REVIEW

Chlorophyll *d* and *Acaryochloris marina*: current status

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Abstract The discovery of the chlorophyll *d*-containing cyanobacterium *Acaryochloris marina* in 1996 precipitated a shift in our understanding of oxygenic photosynthesis. The presence of the red-shifted chlorophyll *d* in the reaction centre of the photosystems of *Acaryochloris* has opened up new avenues of research on photosystem energetics and challenged the unique status of chlorophyll *a* in oxygenic photosynthesis. In this review, we detail the chemistry and role of chlorophyll *d* in photosynthesis and summarise the unique adaptations that have allowed the proliferation of *Acaryochloris* in diverse ecological niches around the world.

Abbreviation

Acaryochloris	*Acaryochloris marina*
APC	Allophycocyanin
Chl	Chlorophyll
EPR	Electron paramagnetic resonance
ESE	Electron spin echo
FTIR	Fourier transform infrared spectroscopy
ORF	Open reading frame
PBP	Phycobiliprotein
PC	Phycocyanin
PE	Phycoerythrin
PEC	Phycoerythrocyanin
Phe	Pheophytin
PS	Photosystem

Q_A	The primary quinine electron acceptor of photosystem II
Y_Z	Redox-active tyrosine in photosystem II

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Chemistry of chlorophyll

Chlorophylls (Chls) are a group of tetrapyrrolic pigments essential for oxygenic photosynthesis, participating in photon capture, transduction and conversion into chemical energy. Currently five Chls have been identified in nature—Chl *a*, *b*, *c*, *d* and the recently discovered *f* (Chen et al. 2010) (Fig. 1) isolated from the filamentous cyanobacterium *Halomicronema hongdechloris* (Chen et al. 2012). Chlorophylls all share common structural features: a tetrapyrrole ring which coordinates a Magnesium (Mg) atom, with an isocyclic fifth ring adjacent to the third ring (Fig. 1). Attached to this tetrapyrrolic ring is a long hydrophobic phytol tail (which is almost always absent in the Chl *c* family (Zapata et al. 2006); Fig. 1b). The degree of saturation of the tetrapyrrolic ring differs amongst chlorophylls; Chlorophyll *c*-type pigments have a completely unsaturated phytoporphyrin (double bond at C17–C18), whereas the other Chls are phytochlorins (reduced C17–C18 double bond; Fig. 1). Anoxygenic photosynthetic bacteria utilise bacteriochlorophylls, most of which are bacteriochlorins which are like phytochlorins but with a reduced C7–C8 double bond (Scheer 2006). These differences in saturation of the chlorophyll macrocycle have profound consequences on their electronic absorption spectra. For example the phytochlorins Chl *a*, Chl *d*, and Chl *f* have approximately equal absorption intensities around 400–450 nm (blue, Soret bands) and 650–700 nm
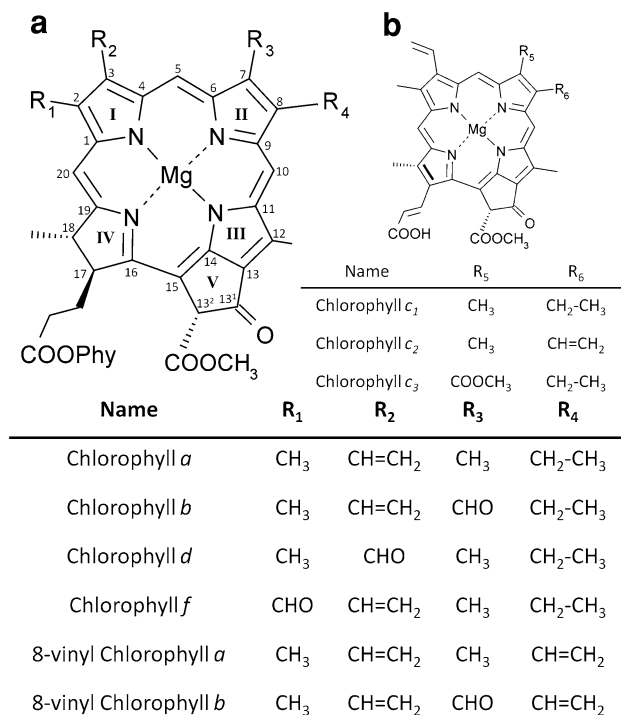
Fig. 1 Molecular structures of chlorophylls. **a** Structures of phytochlorin chlorophylls with variable side chains (R1–R4) listed below. **b** Structures of the three most common phytoporphyrin (Chl c-type) chlorophylls with the variable side chains (R5–R6) are shown below. Carbon atom and ring numbering are as for the phytochlorin. Note the C17–C18 double bond (*bold*) and the absence of the phytyl tail in the Chl c structure. *Phy* phytyl tail ($C_{20}H_{39}$)

Name	R5	R6
Chlorophyll c_1	CH_3	CH_2-CH_3
Chlorophyll c_2	CH_3	$CH=CH_2$
Chlorophyll c_3	$COOCH_3$	CH_2-CH_3

Name	R1	R2	R3	R4
Chlorophyll a	CH_3	$CH=CH_2$	CH_3	CH_2-CH_3
Chlorophyll b	CH_3	$CH=CH_2$	CHO	CH_2-CH_3
Chlorophyll d	CH_3	CHO	CH_3	CH_2-CH_3
Chlorophyll f	CHO	$CH=CH_2$	CH_3	CH_2-CH_3
8-vinyl Chlorophyll a	CH_3	$CH=CH_2$	CH_3	$CH=CH_2$
8-vinyl Chlorophyll b	CH_3	$CH=CH_2$	CHO	$CH=CH_2$

photosystem integrity and correctly positioning the macrocycles within the antenna and reaction centre.

Detection of chlorophylls

Chlorophylls can readily be distinguished from one another based on their spectral properties (Fig. 2a). The detection of pigments such as chlorophylls relies on the absorption and/or fluorescence properties of the molecule, whether in situ as pigment-protein complexes, or extracted in a monomeric state in a solvent. Combined with a suitable separation technology such as HPLC, the spectral properties of a chlorophyll offers power tools for identification and classification as well as provide information regarding their functions. The extinction coefficient of a chlorophyll is an important measure, allowing fast and accurate quantitation based on its absorbance. The extinction coefficients of Chl a and Chl b were initially reported by Mackinney (1941), followed by a report for simultaneous Chl a and Chl b determination by Arnon (1949). These extinction coefficients have since been revised by Smith and Benitez (1955), the accuracy of which has been confirmed by atomic mass spectrometry of the magnesium atom (Porra et al. 1989). The extinction coefficient for Chl d has recently been revised, as well as the first report detailing the extinction coefficient for Chl f (Li et al. 2012).

Chlorophyll d—the major photopigment of Acaryochloris marina

Chl d was discovered 70 years ago (Manning and Strain 1943) and was initially believed to be a minor chlorophyll in red algae. Although its structure was proposed over 50 years ago (Holt and Morley 1959; Holt 1961), it was only in 1996, with the discovery of the predominantly Chl d-containing cyanobacterium *Acaryochloris marina* (named as *Acaryochloris* through the text) that the significance of Chl d became apparent, with Chl d constituting up to 90–99 % of total chlorophylls(Miyashita et al. 1996). The structure of Chl d is identical to Chl a, apart from a substitution of the C3 vinyl group in ring I of Chl a for a formyl group (Fig. 1a), causing a red shift in the Q_y maximum absorbance peak, from 665 nm for Chl a to 697 nm for Chl d and a characteristic 'double' soret band at 400 and 455 nm in methanol (Fig. 2a). These spectral shifts are the result of an electronic redistribution along the y-axis of the tetrapyrrole ring, caused by the electronegative C3 formyl group (Hoober et al. 2007).

