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A NOVEL EPIPHYTIC CHLOROPHYLL *D*-CONTAINING CYANOBACTERIUM ISOLATED FROM A MANGROVE-ASSOCIATED RED ALGA[1]

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A new habitat and a new chlorophyll (Chl) *d*-containing cyanobacterium belonging to the genus *Acaryochloris* are reported in this study. Hyperspectral microscopy showed the presence of Chl *d*-containing microorganisms in epiphytic biofilms on a red alga (*Gelidium caulacantheum*) colonizing the pneumatophores of a temperate mangrove (*Avicennia marina*). The presence of Chl *d* was further proven by high performance liquid chromatography (HPLC)-based pigment analysis and by confocal imaging of cultured cells. Enrichment of mangrove biofilm samples under near-infrared radiation (NIR) yielded the new *Acaryochloris* sp. MPGRS1, which was closely related in terms of 16S rRNA gene sequence to an isolate from the hypertrophic Salton Sea, USA. The new isolate used Chl *d* as its major photopigment; Chl *d* and Chl *a* contents were ~98% and 1%–2% of total cellular chlorophyll, respectively. These findings expand the variety of ecological niches known to harbor Chl *d*-containing cyanobacteria and support our working hypothesis that such oxyphototrophs may be ubiquitous in habitats depleted of visible light, but with sufficient NIR exposure.

Key index words: **chlorophyll *d*; Cyanobacteria; epiphyte; mangrove; photosynthesis**

The spectral range of solar radiation employed for oxygenic photosynthesis has been extended to the near infrared by cyanobacteria containing red-shifted chlorophylls, i.e., chlorophyll (Chl) *d* and *f* (Chen and Blankenship 2011). Chl *f* has so far only been found in a cyanobacterial enrichment from a stromatolite (Chen et al. 2010), and its importance in light harvesting remains to be resolved. In contrast, Chl *d* has been clearly assigned to the cyanobacterium *Acaryochloris marina*, which was originally isolated and described as a symbiont of a didemnid ascidian from shallow waters in Palau (Miyashita et al. 1996, 2003). It is a small, sub-spherical to partially elongate cyanobacterium, ~1–2 μm long, containing Chl *d* as its major photopigment. The discovery is of general interest since in *A. marina* Chl *a* is largely replaced by Chl *d* in the reaction centers and light-harvesting complexes (Hu et al. 1998, Chen et al. 2002, Schlodder et al. 2007). Some evidence indicates that Chl *a* may still play a role in the reaction center of photosystem II in *A. marina*, although the details have yet to be determined (Chen et al. 2005, Razeghifard et al. 2005, Schlodder et al. 2007, Tomo et al. 2007). Nevertheless, Chl *d* seems to have taken over the major roles of Chl *a* in this organism, where it accounts for >95% of the total chlorophyll (Miyashita et al. 1996).

The initial discovery of Chl *d* was made in 1943 in a variety of benthic red algae (Manning and Strain 1943). It seems likely that these red algae were the site of epiphytic colonies of *Acaryochloris* strains, since such Chl *d*-containing *Acaryochloris* strains have been detected, as colonies, on a number of benthic red algae in Japan (Murakami et al. 2004). Since the initial isolation of *A. marina* from didemnid ascidians, where it has now been shown to be

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abundant in epizoic biofilms on the undersides of the ascidians (Kühl et al. 2005, Behrendt et al. 2012), further discoveries of *Acaryochloris* strains have been made. Miller et al. (2005) isolated an *Acaryochloris* strain from a benthic community in an eutrophic, man-made lake in California. de los Rios et al. (2007) reported the occurrence of a cyanobacterium inside cracks below rocks in Antarctica, which was very similar with respect to ultrastructure and 16S rRNA gene sequence to *Acaryochloris*. Other environmental DNA surveys have detected the presence of *Acaryochloris*-like organisms in stromatolites from Shark Bay, Western Australia (Goh et al. 2009) and epilithic biofilms from Mayan archaeological sites in Mexico (McNamara et al. 2006).

More recently, Mohr et al. (2010) isolated a free-living *Acaryochloris* sp. HICR111A, from coral surfaces, and Behrendt et al. (2011) showed that an *Acaryochloris* strain with Chl *d* was abundant in an endolithic niche under crustose coralline algae; both found at Heron Island on the Great Barrier Reef. Furthermore, the association of *Acaryochloris* with stromatolites from Shark Bay, Australia has recently been confirmed by the isolation of a pure culture (Li et al. in press). Finally, a new and apparently symbiotic *Acaryochloris* member, *Candidatus Acaryochloris bahamiensis* was found inside Bahamian didemnid ascidians (López-Legentil et al. 2011). The presence of Chl *d* in such different marine and terrestrial systems (reviewed in Behrendt et al. 2011) suggests that *Acaryochloris* is globally widespread in habitats depleted in visible light, but with sufficient near infra-red radiation (NIR) availability.

