ORIGINAL ARTICLE

A new chlorophyll *d*-containing cyanobacterium: evidence for niche adaptation in the genus *Acaryochloris*

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Chlorophyll *d* is a photosynthetic pigment that, based on chemical analyses, has only recently been recognized to be widespread in oceanic and lacustrine environments. However, the diversity of organisms harbouring this pigment is not known. Until now, the unicellular cyanobacterium *Acaryochloris marina* is the only characterized organism that uses chlorophyll *d* as a major photopigment. In this study we describe a new cyanobacterium possessing a high amount of chlorophyll *d*, which was isolated from waters around Heron Island, Great Barrier Reef (23° 26′ 31.2″ S, 151° 54′ 50.4″ E). The 16S ribosomal RNA is 2% divergent from the two previously described isolates of *A. marina*, which were isolated from waters around the Palau islands (Pacific Ocean) and the Salton Sea lake (California), suggesting that it belongs to a different clade within the genus *Acaryochloris*. An overview sequence analysis of its genome based on Illumina technology yielded 871 contigs with an accumulated length of 8 371 965 nt. Their analysis revealed typical features associated with *Acaryochloris*, such as an extended gene family for chlorophyll-binding proteins. However, compared with *A. marina* MBIC11017, distinct genetic, morphological and physiological differences were observed. Light saturation is reached at lower light intensities, Chl *d/a* ratios are less variable with light intensity and the phycobiliprotein phycocyanin is lacking, suggesting that cyanobacteria of the genus *Acaryochloris* occur in distinct ecotypes. These data characterize *Acaryochloris* as a niche-adapted cyanobacterium and show that more rigorous attempts are worthwhile to isolate, cultivate and analyse chlorophyll *d*-containing cyanobacteria for understanding the ecophysiology of these organisms.

Introduction

Chemical sediment analysis has shown only recently that the distribution and contribution of chlorophyll *d* (Chl *d*)-producing organisms to marine primary productivity has been seriously underestimated so far (Kashiyama *et al.*, 2008). These analyses suggested that such organisms potentially thrive in most aquatic environments on Earth, offshore in hemipelagic to pelagic marine conditions, in saline as well as freshwater lakes. However, despite this recently recognized ecological relevance, there have been few attempts to isolate, cultivate and analyse the organisms involved. The history of Chl *d* and of the organisms producing it has been enigmatic for several aspects.

Discovered as early as in 1943, Chl *d* was first attributed to marine macrophytic red alga (Manning and Strain, 1943). More than half a century later, Miyashita *et al.* (1996) succeeded in isolating *Acaryochloris marina* (strain MBIC11017), a unicellular cyanobacterium that uses Chl *d* as its main photopigment. By now, it could be shown that the Chl *d* found in red algae species originates from colonial *Acaryochloris* living on the surface of the algae (Miyashita *et al.*, 2003). A second isolate belonging to the *A. marina* clade (strain CCMEE5401) was characterized only in 2005, isolated from a site very different from the first one, the Salton Sea saltwater lake in California (Miller *et al.*, 2005).

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However, comparing the 16S rRNA sequences of both strains, these were found to be surprisingly similar (identity 99.2%) and to contain a characteristic insertion, most likely from a γ-proteobacterium, characterizing the small-subunit rRNA gene of *Acaryochloris* as a proteobacterial–cyanobacterial hybrid (Miller *et al.*, 2005).

Unlike most other photooxygenic organisms, *Acaryochloris* uses Chl *d* as a main pigment in the reaction centres and for photosynthetic light harvesting, instead of Chl *a*. Chl *d* absorbs light in the near-infrared region and is believed so far to be unique to the genus *Acaryochloris*. Because of the photophysiological properties of Chl *d*, *Acaryochloris* occupies an ecological niche rich in far-red light (Kühl *et al.*, 2005). Today, *Acaryochloris* is considered to be the source of Chl *d*-based photosynthesis. It is often found in proximity to other organisms such as red algae or ascidians such as *Lissoclinum patella* (Murakami *et al.*, 2004; Kühl *et al.*, 2005) and seems to be widespread according to the analysis of partial 16S rRNA sequences from environmental DNA samples (McNamara *et al.*, 2006; de los Rios *et al.*, 2007; Goh *et al.*, 2009). The genome of the *A. marina* MBIC11017 was recently analysed and was found to be one of the largest cyanobacterial genomes ever studied. It encompasses a total of 8.3 million base pairs that are distributed between one master chromosome of 6 503 724 base pairs and an additional 9 plasmids (Swingley *et al.*, 2008). This unusual genome organization and the fact that approximately 25% of its genes reside in the plasmids gave rise to the idea of an increased lateral gene transfer within the family (Swingley *et al.*, 2008). Despite these intriguing features, the *Acaryochloris* species complex is still poorly described.

Using red light-emitting diode lights during the enrichment phase (Duxbury *et al.*, 2009), we succeeded in isolating a new free-living cyanobacterium that is dominated by Chl *d*. In this study we present a polyphasic approach to describe this new strain, referred to as *Acaryochloris* sp. HICR111A, which shows morphological, physiological and genomic differences with the *A. marina* isolate. *Acaryochloris* sp. HICR111A has been isolated from Heron Island in the Capricornia Cays of the Australian Great Barrier Reef. It grows in tight cell aggregates and lacks phycocyanin but not allophycocyanin, which is different from other isolates. The 16S rRNA identity between strain HICR111A, MBIC11017 and CCMEE5401 is only 98%. Moreover, the distribution and arrangement of crucial photosynthetic genes is different and the light saturation of *Acaryochloris* sp. HICR111A is reached at lower light intensities compared with *A. marina*.

We conclude that *Acaryochloris* sp. HICR111A is a new representative of the *Acaryochloris* clade that seems to be distinct with regard to habitat, morphology and genomic features, underlining that the *Acaryochloris* group is still strongly undersampled. Our data emphasize the importance of isolation and cultivation of more isolates of this outstanding family of cyanobacteria. A greater number of isolates and additional genomic data will facilitate understanding a fascinating piece of microbial diversity, the scope and mechanisms of *Acaryochloris* genomic diversity and the role of Chl *d*-driven photosynthesis in various habitats.

Materials and methods

Isolation and culture conditions

Samples were isolated on Heron Island, Great Barrier Reef (23° 26′ 31.2″ S, 151° 54′ 50.4″ E). The sample material was a dead coral skeleton that was overgrown with algae and cyanobacteria and was found in shallow water on the southern reef flat of Heron Island. After growing in mixed enrichment cultures in ASN (artificial seawater + BG-11 nutrients), subcultures were grown in near-infrared light-emitting diode light (700–740 nm, centred at 720 nm with 20 nm half width; Roithner Laser Technik, Vienna, Austria) as recently described (Duxbury *et al.*, 2009). The presence of Chl *d* was monitored spectrophotometrically and by high performance liquid chromatography (HPLC) in 100% methanol extracts. Those cultures showing relevant amounts of Chl *d* were further subcultured. All cultures were grown in ASN at 25 °C. The isolated unicyanobacterial strain was grown in both continuous red and low-intensity white light (20–30 μmol photons m^{-2} s^{-1}). It has been deposited in the Roscoff Culture Collection from where it is publicly available under the accession number RCC1983.

Microscopy

Light and fluorescence microscopy pictures were taken using a Zeiss Axioskop 40 FL (Carl Zeiss MicroImaging GmbH, Göttingen, Germany) with a HB50 mercury lamp. The fluorescence filter excites at 450–490 nm and allows long-path emission (>515 nm).

Light saturation measurements

Chl *d* concentrations were determined according to Ritchie (2006). Light saturation curve measurements were performed in triplicates with a red diode pulse amplitude modulation (Heinz Walz GmbH, Effeltrich, Germany) fluorometer with electron transfer rate monitored versus increasing intensities of photosynthetically active radiation. Relative electron transfer rate could be calculated based on the known amounts of Chl *d* on the measured surface. We applied the correlation of relative electron transfer rate and oxygen evolution as shown for *A. marina* by Gloag *et al.* (2007). The obtained data points were fitted using the waiting-in-line model as in Ritchie (2008).

