Microenvironment and Phylogenetic Diversity of *Prochloron* Inhabiting the Surface of Crustose Didemnid Ascidians

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ABSTRACT

The cyanobacterium *Prochloron didemni* is primarily found in symbiotic relationships with various marine hosts such as ascidians and sponges. *Prochloron* remains to be successfully cultivated outside of its host, which reflects a lack of knowledge of its unique ecophysiological requirements. We investigated the microenvironment and diversity of *Prochloron* inhabiting the upper, exposed surface of didemnid ascidians, providing the first insights into this microhabitat. The pH and O_2 concentration in this *Prochloron* biofilm changes dynamically with irradiance, where photosynthetic activity measurements showed low light adaptation (E_k ~80±7 µmol photons m^{-2} s^{-1}) but high light tolerance. Surface *Prochloron* cells exhibited a different fine structure to *Prochloron* cells from cloacal cavities in other ascidians, the principle difference being a central area of many vacuoles dissected by single thylakoids in the surface *Prochloron*. Cyanobacterial 16S rDNA pyro-sequencing of the biofilm community on four ascidians resulted in 433 operational taxonomic units (OTUs) where and average of ~85% (65-99%) of all sequence reads, represented by 136 OTUs, were identified as *Prochloron* via BLAST search. All of the major *Prochloron*-OTUs clustered into independent, highly supported phylotypes separate from sequences reported for internal *Prochloron*, suggesting a hitherto unexplored genetic variability among *Prochloron* colonizing the outer surface of didemnids.

40 INTRODUCTION

41 *Prochloron didemni* (Lewin, 1977) is a large (7-25 μm in diameter), spherical
42 cyanobacterium (Cox, 1986) commonly found as an obligate extracellular symbiont
43 associated with mainly didemnid ascidians (Lewin and Cheng, 1975; Newcomb and Pugh,
44 1975), but also holothurians and sponges (Cheng and Lewin, 1984; Parry, 1986). The
45 photosystems of *Prochloron* are typical of a cyanobacterium (Hiller and Larkum, 1985;
46 Christen *et al.*, 1999); however, its major pigment protein complex that binds both Chl *a* and
47 *b* (Hiller and Larkum, 1985) belongs to a special group shared with *Prochlorococcus*,
48 *Prochlorothix* and the Chl *d*-containing cyanobacterium, *Acaryochloris marina* (Chen *et al.*,
49 2005). *Prochloron* is typically located inside the cloacal compartment of didemnid ascidians,
50 but may also occur in other parts such as on the outer surface of asymbiotic ascidians (e.g.
51 *Didemnum candidum)* (Cox, 1986; Lewin and Cheng, 1989) and even intra-cellularly (Hirose
52 *et al.*, 1996; Hirose, 2014). *Prochloron* from the external and internal parts of ascidians have
53 been shown to exhibit significantly different morphological characteristics (Cox, 1986),
54 leading to the suggestion that at least two and potentially more species of *Prochloron* exist.
55 However, phylogenetic studies based on sequencing of the 16S rRNA gene have so far
56 indicated that *Prochloron* are conspecific, with *Prochloron didemni* being the only species in
57 the *Prochloron* genus (Stackebrandt *et al.*, 1982; Stam *et al.*, 1985; Holton *et al.*, 1990;
58 Münchhoff *et al.*, 2007; Donia *et al.*, 2011b).

59 The symbiotic relationship between *Prochloron* and its host is still poorly understood.
60 None of the ascidians with external *Prochloron* are known to harbour morphological
61 adaptations to accommodate the symbionts, and often these ascidians can be found entirely
62 without *Prochloron* cover, suggesting that this particular association is non-obligatory to the
63 host (McCourt *et al.*, 1984; Cox, 1986). In other cases, the photosynthetic activity of
64 *Prochloron* has been shown to enhance host respiration (Pardy, 1984; Koike *et al.*, 1993) and

growth (Olson, 1986), with *Prochloron* photosynthates contributing up to ~60% of the hosts' carbon demand (Alberte *et al.*, 1987). The disjunct positioning of the symbionts in the hosts cloacal cavity, in the test or on the outer test surface, seems counter intuitive to their function in providing a source of carbon, and suggests the presence of an as yet undiscovered nutrient uptake mechanism in the ascidian, facilitating translocation of low molecular weight compounds from the symbionts into the host (Pardy and Lewin, 1981; Griffiths and Thinh, 1983; Hirose and Maruyama, 2004).

There have been many attempts to unravel the physiology and symbiotic nature of *Prochloron* (Lewin and Cheng, 1989; Kühl and Larkum, 2002) (and references therein) but progress has been limited as *Prochloron* has never been successfully cultivated outside its host, and only a single non-confirmed report of short-term culture success with the use of the amino acid tryptophan has been published (Patterson and Withers, 1982). The genome of *Prochloron didemni*, extracted from the cloacal cavity of the ascidian *Lissoclinum patella*, was recently sequenced, revealing a complex set of primary metabolic genes, gene encoding for secondary metabolites and absence of genome reduction (Donia *et al.*, 2011b). This suggests that *Prochloron didemni* may be able to thrive independently of the ascidian host and that this putative symbiotic interaction might in fact not be obligatory for the symbiont, although reports of free-living *Prochloron* are scarce (Cox, 1986; Münchhoff *et al.*, 2007).