Chl *d* extends the range of photosynthetic light capture to ~750 nm, due to the extended range of its Q_y band (peak at ~710 nm in vivo; Manning and Strain 1943, Miyashita et al. 1996, Duxbury et al. 2009). It is this extended light capture range that provides a niche for *Acaryochloris* in situations such as beneath didemnid ascidians (Kühl et al. 2005), coralline algae (Behrendt et al. 2011) and on red algae (Murakami et al. 2004), where little visible light in the range of 400–700 nm is available. Such situations may be commonplace in shallow water marine habitats and this may account for the reports of Chl *d* from a wide range of marine sites (Kashiyama et al. 2008).

In this study, we investigated the possibility that *Acaryochloris*–like cyanobacteria thrive in temperate marine systems around Sydney, Australia. Estuarine waters around Sydney are particularly appropriate for such studies because the high levels of yellow substance (Gelbstoff) in these waters, arising from mangroves and salt marshes significantly reducing the visible light levels. We investigated macroalgae, surface sediments as well as mangrove pneumatophores at the sampling site. Here we present evidence for a new epiphytic *Acaryochloris* strain growing in biofilms on a marine red alga colonizing the pneumatophores of the gray mangrove, *Avicennia marina*. A preliminary account of part of the molecular analysis was presented in a proceedings paper (Li et al. in press).

MATERIALS AND METHODS

Sampling site and mangrove-associated algae. The pneumatophores of the mangrove, *A. marina* (Forsk.) Vierh. were sampled on the southern side of Salt Pan Creek, Georges River, NSW, Australia (34°56′59″S 151°02′32″E; Fig. 1) in a mixed population of the mangroves *A. marina* and *Aegiciras corniculatum* (L.) Blanco. Pneumatophores were collected at extreme low tides, ~2 m outside the canopy, at the edge of the canopy, and ~2 m inside the canopy, respectively. The pneumatophores at this site were colonized mainly by the red alga *Gelidium caulacantheum* J. Agardh (identified from rbcL sequence data) and lesser numbers of the red algae *Caloglossa vieillardii* (Kützing) Setchell and *Catenella nipae* Zanardini and even smaller numbers of various species of brown and green algae (cf. King 1981).

Hyperspectral imaging and microscopy. Small pieces of red algae (scrapings, intact pieces or cross sections) from the sampled pneumatophores were mounted on microscope slides, and observed on a compound epifluorescence microscope (BX51; Olympus, Tokyo, Japan). Bright field and autofluorescence images were recorded using a digital camera (Coolpix 4500; Nikon, Tokyo, Japan) mounted on a microscope with an ocular adapter (Optem, Sandia, TX, USA). Images of UV-induced fluorescence of samples were recorded using the mercury lamp of the microscope with a 350–390 nm excitation filter, a 510 nm beam splitter, and a 520 nm barrier filter in the light path.

Hyperspectral microscopic imaging of transmitted light was recorded using a hyperspectral camera system (VNIR-100;

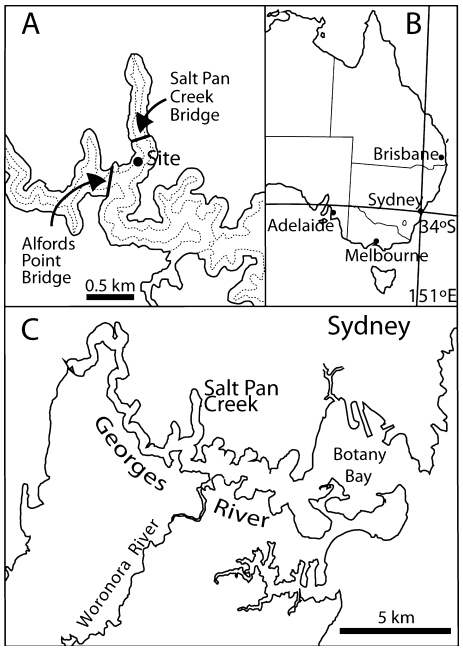

FIG. 1. The location of the mangrove sampling site on Salt Pan Creek, Georges River, Sydney, Australia. (A) The location of the site in Salt Pan Creek. (B) Map of Eastern Australia. (C) Map of the Georges River and Botany Bay in relation to the City of Sydney.

Themis Vision Systems, St Louis, MO, USA; Kühl and Polerecky 2008) mounted on the C-mount of the microscope. The halogen lamp of the microscope was used as light source for the hyperspectral imaging. Hyperspectral image stacks were recorded in darkness, in regions on the slide without objects (100% reference), and in regions with sample material.