High performance liquid chromatography
HPLC analysis was performed using a Shimadzu VP series HPLC system with a SCL-10A system controller and a SPD-M10A diode array detector (Shimadzu, Kyoto, Japan). All HPLC data were integrated and analysed using Class-VP 6.14 software (Shimadzu). For all analytical work, a reverse-phase C18 column (Synergi Fusion-RP 80A, 250 mm × 4.6 mm, 4 μm pore size; Phenomenex, Torrance, CA, USA) was used. Mobile phase was a gradient mixture of acetonitrile (40–0%), methanol (50–100%) and 0.1 M NH_4Ac (10–0%) with a flow rate of 1 ml min^{-1}. Column was equilibrated 30 min before each run with equilibration buffer: 50% methanol, 40% acetonitrile and 10% 0.1 M NH_4Ac. After each run the column was cleaned with five volumes of 100% ethyl acetate. All solvents used were HPLC grade (Malinckrodt Baker, Phillipsburg, NJ, USA) and degassed by vacuum filtration and ultrasonication just before use. Calculation of ratio values was based on HPLC chromatogram peak areas. Each peak was evaluated individually *in silico* at its published absorption maximum wavelength and normalized to its extinction coefficient ε at this wavelength in methanol (ε = 77.62 mol^{-1} cm^{-1} at 696 nm for Chl *d*; ε = 68.72 mol^{-1} cm^{-1} at 665 nm for Chl *a*; ε = 44.6 mol^{-1} cm^{-1} at 667 nm for phaeophytin *a*; and ε = 133 mol^{-1} cm^{-1} at 452 nm for zeaxanthin; Jeffrey and Vesk, 1997; Ritchie, 2006).

Spectroscopic measurements in the absence or presence of glycerol
The spectrofluorimetric assay of Wyman *et al.* (1985) was adapted to measure the possible detachment of phycobiliproteins from the photosynthetic membrane. Fluorescence emission spectra were determined using an Aminco-Bowman Series 2 luminescence spectrometer (Thermo Spectronic, Madison, WI, USA) equipped with a red-sensitive photomultiplier. For measurements, 1 ml of thoroughly resuspended cell culture was mixed with 1 ml of glycerol (to a final concentration of 50%, v/v) or culture medium to yield similar Chl concentrations, transferred to the measuring cuvette and frozen in liquid nitrogen immediately. Fluorescence was excited at 470, 495 and 550 nm in the presence or absence of glycerol.

DNA isolation and PCR
A 50 ml cell culture was harvested by centrifugation and washed twice with SET buffer (40% w/v sucrose, 50 mM Tris-HCl pH 8.0 and 1 mM EDTA). The pellet was frozen and thawed, followed by a proteinase K digestion at 50 °C overnight followed by phenol–chloroform phase separation (Finsinger *et al.*, 2008). Taq polymerase (Promega, Madison, WI, USA) and primers 16SCYfw (5′-GGCTCAGGAT GAACGCTGGCGG-3′) and 16SCYrv (5′-ACCTTGT TACGACTTCACCCCAGTC-3′) were used to amplify the 16S rRNA gene in 35 cycles of denaturation for 30 s at 93 °C, annealing at 60 °C for 30 s and elongation for 60 s at 72 °C. Amplified 16S rRNA gene fragments were cloned into the pGEM-T vector System 1 (Promega), transformed into *Escherichia coli* and sequenced.

Field emission gun scanning electron microscope
Samples were fixed with 2.5% (vol/vol) glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 2.5 h. Fixed cells were washed twice, for 30 min each time, in 0.1 M phosphate buffer, and postfixed with 1.0% (w/v) osmium tetroxide in 0.1 M phosphate buffer. The cells were then dehydrated by 30-min washes in a graded acetone series of 20% (v/v), 40%, 60%, and 80% and a final two washes in 100%. Samples were critical-point dried, mounted on aluminium pin stubs, and coated with a 5-nm layer of platinum and palladium using an Agar Scientific high-resolution sputter coater fitted with an Agar Scientific (Agar Scientific Ltd., Stansted, UK) thickness monitor. Samples were examined with an LEO (LEO Elektronenmikroskopie GmbH, Oberkochen, Germany) 1530 series field emission gun scanning electron microscope instrument operating at 3 kV.

Transmission electron microscopy
Samples for electron microscopy were prepared as in Fiedler *et al.* (1998) with slight modifications: cell cultures were harvested and the cells were fixed overnight with 2% glutaraldehyde and incubated in 2% $KMnO_4$ at 4 C for 16 h. The cells were embedded in 1% Seakem agarose, which was cut in pieces of 1 mm². The samples were dehydrated by a 20-min graded ethanol series of 70% (v/v), 80%, 90%, 95% and a final three washes in 100% ethanol, followed by infiltration with ethanol/Epon (1:2) for 30 min, and 90-min incubation in ethanol/Epon (1:3). Finally, the agarose cubes were embedded in pure Epon by incubation at 40 °C for 24 h and at 60 °C for 48 h. Ultrathin sections (70–80 nm) were mounted on pioloform-coated copper grids and poststained with 2% uranyl acetate in ethanol and lead citrate (Reynolds, 1963). Electronmicrographs were imaged using a Philips Tecnai electron microscope (FEI EUROPE, Eindhoven, The Netherlands) at 80 kV.

Genome analysis
The DNA was extracted as mentioned above. As an additional step the DNA was treated with 10 μg ml^{-1} RNase A at room temperature for 30 min followed by another phenol–chloroform phase separation to remove the RNase. The DNA was precipitated with 70% ethanol with sodium acetate and checked for purity on a 1% agarose gel. For overview sequencing, a single paired-end (200 bp distance) run on an Illumina (San Diego, CA, USA) (Solexa) sequencer was performed, yielding ∼85 million reads with a length of 76 nt. The Velvet collection of

algorithms (Zerbino and Birney, 2008) was used for the *de novo* assembly of obtained sequences using de Bruijn graphs. The initial *de novo* assembly (parameters: kmerlength = 31, exp_cov = auto and cov_cutoff = auto) resulted in two distinct populations of contigs, characterized by their different GC content, centred either between 58% and 66% GC or between 40% and 48% GC (Figure 1). Refining the assembly parameters (kmerlength = 61, exp_cov = 350 and cov-cutoff = 10) gave a significantly better result (in terms of contig lengths) for the low GC population, while excluding the population of high GC reads from the assembly. A separate assembly of all unused reads, again, gave two contig populations, one with low and one with high GC (Figure 1). Thus, there seems to be more than one origin of contaminating DNA, and preliminary analyses showed that these bacteria are affiliated to the α-proteobacteria. From now on we focused on the refined assembly that produced 871 contigs, ranging in length from 121 to 241276 nt. The total size of the assembly was 8 371 965 nt, and the N50 value was 40 719 nt. The N50 value indicates the length of the contig, in which half of the total length is reached. As the size of the assembly is very similar to the genome size of *A. marina*, most genomic information can be expected to be found within this data set. After gene modelling and automatic annotation using RAST (Rapid Annotation using Subsystem Technology; Aziz *et al.*, 2008) we found genes with high similarity to *Acaryochloris* genes.
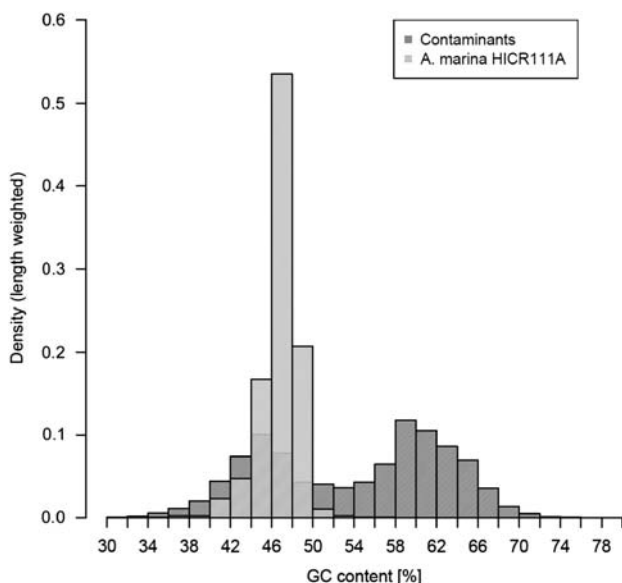

Figure 1 Comparison of the final *A. marina* HICR111A sequence assembly (red) and the assembled contaminant sequences (green) based on GC content. To ensure comparability, the densities have been length-weighted. Although the *A. marina* contigs are centred around a GC of 47%, the contaminant contigs are distributed between 32% and 76% with peaks around 45% and 59%, respectively (The color reproduction of this figure is available on the html full text version of the manuscript).