Function of chlorophyll d

Chlorophylls can broadly be divided into two groups based on their function in either the antenna system only or in

(red, Q_Y bands), with an absorbance 'trough' in the green spectral region (Fig. 2a). The more electronically symmetrical phytoporphyrins (Chl c-type) on the other hand, absorb only weakly in the red spectrum, and much more intensely around 450 nm (Zapata et al. 2006). The absorbance spectrum of the phytochlorin Chl b is intermediate between other phytochlorins and the phytoporphyrins, with a Soret:Q_Y ratio of 2.8 in methanol (Fig. 2a). Different macrocycle peripheral groups can also modulate the electronic configuration, resulting in significant, biologically relevant changes in their absorbance spectra (Hoober et al. 2007) (Fig. 2a). The Mg atom coordinated by the macrocycle is important in mediating interactions with proteins as well as maximising the excited state lifetime of the molecule. Exceptions to this ubiquitous Mg atom can be found in two unrelated bacteria *Acidiphilium rubrum* and *Candidatus* Chloracidobacterium thermophilum, both of which contain bacteriochlorophyll a with a chelated zinc (Zn) atom rather than Mg (Wakao et al. 1996; Tsukatani et al. 2012). Less is known about the role of the phytyl tail of chlorophylls. Mutants in phytyl synthesis are sensitive to photo-oxidative stress and have reduced photosytsem stabilities (Bollivar et al. 1994; Shpilyov et al. 2013), suggesting a role for the flexible phytyl tail in maintaining
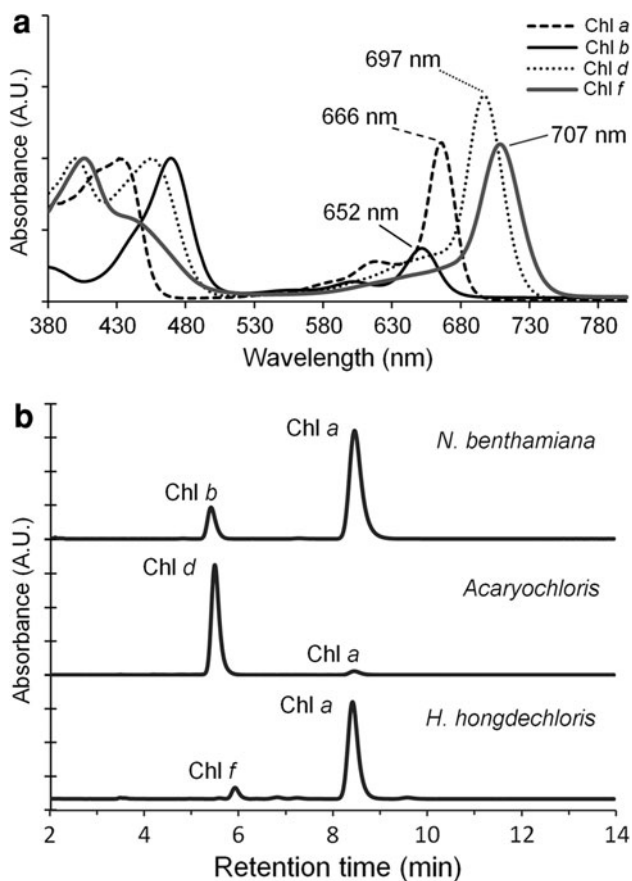
Fig. 2 Online spectra and HPLC chromatography of chlorophylls. Total pigment was extracted from ground *Nicotiana benthamiana* leaves, *Acaryochloris* or H. *hongdechloris* cells with 100 % methanol and directly subjected to RP-HPLC analysis. **a** Online spectra were collected for each chlorophyll peak detected and plotted with data normalised to the Soret maxima. The Q_y maximum for each chlorophyll is indicated. **b** Maximum absorbance between 620 and 720 nm of total pigment from the photosynthetic organisms was plotted against time. The identity of each chlorophyll peak is indicated

both the antenna and reaction centre of the photosystems. Chl *b*, Chl *c* and most likely Chl *f* (Akutsu et al. 2011; Chen et al. 2012) are accessory pigments found in the antenna system of the photosynthetic organism, harvesting light and transferring energy; however, they are not involved in charge separation or electron transfer. The primary role of accessory pigments is to extend the photosynthetically active range of an organism. For example, Chl *b* is found in photosynthetic eukaryotes and prochlorophytes and its absorbance spectrum (Fig. 2a) allows these organisms to absorb light towards the middle (green) part of the visible spectrum. On the other hand Chl *f*, which has been found in the marine filamentous cyanobacterium *H. hongdechloris* (Chen et al. 2012) and a freshwater unicellular cyanobacterium from Lake Biwa, Japan (Akutsu et al. 2011) extends the photosynthetically available

light for these organisms further into the far-red region of the spectrum (Fig. 2a).

Until relatively recently it was believed that the only redox active chlorophyll in the reaction centre was Chl *a* (and its derivatives). Following the 'rediscovery' of Chl *d*, however, there has been a shift in this paradigm and Chl *d* has been shown to act as a primary electron donor in the reaction centre of the *Acaryochloris* photosystem I (PS I) (Hu et al. 1998; Tomo et al. 2008) and probably photosystem II (PS II) (Nieuwenburg et al. 2003; Chen et al. 2005d; Tomo et al. 2007; Itoh et al. 2007). Analogous to the Chl *a* special pair in plant and other cyanobacterial PS I, the Chl *d* special pair is a Chl *d*/Chl *d'* (a Chl *d* 13^2-C epimer) heterodimer (Tomo et al. 2008). Despite the fact that 90–99 % of the chlorophyll in *Acaryochloris* is Chl *d* (Miyashita et al. 1996), Chl *a* still plays a critical role in *Acaryochloris*, with small amounts of pheophytin *a* (Phe *a*; demetalated Chl *a*) being detected in PS II, and Chl *a* being detected in both PS I and PS II (Tomo et al. 2007, 2008). There is still some debate regarding the exact stoichiometry and role of Chl *a* in the *Acaryochloris* photosystems (Tomo et al. 2007, 2008, 2011; Tsuchiya et al. 2012a); however, a clearly defined role for Phe *a* acting as the primary electron acceptor in *Acaryochloris* PS II has being confirmed (Razeghifard et al. 2005). Furthermore, there have been no reports of Phe *d* being detected in *Acaryochloris* to date. *In vitro* redox potential measurements have demonstrated the higher oxidative potential of Phe *d* compared with Phe *a* which may explain the presence of Phe *a* and not Phe *d* in *Acaryochloris* (Kobayashi et al. 2007).

Synthesis of chlorophylls

The total synthesis of Chl *a* was described in exquisite detail in 1990 by Woodward and colleagues (Woodward et al. 1990). In brief, the first step in the process involves synthesising each of the four rings. 'Left hand' (rings I and IV) and 'right hand' (rings II and II) components are then synthesised and combined to generate a (porphyrin) tetrapyrrole ring. The remaining steps in the synthesis focus on reduction of the porphyrin to a chlorin, various side chain substitutions, and the formation of the isocyclic ring V. This chemical synthesis of Chl *a* requires 46 steps in total, with numerous acids, reducing agents, catalysts and solvents necessary for completion. From Chl *a* to Chl *d* the relatively simple C3 vinyl group substitution for formyl can be obtained using either a strong oxidising agent (Holt and Morley 1959) or a thiol under acidic conditions (Fukusumi et al. 2012). Fukusumi et al. (2012) achieved a Chl *d* yield of 31 % using reaction conditions, where Chl *a* was dissolved in tetrahydrofuran to which thiophenol and acetic acid were added. These methods in synthesising Chl *d* from Chl *a* demonstrate the general susceptibility of the

C3 side chain to nucleophilic attack and oxidative cleavage, and offer hints as to how Chl *d* may be synthesised in vivo.

Biosynthesis of chlorophylls

Biosynthesis of Chl *a*

The synthesis of biological tetrapyrroles, including chlorophylls, is a metabolically expensive process. The first committed precursor in tetrapyrrole synthesis is 5-aminolevulinic acid (5-ALA), derived from either glutamate or glycine and succinyl-coenzyme A (Beale 2006; Jahn et al. 2006). Eight 5-ALA molecules are condensed through a series of reactions to form protoporphyrin IX, a tetrapyrrolic ring molecule from where heme and phycobilin synthesis branches from chlorophyll synthesis through the chelation of either Fe or Mg, respectively (Yaronskaya and Grimm 2006). For Chl *a* synthesis, the final few reactions catalyse the oxidative formation of the fifth isocyclic ring, reduce the C17–C18 and $C8^1$–$C8^2$ double bonds, and phytylate the molecule (Rudiger 2006). The genes encoding all the enzymes in this biosynthetic pathway have been elucidated in cyanobacteria (Bollivar 2006) and more recently higher plants, with the characterisation of a 3,8-divinyl protochlorophyllide *a* 8-vinyl reductase (DVR) from *Arabidopsis thaliana* (Nagata et al. 2005). Using an elegant bioinformatics approach, Ito and colleagues were also able to identify an unrelated gene in *Synechocystis*, a cyanobacterium which does not have an orthologue of DVR, that was essential for the reduction of the 8-vinyl group (Ito et al. 2008). It is of interest to note that the marine cyanobacterium *Prochlorococcus* lacks this reductase and uniquely uses 8-divinyl chlorophyll *a* and *b* for photosynthesis (Chisholm et al. 1992; Nagata et al. 2005) (Fig. 1a). There have been no reports to date describing 8-divinyl variants of Chl *d* or Chl *f*.