After image acquisition, hyperspectral stacks were dark corrected and normalized to the reference hyperspectral stack using the system software (Hypervisual, Themis Vision Systems). Such corrected image stacks contained values of spectral transmittance for every pixel in % of incident light and transmission spectra in particular areas of interest could be extracted. After file format conversion in ENVI (Exelis Visual Information Solutions, Boulder, CO, USA), further processing of corrected data were done according to Polerecky et al. (2009) using the freeware Look@MOSI (http://www.microsen-wiki.net/doku.php?id=lookatmosi_howto). Briefly, we used the fourth derivative of log-transformed hyperspectral stacks of transmittance to quantify the strength of light attenuation at particular wavelengths characteristic of Chl *a* (675–80 nm) and Chl *d* (710 nm) absorption. This analysis enabled us to generate false-color images showing the relative intensity of Chl *a* and Chl *d* absorption over the investigated sample, which could be spatially related to microscope images of the same sample.

Confocal microscopy. Laser scanning confocal microscopy was used to image the morphology and to characterize the cellular micro-localisation of photopigments of the cultured *Acaryochloris* sp. MPGRS1. A confocal laser scanning system (e.g., Leica TCS SP5 AOBS; Leica Microsystems, Wetzlar, Germany) on an inverted microscope (Leica DMI6000; Leica Microsystems) was used to image *Acaryochloris* cells mounted in culture K+ES seawater medium (Miyashita et al. 1996) under a glass coverslip on a glass slide (see culture method, below). Cells were imaged using the HCX APO UV1 100× N.A. 1.30 oil immersion objective and excitation by the Argon laser 488 nm line, capturing emission spectra from photosynthetic pigments at 650–740 nm. To test for the presence of different chlorophylls, emissions at 488 nm excitation were imaged separately into two different spectral bands and detected in two different photomultiplier tubes (PMTs), eventually selecting emissions at 680–720 nm (Chl *a*) in the PMT1 detector channel and at 730–760 nm (Chl *d*) in PMT2 detector channel. A three-dimensional (3D) reconstruction of cells was made by serial image sectioning in the xyz scan mode using two averaged frames and four averaged lines per section, at 0.17 μm increments and to a total depth of 4.36 μm. The 3D images of cells were made using Leica LAS AF2.2.1 software (Leica Microsystems).

Isolation and cultivation of Acaryochloris *sp. MPGRS1.* Cell aggregates of the putative Chl *d*-containing organisms were scraped off the ramuli of *G. caulacantheum* using a sharpened dissection needle; cells were concentrated and washed in filtered seawater in a microcentrifuge tube. The collected cells were then placed in an Erlenmeyer flask (20 mL) in K+ES seawater medium (Miyashita et al. 1996) and placed in a custom-built incubation chamber illuminated with 720 nm light (20 μmol photons $\cdot$ m^{-2} $\cdot$ s^{-1}) provided by a NIR LED array (Cat. L720-04AU; Epitex, Kyoto, Japan). The samples were grown at 27°C in continuous NIR light for several weeks and were then subcultured with fresh K+ES medium every 30 d. The green cells obtained by this cultivation technique contained >95% Chl *d* with the remaining Chl being Chl *a*, as determined using high performance liquid chromatography (HPLC) analysis. HPLC analysis was performed on a Shimadzu VP series HPLC system with a reverse phase C18 column 250 mm × 4.6 mm (Phenomenex, Torrance, CA, USA) at a 100% methanol flow rate of 1 mL $\cdot$ min^{-1}. Isolated pigment was identified using on-line absorbance spectra and known retention times of Chl *a* and Chl *d*.

Genome DNA extraction. Cells from the type strain *A. marina* (MBIC11017) and *Acaryochloris* sp. MPGRS1 were harvested from 1.5 mL culture medium by centrifugation and rinsed twice in water. The cell pellet was resuspended in 250 μL Tris-EDTA buffer (pH = 8.0; TE, BIOTHEMA, Handen, Sweden) containing lysozyme (20 mg $\cdot$ mL^{-1}). After resuspension, 0.2 μL proteinase K (20 mg $\cdot$ mL^{-1}; FINNZYMES, Vantaa, Finland) and 20% SDS (final concentration 2%) were added. The mixture was incubated at 50°C until the cell suspension color changed from green to yellowish brown. Subsequently, RNAse A (final concentration 0.16 mg $\cdot$ μL^{-1}; R6148, SIGMA, St Louis, MO, USA) was added and incubated at 65°C for 40 min, followed by phenol-chloroform phase separation. Extracted DNA was stored in nuclease-free water at −20°C.