Results and discussion

Morphology
Unlike *A. marina* MBIC11017, *Acaryochloris* sp. HICR111A was not isolated from a cell suspension squeezed out from the colonial ascidian *L. patella* (Miyashita *et al.*, 1996) or its vicinity (Kühl *et al.*, 2005), but was scraped off a dead coral skeleton overgrown with other algae and cyanobacteria. Applying defined near far-red light conditions to mixed algal cultures enabled us to obtain a unicyanobacterial culture of a Chl *d*-dominated prokaryote. The novel isolate is related to *A. marina* MBIC11017, yet morphological differences were observed. The culture consistently appears light green, whereas *A. marina* cultures are blue-green under moderate and low light conditions (Figure 2a). The novel strain grows in aggregates, (Figures 2b–d), whereas cells of *A. marina* are dispersed as single cells in the medium (Figures 2e–g). Figures 2d and g show field emission gun scanning electron microscopy pictures of *Acaryochloris* sp. HICR111A and *A. marina,* respectively, suggesting that the cell surface of both strains is alike with regard to fibril size and structure. The individual cells are rod shaped and between 1 and 2 μm long with a diameter of 0.75–1 μm, which is somewhat smaller than the 2–3 μm in length and the diameter of 1.5–2.0 μm reported for *A. marina* (Miyashita *et al.*, 1996) and for other isolates of the complex (Miller *et al.*, 2005; Akimoto *et al.*, 2006). The electron micrographs also show that *Acaryochloris* sp. HICR111A cells are surrounded by an intercellular matrix that helps the cells to stay attached to each other, which we did not observe for *A. marina*. We suppose that growing in aggregates facilitates attaching to surfaces and settling in near far-red light niches. To a certain extent, this feature can also be observed for the MBIC11017 isolate, but only when they reach stationary phase without shaking. *Acaryochloris* sp. HICR111A cultures are unicyanobacterial but not axenic. Therefore, the observed intercellular matrix might in part be because of the presence of other bacteria, trying to form a consortium with the phototrophic *Acaryochloris*.

Pigmentation and photosynthetic parameters
Figure 3 shows *in vivo* spectra of *Acaryochloris* sp. HICR111A and *A. marina*. Both look very similar to each other, showing the typical pattern for a Chl *d*-dominated organism. Compared with Chl *a* cyanobacteria, the Q_y band is shifted to the far red with a maximum at 710 nm for MBIC11017 and 707 nm for the novel strain. The Soret band is shifted towards the blue-green at 460 nm. Carotenoids absorb at a maximum of 500 nm. Measuring *in vivo* spectra for *Acaryochloris* sp. HICR111A is particularly difficult because of scattering and sinking of the cell aggregates, which might have contributed to the slightly broadened peak of the Q_y band.
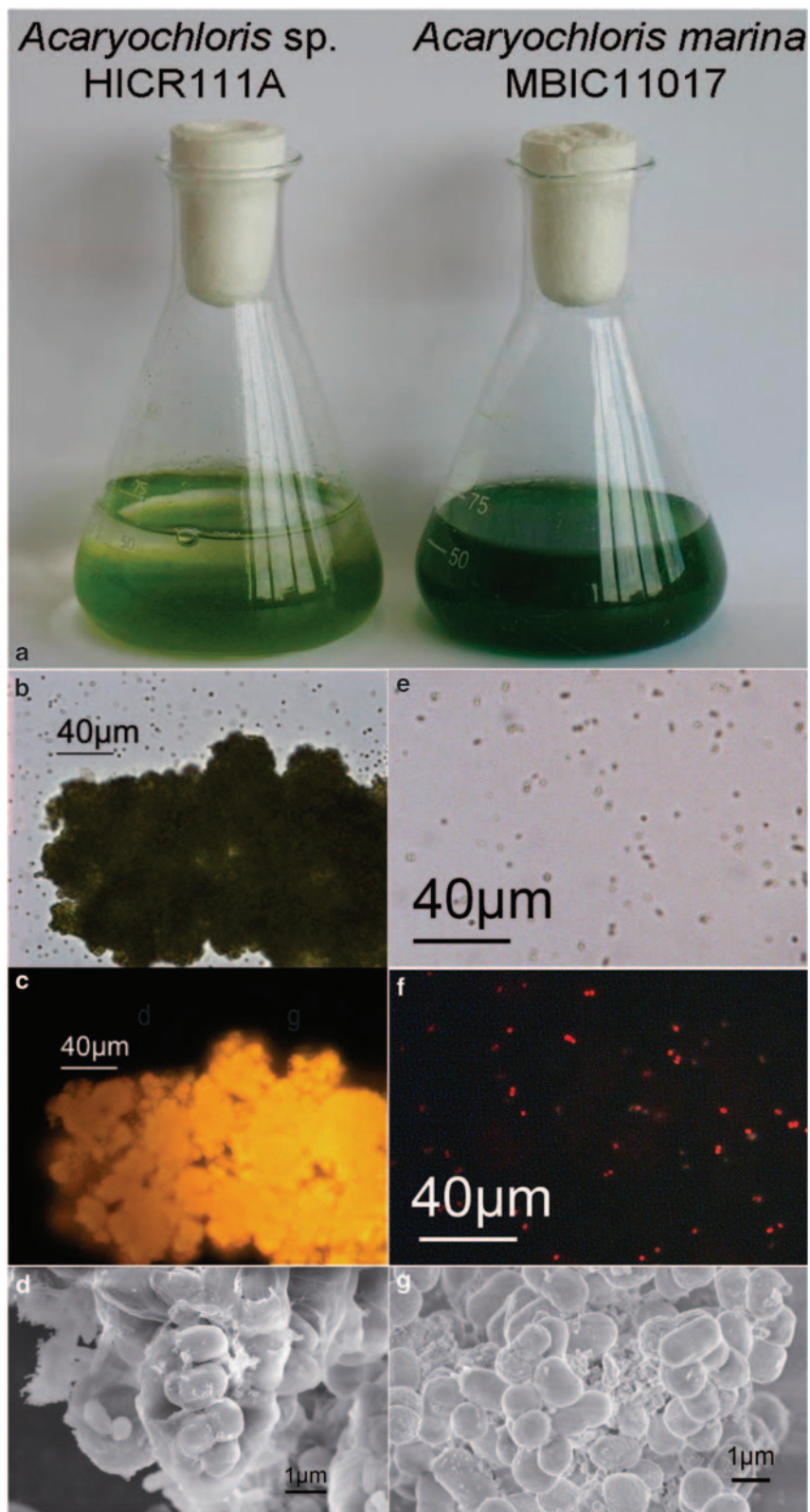
Figure 2 (**a**) Cultures of *Acaryochloris* sp. HICR111A appear yellow-green (left flask), and those of *A. marina* MBIC11017 blue-green (right flask). (**b**) Light microscopy, (**c**) fluorescence microscopy and (**d**) field emission gun scanning electron microscopy (FEGSEM) of aggregated *Acaryochloris* sp. HICR111A. (**e**) Light microscopy, (**f**) fluorescence microscopy and (**g**) FEGSEM of non-aggregated *A. marina* MBIC11017. Both cultures were grown under medium white light at 25 °C.
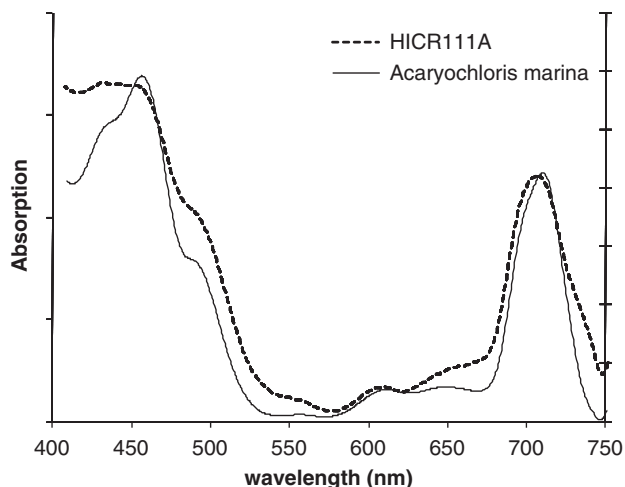
Figure 3 *In vivo* absorption spectra of *Acaryochloris* sp. HICR111A and *A. marina* MBIC11017 show the typical features of Chl *d*-dominated organisms. The Q_y maxima of Chl *d* were detected at 710 nm for *A. marina* MBIC11017 and at 707 nm for the novel strain.
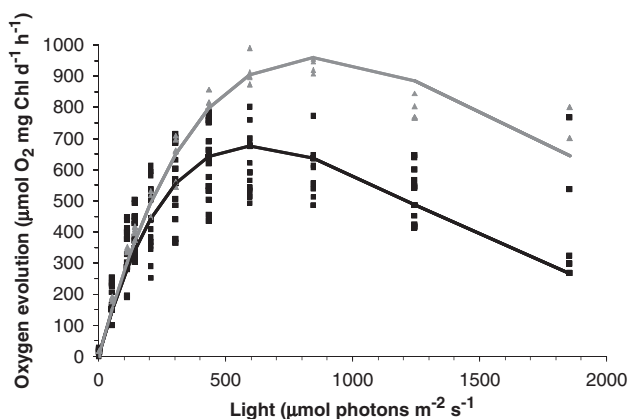