A recent study of the *in-hospite* microenvironment of *L. patella* (Kühl *et al.*, 2012) showed an extremely dynamic system with steep spatial and temporal O_2 and pH gradients resulting from photosynthetic activity of the symbiont, as modulated by ambient irradiance levels, and a high holobiont respiration rate. *Prochloron* therefore seems to thrive in environments with strong diurnal fluctuations of the chemical environment, much akin to other cyanobacteria found in highly productive biofilm ecosystems (Kühl *et al.*, 1996). Knowledge of the phylogenetic diversity of *Prochloron* and the environmental conditions of

its microhabitat may provide fundamental new insight into its putative symbiotic interaction with its ascidian hosts. In this respect, the primary outstanding questions regarding ascidian-*Prochloron* symbioses are: (i) whether the different locations inside or on the ascidians host tissue result in different microenvironmental conditions surrounding the *Prochloron*, and (ii) to what extent the *Prochloron* associated with these different tissue specific locations are different. Here we present the first study describing the microhabitat and phylogeny of *Prochloron* located on the outer surface of didemnid ascidians.

RESULTS

Microscopy imaging: Green *Prochloron* biofilms were observed on a fraction of the ascidians found in this study, and in most cases the biofilm only covered part of the test surface (Fig. S1). *Prochloron* biofilms were not observed inside or below the investigated ascidians (data not shown). One ascidian sample was examined in further detail (Fig. S2): The *Prochloron* biofilm patch was homogenous and only a few cell layers thick (Fig. 1A). Microscopy investigations of the biofilm revealed only cell morphotypes resembling that of *Prochloron*. Fluorescence imaging of ascidian cross-sections revealed the presence of a transparent exo-polymeric, mucoid layer (m), extruding from the ascidian surface and engulfing the *Prochloron* cells (Fig. 1B; seen as dark structures in the image on a background of fluorescing resin). At high magnification transmission electron microscopy (TEM), the mucus appeared as a fibrous substance, containing some bacteria-like inclusions (Fig. 1C and F). The relatively small *Prochloron* cells (7-10 µm in diameter) exhibited a typical peripheral band of loosely stacked thylakoid (t) membranes (Fig. 1D and E), which expanded into numerous small “vacuoles” (v) in the centre of the cell (Fig. 1D). Further observations indicated that up to 10 thylakoid membranes were stacked in peripheral bands, some of them

running among the many central vacuoles. The surface of all examined *Prochloron* cells was covered with virus-like structures approximately 100 nm in length (Fig. 1D and E, arrow).

Oxygen and pH dynamics: Oxygen and pH dynamics was investigated on a white ascidian with approximately 50% *Prochloron* coverage (depicted in figure S2). The O_2 concentration at the *Prochloron* covered surface of the ascidian rapidly increased upon illumination; from hypoxic in the dark to hyper-oxic at higher light levels (Fig. 2A). For each irradiance step, a new steady state O_2 concentration and pH was reached within 5 minutes of illumination. An increase in O_2 concentration was also observed at *Prochloron*-free areas although to a lesser extent (Fig. 2A). Profiles of O_2 concentration towards the *Prochloron*-covered surface showed a net efflux of O_2 reaching a maximum of 0.15 ± 0.01 nmol O_2 cm^{-2} s^{-1} at a photon irradiance (PAR, 400-700 nm) of 155 µmol photons m^{-2} s^{-1}, which remained fairly constant at higher light levels (Fig. 2C). Locally, pH increased up to 8.33 ± 0.02 during illumination and decreased to a minimum of 7.90 ± 0.09 in the dark (Fig. 2D). The *Prochloron*-free ascidian surfaces consumed O_2 from the water column at low light, but exhibited net O_2 efflux at high irradiance, reaching a maximum value of 0.06 nmol O_2 cm^{-2} s^{-1} at 380 µmol photons m^{-2} s^{-1} (Fig. 2C). In the absence of symbionts, pH decreased towards the surface in both dark and light to 8.00 ± 0.03 and 8.11, respectively (Fig. 2E). Only one pH profile was obtained in the light due to damage to the fragile pH electrode.