Polymerase chain reaction. The 16S rRNA gene sequence was amplified using isolated genomic DNA as template with cyanobacteria-specific primer sets 27F 5′-AGAGTTTGATC CTGGCTCAG-3′/809R 5′-GCTTCGGCACGGCTCGGGTCGAT-A-3′, and 740F 5′-GGC (TC)(AG) (AT)A(AT)CTGACACT (GC)AGGGA-3′/1494R (5′-TACGGCTACCTTGTTACGAC-3′). All PCR reactions were prepared in a volume of 20 μL containing 1x Mango Taq Polymerase Reaction buffer, 2 mM $MgCl_2$, 0.2 mM of each dNTPs (BIOLINE, London, UK), 1.25 U Mango Taq Polymerase (BIOLINE), 20 ng template DNA and 5 pmol of each primer. Thermal cycling was performed with an initial denaturation at 94°C for 2 min, followed by 35 cycles of DNA denaturation at 94°C for 10 s, annealing at 54°C for 20 s, and extension at 72°C for 60 s. Amplification was finalized with an extension step at 72°C for 7 min.

For the detection of the presence of the gene for phycocyanin, cpcG, PCR was similarly employed using specifically designed primers (forward: CCAGGCAGATTACCGTACAG, and reverse: AAATTCCTCACCAAAACGAGTG).

Sequencing. Amplified 16S rRNA gene fragments were separated on a 1.5% agarose gel and the major product was purified for sequencing. Purified DNA fragments were sequenced at the Australian Genome Research Facility Ltd (AGRF; www.agrf.org.au) using the purified DNA (PD) Service. Both sequences using primer pair 27F/809R and 740F/1494R were used for sequencing. Complete 16S rRNA gene sequences were obtained by combining these two partial gene sequences through 100% overlapping regions (16S rRNA gene position ~ 740–809).

Phylogenetic analysis. 16S rRNA gene sequence data were analyzed using multiple sequence alignment tools (ClustalW), where the sequence alignment was modified manually using the previously published alignment of Mohr et al. (2010). Phylogenetic relationships were inferred using the neighbor-joining (NJ) method with the Jukes-Cantor model using molecular evolutionary genetic analysis (MEGA) software version 5.0. The phylogenetic tree stability was evaluated by bootstrap replication at 1000 times. The sequence data were obtained directly in the case of *A. marina* (MBIC11017) and *Acaryochloris* sp. MPGRS1 or were obtained from Mohr et al. (2010).

RESULTS

Detection of Chl d*-containing cells on* Gelidium caulacantheum. Brightfield and fluorescence microscopy of small intact thallus samples, as well as hand-cut cross sections of *G. caulacantheum*, and surface scrapings of the alga revealed the presence of epiphytic aggregates of unicellular greenish cells that exhibited various degrees of red fluorescence upon UV-excitation (Fig. 2, A, B, D, E and G). These epiphytes were small (~1 μm diameter)

unicellular cells growing in thin layers on the lower and mid parts (and never at the tip) of the ramuli of *G. caulacantheum*. Stronger UV-induced red autofluorescence of the red algal chloroplasts were also observed. Hyperspectral imaging of the same samples revealed spectral signatures characteristic of Chl *d* with a trough in the spectral transmission at 710 nm, corresponding to the in vivo absorption maximum of Chl *d* (Fig. 2C). Color mapping of Chl *a* (675 nm) and Chl *d* (710 nm) distribution over the sample showed that Chl *d* was distributed in thin epiphytic biofilms and patches on the red alga (Fig. 2, F, H and I).

G. caulacantheum was predominant on the surface of pneumatophores of the gray mangrove, *A. marina* that were air-exposed at low tide (most abundant on