Figure 4 Light saturation curve of *Acaryochloris* sp. HICR111A (black line) and *A. marina* MBIC11017 (grey line). The graphs were fitted using the waiting-in-line model (Gloag *et al.*, 2007), and the oxygen evolution was calculated based on the relative electron transfer rate (ETR) (Gloag *et al.*, 2007).

The phycobiliproteins, phycocyanin and allophycocyanin, were detected previously in *A. marina* (Marquardt *et al.*, 1997; Hu *et al.*, 1999), in which they are associated with the photosynthetic membrane in an unusual way (Chen *et al.*, 2009). However, in this study both strains showed very low absorption in the area in which one would typically expect phycobiliproteins to absorb (615 nm for phycocyanin and 650 nm for allophycocyanin). The low signals observed in this study in the *in vivo* spectra may partially belong to vibrational bands or constitute derivative bands of chlorophylls. There is neither spectroscopic nor genetic evidence for the presence of phycoerythrin in either strain.

Light saturation curves in Figure 4 show that *Acaryochloris* sp. HICR111A reaches light saturation at approximately 600 μmol photons m^{-2} s^{-1},

whereas the MBIC11017 strain becomes saturated only at an illumination of 850 μmol photons m^{-2} s^{-1}. Moreover, both strains differ in the maximum gross photosynthesis rate. Therefore, *Acaryochloris* sp. HICR111A cannot use light of higher intensities as efficiently as *A. marina*. Furthermore, in the latter, distinct differences in the pigmentation occurred under different light conditions, whereas *Acaryochloris* sp. HICR111A showed only minor changes (Figure 5). The Chl *a*/Chl *d* ratio in *A. marina* MBIC11017 increased significantly with raised light intensity, from < 2% under low light to approximately 6% under high light. In *Acaryochloris* sp. HICR111A, the Chl *a*/Chl *d* ratio changed only from ~2% to 2.8% under the same conditions.

A. marina MBIC11017 and *Acaryochloris* sp. HICR111A are distinct with regard to the presence of phycobiliproteins

Figure 6 compares 77 K fluorescence emission spectra of both strains. *A. marina* shows emission maxima at 730 and 758 nm after Chl *d* excitation at 470 nm (Figure 6a). In contrast, *Acaryochloris* sp. HICR111A shows a single maximum at 750 nm (Figure 6b). Moreover, the ratio of the peaks changed in the MBIC11017 strain when phycocyanin was specifically excited at 550 nm. The emission at 758 nm decreased, whereas signals at 647 and 669 nm increased compared with the main peak at 730 nm used for normalization. The peak pattern of the novel strain did not change, suggesting the absence of phycocyanin.

The relatively low amounts may render the direct spectroscopic detection of phycobiliproteins too insensitive. Therefore, we adapted the assay of Wyman *et al.* (1985) to show the detachment of phycobiliproteins from the photosynthetic membrane by strongly enhanced fluorescence peaks in the presence of glycerol. When fluorescence was excited at 550 nm (absorbed by phycocyanin), a dramatic increase of phycobiliprotein autofluorescence at 647 nm was observed in MBIC11017 (Figure 6c) but not in HICR111A (Figure 6d). This observation can be explained assuming glycerol-induced detachment of originally coupled phycobiliprotein complexes from the photosynthetic apparatus, as was shown previously for the phycobiliproteins of *Synechococcus* (Wyman *et al.*, 1985) and *Prochlorococcus* (Lokstein *et al.*, 1999; Steglich *et al.*, 2003). Hence, phycocyanin excitation—no longer quenched by energy transfer—is re-emitted as fluorescence in case of the MBIC11017 strain. As the addition of glycerol to *Acaryochloris* sp. HICR111A did not cause any change in the 77 K spectra, these results suggest the absence of physiologically relevant amounts of phycobiliproteins in this strain. However, at the ultrastructural level transmission electron microscopy images indicated both strains to be very similar, with condensed thylakoids located close to the cell wall (Figure 7).
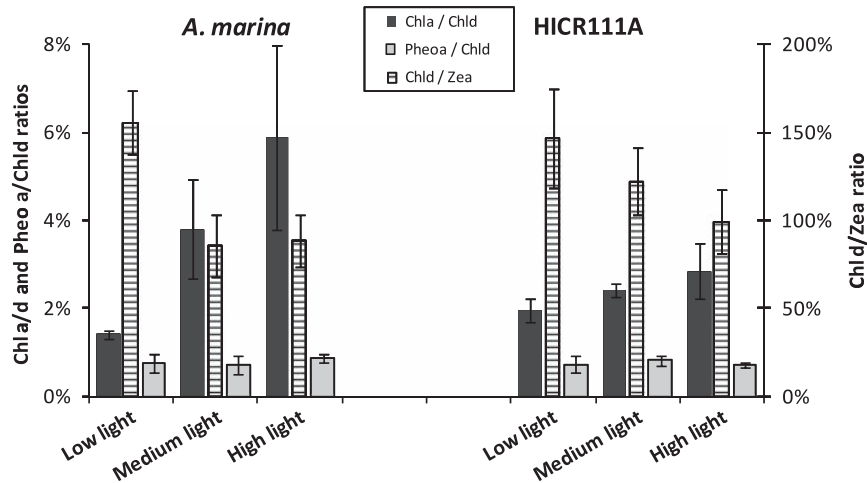
Figure 5 Pigment ratios from *A. marina* cells (left side) compared with *Acaryochloris* sp. HICR111A cells (right side) grown under white light of low (5–10 μmol photons m^{-2} s^{-1}), medium (25–30 μmol photons m^{-2} s^{-1}) and high (75–100 μmol photons m^{-2} s^{-1}) intensity. Left *y* axis describes Chl *a/d* (dark grey bars) and Pheo *a*/Chl *d* (light grey bars) ratios, and right *y* axis describes the Chl *d*/Zea (striped bars) ratio. The data shown are the average of three (*A. marina*) and two (*Acaryochloris* sp. HICR111A) experiments with standard errors. Pigment amounts were measured after separation by HPLC.
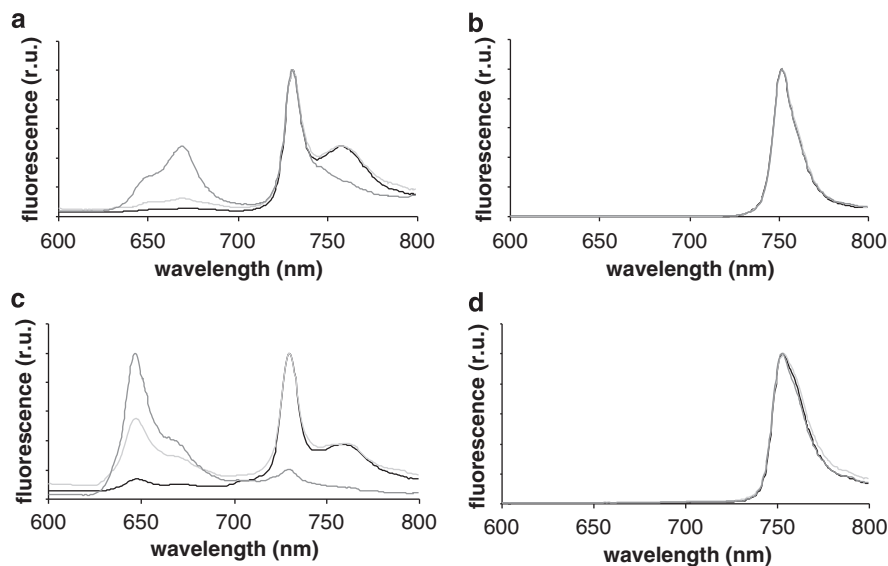