Gross photosynthesis (Fig. 2B) measured at the *Prochloron*-covered surface revealed a light utilisation efficiency of α = 0.013 ± 0.002 mol O_2 per mol photons, with a rapid increase in O_2 production at low light levels and a minimum saturating irradiance (E_k) of ~80 (±7) µmol photons m^{-2} s^{-1} according to the model fit. The volume-specific gross O_2 production rate reached a maximum of 7.6 (±1.3) nmol O_2 cm^{-3} s^{-1} (model fit: P_{max} = 8.1 (±0.3) nmol O_2 cm^{-3} s^{-1}), corresponding to an areal gross photosynthesis rate of 0.16 (±0.01)

nmol O_2 cm^{-2} s^{-1} at 380 µmol photons m^{-2} s^{-1} (assuming that the measured change in O_2 concentration is averaged over a volume corresponding to a sphere with a diameter of 0.2 mm), followed by a slight decrease in photosynthesis at the highest irradiance (800 µmol photons m^{-2} s^{-1}).

Cyanobacterial phylogeny: Pyrosequencing of the 16S rDNA gene from biofilm of four individual ascidian samples with surface *Prochloron* (Fig. S1), yielded a total of 8,285 high quality sequences grouping into 433 operational taxonomic units (OTUs) with >3% raw sequence divergence. Of these, 136 OTUs were most similar to *Prochloron* sp. sequences. 297 OTUs, representing ~15% of all obtained sequences, were of non-*Prochloron* origin and dominated by nitrogen fixing marine cyanobacteria such as *Pleurocapsa* sp. and *Symploca* sp. (Fig. 3). The four biofilm samples grouped two and two with respect to sequence diversity, with the two red ascidians showing much lower sequence diversity (total of 29 and 34 OTUs) and higher percentage *Prochloron* cover (>98%) than the two gray samples (total of 151 and 358 OTUs and >65% *Prochloron* cover) (Fig. 3). The 12 *Prochloron* OTUs included in figure 4 represent >92% of all the *Prochloron*-like sequences obtained and were selected for inclusion based on their read length (>437bp) and their relative abundance (≥25 highly similar sequences per OTU). Phylogenetic mapping of the representative sequence of each of the 12 OTUs supported the presence of separate phylotypes of *Prochloron* (bootstrap values >80%) (Fig. 4), with all *de novo* sequences clustering away from all but two previously published sequences (GenBank accession number DQ357967 and DQ385852), which were both isolated from within the tunic of the colonial ascidian *Trididemnum miniatum* (Hirose *et al.*, 2006; Münchhoff *et al.*, 2007). A strong separation (bootstrap = 100%) was found between sequences representing *Prochloron* from the cloacal cavity of ascidians and the

163 presented surface sequences including the only other published surface *Prochloron* sequence
164 (JX099360, Hirose *et al.*, 2012).
165
166 DISCUSSION
167 External, surface-associated *Prochloron* has generally been considered of less importance to
168 their host than their internal counterpart (Cox, 1986), mainly because of the apparent lack of
169 morphological adaptations of the host to support the symbiosis and the lack of vertical
170 transfer of symbionts from parent to progeny in these ascidians (Hirose, 2014). However, the
171 exo-polymeric matrix observed on the surface of the investigated ascidian, clearly extending
172 from the host surface, encapsulated the surface *Prochloron* cells (Fig. 1B, C) and may
173 represent an important means of attachment; forming a stabilised environment for growth in
174 an otherwise flow-exposed habitat, much in line with the function of exo-polymeric
175 substances in other microbial biofilms (Wotton, 2004). A similar fibrous, exo-polymeric
176 matrix at the host/symbiont interface has also been observed in the more protected
177 environment of the cloacal cavity of *L. patella*, although at a much smaller scale (Kühl *et al.*,
178 2012). While more ultra-structural work is necessary to confirm the general presence of such
179 a matrix on ascidians with surface-associated *Prochloron*, this could represent a previously
180 unrecognised morphological adaptation of the ascidian to accommodate *Prochloron*
181 specifically, and as such may embody an important structural link in the establishment and/or
182 maintenance of this symbiosis.
183 The surface-associated *Prochloron* responded rapidly to changing light conditions
184 (Fig. 2A, B), and exhibited efficient photosynthesis at low irradiance levels with saturation at
185 ~200 µmol photons m^{-2} s^{-1} (Fig. 2B). This is in agreement with the conditions under which
186 the host ascidian is typically found, i.e. on the underside of stable coral rubble, where only
187 diffuse, reflected light may be available for photosynthesis (Brakel, 1979; Kühl *et al.*, 2007).

188 A similar photosynthetic response was found previously with *Prochloron* located in the
189 cloaca of the didemnids *Diplosoma virens* (Kühl *et al.*, 2005) and *L. patella* (Alberte *et al.*,
190 1986; Kühl *et al.*, 2012). While *D. virens* inhabits low light, cryptic reef habitats, *L. patella* is
191 commonly found exposed to full sunlight. However, the spiculous tunic of *L. patella* has been
192 shown to strongly attenuate the light reaching the cloacal cavity, so that the symbionts
193 residing there mostly experience relatively low light conditions (Kühl *et al.*, 2012). The
194 present study indicates some level of photoinhibition at 800 µmol photons m^{-2} s^{-1} (Fig. 2B)
195 which is comparable to what has been observed previously for *Prochloron* cells extracted
196 from the cloacal cavity of low-light acclimated *L. patella* colonies (Alberte *et al.*, 1986),
197 although Kühl *et al.* (2012) observed no such inhibition below 1000 µmol photons m^{-2} s^{-1}.
198 Thus, despite being adapted to low light habitats, the *Prochloron* cells are able to cope with
199 much higher light levels than normally encountered and can adapt to full sunlight exposure
200 (Alberte *et al.*, 1986). This flexibility could be of benefit during a free living stage with
201 exposure to more variable irradiance levels.