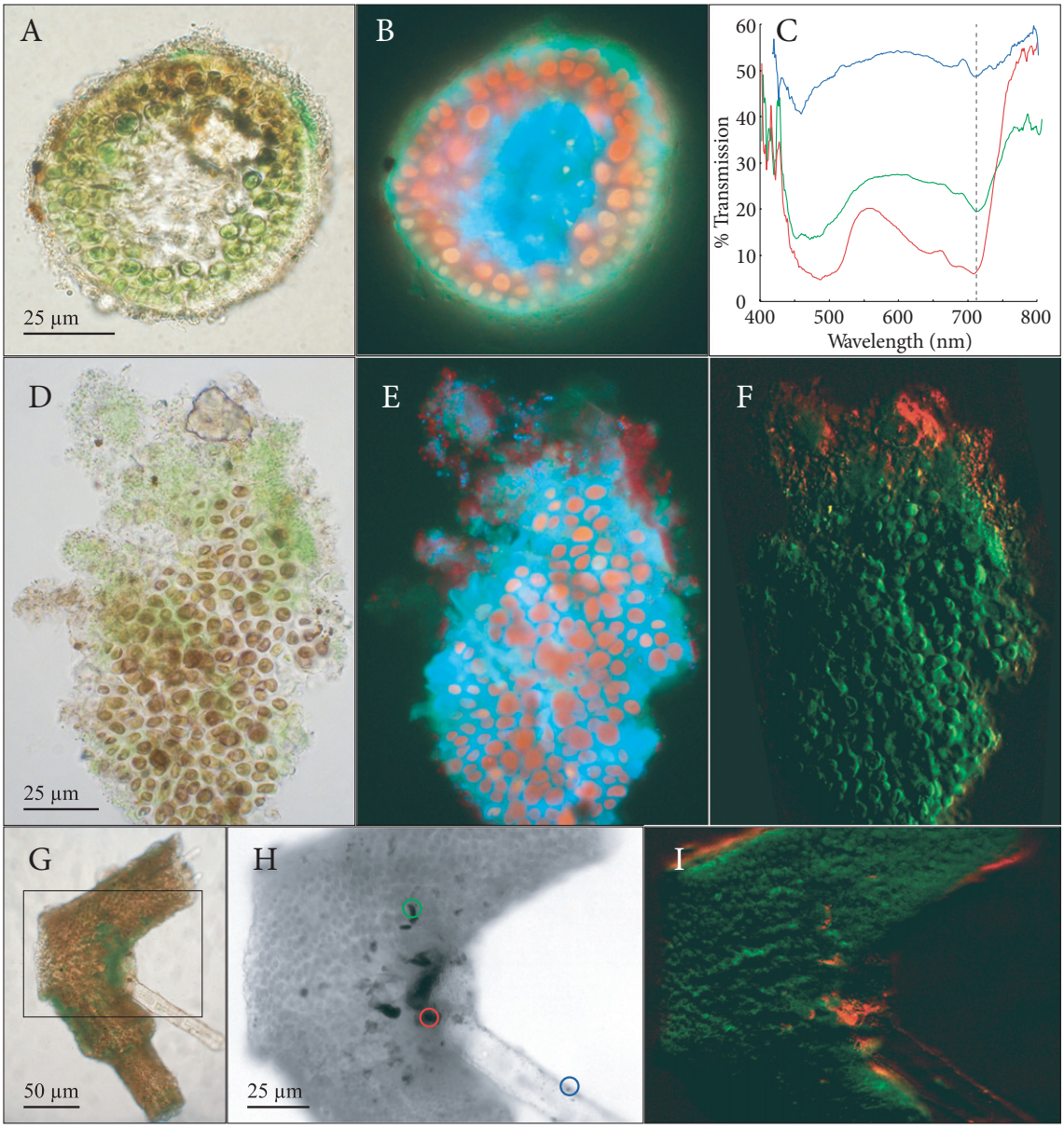

FIG. 2. Bright field, epifluorescence and hyperspectral images of *Acaryochloris*-like cells on the outer surface of *Gelidium caulacantheum* ramuli. Larger red algal chloroplasts appear bright red in the fluorescence images. (A) and (B) Brightfield and UV-induced autofluorescence of a *G. caulacantheum* cross section indicating *Acaryochloris*-like cells around the periphery. (C) Spectral transmission spectra from three areas of interest (position indicated in panel H) containing small unicellular epiphytes showing a distinct Chl *d* absorption maximum at ~710 nm. (D) and (E) Brightfield and UV-induced autofluorescence images of a surface scraping of cells from *G. caulacantheum*. (F) Color coded hyperspectral image of the same sample, where green quantifies Chl *a* (675 nm) absorption and red quantifies Chl *d* (710 nm) absorption. (G) Brightfield image of a *G. caulacantheum* section with green epiphytes. (H) and (I) Hyperspectral image of the same sample showing transmission at 710 nm (H) and a color coded image of the same sample quantifying Chl *a* (675 nm) and Chl *d* (710 nm) absorption over the sample.

pneumatophores just outside the mangrove foliage vertical profile). Other, less abundant algae (mainly red algal species) also colonized the mangrove pneumatophores, but Chl *d* was only detected in the hyperspectral analysis of *G. caulacantheum*.

Isolation and characterisation of a new Acaryochloris *sp.* On the basis of enrichments under NIR (Duxbury et al. 2009) from surface scrapings of *G. caulacantheum*, we obtained a final culture containing subspherical dull greenish cells, which were ~1 μm or less in diameter and with similar morphology as other reported *Acaryochloris* strains. The 16S rRNA sequence of the new *Acaryochloris* strain was compared with other near full-length sequences of other *Acaryochloris* strains (as described in Mohr et al. 2010); the sequences included the unique VI region, which was specifically targeted employing the primers detailed in Mohr et al. (2010). Alignment was done using ClustalW followed by manually checking against the published alignments in Mohr (2010). The phylogenetic trees were constructed using 16S rRNA alignment of 1476 bp including gaps and showed that *Acaryochloris* sp. MPGRS1 clustered with other *Acaryochloris* strains (Fig. 3) and was most closely related to *Acaryochloris* CCMEE5410 obtained from the Salton Sea, a hypertrophic man-made lake (Miller et al. 2005). We named this new strain of *Acaryochloris* "mangrove pneumatophore, Georges River, Sydney, strain 1" (MPGRS1).