Figure 6 The 77 K fluorescence emission spectra of *A. marina* (**a**, **c**) and *Acaryochloris* sp. HICR111A (**b**, **d**) cells after excitation at 470 nm (black), 495 nm (light grey) and 550 nm (grey). Cells were grown under medium light conditions (25–30 μmol photons s^{-1} m^{-2}). (**c**, **d**) The effect of glycerol according to Wyman *et al.* (1985) for the respective strains (relative units (r.u.)) is shown.

Phylogeny

Figure 8 shows the phylogenetic relationship of *Acaryochloris* sp. HICR111A within the cyanobacterial kingdom based on a 16S rRNA sequence comparison. All three sequences from the genus *Acaryochloris* group together within the unicellular cyanobacteria group, with *Cyanothece* PCC7425 being their closest relative. At the 16S rRNA level, *Acaryochloris* sp. HICR111A shows 98.00% sequence identity with *A. marina* MBIC11017 and 98.69% identity with *Acaryochloris* sp. CMEE5410. Interestingly, *Acaryochloris* sp. HICR111A forms a separate clade from isolates MBIC11017 and CCMEE5410, which are the two strains of *Acaryochloris*

that have or are having their genomes sequenced (Swingley *et al.*, 2008). With regard to these two strains, *Acaryochloris* sp. HICR111A represents a more deeply branching isolate. Considering the phylogenetic distance we speculate that diversity of *Acaryochloris* is high. It seems comparable to the distance between distinct *Prochlorococcus* and *Synechococcus* strains that belong to clearly distinct ecotypes, here located in the lower part of the tree. In these picocyanobacteria, a 16S rRNA divergence of 2% is characteristic of a very different ecophysiology and of genomes that differ in the presence of several hundred genes from each other, both in *Synechococcus* (Dufresne *et al.*, 2008;
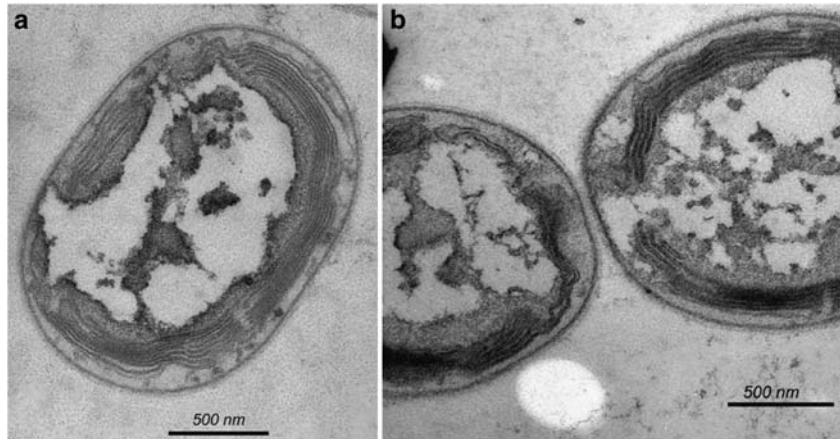
Figure 7 Transmission electron microscopy images of *A. marina* (**a**) and *Acaryochloris* sp. HICR111A (**b**).

Scanlan *et al.*, 2009) and in *Prochlorococcus* (Rocap *et al.*, 2003; Kettler *et al.*, 2007). For *Acaryochloris*, much less data are available but similar differences might be assumed.

Complete 16S rRNA sequences of >1300 bp are available for very few strains only, mainly because of the lack of adequate isolates. To take environmental and additional sequences into account that might belong to the *Acaryochloris* clade, we constructed a separate phylogenetic tree based on an alignment of much shorter sequences (Figure 9). Despite not being sufficiently long for a proper phylogenetic analysis, we get a glimpse of the diversity within the *Acaryochloris* group. All other *Acaryochloris* strains in culture, such as MBIC11017, Awaji-1 and CCMEE5410, are in one part of the tree, whereas the novel strain HICR111A is the only cultivated representative in another part (Figure 9). Here, *Acaryochloris* sp. HICR111A forms a shallowly branching clade together with the 'uc clone 11A2 9' sequence. The latter, in fact, originates from the same pre-culture that led to the isolation of *Acaryochloris* sp. HICR111A and thus was isolated from the very same location, supporting the idea that the new isolate is a representative of a genetically distinct clade.

Analysing more intensely studied cyanobacteria, such as *Synechococcus* and *Prochlorococcus*, we suggest that the diversity within the *Acaryochloris* group might be similar to other picocyanobacteria, which is in line with observations of global distribution of Chl *d* and of *Acaryochloris* itself (McNamara *et al.*, 2006; de los Rios *et al.*, 2007; Kashiyama *et al.*, 2008; Goh *et al.*, 2009).

Genomic data support the absence of phycocyanin genes and show features of photosynthetic genes typical for Acaryochloris
To characterize *Acaryochloris* sp. HICR111A in more detail, an overview sequencing analysis was performed using an Illumina sequencer. The obtained ∼85 million reads of length 76 nt would correspond to a theoretical 375 × genome coverage if a genome size similar to *A. marina* (8 361 608 nt) is assumed. However, despite attempts over several years we did not succeed in obtaining an axenic culture of the novel strain. Therefore, the genomic data were also expected to contain parts of contaminating sequences. Indeed, during the assembly, two major populations of contigs emerged, characterized by their different GC content, centred either between 58% and 66% GC or between 40% and 48% GC (Figure 1). Preliminary analyses showed that the reads from the non-*Acaryochloris*-like bacteria are affiliated to the α-proteobacteria, one related to *Rhodospirillum* spp. and another to the marine *Parvularcula bermudensis* (Cho and Giovannoni, 2003). Finally, we obtained 871 contigs, with a total size of the assembly of 8 371 965 nt, which is very similar to the genome size of *A. marina* MBIC11017. After gene modelling and automatic annotation, we found genes with high similarity to *Acaryochloris* genes. Among these genes is the 16S rRNA gene of *Acaryochloris* sp. HICR111A (100% identity), as well as the widely conserved photosynthetic genes *psbA* (for the D1 core subunit of photosystem II) and *psbC* (encoding the CP43 protein of photosystem II). Thus, a vast majority of genes are found in fragments of substantial length and a more refined analysis seemed promising. For all HICR111A genes mentioned below, Genbank accession numbers are given in Table 1; a full description of this data set is to be published elsewhere.