Biosynthesis of Chl *b* and Chl *d*

Chl *a* is found in almost all oxygenic photosynthetic organisms identified to date and is thought to be the progenitor chlorophyll, uniquely suited for its roles in photosynthesis(Björn et al. 2009). The other chlorophyll molecules are also most likely synthesised along the same biosynthetic pathway as Chl *a*, diverging at the last few steps of biosynthesis or alternatively being synthesised from Chl *a* itself. Chl *b*, Chl *d* and Chl *f* are identical to Chl *a* apart from a formyl substitution at the C7, C3 or C2 position in the macrocycle respectively (Fig. 1a). These substitutions affect the polarity of the molecule similarly (Fig. 2b) but distinctly perturb the electronic configuration in the macrocycle, as evidenced by their different absorption spectra (Fig. 2a). The enzyme responsible for the synthesis of Chl *b*, chlorophyll(lide) *a*-oxygenase (CAO) has been identified and characterised (Tanaka et al. 1998; Espineda et al. 1999; Oster et al. 2000). CAO is specific for Chlide *a* in vitro (Oster et al. 2000) however in vivo it may be that both Chl *a* and Chlide *a* act as a substrate and there is some evidence that protochlorophyllide *a* can also act as a substrate under unusual physiological conditions (Xu et al. 2002). Surprisingly, heterologous expression of a CAO gene in *Acaryochloris* led to the synthesis of a novel chlorophyll, termed P_{672} (or [7-formyl]-Chl *d*) based on its Q_y maximum (Tsuchiya et al. 2012b). This new chlorophyll has formyl groups at the C3 and C7 positions, similar to Chl *d* and Chl *b* respectively, suggesting that both the as yet uncharacterised 'Chl *d* synthase' and the CAO work in concert to form P_{672}. [7-formyl]-Chl *d* is incorporated into the antenna-PS II of CAO-expressing *Acaryochloris* however it does not function in primary electron transfer (Tsuchiya et al. 2012a). The amount of Chl *d* per PS II is reduced in these cells, whereas Chl *a* and Phe *a* levels remain unchanged (Tsuchiya et al. 2012a), providing strong evidence for the essential role of Chl *a* in *Acaryochloris* photosystems (see "PS I" section).

Chl *a* has five oxygen atoms in its structure, four of which are derived from H_2O and the fifth from molecular oxygen (Walker et al. 1989; Porra and Scheer 2000). Based on the incorporation of two ^{18}O-labelled oxygens from molecular oxygen into the Chl *d* molecule, Schliep et al. (2010) proposed that the oxygen in the C3 formyl group of Chl *d* is added via an oxygenase-type reaction. Furthermore, the timing of incorporation of the labelled oxygen into Chl *a* and Chl *d* indicates that Chl *d* is synthesised directly from Chl *a* and probably not from a Chl *a* precursor. Perhaps more evidence to support the synthesis of Chl *d* directly from Chl *a* may be found from the CAO-expressing *Acaryochloris* described by Tsuchiya et al. (2012a, b). They detected no decrease in the Chl *a* in these cells despite the newly synthesised [7-formyl]-Chl *d* constituting up to 10 % of the total chlorophyll. There was, however, a reduction in Chl *d* levels, suggesting that the substrate for the CAO enzyme is downstream of Chl *a* biosynthesis (but not Chl *d* biosynthesis). This interpretation would mean that Chl *d* is synthesised from Chl *a,* and that the CAO enzyme acts on Chl *d* which is somewhat controversial as the only confirmed substrate for CAO is Chlide *a* (Oster et al. 2000). Alternatively, CAO may be converting Chlide *a* to Chlide *b*, reducing the available Chlide *a* for Chl *a* biosynthesis. The 'Chl *d* synthase' enzyme(s) has yet to be characterised, however it has been suggested a cytochrome P450, which commonly catalyse mono-oxygenase type reactions, may be involved (Chen and Blankenship 2011). It is unknown whether Chl *f* is

synthesised from Chl(ide) *a* or some other intermediate in the Chl *a* biosynthetic pathway however it seems logical that the reaction may be similar to Chl *b* synthesis, with the C2 methyl group being substituted for a formyl, possibly with a hydroxymethyl intermediate (Kräutler 2011).

Biology of *Acaryochloris marina*

Ecological distribution

Acaryochloris was first isolated from a squeezed extract of didemnid ascidians, *Lissoclinum patella*, collected from Palau Island (Miyashita et al. 1996). The phylogenetic study on small subunit rDNA sequences showed that *Acaryochloris* falls into the cyanobacteria radiation and forms a divergent clade, a new genus in cyanobacteria (Fig. 3) (Miyashita et al. 2003). Miyashita et al. were originally attempting to isolate and culture *Prochloron*, a symbiotic cyanobacterium containing Chl *a* and Chl *b* (Lewin and Withers 1975; Takaichi and Mochimaru 2007). Instead of *Prochloron*, they found small yellowish-green colonies formed by the Chl *d*-containing cyanobacterium *Acaryochloris marina*. For a long time after the discovery of *Acaryochloris*, it was presumed that it was a symbiont, similar to *Prochloron*, although it could be cultured and grown autophototrophically (Miller et al. 2005; Larkum and Kühl 2005). A microphotometric survey revealed that *Acaryochloris* grows on the underside of the didemnid ascidians where the visible light was strongly depleted by ascidian tissue and its symbiotic *Prochloron*. It is likely this far-red light enriched environment provides the selective pressure for the Chl *d*-containing organism (Kühl et al. 2005).

In 2004, a free living *Acaryochloris* strain was isolated as an epiphytic cyanobacterium from a range of red algae: *Ahnfeltiopsis flabelliformis*, *Callophyllis japonica* and *Carpopeltis prolifera* (Murakami et al. 2004). This discovery corrected the previous understanding that Chl *d* is a minor photopigment associated with red-algae (Manning and Strain 1943) and also raised the question of how widely Chl *d* and its containing organisms are distributed. We now know *Acaryochloris* spp, and therefore Chl *d*, are found widely through both aquatic and terrestrial ecological systems including: the intertidal zone of tropical reefs (Mohr et al. 2010), epibolic biofilms on colonial ascidians (Martínez-García et al. 2011), inside the tissues of didemnid ascidians (López-Legentil et al. 2011), eutrophic hypersaline lake (Miller et al. 2005), temperate fresh water lake and saline lakes (Kashiyama et al. 2008), high altitude lakes (Fleming and Prufert-Bebout 2010), terrestrial epilithic and endolithic biofilms (McNamara et al. 2006; De Los Ríos et al. 2007), endolithic biofilm under crustose

coralline algae (Behrendt et al. 2011), sea sediments (Kashiyama et al. 2008) and stromatolites (Goh et al. 2008; Li et al. 2013). In addition, a recent environmental survey examining *Acaryochloris* associated with different marine invertebrates from the coast of the Republic of Palau identified numerous *Acaryochloris* phylotypes, even from the same invertebrate, and found no clear relationship between specific phylotypes associating with specific invertebrates, nor any clear geographical bias (Ohkubo and Miyashita 2012). Up to now, five strains of *Acaryochloris* have been successfully isolated and cultured: *Acaryochloris* MBIC11017 isolated from the squeezed extract of didemnid ascidian in Palau Island (Miyashita et al. 1996, 2003), *Acaryochloris* AWAJI-1 isolated as an epiphyte form red algae in Japan (Murakami et al. 2004), *Acaryochloris* sp.CCMEE 5410 isolated form eutrophic hypersaline lake, Salton Sea (Miller et al. 2005), *Acaryochloris* sp HICR111A isolated form intertidal zone surrounding dead coral in Heron Island, Australia (Mohr et al. 2010) and the recently isolated *Acaryochloris* sp MPGRS1, an epiphyte from red algae *Gelidium caulacantheum* collected from Georges River, Australia (Larkum et al. 2012). The 16s rDNA phylogenetic tree demonstrates the monophyletic nature of all five cultured *Acaryochloris* which are all clustered together, distinct from other cyanobacterial classes (Fig. 3).

The ecological significance of *Acaryochloris* and the contribution of Chl *d*-photosynthesis in the biosphere are still largely unknown. The diversity of ecological niches that are associated with *Acaryochloris* spp or *Acaryochloris*-like organisms causes difficulties in understanding the roles of Chl *d*-photosynthesis as global primary producers. All *Acaryochloris* spp are found to be associated with other organisms in their natural habitats although some of them are successfully cultured in the laboratory. More robust growth, at least in the case of *Acaryochloris* MBIC 11017, was observed when grown with some coexisting bacterial contaminations in the medium (Swingley et al. 2005).

Cell structure

Acaryochloris is a unicellular cyanobacterium, normally 1.8–2.1 μm × 1.5–1.7 μm in size (Fig. 4a, b). The cells are either spheroidal or ellipsoidal in shape and are yellow-greenish in colour due to the chlorophyll-binding protein complexes in thylakoid membranes (Fig. 4c). Amongst five cultured strains, *Acaryochloris sp.* HICR111A and *Acaryochloris sp.* MPGRS1 are relatively smaller in size, being 1–2 μm × 0.75–1 μm and 1.67 ± 0.23 μm × 1 ± 0.14 μm, respectively (Mohr et al. 2010; Larkum et al. 2012).