The MPGRS1 strain exhibited the same pigmentation profile as other isolated *Acaryochloris* cultures (Fig. 4). Chl *d* was the major photopigment, accounting for up to 98% of total cellular chlorophyll, whereas Chl *a* accounted for only 1%–2% of total chlorophylls in MPGRS1. Like other *A. marina* strains, the MPGRS1 strain contained zeaxanthin and alpha-carotene as its main carotenoids, and no other carotenoids were detected in the HPLC analysis. PCR primers for phycocyanin (cpcG) confirmed the presence of this phycobiliprotein in *Acaryochloris* sp. MPGRS1.

Confocal microscopy of the unicellular greenish cells showed that they were oval in shape, with an average size of 1.67 ± 0.23 μm length and 1 ± 0.14 μm width. Chlorophyll emissions arose from two separate and clearly defined regions in each cell—a band (400–600 nm width) encircling each cell with far-red emissions imaged at 730–750 nm, corresponding to Chl *d*, augmented in patches by red emissions, imaged at 680–720 nm, corresponding to more blue-shifted spectral forms of Chl *d* (Fig. 5).

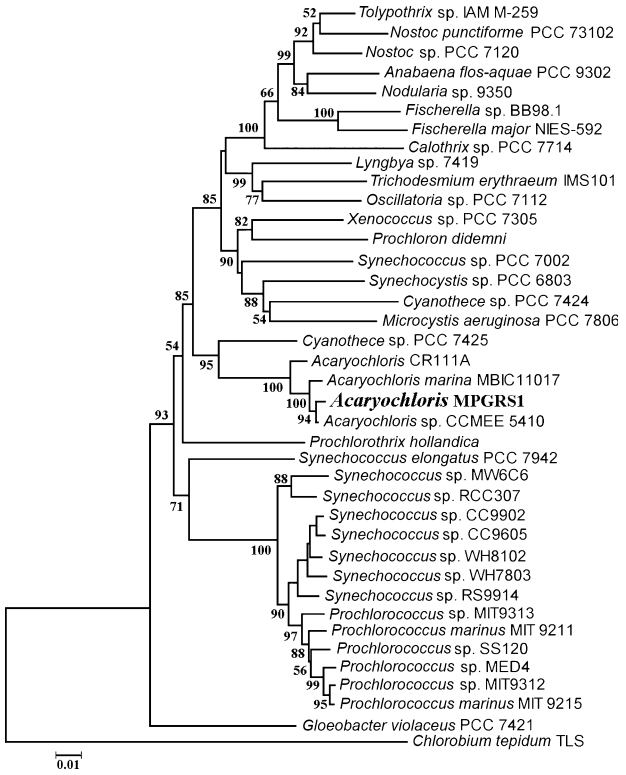

FIG. 3. Phylogenetic position of the new *Acaryochloris* sp. MPGRS1. Neighbor-joining phylogenetic tree analysis based on the comparison of 1476 bp 16S rRNA genes. The green sulfur bacterium *Chlorobium tepidum* TLS was used as an out group. The phylogenetic relationship was constructed using the Neighbor-Joining methods, where support values were calculated using molecular evolutionary genetics analysis (MEGA) software version 5.0 (MEGA 5). Scale represents 0.01 substitutions per nucleotide position. Only bootstrap values >50% of 10,000 re-samplings of the data were illustrated at nodes.

DISCUSSION

The discovery of a new *Acaryochloris* sp. MPGRS1 strain, on the red alga, *G. caulacantheum,* growing on the pneumatophores of the gray mangrove, *A. marina,* extends the range over which these Chl *d*-containing cyanobacteria have been found. Following Murakami et al. (2004), this represents only the second report of *Acaryochloris* on a red alga. In the investigated mangroves, the dominant alga was the red alga *G. caulacantheum*. Other algae, some more characteristic of pneumatophores in this region, e.g., *Caloglossa vieillardii* (=*C. leprieurii*), *Catenella nipae* and *Bostrychia* spp. (King 1981) were not observed to have *Acaryochloris* on their surfaces. However, we did not examine pneumatophores exhaustively throughout the region and it is possible that a more careful examination would reveal them. Interestingly, a recent survey of the cyanobacterial diversity in the phyllosphere of a Brazilian mangrove system (Rigonato et al. 2012) showed the presence of 16S rRNA gene sequences closely related to *Acaryochloris* in some of their samples, thus further pointing to mangroves as a habitat for *Acaryochloris*-like cyanobacteria.