202 While tempting to suggest, production of O_2 by the surface *Prochloron* biofilm is
203 unlikely to be a major driver in the symbiosis. *Prochloron* is the only phototroph reported to
204 form coherent biofilm patches on ascidians, and didemnid ascidians are the only colonial
205 ascidians on which these patches occur; and even then the occurrence of patches is sporadic.
206 Ascidian zooids are efficient filter feeders and can presumably ventilate their immediate
207 microenvironment by advective O_2 transport. The net O_2 efflux detected on areas of the
208 ascidians with no *Prochloron* cover (Fig. 2E), was found to be a result of O_2 production by
209 the substratum below the thin (~1.5 mm) ascidian colony. This was evident from the
210 cessation of O_2 evolution when the ascidian was removed from its substratum, as well as
211 from a high O_2 production by algae in the substratum itself (data not shown). The identity of
212 the algae in the coral rock was not investigated. As it was not possible to measure the flux of

O_2 into the ascidian due to the presence of hard spicules in the tunic, the presented net flux of O_2 represents an underestimation of the total flux of O_2 away from the biofilm layer. As such, a reliable number for the holobiont (animal and symbionts) respiration (calculated as: respiration = gross production – net production) cannot be obtained from the presented data. The production of O_2 in the coral rock underneath the ascidian likely further biased the net flux and can explain the little apparent difference in gross production and net efflux at the ascidian surface. Therefore, in future studies, the ascidian should be removed from its substratum in order to obtain reliable respiration measurements and thereby photosynthesis to respiration (P:R) ratios, albeit with attention to the risk of stressing the host during this procedure.

The pH conditions in the external *Prochloron* biofilm showed limited variation with photosynthetic activity (Fig. 2D), which is in stark contrast to that observed in *L. patella*, where pH varies from pH <6 in darkness to pH >10 under light saturation (Kühl *et al.*, 2012). The more constant pH in the surface *Prochloron* biofilm is likely a result of (i) the lower biomass density (biofilm ~100 µm thick, Fig. 2A), and (ii) the more exposed position of the biofilm, resulting in more efficient equilibration of CO_2 with the surrounding seawater compared to inside the cloacal cavities. The optimum pH for photosynthesis in *Prochloron* isolated from *L. patella* has been shown to range between pH 8 and 9 (Dionisio-Sese *et al.*, 2001), so the conditions on the ascidian surface would seem more lenient in comparison to the extreme environment within *L. patella*. As such, it appears that the photosynthetic capacity of the external *Prochloron* closely resembles that of internal *Prochloron* isolated from *L. patella*. However, the strong difference in the pH climate of the two environments could be responsible for differences in metabolic functions and morphology of the associated *Prochloron* phylotypes. One pH-dependent difference between internal and external

237 *Prochloron* habitat could e.g. be related to the extent of inorganic carbon limitation for
238 photosynthesis under high irradiance but this remains to be further explored.

239 The lack of vertical transfer of symbionts in ascidians with surface associated
240 *Prochloron* (Hirose, 2014) indicates that colonisation of the ascidian is a result of seeding
241 from the water column (Cox, 1986), which evidently could allow for colonisation by
242 cyanobacteria other than *Prochloron*. Despite this, in two of the four biofilm samples
243 investigated, only less than 3% of all sequences were related to non-*Prochloron*
244 cyanobacteria (Fig. 3). In the two other samples, a larger diversity was observed while
245 *Prochloron* sequences still dominated (65 to 79% of cyanobacterial 16s rDNA sequences). Of
246 sequences belonging to non-*Prochloron* cyanobacteria, the nitrogen fixing (diazotrophic)
247 *Pleurocapsa sp.* and *Symploca sp.* dominated. The presence of diazotrophic organisms has
248 also been observed in the microbial communities of *L. patella* (Behrendt *et al*., 2012).
249 Association with nitrogen fixing organisms could be of benefit to the ascidian host in the
250 generally nitrogen limited waters of coral reefs, and similar associations are also known from
251 other organisms living on the reef such as corals (Lema *et al.*, 2012) and sponges (Wilkinson
252 and Fay, 1979). Considering the reported lack of nitrogenase genes in the *Prochloron*
253 genome (Donia *et al*., 2011b), the presence of diazotrophs in *Prochloron* biofilms gives
254 weight to previous reports of nitrogen fixation by *Prochloron* communities (Paerl, H. W.,
255 1984; Kline *et al*., 1999). The dichotomous community structure observed between the
256 ascidian samples investigated here highlights a new and potentially important study area
257 investigating the host-specificity of cyanobacterial biofilms on crustose ascidians. In a recent
258 study , it was found that the microbial diversity on the surfaces of two colonial ascidians was
259 lower than in the surrounding seawater (da Silva Oliveira *et al.*, 2013), demonstrating
260 selective pressure for or against certain prokaryotes. Similarly, in a survey of the microbial
261 diversity in *L. patella*, Behrendt *et al.* (2012) found lower cyanobacterial diversity in the