Two copies of *psbA* were found in *Acaryochloris* sp. HICR111A. *A. marina* contains three copies of *psbA* (Swingley *et al.*, 2008). From these, two share 100% amino acid identity (AM1_2166 and AM1_2889), whereas the third (AM1_0448) codes for a divergent D1 protein and shares only 61% amino acid identity with the first two (Swingley *et al.*, 2008). Phylogenetic analysis of the two HICR111A D1 proteins showed that both copies
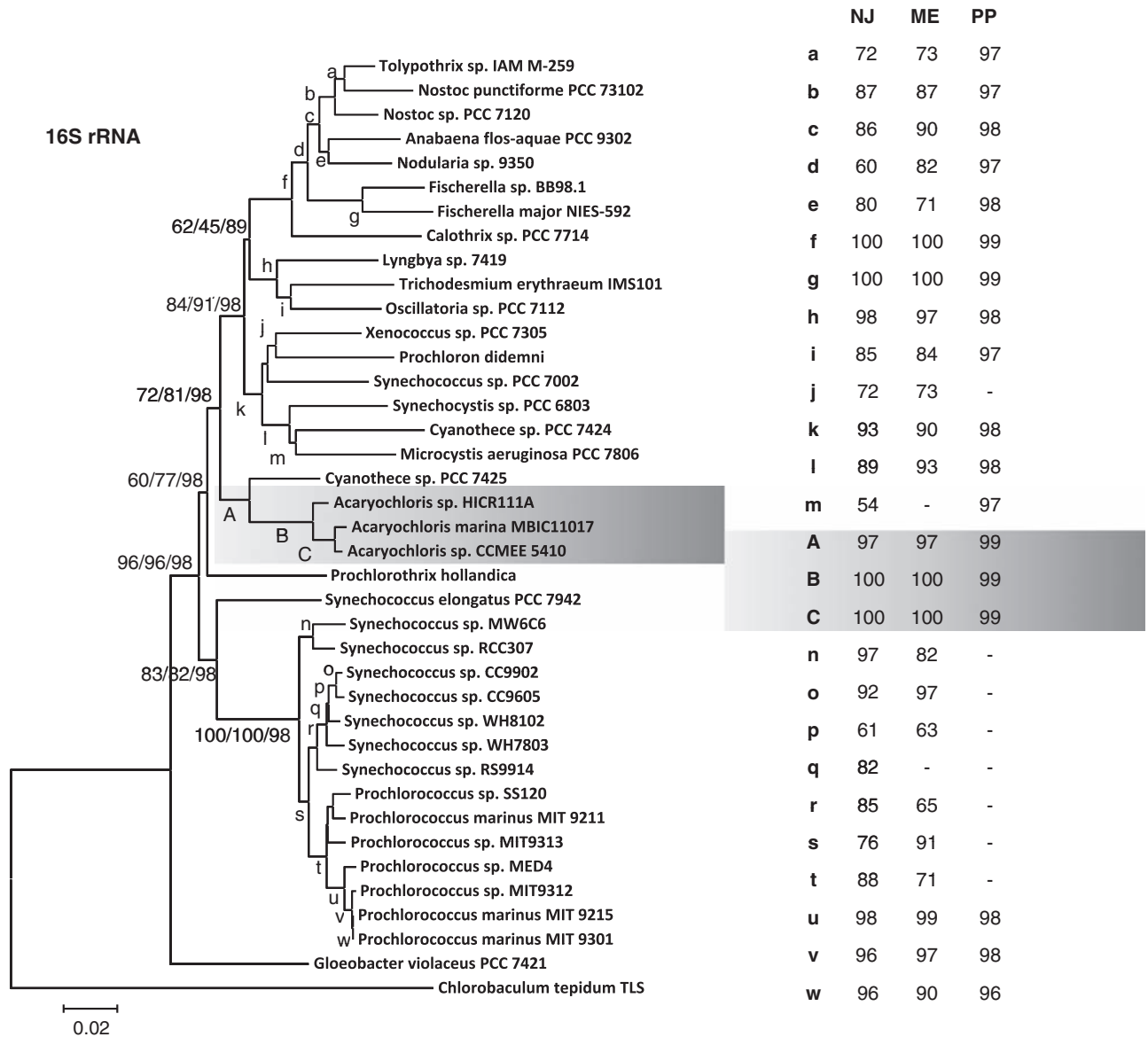
Figure 8 Phylogenetic tree (neighbour joining (NJ)) based on 16S rRNA sequence comparison (1363 bp alignment length; all alignments available upon request) showing the relationship of *Acaryochloris* HICR111A with other members of the species complex and the position of *Acaryochloris* within the cyanobacterial kingdom. The comparison contains 39 cyanobacterial taxa, and the green sulphur bacterium *Chlorobaculum tepidum* as out group. Minimal evolution (ME) and NJ support values with Jukes–Cantor distances, and 10 000 bootstrap replicates were calculated using molecular evolutionary genetics analysis (MEGA) software version 4.0 (MEGA 4; Tamura *et al.*, 2007). Bayesian posterior probability (PP) values were calculated using MrBayes 3.1.2. (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). NJ, ME and PP support values are shown in the table next to the tree indicating strong support for most nodes.

group closely with their respective *A. marina* MBIC11017 homologues (Figure 10). One *Acaryochloris* D1 protein is closely related to its orthologues from *Prochlorococcus*, *Synechocystis* and plant plastids; however, the other is not. The presence of two very different D1 forms thus seems as a distinct feature of *Acaryochloris*. However, the divergent form is closely related to a similarly divergent D1 protein in *Anabaena variabilis*, two *Synechococcus* and several *Cyanothece* strains, all nitrogen-fixing cyanobacteria (Figure 10).

A. marina possesses a set of 12 genes for chlorophyll-binding proteins (CBPs), with 11 in the chromosome and 1 in plasmid pREB3. The major light-harvesting proteins in *Acaryochloris*, which bind primarily Chl *d* and a small amount of Chl *a*, belong to this class (Chen *et al.*, 2002). These proteins are homologous to the Pcb-type light-harvesting proteins with six-membrane-spanning helices as originally defined by LaRoche *et al.* (1996) for the prochlorophytes. In *Acaryo-chloris*, two Pcb types exist (Chen *et al.*, 2005): PcbA
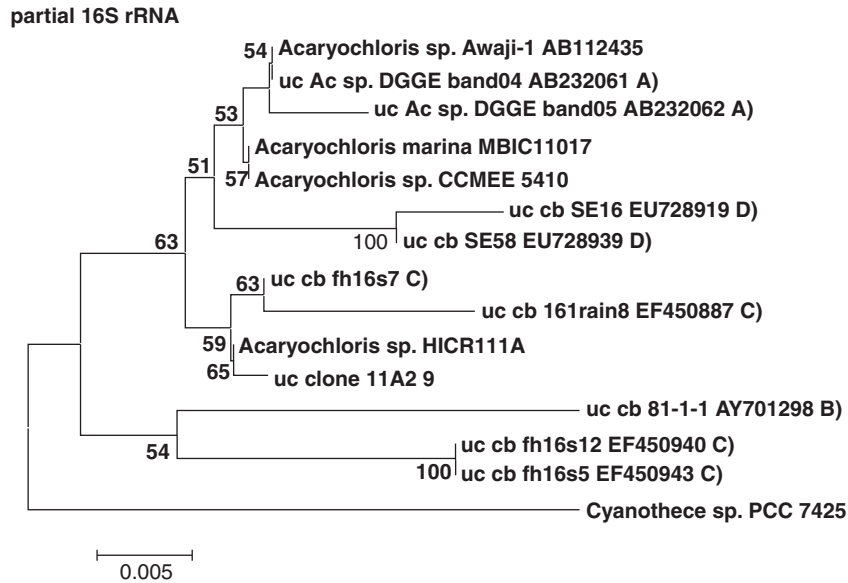
partial 16S rRNA

Figure 9 Phylogenetic tree based on 376 bp of the 16S rRNA gene (uc cb, uncultured cyanobacterium; uc Ac, uncultured *Acaryochloris*). Capital letters indicate the sample origin; A, red algae, Japan; B, *Pseudoaxinella flava* (sponge)-associated, Bahamas; C, *Lyngbya*-associated (Fern Hammick Spring, Florida); and D, Atacama Desert, Bolivia. The evolutionary history was inferred by neighbour joining (NJ; Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.