Estuarine waters in Australia contain high levels of yellow substance (Gelbstoff; Kirk, 2010) and this reduces the penetration of visible light in such waters. A spectrophotometric examination of the waters in Salt Pan Creek, Georges River in 2004

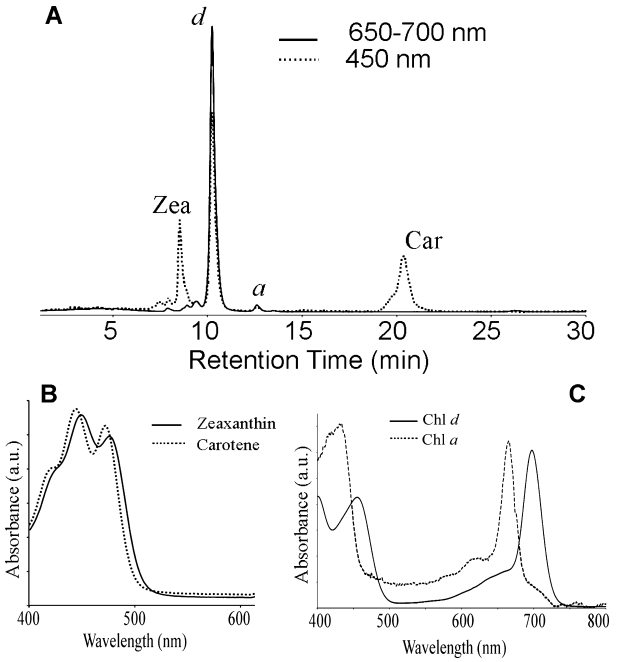

FIG. 4. High performance liquid chromatography and absorption spectra of resolved pigments in the new *Acaryochloris* sp. MPGRS1. (A) Chromatograph recorded at 450 nm (dotted line) and at the maximal spectral reading between 650 and 750 nm (solid line) (B) absorption spectra of resolved carotenoids: zeaxanthin (solid line) and alpha-carotene (dashed line) (C) absorption spectra of resolved chlorophylls: Chl *a* (dashed line) and Chl *d* (solid line).

showed that all visible light was reduced from ~2,100 μmol photons · m^{-2}·s^{-1} (summer) to levels <1% within the upper 1.2 m of the water column (Drew and Larkum, unpublished results). The tidal range in the Georges River estuary is ~2 m under neap tides and ~4 m under spring tides, thus visible light would be greatly restricted over large periods of the tidal cycle. Red algae have an abundance of phycobiliproteins, which are capable of harvesting the green light maximally transmitted by yellow substance, and this must be an important factor in their abundance on the pneumatophores. However, green and brown algae are also present and these may use efficient light-harvesting strategies during low tide when they can be air-exposed for 1–3 h. Cyanobacteria including *Acaryochloris* MPGRS1, as we show here are also present.

Many, but not all strains of *Acaryochloris*, contain the phycobiliprotein, phycocyanin (Chen et al. 2009), which can function as an antenna pigment (Petrasek et al. 2005, Duxbury et al. 2009). *Acaryochloris* sp. MPGRS1 possesses the gene for phycocyanin too and, if expressed, it can thus harvest visible light and near-infra-red radiation during most periods of the day. The presence of a dense covering of red algae in the vicinity of *Acaryochloris* MPGRS1 would reduce the visible light during emergence but this would be offset by the high light levels and the location of *Acaryochloris* sp. MPGRS1 on the surface of ramuli of *G. caulacantheum*.

A recent study of the energy storage efficiency of *Acaryochloris* MBIC11017 (type strain) showed that its Chl *d*-driven photosynthesis exhibits a similar or even higher quantum efficiency compared to other oxygenic phototrophs that contain Chl *a* (Mielke et al. 2011). Also, *Acaryochloris* MBIC11017 was shown to grow and photosynthesize effectively in biofilms under NIR illumination (Behrendt et al. 2011, 2012). Thus, these Chl *d*- driven phototrophs are apparently not experiencing photon energetic limitations per se in their photosynthetic activity by expanding their major light harvesting into the NIR. However, the typical natural habitat and growth mode of *Acaryochloris* seems to be growth in biofilms on various biotic or abiotic surfaces (Behrendt et al. 2011, 2012) and in such surface-associated communities where the realized energy storage efficiency at the community level may be less (see Al-Naijar et al. 2010). Further studies of photosynthetic activity and efficiency of Chl *d*-containing cyanobacteria in their natural habitat are thus needed to better understand their ecological niche and the environmental controls thereof.

A number of strains of *Acaryochloris* have so far been typified by 16S rRNA gene analysis of near full-length sequences. This includes the VI region,

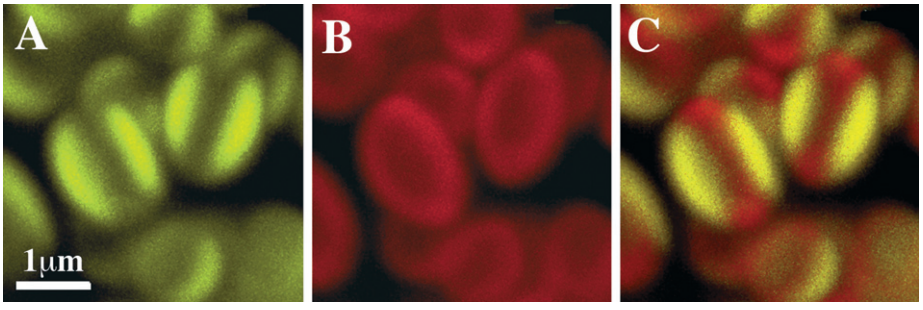

FIG. 5. Confocal imaging of spectral fluorescence of cultured cells of the new *Acaryochloris* sp. MPGRS1 excited at 488 nm showing distinct differences in proportional localisation of the photosynthetic pigments. (A) Emissions imaged at 680–720 nm (B) emissions imaged at 730–760 nm, corresponding mainly to Chl *d* (C) overlay of images (A) and (B).

