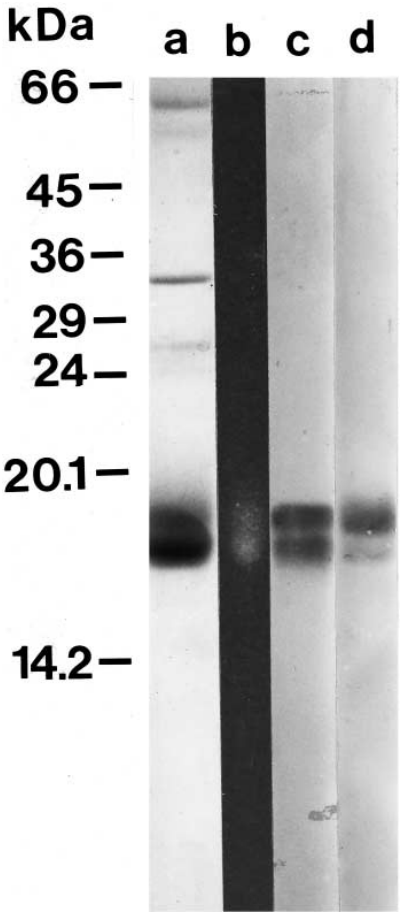

Fig. 3. Electrophoretic fractionation on 12% polyacrylamide gels of the phycobiliprotein aggregates of *Acaryochloris marina*. a: Coomassie-stained gel; (b) fluorograph of a gel after incubation in a solution of 2 mol/l glycine with 0.2 mol/l $ZnSO_4$ to visualize bilin-carrying subunits; (c) Western blot immunodecorated with an antibody against C-phycocyanin; and (d) Western blot immunodecorated with an antibody against allophycocyanin.

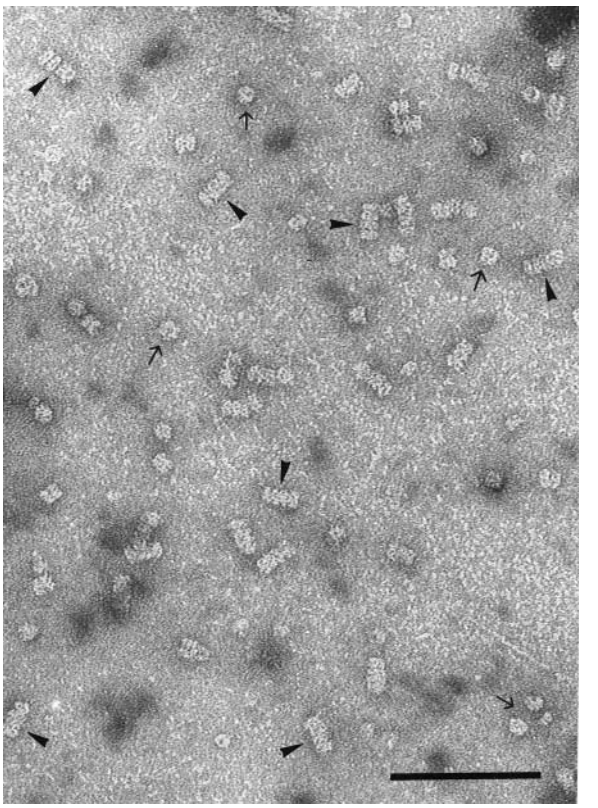

Fig. 4. Negatively stained phycobiliprotein aggregates of *Acaryochloris marina*. Samples were fixed with 0.1–0.3% glutaraldehyde and contrasted with 2% aqueous uranyl acetate. Intact aggregates (arrowheads) are rod-shaped and composed of four hexameric biliprotein units. Face-on views of dissociated biliprotein aggregates are indicated by arrows. Bar: 100 nm.

an early phycobilisome precursor [21], even though it may be a secondary degenerated phycobilisome. Taking into account the ultrastructural similarities between *Acaryochloris* and *Prochloron*-like organisms, one can speculate that biliproteins (when present) are organized in a similar way in other cyanobacteria with stacked thylakoids. The biliprotein aggregates are thought to be attached to thylakoid membranes because they are found in the non-soluble fraction of the cells before detergent treatment. They might be bound to unstacked thylakoid domains or be located within the thylakoid lumen. Their definite location and specific attachment sites have to be elucidated by further investigations.

Acaryochloris is a very unusual organism in two respects because it synthesizes Chl *d* as the main accessory pigment [1], and additionally the biliproteins PC and AP. To our knowledge, only one organism, the *Prochloron*-like cyanobacterium, *Prochlorococcus marinus* CCMP 1375 [9], shows a similarly complex pigmentation pattern, containing a Chl *a*/*b*/*c* antenna and PE simultaneously. Both investigations cast a new light on the evolution of oxygenic prokaryotes and chloroplasts. They support the view that the radiation of ancient oxygenic bacteria developed a lineage, or lineages, of organisms containing several types of Chl and biliprotein antennae simultaneously. A loss of selected antenna systems occurred during further evolution [22], thus leading to the occurrence of the Chl *a*/*b* lineage as well as the Chl *a*/*c*, Chl *a*/*b*/*c*, Chl *a*/biliprotein and Chl *a*/*c*/biliprotein branches.

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