Prochloron-inhabited cloaca, with a Shannon index of diversity of just 1.8 ± 0.7 compared to 4.4-5.4 reported in marine planktonic habitats (Schloss *et al.*, 2009). Thus, some unidentified mechanisms seem to ensure primary colonisation by *Prochloron*, emphasising the connection between didemnid ascidians and this particular taxon of cyanobacteria. The frequent formation of bioactive secondary metabolites such as patellamide and other cyanobactins in didemnid-*Prochloron* associations may play an important role in such allelopathy against other cyanobacteria (Donia *et al.*, 2011b; Donia *et al.*, 2011a).

Until now, significant genetic divergence in the *Prochloron* genus has not been reported even amongst geographically widely separated samples (Münchhoff *et al.*, 2007; Donia *et al.*, 2011b). In present study, one OTU (denovo385, Fig. 4) dominated all four biofilm samples, containing 56-92% of all *Prochloron* sequences. This phylotype was closely related to *Prochloron* inhabiting the tunic of the ascidian *Trididemnum miniatum* (Hirose *et al.*, 2006; Münchhoff *et al.*, 2007). However, a rare biosphere of *Prochloron* with notable sequence diversity was also detected, all clustering separately from previously reported internal (cloaca) *Prochloron* sequences obtained from diverse geographical and host origin (see supplementary table S4). Importantly, these results suggest a hitherto undiscovered speciation of the *Prochloron* genus into several separate phylotypes. This potential speciation of the *Prochloron* genus might have been hidden till now due to the almost exclusive focus on *Prochloron* living inside ascidians as opposed to the potentially less specialised externally associated types. The phylogenetic separation of the internal and external *Prochloron* shown here finally provides a phylogenetic basis for the long known morphological differences between *Prochloron* from these two environments (Cox, 1986; Hirose *et al.*, 2012): External *Prochloron* exhibit a reticulate type of vacuolation, i.e., a central region filled with vacuoles from expanded portions of the thylakoids (see figure 1A), which differs from the single large central thylakoid vacuole observed in internal *Prochloron* (Cox, 1986, see figure 4).

Interestingly, the intra-tunic dwelling *Prochloron* (represented by the sequences DQ357967 and DQ385852 in figure 4) from the ascidian *Tridemnum miniatum* (Hirose *et al.*, 2006), which clusters with the surface *Prochloron* sequences presented in this study, exhibit a reticulate vacuolation similar to that of the surface *Prochloron*. While the structural differences between external, surface-associated and internal *Prochloron* provide support for phylogenetic divergence, the only database sequence that is reported to have originated from an external surface-associated *Prochloron* (JX099360, Hirose *et al.*, 2012) is phylogenetically affiliated with the sequences reported here and the sequences from internal *Prochloron* (Fig. 4). However, the lower bootstrap values (77-79%) separating this sequence from the sequences obtained in this study suggests that these are more closely related than to the internal *Prochloron* sequences.

The lack of success in cultivating *Prochloron* has been taken to indicate that these cyanobacteria rely on as yet unidentified compounds excreted from the ascidian (Yellowlees *et al.*, 2008). Specifically, a significant decrease in photosynthesis of internal *Prochloron* over just a few hours after isolation from the host has led to the suggestion that the carbon uptake mechanism is directly affected (Critchley and Andrews, 1984; Alberte *et al.*, 1987; Christen *et al.*, 1999). Interestingly, however, we found that external *Prochloron* cells retained their photosynthetic potential, as measured by variable chlorophyll fluorescence, for 45 h after isolation with no indication of a decline (see supplementary information Fig. S2). This indicates that external *Prochloron* may be less dependent on its host than its internal counterpart, which is also in line with their comparatively looser association.

In conclusion, we provide the very first insights into the phylogenetic diversity and photosynthetic performance of *Prochloron* inhabiting the exposed surface of crustose didemnid ascidians. Our results indicate that external surface-associated *Prochloron* have a similar photosynthetic capacity as reported for *Prochloron* thriving inside the cloacal cavity

of other didemnid ascidians, and they can retain their photosynthetic capacity for much longer when isolated form the ascidian surface. However, the microenvironmental conditions experienced by the surface *Prochloron* differ substantially from that experienced by internal *Prochloron*, suggesting a significant plasticity in the environmental requirements of these cyanobacteria. The phylogenetic divergence between the surface *Prochloron* investigated in the present study and previously published sequences from internal *Prochloron* strongly suggests an evolutionary adaptation to these different environmental conditions and the existence of multiple *Prochloron* phylotypes or species. Given the fact that *Prochloron* lives in association with numerous other marine hosts such as holothurians and sponges, this work further emphasizes the need to explore the diversity of *Prochloron* from an evolutionary point of view in order to better understand where and when this symbiosis evolved.