Table 1 Genbank accession numbers for all mentioned *Acaryochloris marina* sp. HICR111A genes

Figure/ID	Definition	Accession
Figures 8 and 9		
Acaryochloris sp. HICR111A	*Acaryochloris* sp. HICR111A 16S rRNA gene	EU873540
uc clone 11A2_9	Uncultured *Acaryochloris* sp. 16S rRNA gene, clone HICR111A2_9	GQ370819
Figure 10 *psbA*		
Acaryochloris sp. HICR111A	*psbA1* gene and D1 protein sequence	GU354023
Acaryochloris sp. HICR111Ab	*psbA2* gene and D1 protein sequence	GU354024
Figure 11 *psbC*		
PsbC_HICR111A	*Acaryochloris* sp. HICR111A PsbC	GU354025
IsiA_HICR111A	*Acaryochloris* sp. HICR111A IsiA	GU354026
CBP_h1_HICR111A	*Acaryochloris* sp. HICR111A CBP_h1	GU354027
CBP_h2_HICR111A	*Acaryochloris* sp. HICR111A CBP_h2	GU354028
CBP_h3_HICR111A	*Acaryochloris* sp. HICR111A CBP_h3	GU354029
CBP_h4_HICR111A	*Acaryochloris* sp. HICR111A CBP_h4	GU354030
CBP_h5_HICR111A	*Acaryochloris* sp. HICR111A CBP_h5	GU354031
CBP_h6_HICR111A	*Acaryochloris* sp. HICR111A CBP_h6	GU354032
CBP_h7_HICR111A	*Acaryochloris* sp. HICR111A CBP_h7	GU354033
CBP_h8_HICR111A	*Acaryochloris* sp. HICR111A CBP_h8	GU354034
Figure 12 *Apc*		
AH ApcB	*apcB* gene and protein sequence	GU354018
AH ApcA1	*apcA* gene and protein sequence	GU354019
AH ApcA2	*apcA2* gene and protein sequence	GU354020
AH ApcA3	*apcA3* gene and protein sequence	GU354021
AH ApcA4	*apcA4* gene and protein sequence	GU354022

(renamed to CBPII), which are predominantly associated with the photosystem II reaction centre core to form a giant supercomplex (Chen *et al.*, 2008) and PcbC (renamed to CBPI) associated with PSI. The other members of this superfamily are the *psbC* gene coding for the CP43 protein of photosystem II and the gene *isiA* for the iron stress-induced protein IsiA (CBPIII).

The novel strain possesses the genes *psbC* and *isiA* and eight *cbp* homologues belonging to this superfamily. For most CBPs, exactly one matching homologue was found (Figure 11), which indicates
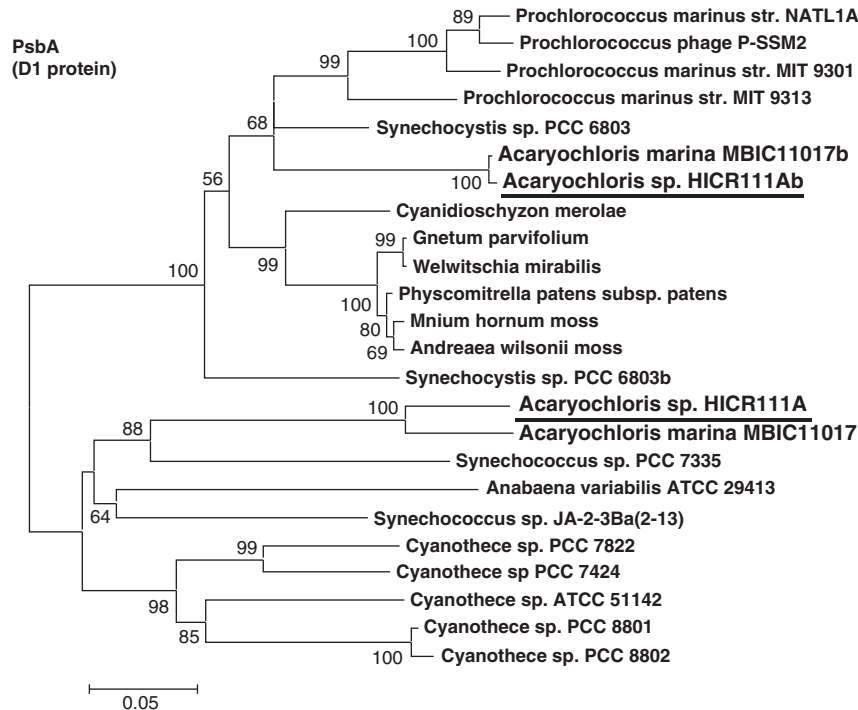
Figure 10 Phylogenetic tree (neighbour joining (NJ)) for the amino acid sequences of the photosynthetic core protein D1 encoded by *psbA,* calculated using MEGA 4 (Tamura *et al.*, 2007). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Bootstrap values of $\geqslant 50$ indicate support.

similar strategies in all these strains in constituting the photosynthetic machinery. The missing CBP homologues are CBPIIIb, which belongs to the IsiA subfamily and is plasmid encoded in *A. marina*, and CBPIa/d.

As expected, the genomic data confirmed the absence of phycoerythrin genes. However, we were surprised to find not a single copy of a phycocyanin-related gene. In *A. marina* MBIC11017, the genes for the phycocyanin α- (*cpcA*) and α-subunits (*cpcB*) as well as the gene for the phycobilisome rod-core linker polypeptide (*cpcG*) are located in a single cluster on plasmid pREB3. None of these genes is present in *Acaryochloris* sp. HICR111A, despite the fact that other parts of plasmid pREB3 are well conserved. In contrast, all allophycocyanin-related genes are present. We even found an additional fourth copy of the allophycocyanin α-subunit gene (*apcA*). The phylogenetic tree in Figure 12 visualizes their relationship to each other and to other phycobiliproteins of cyanobacteria and red algae. The fourth copy of ApcA is distinct from the other three. Instead of grouping with homologous ApcA from *Acarychloris marina,* it seems to share more sequence similarity with *Cyanothece* and *Crocosphaera* ApcA. With all subunits present we conclude that allophycocyanin is present in the novel strain. As there is no evidence for phycocyanin, the photosynthesis apparatus is clearly distinct between the new *Acaryochloris* isolate HICR111A and *A. marina* MBIC11017.