Cyanobacteria are Gram-negative prokaryotes, having a layer of peptidoglycans as a part of the cell envelope. Most
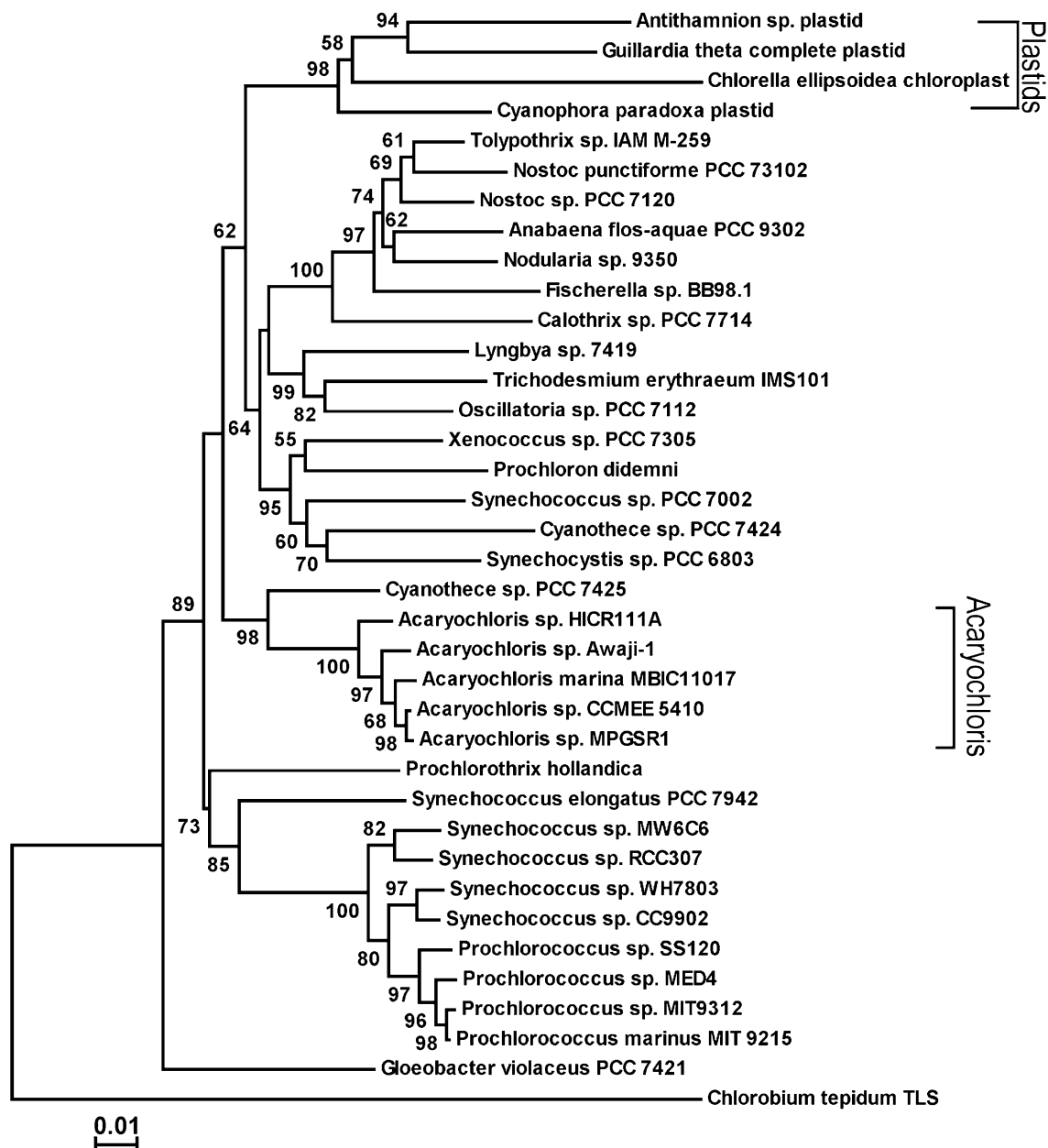
Fig. 3 16S rRNA phylogenetic tree of *Acaryochloris* spp with other cyanobacteria and eukaryotic plastids. It was constracted with MEGA (molecular evolutionary genetics analysis) using Neighbour-joining method. The 16S rRNA sequences are aligned with multiple sequence aligment tools (ClustalW). The alignment was then manually edited based on the alignment of Mohr et al. (2010). The evolution distance was calculated with Jukes–Cantor model. The phylogenetic tree stability was evaluated by bootstrap replication at 10000 times. The sequence data of *Acaryochloris* sp. Awaji-1 and *Acaryochloris* sp. HICR111A are obtained from NCBI with accession number of AB112435 and EU873540, respectively. The sequence data of *Acaryochloris* sp. MPGRS1 is kindly provided by Yaqiong Li. The sequences of four plastids and the other cyanobacteria are downloaded form NCBI as described by Miyashita et al. (2003) and Mohr et al. (2010)

Gram-negative bacteria possess a peptidoglycan layer ∼2–6 nm in thickness. Unicellular cyanobacteria on the other hand, have a thicker peptidoglycan layer of ∼10 nm and filamentous cyanobacteria possess an even thicker peptidoglycan layer of ∼15–35 nm (Hoiczyk and Hansel 2000). The peptidoglycan layer in *Acaryochloris* is about 10 nm, which is similar to other unicellular cyanobacteria,

is sandwiched between the cytoplasmic membrane and the outer membrane (Marquardt et al. 2000).

There are 7–11 layers of thylakoid membranes peripherally surrounding the cytoplasm. These membranes are predominantly stacked and phycobiliprotein (PBP) arrays are located between the membranes in non-stacked area, although it is uncertain as whether they are attached to
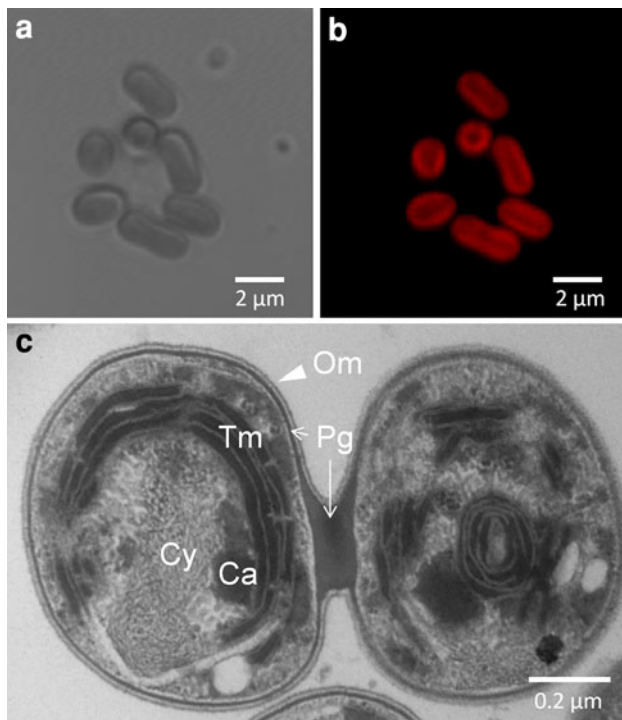
Fig. 4 Confocal light and electron microscopic photography of *A. marina* cells. **a** Transmitted light and **b** corresponding confocal light microscopic images of *Acaryochloris* cells ($\lambda_{ex} = 488$ nm, $\lambda_{em} > 692$ nm). **c** Transmission electron micrograph of a dividing *A. marina* cell. The majority of chlorophyll-derived fluorescence is observed around the periphery of the cells in **b**, corresponding to the thylakoid membrane (Tm) **c**. *Cy* cytoplasm, *Ca* carboxysome, *Pg* peptidoglycan (*arrows*), *Om* outer membrane (*arrowhead*)

cytoplasm side of the membranes (Chen et al. 2002, 2009; Swingley et al. 2005). Instead of a usual phycobilisome in other cyanobacteria, an array structure of PBP was observered. This PBP array structure is present in *Acaryochloris* sp MBIC 11017, but absent in *Acaryochloris* CCMEE 5410 which lacks PBP proteins (Chen et al. 2009). The effect of iron availability on thylakoid membrane has been examined in *Acaryochloris* MBIC 11017. Under iron-enriched conditions, thylakoid membranes were evenly spaced from 20 to 35 nm apart by PBP arrays and the stacked structure was only observed around the end and 'corners' of the cell occasionally (Swingley et al. 2005). This dramatic change in thylakoid membrane and photosynthetic apparatus is in agreement with the previous study that Chl *d*-binding light-harvesting proteins are the major light-harvesting system for PS I and PS II under iron-stressed conditions (see 'Light-harvesting system in *Acaryocloris*' section). Carboxysomes with a typical polyhedral shape ranging from 170 to 230 nm are observed in the central cytoplasmic part (Marquardt et al. 2000). Interestingly, the thylakoid membrane stacks in *Acaryochloris* are perforated by channel-like structures connecting central and peripheral

cell portions. This ultrastructural feature has not been found in other organisms (Marquardt et al. 2000).