EXPERIMENTAL PROCEDURES

Sampling and sample conservation: Individuals of encrusting ascidians covered with green patches of *Prochloron* cells were sampled during low tide at the border of the reef flat and the inner reef crest of the Heron Island lagoon, Great Barrier Reef, Australia (151° 55’ 00 E, 23° 26’ 07 S during 2013 and 2014 field trips. The ascidians (Red2013, Red2014, Gray2014a, Gray2014b; see supplementary figure S1) were all found in cryptic habitats on the underside of dead coral rubble patches, which were partly air-exposed during low tide. Coral substrate with adherent specimens of the particular ascidian was transferred to a container with seawater and immediately transported to Heron Island Research Station, where the samples were maintained in shaded, outdoor aquaria under a continuous flow of fresh seawater. Prior to sampling of the *Prochloron* biofilm for phylogenetic analysis, each individual ascidian was placed in 0.2 µm filtered seawater for at least 1 hour in order to reduce contamination by non-associated cells. A soft paint brush was then used to gently brush off the biofilm from the

surface of the ascidian into fresh 0.2 µm filtered seawater. The collected cells were spun down at 500g for 1 minute and the supernatant replaced with RNAlater™ (Ambion, Inc., US). Fixed cells were stored at 4°C until further analysis. For transmission electron microscopy (TEM), a small piece of ascidian with a *Prochloron* surface biofilm (~1 cm^2) was cut out with a scalpel and transferred to an Eppendorf tube containing 1.5 mL TEM fixative (1X phosphate buffered saline [PBS, pH 7.2], 0.8% paraformaldehyde solution, 2.5% glutaraldehyde, 0.65 M sucrose) for 24 h at 4°C before being transferred to a clean container and washed several times in 1X PBS containing 0.65 M sucrose.

Microscopy: For bright field imaging, a thin (<1 mm) ascidian cross section was excised with a scalpel, positioned on a microscope slide and covered with a coverslip. Imaging was done on a compound microscope equipped with a camera (Olympus BX51 with DP71 CCD). For TEM, a piece of pre-fixed ascidian (~4 mm^2) (see above) was dehydrated step wise in ethanol at increasing concentrations (50%, 70%, 90% and 100%) and embedded in Spürr resin after pre-staining with Osmium as described previously (Pernice *et al.*, 2012). Briefly, resin blocks were cut into 100-120 nm sections using a microtome (Ultracut E Leica Microsystems, Australia) with a diamond knife, mounted onto TEM grids and counterstained with 2% uranyl acetate (10 min) and Reynold's lead citrate (10 min). Tissue sections were imaged at the Centre for Microscopy and Microanalysis (University of Sydney, Australia) using a transmission electron microscope (JEOL JEM1400, Korea LTD) operated at 80 kV accelerating voltage. In order to visualise faint structures, fluorescence imaging of thin sections (2 µm), prepared as described for TEM but with no counter stain, was carried out on an inverted fluorescence microscope (Eclipse-T*i*, Nikon, US), using blue light (FITC filter settings) for excitation of the resin.

Flow cell and microsensor setup: Measurements were conducted on an intact ascidian (3 cm^2) attached to a pieces of substrate (coral rubble). The ascidian was left on its substrate in order to avoid disturbing the fragile tissue and surface biofilm. The sample was placed in an acrylic flow chamber supplied with seawater (salinity 35 psu) at a flow velocity of approximately 3 cm s^{-1}, and maintained at a constant temperature of 26°C (± 0.5°C) with an aquarium heater (Sonpar Aquarium Equipment, China). Illumination was provided by a fibre optic tungsten-halogen light source (Schott KL 2500, Germany) equipped with a collimating lens. Incident irradiance levels were measured in µmol photons m^{-2} s^{-1} (PAR, 400-700 nm) with a miniature scalar irradiance quantum sensor (Walz GmbH, Germany) positioned over a black, non-reflecting surface at a distance from the light source equal to the distance of the sample during measurements. A microsensor (optode or electrode, see details below) was mounted above the flow chamber on a motorised micromanipulator for automated profiling (MMS, Unisense A/S, Denmark).