which being longer than in other cyanobacteria can be taken as a diagnostic feature of *Acaryochloris* strains (Miyashita et al. 2003, Miller et al. 2005, Mohr et al. 2010, Behrendt et al. 2011). The whole genome of *A. marina* (MBIC11017) has been sequenced (Swingley et al. 2008) and a provisional genome of *Acaryochloris* CCMEE5410 has been published (Miller et al. 2011). In addition, extensive genome sequencing has been carried out on a recently isolated strain, *Acaryochloris* CR111A from shaded coral habitats (Mohr et al. 2010). Phylogenetic analysis of the near-complete 16S rRNA gene sequences of *Acaryochloris* strains showed that all strains form a distinct cluster in the cyanobacterial phylogenetic tree with closest relation to *Cyanothece* (Li et al. in press). These strains included the type strain isolated in Palau (MBIC11017), the strain isolated on the reef flat at Heron Island, Great Barrier Reef (CR111A), and a strain isolated from stromatolites in Shark Bay, Western Australia (ssball1). Our phylogenetic analysis showed that the new pneumatophore *Acaryochloris* sp. MPGRS1 was closely (98%) related to the Salton Sea strain of *Acaryochloris* CCMEE5410 (Fig. 3); but unlike the latter strain *Acaryochloris* sp. MPGRS1 does contain a gene for the phycobiliprotein, phycocyanin.

Recently, a new *Acaryochloris* member, i.e., *Candidatus Acaryochloris bahamiensis* was proposed based on microscopic evidence and phylogenetic analysis of partial 16S rRNA gene and 16S-23S ITS sequences that exhibited somewhat higher sequence divergence (>5%) when compared to *Acaryochloris* MBIC11017 (López-Legentil et al. 2011). Other shorter sequences with homology to parts of the *A. marina* 16S rRNA gene sequence are deposited in NCBI (e.g., Ohkubo et al. 2006, Green and Barnes 2010, Lins-de-Barros et al. 2010) and reinforce the idea that *Acaryochloris* is a globally distributed cyanobacterial taxon.

Confocal imaging of the new *Acaryochloris* sp. MPGRS1 isolate from mangrove samples confirmed the presence of Chl *d* as the main chlorophyll in these cells. However, we discovered that chlorophylls apparently localized in two distinct regions in each cell, with the more far-red shifted Chl *d* (emissions imaged at 730–760 nm) forming an encircling band around the cell, presumably in thylakoids, while shorter wavelengths emissions imaged at 680–720 nm were restricted to certain regions of the encircling band. Chl *a* was rare in all regions of this encircling band. Previously, we reported that Chl *d* bound to the special light-harvesting pcb-type pigment-protein of *Acaryochloris* gave rise to fluorescence emission between 680 and 720 nm, with a peak at 713 nm (Chen et al. 2002), while photosystem I isolates gave rise to longer wavelength fluorescence. It is therefore possible that our heterogeneity is the result of distinct regions of Chl *d* localization in the encircling band. Spatial heterogeneity in photopigment distribution at the single cell level was found in *Candidatus A. bahamiensis* (López-Legentil et al. 2011), where the fluorescence of photosynthetic pigments of *Candidatus A. bahamiensis* localized in two distinct peripheral regions of the cell when excited with a 635 nm laser line: one area emitting fluorescence between 700 and 750 nm corresponding to Chl *d*, and another area emitting at 640–670 nm corresponding to phycobiliproteins (López-Legentil et al. 2011). Whether such distinct spatial localisation of chlorophylls and phycobiliproteins is a characteristic of *Acaryochloris* is yet not known. We did not analyze for fluorescence of phycobiliproteins. Further work on other *Acaryochloris* strains is needed to reveal whether this feature of heterogeneous pigment distribution is a unique morphological characteristic of cells of the *Acaryochloris* genus.

Our study adds to the growing evidence that Chl *d*-containing cyanobacteria in the genus *Acaryochloris* have a much wider geographic distribution than initially thought, when the type strain was first isolated from tropical ascidians. In line with other recently described habitats of *Acaryochloris*, a major niche-defining trait on the mangrove pneumatophores is strong depletion of visible light but sufficient NIR exposure. Searches for Chl *d*–containing oxyphototrophs in habitats with similar characteristics would thus appear to hold out much promise.

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