Pigment composition

Acaryochloris has an exceptional pigment complement. As well as containing Chl *d* as its major pigment, *Acaryochloris* also contains Chl *a*, zeaxanthin, α-carotene and phycobilins as minor pigments (Miyashita et al. 1997). MgDVP-like pigment and allophycocyanin are also detected as trace pigments under certain cultural conditions. Chl *d'* and Phe *a* are present as minor components, but neither Chl *a'* nor Phe *d* is found in *Acaryochloris* (Akiyama et al. 2001). Chl *d* makes up about 80 % of the total lipid-soluble pigment of the cell and more than 2 % of the cell dry weight (Miyashita et al. 1997). Chl *a* only presents as a minor pigment in *Acaryochloris*. Its content varies in different light conditions from 1 to 10 % of the total lipid-soluble pigment. The Chl *a*/Chl *d* ratio also varies, between 0.03 and 0.1, depending on the growth conditions (Mimuro et al. 2004), with cells grown under high light intensity and micro-aerobic condition showing a higher Chl *a*/Chl *d* ratio (Mimuro et al. 2004; Lin et al. 2013).

In all prokaryotes, α-carotene and its derivatives are only found in two genera of cyanobacteria: the 8-vinyl Chl *a* and 8-vinyl Chl *b*-containing *Prochlorococcus* and *Acaryochloris*. α-carotene in *Acaryochloris* replaces all the functions of ß-carotene in cyanobacteria. It has a unique C-6′(6′S)-chirality, which is the first evidence of the natural occurrence of (6′S)-α-carotene (Takaichi et al. 2012).

Genome diversity and regulation of gene expression

The genomes of *Acaryochloris* strain MBIC 11017 and CCMEE 5410 were sequenced in 2008 and 2011, respectively (Swingley et al. 2008; Miller et al. 2011). Both strains have enormous genomes compared to other unicellular cyanobacteria. The strain MBIC11017 has a genome of 8.36 Mb, with 8528 predicted ORFs, while the strain CCMEE 5410 genome is slightly smaller than MBIC11017, with a size of 7.88 Mb and 8383 predicted ORFs. They share 6112 putative orthologs, although there are more than 25 % predicted ORFs in each genome which was absent from the other (Miller et al. 2011). A preliminary genome study on *Acaryochloris* sp. HICR111A also showed a surprisingly expanded genome, estimated as ∼8.37 Mb, however due to contamination the sequencing could not be completed (Mohr et al. 2010). In strain MBIC11017, along with the main circular chromosome DNA, there are 9 distinct plasmids which harbour >20 % of the total genome content. A comparison of *Acaryochloris* and other bacterial genome sizes and gene numbers are

show in Table 1. The *Acaryochloris* genome is approximately four-fold larger than the *Prochlorococcus* genome, more than two times the size of *Synechocystis* PCC 6803, and of similar size to the genome of the filamentous cyanobacterium, *Nostoc* ATCC 29133.

The genomes of MBIC11017 and CCMEE 5410 are conserved and share a similar GC content of 47 %. About 89 % of ORFs on the strain MBIC11017 chromosome are homologous to strain CCMEE 5410. Differences, however, are concentrated on ORFs from plasmids with approximately 77 % of ORFs in MBIC11017 plasmids having no homologues in CCMEE 5410 genome (Swingley et al. 2008; Miller et al. 2011).

Comparative analysis of the strain MBIC 11017 and CCME 5410 genomes suggest both have an unusually high number of gene duplicates (Miller et al. 2011). They hypothesise that one reason for these gene duplicates is the presence of multiple copies of *rec A*, which encodes an enzyme necessary for homologous recombination. However, they also observe that these duplicates are rapidly removed from the genome (Swingley et al. 2008; Miller et al. 2011). Interestingly the rate of duplication and duplicate loss in *Acaryochloris* falls in the range of eukaryotes rather than bacteria and many of these duplicated genes may come from foreign origin through horizontal gene transfer (Miller et al. 2011). Irrespective of why, the expanded genome clearly allows a high level of gene expression plasticity and the large number of gene duplications provides the raw materials for neofunctionalization (Miller et al. 2011). The

Table 1 Genome comparison of *Acaryochloris* spp to other cyanobacteria or bacteria

Strains	Genome size (Mbp)	Gene number	Description
Escherichia coli str. K-12 substr. MG1655[a]	4.64	4496	Bacteria
Synechocystis sp. PCC6803[b]	3.96	3725	Cyanobacteria
Nostoc punctiforme ATCC 29133[b]	9.06	6794	Cyanobacteria
Prochlorococcus marinus MED4[b]	1.66	1756	Cyanobacteria
Prochlorococcus marina MIT9313[b]	2.41	2326	Cyanobacteria
Acaryochloris marina MBIC11017[b]	8.36	8462	Cyanobacteria
Acaryochloris sp. CCMEE 5410[a]	7.88	7587	Cyanobacteria
Acaryochloris sp. HICR111A[c]	8.37	N/A	Cyanobacteria

[a] Genome information from NCBI Genbank

[b] Genome information form Cyanobase http://genome.kazusa.or.jp/cyanobase/

[c] Genome information form Mohr et al. (2010)

gene(s) encoding the 'Chl *d* synthase' may have evolved through such means. A large repertoire of 'genetic options' that comes with a large genome is probably important for the ability of *Acaryochloris* to exploit diverse ecological niches. It has also been proposed that organisms with a large genome are more likely to occupy a non competitive environment where resources (in case of *Acaryochloris*, the light source) are scarce but diverse in nature (Swingley et al. 2008; Konstantinidis and Tiedje 2004).

Important cellular metabolism

Nitrogen-fixation

The capacity of nitrogen-fixation has been reported in many cyanobacteria (Zehr 2011). Since the nitrogen-fixing enzyme complex, nitrogenase, is extremely oxygen sensitive, oxygen-evolving cyanobacteria have had to adopt strategies to achieve an anaerobic environment for this enzyme (Bergman et al. 1997).

For heterocystous cyanobacteria, oxygenic photosynthesis and nitrogen-fixation are separated spatially, with photosynthesis carried out in vegetative cells, whereas nitrogen-fixation takes place in specialised cells called heterocysts which have low oxygen permeability (Fay 1992). Other non-heterocystous solve this problem by separating photosynthesis and nitrogen- fixation by time. In these cyanobacteria, photosynthesis is performed during the light period (day time) and nitrogen-fixation happens in the dark periods (night) (Gallon 2005).

The best studied *Acaryochloris* strains, sp. MBIC11017 and sp. CCMEE 5410, do not have complete set of genes for the nitrogenase enzyme complex (Swingley et al. 2008; Miller et al. 2011) However, a complete set of genes encoding the nitrogenase complex was reported in the unfinished *Acaryochloris* sp HICR111A genome sequence (Mohr et al. 2010; Pfreundt et al. 2012). Phylogenetic comparison revealed that they are more closely related to the nitrogenase in *Trichodesmium erythraeum*, which suggest the genes may be recruited recently by horizontal gene transfer (Pfreundt et al. 2012). By measuring the gene expression and the nitrogenase activities, Pfreundt et al. (2012) confirmed nitrogenase activity in *Acaryochloris* sp HICR111A.

Although strains MBIC11017 and CCMEE 5410 do not have the essential set of nitrogenise genes, little is known regarding nitrogen metabolism in these strains. Recently, a new class of cyanobacterial PS II D1 protein was identified and designated as 'rogue' D1 proteins (rD1). Genes encoding the rD1 protein are almost exclusively found in dizaotroph or organisms that contain nitrogenase complexes, including *Acaryochloris* sp. HICR111A. Interestingly, *Acaryochloris* strain MBIC11017 which does not have nitrogenase genes is the only exception known by so

far. The details on nitrogen metabolism are required in the future for understanding the relationship between photosynthesis and nitrogen metabolism, and the role of rD1 (Murray 2012).