O_2 and pH measurements: A fast responding O_2 optode (tip size 50 µm, 90% response time <0.5 s; Pyroscience GmbH, Germany) was connected to a logger (Firesting, Pyroscience GmbH, Germany) and the O_2 signal was monitored and saved on to a PC via the Firesting logger software (v. 2.30). A two-point calibration of the optode was done at atmospheric O_2 concentration and at zero % O_2 in a solution of Na_2SO_3 (20% w/v) at experimental temperature. For gross photosynthesis measurements and profiling of O_2 concentration, the following Firesting software settings were employed: 0.25 seconds per measurement, 10 ms flash time, 100% flash intensity, 200% signal amplification.

pH measurements were made with pH glass microelectrodes (50 µm tip size, Unisense A/S, Denmark), connected to a high impedance millivolt meter (Unisense A/S, Denmark) with an external standard 2 mm reference electrode (Ionode LLC, Australia). The pH electrodes were

calibrated via a linear fit to the millivolt output measured in pH 4, 7 and 10 buffers, resulting in a slope of ~51 mv/pH units, and the signal was logged during measurements (SensorTrace PRO Unisense A/S, Denmark).

Before each profile measurement, the microsensor was manually positioned at the surface of the ascidian/*Prochloron*-layer, as observed through a dissection microscope mounted on an articulating arm (American Scope, United States). This position was set as zero depth and the microsensor was then retracted vertically a set distance to the starting point of the profile. Each of the O_2 and pH profiles was commenced 1.0 and 2.0 mm from the ascidian surface, respectively, and performed towards the surface in vertical steps of 100 µm. At each position, the sensor signal was allowed to stabilise for 5 seconds (95% signal response <5s) before logging the O_2 concentration. Four replicate profiles were recorded at each light level (down-welling photon irradiance: 19, 46, 83, 155, 380 and 800 µmol photons m^{-2} s^{-1}) in areas with and without *Prochloron* biofilm (see figure S1), respectively, . Between each light level, an acclimation period of ~10 minutes was included, which was observed to be sufficient for establishing a steady state O_2 concentration and pH after a change in light (see Fig. 2). The net O_2 efflux, i.e., the net photosynthesis, was calculated from measured steady-state concentration profiles using Fick's first law of diffusion with a diffusion coefficient of 2.30 x 10^{-5} cm^2 s^{-1} (calculated for an experimental temperature of 26°C and salinity of 35 psu; (Garcia and Gordon, 1992). Rapid light-dark shifts were conducted with the O_2 optode tip placed at the surface of the ascidian following the procedure of Revsbech and Jørgensen (1983).

In order to take into account any photosynthesis resulting from the pulsed red LED light (wavelength 620 nm) emitted by the optode during the operation, a set of O_2 measurements were carried out at steady state in the dark with identical optode settings as used for measuring gross photosynthesis. To minimise O_2 production from light emitted by

412 the optode, the measuring rate was reduced to once every 2 seconds. As a result of the
413 optode's flashing LED measurement light, O_2 was produced in the dark at a rate of 0.8 ± 0.27
414 nmol cm^{-3} s^{-1}. This would result in a proportional under-estimation of the gross
415 photosynthesis in the light, and therefore this value was added to the measured gross
416 photosynthetic rate of the *Prochloron* layer at each light level. Curve fitting of experimental
417 P vs. E curves were performed according to Platt *et al.* (1980) and Ralph and Gademann
418 (2005) and allowed estimation of light utilisation efficiency (alpha), maximum
419 photosynthetic rate (P_{max}), and the minimum saturating irradiance ($E_k = P_{max}$/alpha) and. To
420 measure longer term O_2 and pH dynamics, the sensor was positioned and left at the surface of
421 the ascidian for the duration of the experiment (45 minutes), while the irradiance level was
422 increased every 5 minutes.
423
424 **DNA extraction, sequencing and data analysis:** Biofilm material from four individual
425 ascidians was analysed as follows: Prior to DNA extraction, cells were pelleted at 1500 g for
426 5 min after which the DNA was extracted according to the manufacturer's instructions with
427 the Powersoil DNA isolation kit (MO BIO, USA). The extracted DNA was resuspended in
428 100 µL of sterile water. The DNA was sequenced at Molecular Research LP (Shallowater,
429 Texas, USA) using standard protocols (Dowd *et al.*, 2008). Briefly, the sample was amplified
430 using the cyanobacterial-specific primer pair 357F (CCTACGGGAGGCAGCAG) / 809R
431 (GCTTCGGCACAGCTCGGGTCGATA) (modified from (Jungblut *et al.*, 2005)). A single-
432 step 30 cycle PCR using HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA) was used
433 under the following conditions: 94^{o}C for 3 minutes, followed by 28 cycles of 94°C for 30
434 seconds; 50°C for 40 seconds and 72^{o}C for 1 minute; after which a final elongation step at
435 72°C for 5 minutes was performed. Following PCR, the amplicon product was purified using
436 Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA). The sample was

sequenced utilizing Roche 454 FLX titanium instruments and reagents and following manufacturer's guidelines. DNA sequences were processed using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline (Caporaso *et al.*, 2010). Sequences were de-multiplexed and reads shorter than 200 bp as well as reads with a quality score <25 or containing homopolymers exceeding 6 bp were discarded. ACACIA (Bragg *et al.*, 2012) was used to de-noise and error correct the dataset. Following chimera removal using UCHIME (Edgar *et al.*, 2011), Operational Taxonomic Units (OTUs) were defined at 97% sequence identity using UCLUST (Edgar, 2010) and assigned taxonomy against the SILVA database (version 111), (Pruesse *et al.*, 2007) using BLAST (Altschul *et al.*, 1990).