Conclusions

We isolated a novel cyanobacterial strain that groups within the genus *Acaryochloris*. It uses Chl *d* as a main photopigment and is similar to *A. marina* in cell size and shape. Electron micrographs show that the novel strain *Acaryochloris* sp. HICR111A grows in cell aggregates that are surrounded by an extracellular matrix. This property has been observed for other isolates and we suggest that aggregated growth might be common for the family *Acaryochloris*, provided it is not influenced or triggered by habitat conditions or symbiotic factors. Allophycocyanin-coding genes were observed in HICR111A, but phycocyanin genes were not. In fact, *A. marina* MBIC11017 seems to be the only strain that expresses phycobiliproteins on a physiologically relevant level (Chan *et al.*, 2007). So far, *A. marina* MBIC11017 is the only member of the genus with a fully annotated genome. In *A. marina,* all phycocyanin-related genes are located on plasmid pREB3 and seem to be absent in *Acaryochloris* sp. HICR111A with strong support from our genomic data set. One factor that may contribute to genetic diversity within the group is the high number of transposable elements in *Acaryochloris*. Phylogenetic data support the idea of high diversity; however, it has to be pointed out that the *Acaryochloris* clade needs to be sampled and cultured more intensively on a global scale. A major consideration should be whether new species should be defined
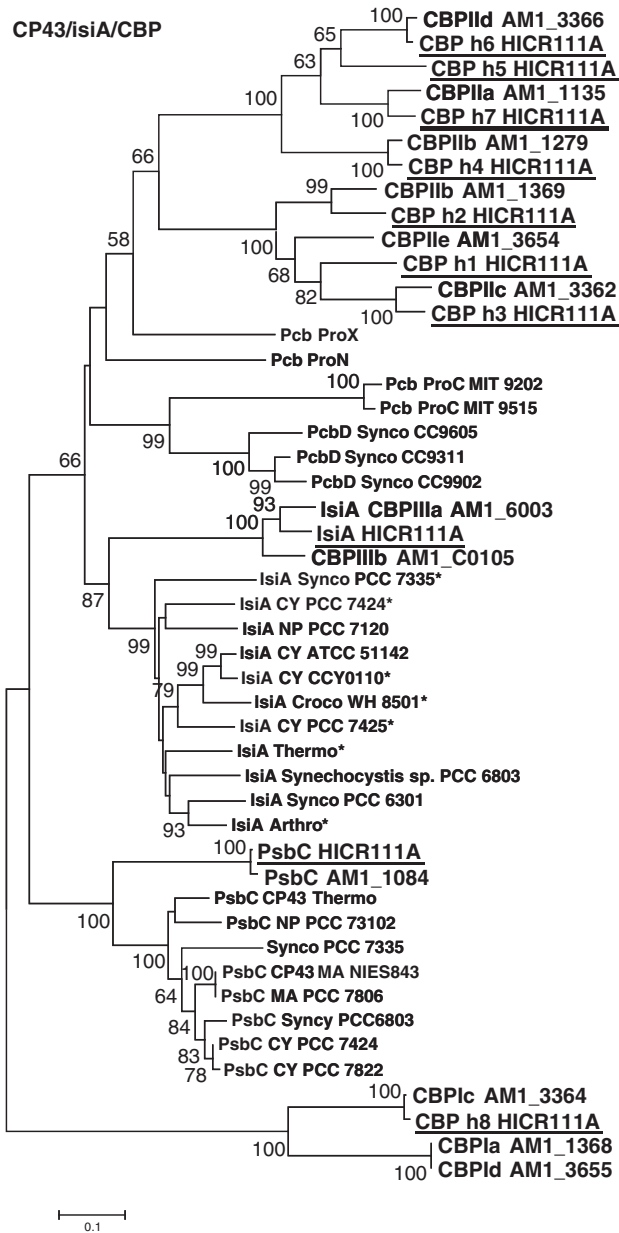
Figure 11 Phylogenetic tree of chlorophyll-binding proteins belonging to the CP43 superfamily, encoded by *psbC, isiA, pcb* and their homologues in *Acaryochloris* and other cyanobacteria. Protein IDs are given for the *A. marina* sequences, and the positions of *Acaryochloris* sp. HICR111A proteins are underlined (and see Table 1 for additional details). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test is shown next to the branches when >50. Other taxa: *Prochlorothrix hollandica* (ProX), *Prochloron didemni* (ProN), *Prochlorococcus marinus* (ProC), *Synechococcus* sp. (Synco), *Cyanothece* sp. (CY), *Nostoc punctiforme* (NP), *Crocosphaera watsonii* (Croco), *Thermosynechococcus elongatus* BP-1 (Thermo), *Arthrospira maxima* CS-328 (Arthro), and *Microcystis aeruginosa* (MA). Asterisks indicate annotation inferred by sequence alignment.

Figure 12 Phylogenetic tree showing the relationships among allophycocyanin subunits (ApcA or ApcB) and selected other phycobiliproteins in *Acaryochloris*, other cyanobacteria and red algae. The positions of *Acaryochloris* sp. HICR111A proteins are underlined. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches when ⩾50. If present, the original annotation is given. Abbreviations: Cpc, cyanobacterial phycocyanobilin; Cpe, cyanobacterial phyco-erythrin; Hyp, hypothetical protein; Phyc, phycobili-protein; Rcpe, red algal phycoerythrin. Taxon abbreviations: *Acaryochloris marina* (AM), *Acaryochloris* sp. HICR111A (AH), *Synechococcus* sp. (Syn), *Synechococcus* sp. JA-2-3B'a(2-13) (JA-2-3Ba(2-13)), *Synechococcus* sp. JA-3-3Ab (JA-3-3Ab), *Gloeobacter violaceus* PCC 7421 (Gloe), *Gracilaria tenuistipitata* var. *liui* (Grac) or as in Figure 10.

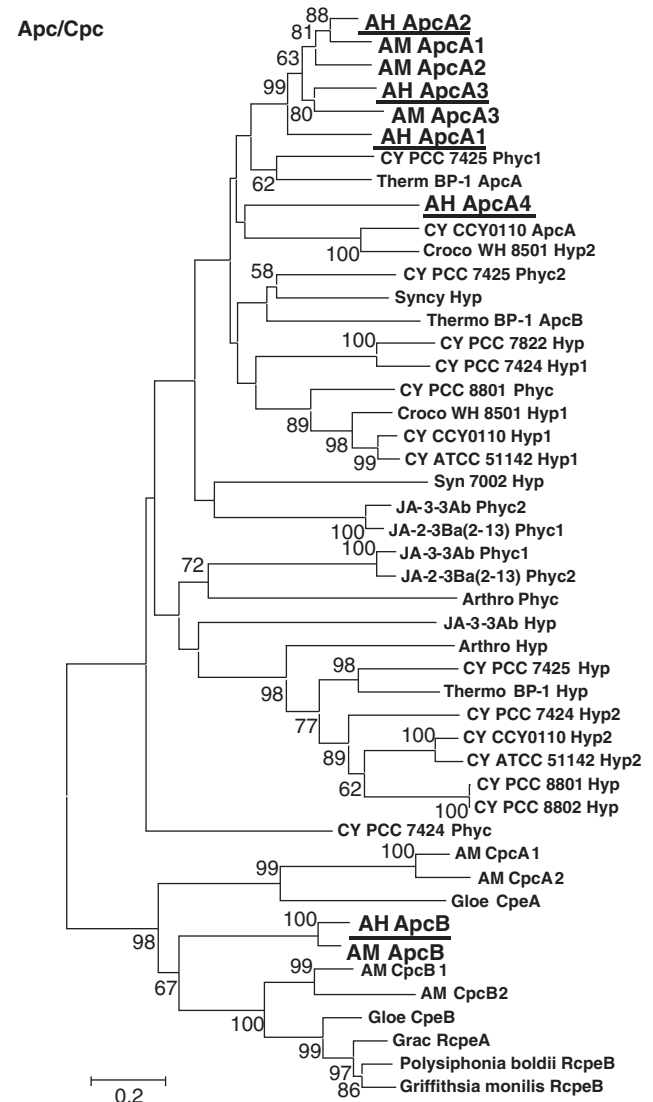within the clade, as habitat, morphology and photochemical properties seem to be diverse. The characterization of such microbes will facilitate the understanding of life in an ecological niche depleted in visible light but abundant in infrared light. The *Acaryochloris* sp. HICR111A described in this study has been deposited in the Roscoff Culture Collection from where it is publicly available under the accession number RCC1983.

Acknowledgements

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