Fatty acid content

Cyanobacteria are diverse in their fatty acid composition. Based on the pattern of desaturation of their fatty acids, cyanobacteria are divided into five groups: group 1 cyanobacteria- contain saturated and mono-unsaturated fatty acids; group 2 and group 3—cyanobacteria contain either α-linolenic acid (C18.3ω3) or γ-linolenic acid (C18.3ω6), respectively; group 4 cyanobacteria produce both α-linolenic acid (C18.3ω3) and γ-linolenic acid (C18.3ω6) as well as stearidonic acid (C18.4ω3) and group 5 cyanobacteria which contain polyunsaturated fatty acid with only two double bonds (Murata et al. 1992; Cohen et al. 1995). Gao et al. analysed the fatty acid composition of *Acaryochloris* MBIC 11017 and reported the most prominent single fatty acid was C16.0 with percentage of 52 % of total fatty acids extraction, although no C16 unsaturated fatty acid was detected. This strain also contains a mixture of C18 unsaturated fatty acids, predominantly stearidonic acid (C18.4ω3), which constitutes 19 % of the total fatty acids extracted. The minor C18 unsaturated fatty acids included C18.3ω3, C18.3ω6, C18.2ω6, C18.2, C18.1ω9, C18.1 and C18.0, with no C20 or higher fatty acids observed (Gao et al. 2010). Based on the presence of C18.4ω3, C18.3ω3, C18.3ω6, *Acaryochloris* MBIC 11017 belongs to group 4 cyanobacteria which including *Synechocystis* PCC 6803. However, these two specieses do not fall in the same group based on their 16s rRNA phylogenetic classification (Fig. 3).

Gao et al. also reported a fusion gene with an N-terminal catalase-related allene oxide synthase domain and a C-terminal lipoxygenase domain. Based on the origin of the oxygen in the 13S-hydroxyl group they concluded the intermediate in this catalase-related allene oxide synthase activity is ionic (Gao et al. 2009). Furthermore, they found the C-terminal lipoxygenase of this fusion gene has a unique catalytic activity that specifically utilizes the terminal pentadiene of omega-3 or omega-6 fatty acids. All omega -3 polyunsaturated fatty acids in *Acaryochloris* are oxygenated at the n-7 position which has never been reported before (Gao et al. 2010). Interestingly the α-carotene in *Acaryochloris* was recently found to have an C-6′(6′S)-chirality, which is also unique in nature (Takaichi et al. 2012). Considering the unique photopigments (Chl *d* and α-carotene) found in *Acaryochloris*, it may be that an unusual membrane environment to harbour them and their associated photosystems is necessary. It has been reported in cyanobacteria that the presence of unsaturated fatty acids accelerate the synthesis of D1 protein de novo, and thus protect the photosystem form high light damage (Gombos et al. 1997).

Hydrogenase and hydrogen production

Hydrogen is a potential energy source for the future. It is clean and one of its sources, water, is ubiquitous on earth. Photosynthetic hydrogen production utilizes the energy from sunshine and produce molecular hydrogen, which is both 'ecofriendly' and renewable. Oxygenic photosynthesis collects the solar energy via an antenna system and extracts electrons and protons from water at the oxygen evolution centre in PS II. The transmembrane proton gradient created during this process is utilized for ATP synthesis and the electrons are passed via an electron transfer pathway to ferredoxin, an iron-sulphur protein that is located at the reaction centre of PSI which further carries electrons to other pathways. Under normal aerobic conditions, these electrons reduce $NADP^+$ to NADPH, an important source of reducing power for CO_2 fixing in the Calvin-Benson cycle. However under anaerobic conditions, hydrogenase (or nitrogenase) can accept electrons from reduced ferredoxin and then convert protons to molecular hydrogen (or fixing N_2).

There are two types of hydrogenase found in prokaryotes: the uptake hydrogenase which consumes the hydrogen generated during the nitrogen-fixation; and the bidirectional or reversible hydrogenase which can either consume or produce hydrogen (Allakhverdiev et al. 2010a).

Some cyanobacteria have both types of hydrogenase, some only have a single type of hydrogenase, either uptake or bidirectional, and others do not have any kind of hydrogenase. All hydrogenases in cyanobacteria have a NiFe reaction centre which is highly sensitive to oxygen only functional under anaerobic conditions. Despite this obstacle, hydrogenase activity has been detected in a wide range of genera in cyanobacteria under various culture conditions (Dutta et al. 2005).

Acaryochloris MBIC 11017 has a single bidirectional hydrogenase encoded by *hoxEFUYH* on the plasmid pREB4 (Swingley et al. 2008). The arrangement of these *hox* genes is similar to that of *Synechocystis* PCC 6803, where the bidirectional hydrogenase is encoded by the *hoxEFUYH* operon with the promoter upstream the *hoxE* gene (Kiss et al. 2009). However RT-PCR of the *hox* genes showed that their regulation pattern in *Acaryochloris* is actually closer to that of *Synechocococcus elongatus* PCC 7942 *where hoxEF* and *hoxUYH* fall into distinct operons with a stronger promoter for the latter one (Kiss et al. 2009, 2013). Anaerobic darkness, low light intensities and far red light illumination conditions all induce expression of the *hox* genes in *Acaryochloris* (Kiss 2012). Under anaerobic

conditions, Boichenko et al. (2000) demonstrated the capability of H_2 production in *Acaryochloris* for the first time, although the rate of H_2 production was very low, approximately 80–250 μmol mol Chl^{-1}. Interestingly, in cells grown under light that is depleted in the active spectral region below 650 nm (i.e. the active spectral region of *Acaryochloris* is 650–750 nm), the H_2 production rate is increased three-fold.

A relatively lower availability of reducing equivalents for hydrogenase under dark hypoxic conditions has been reported (Kiss et al. 2013), although optimized culture conditions may be required for the activity of hydrogenase. The best condition for microorganisms to produce hydrogen was reported to be low irradiation, red light (Allakhverdiev et al. 2010a), which seem the similar cultural condition for *Acaryochloris*. The full set of gene encoding bidirectional hydrogenase located on one plasmid provide intriguing aspect for choosing *Acaryochloris* as a candidate for hydrogen fuel production. However, currently the level of hydrogen production is too low and culture conditions still need to be optimized.

Chl *d*-photosynthesis

Photosynthesis is the utilization of solar energy by plants, algae and certain bacteria for the synthesis of complex organic molecules. The overall reaction of the complicated process of photosynthesis can be expressed by the following equation:

$$CO_2 + 2H_2A \rightarrow [CH_2O] + 2A + H_2O$$

Here H_2A is the reductant, A is the electron acceptor and CH_2O is the general formula of the carbohydrates, where the chemical energy is initially stored as organic carbon. H_2A can be H_2O, H_2S, etc. where $H_2A = H_2O$, O_2 is released, and this process is called oxygen-evolving photosynthesis, also named as oxygenic photosynthesis. Alternatively anoxygenic photosynthesis describes when a reductant other than H_2O is used, i.e. no O_2 is released. Oxygenic photosynthesis is one of the central events in the development of life on Earth because the accumulated free oxygen in the atmosphere is the essential element for the development of advanced eukaryotic life forms. All known oxygen-evolving photosynthetic organisms require two tandemly linked photosystems (PS), PS I and PS II. Each PS is comprised of an accessory antenna system, core-antenna system and a reaction centre. In PS II, light energy is used to drive the splitting of water, production of molecular oxygen and translocation of electrons across the membranes leading to an electrical potential (negative outside), which consequently establishes a proton gradient that in turn drives ATP synthesis. On the other hand, PS I

uses energy from the absorption of a photon to reduce $NADP^+$ to NADPH and also drive cyclic photophosphorylation. The products of this light reaction, ATP and NADPH, are required for CO_2 fixation. The synthesis of stable carbon products (carbohydrates) is a light-independent process and hence known as the dark reactions. The photosynthetic products play an essential role in biomass production and carbon sequestration on Earth.

Light-harvesting system in *Acaryochloris*

Light-harvesting systems function at a very early step of photosynthesis and have developed individual systems to adapt to various light environments (Chen and Scheer 2012). There are two broad classes: peripheral membrane antenna complexes and integral-membrane antenna systems. Most cyanobacteria use the peripheral membrane phycobilin-binding proteins –phycobiliproteins (PBPs)- as their primary light-harvesting system, except prochlorophytes and *Acaryochloris* spp. Prochlorophytes are a unique group of cyanobacteria that carry out oxygenic photosynthesis using Chl *a* and Chl *b*. They lack PBPs and use the membrane-bound Chl *a/b*-binding protein as their major light-harvesting protein complexes (La Roche et al. 1996). The membrane-bound Chl *a/b* binding light-harvesting protein complex was designated as prochlorophyte chlorophyll- binding (Pcb) protein (La Roche et al. 1996) and was re-named as CBP to cover all accessory chlorophyll-binding proteins in cyanobacteria (Chen et al. 2008). The CBP proteins are only found in cyanobacteria, and are different from the light-harvesting complex (LHC) superfamily in plants and algae. This unique light-harvesting system has six tranmembrane helices and binds different types of chlorophylls, including Chl *a, b, d* and 8-vinyl Chl *a* and 8-vinyl Chl *b*.

Phycobiliproteins are made of an aggregated trimer of heterodimers that is composed of an α and a β-subunits and formed into hexamers by a tight face-to-face arrangement. The PBP large complexes are constructed by cylindrical cores with several radiating peripheral rods. There are four main classes of PBP: phycoerythrin (PE), phycoerythrocyanin (PEC), phycocyanin (PC) and allophycocyanin (APC). The cylindrical cores are composed of APC while the peripheral rods are composed of PC alone or with PE or PEC. It is known that the light energy absorbed by peripheral rods (PC, PE or PEC) is transferred to the special pair of Chl *a* in reaction centres through APC in the core, which is adjacent to the thylakoid membranes. There are two antenna systems coexisting in *Acaryochloris*, PBPs (Marquardt et al. 1997) and chlorophyll *d*-binding protein complexes (Chen et al. 2002). The two systems function complementarily to meet the changes of light environments

(Miyashita et al. 1997; Duxbury et al. 2009) although PBPs are absent in two of the five isolated strains, *Acaryochloris* sp CCMEE5410 (Miller et al. 2005) and HICR111a (Mohr et al. 2010).

Acaryochloris has a unique PBP arrangement. It does not contain a typical phycobilisome (PBS). The first transmission electron microscopic observation revealed no detectable PBS in *Acaryochloris*, although there are detectable PC pigments (Miyashita et al. 1996). Hu et al. were the first group to isolate the rod structure of PBP and PBP-PS II complexes in *Acaryochloris*. Each aggregation consists of four discs and each disc is formed by three hexamers ($\alpha_6\beta_6$). The spectral analysis suggested that PBPs are physically and functionally associated with PS II, not PS I (Hu et al. 1999). The PBP rod-arrays are located at stromal side of the thylakoids membranes where they are attached to PS II-antenna supercomplexes (Chen et al. 2009).

The energy transfer from simple PBPs to chlorophylls in the reaction centre is approximatly 70 ps, three times faster than the energy transfer from PBS to PS II in Chl *a*-containing cyanobacteria (Petrášek et al. 2005). No direct evidence support the connection between the PBPs and PS I, although the analogous CpcG2 protein was predicted in *Acaryochloris* based on sequence comparison (Chen and Cai 2007). Interestingly, excepting the putative genes encoded for ApcA and ApcB found in the *Accaryochloris* main chromosome, all PC-associated subunits (CpcA–CpcG) encoding genes are located on one plasmid, pREB3. It has been suggested that ApcA and ApcB form part of bottom disc with CpcA and CpcB together (Hu et al. 1999) and there are no other APC-associated subunits in *Acaryochloris*. It is still uncertain as to how PBPs associate with the thylakoid membranes because *Acaryochloris* lacks the core-membrane linker, ApcE. Notably, three copies of CpcG encoded genes in *Acaryochloris* genome belong to the same protein family, CpcG2, rather than CpcG1 group (Chen and Cai 2007). It is generally understood that CpcG1 plays an important role in attaching the rods to the PBS core when the core is attached to the membrane by core-membrane linker, ApcE. CpcG2 contains a hydrophobic C-terminus, which may play a functional role in attaching the rods to the membranes directly. CpcG2 is involved with the formation of simplified PBP structure that is associated with PS I instead of PS II (Kondo et al. 2007; Deng et al. 2012). The physiological function of CpcG2 in *Acaryochloris* is uncertain.

CBP-type light-harvesting protein complexes in *Acaryochloris* are reported to be associated with PS I and PS II (Chen et al. 2005c, d). PS I-antenna supercomplexes (trimer of PS I with a ring of CBP protein around) are not only detected under iron-stress conditions, as in other cyanobacteria (Bibby et al. 2001a, b), but can also be detected under normal culture conditions although the genes encoding CBP proteins are different from the iron-stress-induced CBP (Chen et al. 2005b, 2008).

PSII-antenna complexes were isolated from *Acaryochloris*. It contains a tetramer of PS II alongside two CBP subunits, the first tetrameric PS II structure reported (Chen et al. 2005a). The existing multiple copies of genes that encode the CBP light-harvesting-proteins suggest the capability to thrive under low light condition (Swingley et al. 2008; Chen et al. 2008). The fact that two isolates of *Acaryochloris* spp (CCMEE5410 and HICR111a) demonstrated the absence of PBPs suggests that PBPs are the result of an environmental adaptation and that the genes may have been recruited recently (Mohr et al. 2010; Miller et al. 2011). Further investigation is required for defining the relationship between the two antenna systems.

Photosystems in *Acaryochloris*

Acaryochloris spp are the only known organisms in which the essential function of Chl *a* in oxygenic photosynthesis has largely been replaced by Chl *d*. Chl *d* and Chl *d'* function as the special pair in PS I instead of Chl *a*/Chl *a'* (Akiyama et al. 2001; Hu et al. 1998) although the special pair of PSII is an ongoing controversy as to whether it is a Chl *d*/Chl *d* homodimer (Chen et al. 2005d; Tomo et al. 2007) or Chl *a*/Chl *d* heterodimer (Schlodder et al. 2007). Both PS II and PS I are multisubunit, pigment-protein complexes. Each PS II of cyanobacteria is composed of approximate 20 subunits and 35 chlorophylls (Umena et al. 2011). The first crystal structure of PS I from cyanobacteria showed that PS I exists as a trimer in vivo (but monomer in higher plants) and one monomer consists of at least 11 protein subunits and 96 chlorophylls (Jordan et al. 2001). The polypeptide composition of both PS I and PS II complexes in *Acaryochloris* are similar to that of well-known Chl *a*-containing cyanobacteria (Hu et al. 1998; Chen et al. 2005d; Tomo et al. 2008; Swingley et al. 2008). However, the pigment profiles are different due to the unique pigment composition in *Acaryochloris*.

PS II

The nature of PS II primary donors is an intriguing question as either way, the answer will challenge our current knowledge of energy balance in PS II. If there is Chl *d* in PS II centre, then whether there will be enough energy for water oxidation which is driven by PS II needs to be addressed. Alternatively, if Chl *a* play the primary donor in PS II, P680, as in other cyanobacteria, the uphill energy mechanism has to be considered because Chl *d* is the major photopigment in light-harvesting system. Mimuro et al. were the first team who studied the nature of the primary

donor in PSII using *Acaryochloris* cell's decay fluorescence measurement. The decay of the fluorescence component in the 685– 695 nm wavelength range with a life time of 15 ns lead to the favoured conclusion that Chl *a* is the primary donor in PS II and an uphill energy scheme must be applied in *Acaryochloris* when the energy absorbed by Chl *d* in light-harvesting complexes is transferred to the accessory Chl *d* in the reaction centre, then moderately uphill energy transferred to Chl *a* in the reaction centre (Mimuro et al. 2000).

The pigment composition provided additional support for the proposed P680 in PS II of *Acaryochloris*. The minimal ratio of Chl *a* to Phe *a* was about 1:1 under various culture conditions and the constant presence of Chl *a* strongly suggested that Chl *a* plays a critical role in *Acaryochloris*, maybe as P680 in PS II. However, these hypotheses on the identity of the primary donor of PSII in *Acaryochloris* were proposed on the basis of pigment analysis combined with whole cell picosecond fluorescence kinetics due to the lack of purified PS II complexes at that time (Mimuro et al. 1999, 2000, 2004; Akiyama et al. 2002). Even with more in depth pigment analysis on isolated *Acaryochloris* PS II there remains uncertainty regarding the ratio of Chl *a* to Phe *a*. An initial report on isolated PS II 'crude cores' determined a Chl *a*: Phe *a* ratio of 0.5, which is less than the two Chl *a* required if the special pair in PS II is composed by Chl *a* (Chen et al. 2005d), however more recent reports suggest this ratio may be closer to 1.5 or 1.0 (Tomo et al. 2007; Tsuchiya et al. 2012a). Fourier transform infrared spectroscopy (FTIR) analysis on purified PS II particle by Tomo et al. demonstrated that P_{713} of Chl *d* is the primary donor of PS II (Tomo et al. 2007). However, Itoh et al. suggested P725 of Chl *d* as the primary donor in PS II based on the laser-induced bleach at 725 nm with the recovery time of 25 μs and 1.2 ms using isolated thylakoid membrane (Itoh et al. 2007). The bleaching at ∼713 nm was not observed by either Itoh et al. or by Schlodder et al. (2007) (Tomo et al. 2008). According to the theoretical calculation, Renger and Schlodder indicated the possibility that Chl *a*/Chl *d* heterodimer drives the water oxidation because Chl *a* is necessary for stabilization of the positive charge, and Chl *d* is the primary electron donor of PS II in *Acaryochloris*, i.e. in the reaction centre core, there are three Chl *d*, one Chl *a* and 2 Phe *a* (Renger and Schlodder 2008).

Electron paramagnetic resonance (EPR) spectral measurements using *Acaryochloris* cells revealed that electron transfer to the oxygen-evolving complex of the donor side of PS II is indistinguishable from Chl *a*-containing oxygenic photosynthetic organisms (higher plants, algae and cyanobacteria) (Razeghifard et al. 2005). The flash-induced oxygen evolution patterns in *Acaryochloris* cells suggested that the redox-potentials and kinetics within the oxygen-

evolution centre of PS II and of redox-active tyrosine site (Yz) are the same as in higher plants (Shevela et al. 2006). The direct measurement on redox potential of Phe *a* and the energy gap between [P_{PSII}·Phe a^-·Q_A] and [P_{PSII}·Phe a·Q_A^-] indicated the overall energetic balance of PS II are similar between Chl *a*-driving PS II and Chl *d*-driving PS II (Allakhverdiev et al. 2010b, 2011). The shifted redox potential of Phe *a* and Q_A produced an energy gap of −325 mV, which is sufficient for water-splitting reaction driving by Chl *d*-PS II in *Acaryochloris*. On the basis of pigment analysis, the accessory Chl *a* and Chl *az* in Chl *a*-containing oxygenic photosynthetic organisms are all replaced with Chl *d* in *Acaryochloris* (Mimuro et al. 2004; Kobayashi et al. 2005), but the controversial results were reported based on spectral analysis (Tomo et al. 2007).

Since it is known that Phe *a* is the primary electron acceptor of PS II and no Phe *d* is detected in *Acaryochloris*, the function of Phe *a* plays the same function as in Chl *a*-containing cyanobacteria, a primary acceptor of PS II, although the nature of the primary donor of PS II of *Acaryochloris* is still under debate.

One type of photoprotection of PS II in cyanobacteria is the induction of additional copies of PsbA and PsbD genes, which encode a D1 and a D2 protein with the same/or highly similar amino acid sequence as the D1/D2 expressed under the normal conditions (Mulo et al. 2009). A large mRNA pool of *psbA* maintains the higher turnover rate of photodamaged D1 protein that is caused by the strongly oxidative chemistry of water splitting. The *Acaryochloris* genome encodes multiple copies of genes encoding PS II subunits, such as three *psbA*, three *psbD* and two *psbE*, four *psbU* and two *psbV* homologues (Swingley et al. 2008). The three copies of *psbA* genes produce two different D1 proteins, a normal one (D1:1) and the isoform (D1:2) (also see rD1 in "Nitrogen-fixation" section). Various stress conditions upregulate the isoform of D1 (Kiss et al. 2012; Murray 2012). Interestingly, *Acaryochloris* possesses a D2 protein as well, most cyanobacteria have two *psbD* copies for the same D2 protein. The isoform of D2 is induced in cells grown under a low visible light/high far-red light regime, indicating the important adaptational role for the D2 isoform in *Acaryochloris*.

PS I

In general, PS I has as special dimeric forms of Chl *a*, P700, and generates a strong reducing power to produce NADPH with electrons supplied from PS II. However, *Acaryochloris* uses Chl *d* as an energy sink in PS I, i.e. the special pair in PS I is Chl *d*, P740 (Hu et al. 1998). The polypeptide composition of PS I and the sequence of PsaA and PsaB in *Acaryochloris* show about 86 % homology which suggests the same architecture of PS I as other

cyanobacteria. The image of isolated PS I supercomplexes confirmed that it has a trimer of PS I that form the mega-supercomplexes of antenna-PS I under certain culture conditions (Chen et al. 2005b). The PS I reaction centre complexes of *Acaryochloris* use Chl *d* both as the major antenna pigment and as the primary electron donor (Hu et al. 1998). The isolated PS I complexes contain ~ 180 Chl *d* and <1 Chl *a* per complex. The ratio of Chl *d*/P740 = 140 in the PS I complexes of *Acaryochloris* (Hu et al. 1998) is in a range similar to that of Chl *a*/P700 = 100–150 in higher plants, algae and cyanobacterial PS I (Akiyama et al. 2002). The FTIR difference spectral analysis strongly supports that P740 consist of two Chl *d* molecules (Sivakumar et al. 2003). Spectral analysis using EPR and Electron Spin Echo (ESE) technologies on isolated PS I complexes confirms the similarity between the Chl *d*-binding PS I in *Acaryochloris* and Chl *a*-binding PS I in other oxygenic photosynthetic organisms (Santa-barbara et al. 2007). The role of Chl *a* as A_0 in PS I was suggested according to the pigment composition found in isolated PS I, one Chl *a* per P740 (Hu et al. 1998). Recently, Tomo et al. (2008) isolated highly purified PS I complexes from *Acaryochloris* and indicated the details of pigment composition of PS I particle. There are 97 Chl *d*, 2 Chl *a* and 25 α-carotene, two phylloquinone per one Chl *d'* (i.e. per monomer of PS I). Interestingly, the redox potential of P740 was estimated as 439 mV, similar to P700 of Chl *a*-PS I.

Electron carriers and carotenoids

Oxygenic photosynthesis requires PSI and PS II working in tandem and through a non-cyclic electron transfer chain (ET) to generate ATP. The same fluorescence transients as Chl *a*-containing species implies there is the same operation of intersystem electron transport chain in *Acaryochloris* (Schiller et al. 1997; Boichenko et al. 2000).

The PS I and PS II complexes in *Acaryochloris* are spatially separated and axenically clustered in thylakoid membrane. This phenomenon is also observed in green plants and prochlorophytes (van der Staay and Staehelin 1994). However, in those organisms the PSII is enriched in stacked thylakoid membranes, whereas in *Acaryochloris* the stacked area is devoid of PSII (Chen et al. 2009; Marquardt et al. 2000). This PS I–PS II spatial separation is also supported by detailed chlorophyll fluorescence measurements which indicate that there is no direct energy transfer from PS II to PS I (Itoh et al. 2007). Furthermore, with the aid of structure modelling Chen et al. (2009) revealed the clustered spatial separation of PS I and PS II in thylakoid membranes of *Acaryochloris*.

Based on the pigment composition of Chl *d'* (representing PS I) and Phe *a* (representing PS II) in *Acaryochloris*, the ratio of PS I and PS II is estimated to be 1:1, which is higher than conventional cyanobacteria (Mimuro et al. 2004). Electron microscopic images also show that ~ 50 % of thylakoid membranes are covered by PBP arrays if they are associated with PS II. However, based on the spin densities on the Y_Z radical and the photo-oxidixed P740, the ratio of PS I to PS II is 1.8:1 (Itoh et al. 2007).

Interestingly, the same Phe *a* molecule in the different organisms demonstrated a different redox potential. The redox potential of Phe *a* in PS II of *Synechocystis* PCC 6803 is -536 ± 8 mV and in PS II of *Acaryochloris* is -478 ± 24 mV (Allakhverdiev et al. 2010b). Such different feature clearly indicates the conservation in the properties of oxygen evolution centre (Details see "PS II" section). The photoacoustic measurements indicate the energy storage efficiency of photosynthetic reactions driven by Chl *d* in *Acaryochloris* is comparable to or higher than the typical reactions driven by Chl *a* in the oxygenic photosynthetic organisms (Mielke et al. 2011).

There are two soluble electron carriers between the cyt b_6f and PS I, plastocyanin (PC) and cyt c_6. The two electron carriers are usually found in a ratio of $\sim 1:3$ according to the amount of PS I (Hope 2000). There are two copies of *petJ* (encoding cyt c_6) and one *petE* (encoding PC) found in *Acaryochloris* genome. The expression of *petE* has been confirmed (Bailleul et al. 2008) although no purified PC is reported in *Acaryochloris*. On the other hand, purified cyt c_6 of *Acaryochloris* indicated that it is the major soluble electron carrier from cyt b_6f to PS I (Bell et al. 2009). The function of isoform of cyt c_6 is unknown.

Conclusion

The discovery of *Acaryochloris* in 1996 precipitated a shift in our understanding of oxygenic photosynthesis. The presence of Chl *d* as its major photopigment, substituting many of the roles previously thought to be unique to Chl *a*, has for the first time allowed the comparative study of the energetics of oxygenic photosystems. The discoveries of red-shifted chlorophylls have highlighted the fundamental question of what is the energy threshold for oxygenic photosynthesis i.e. at what point is the energy of a photon insufficient to drive the process? The red-shifted absorption characteristic of Chl *d* has clear advantages in absorbing light filtered through Chl *a*-containing organisms, however challenges associated with the lower energy of these photons have had to be addressed by *Acaryochloris*. From its comparatively large genome, to its two complementary light-harvesting systems and unusual complement of accessory pigments, the distinctive adaptations of *Acaryochloris* are clearly advantageous for its proliferation in

diverse environments across the planet. The discovery of *Acaryochloris* and more recent discovery of two Chl *f*-containing cyanobacteria raise the question of whether there are other as yet uncharacterised chlorophylls in oxygenic photosynthetic organisms, and whether the discovery of these too will challenge both and add to our understanding of this vital process.

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