Phylogenetic analysis: DNA sequences were aligned using the multiple sequence alignment program MAFFT (Katoh *et al*., 2002) employing the L-INS-I algorithm. In following assessment regions with gaps were eliminated, and alignments were refined using trimAL (Capella-Gutierrez *et al.*, 2009). After re-assessment of alignments, sequences were trimmed to same sequence length (437bp) length and phylogenetic trees were constructed using Maximum Likelihood (ML) and the Generalised Time-Reversible (GTR) model in FastTree (Price *et al.*, 2009; Price *et al.*, 2010) with a bootstrap resampling number of 1000. Finally, the topology of the trees was assessed using PhyML (Guidon et al., 2010) and IQTree (Nguyen *et al.*, 2015). *Prochloron* reference sequences plus the outgroup sequence were obtained from GenBank via BLAST searches and the NCBI Taxonomy Browser. The reads corresponding to this study are available from NCBI archive.

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463 samples were collected under the Great Barrier Reef Marine Parks Permit G12/35118.1 and
464 G14/36977.1. Authors declare no conflict of interest.
465
466

467 FIGURE LEGENDS

468 Figure 1. Microscopy and transmission electron micrographs (TEM) showing: (A) a cross
469 section of an ascidian (as) with *Prochloron* biofilm on the exposed, upper surface (arrow) and
470 a magnified section of the surface layer with individual *Prochloron* cells visible; (B)
471 fluorescence image of the interphase between the ascidian/*Prochloron* layer, showing matrix
472 protrusions (m) from the ascidian, encasing the *Prochloron* cells (p); (C) *Prochloron* cell (p)
473 encased in ascidian exo-polymeric matrix (m); (D) close up of *Prochloron* cell showing
474 internal vacuoles (v) and stacked thylakoid membranes (t); (E) high magnification image of
475 section of *Prochloron* cell, showing 10 thylakoid membranes (t) stacked in peripheral band
476 and virus-like particles (arrow) attached to the outer membrane; (F) Edge (arrow) of ascidian
477 surface matrix showing its fibrous structure and bacteria-like inclusions (b).

478

479 Figure 2. A: Oxygen and pH dynamics at the ascidian surface with and without a *Prochloron*
480 biofilm (black and grey line, respectively). Vertical, dashed lines indicate changes in surface
481 irradiance noted as number in the upper part of the graph (0-350 µmol photons m^{-2} s^{-1}).
482 Shaded areas indicate periods of darkness. The horizontal, dashed line indicates O_2 saturation
483 point at experimental temperature and salinity. Numbers in brackets indicate steady state pH
484 at the surface of the *Prochloron* layer under the different irradiance regimes. No change in
485 pH could be detected at the bare surface (pH 8.05). B: Gross photosynthesis vs. irradiance (P
486 *vs.* E) curve measured in the *Prochloron* layer (closed diamonds) and a bare surface of the
487 ascidian (open circles). Solid line shows a non-linear curve fit according to (Harrison and
488 Platt, 1986). Dashed lines represent 95% confidence interval. (C) Net O_2 flux measured over
489 the *Prochloron* layer (closed diamonds) and bare surface (open circles). Note: for technical
490 reasons no flux rate measurements were obtained in the dark (0 PAR). D: Steady state
491 profiles of pH measured from the overlaying water towards the *Prochloron* layer and bare

ascidian surface (E) in light (closed symbols, 155 µmol photons m^{-2} s^{-1}) and darkness (open symbols). Only one measurement (n=1) was obtained of pH on the bare surface (D) in light. In all graphs error bars indicate standard deviation of replicate profiles (n=4).

Figure 3: Percentage distribution of the dominant cyanobacterial 16s rDNA sequences from the individual ascidian biofilms investigated in this study. Numbers in parenthesis indicate number of high quality sequences obtained from each sample.

Figure 4. The phylogenetic relationships of the *Prochloron* inhabiting the surface of didemnid ascidians inferred from 16S rDNA gene analysis using Maximum Likelihood. Numbers in parentheses after each "denovo" sequence indicate number of sequences represented by that branch from each ascidian. Database sequences are represented by GenBank accession numbers. Bootstrap values for 1000 trees calculated by the Maximum Likelihood method are indicated with open (>80%), closed (>90%) and red (100%) circles at branch points. *Synechocystis* sp. *PCC 6803* (NR_074311.1), *Prochlorococcus marinus* subsp. Pastoris (AF180967) and denovo62 which group closest to *Synechococcus* sp. were used to form an outgroup to root the tree. Right: Published TEM images of *Prochloron* sp. cells representing specific sequences indicated by arrows (included with permission).

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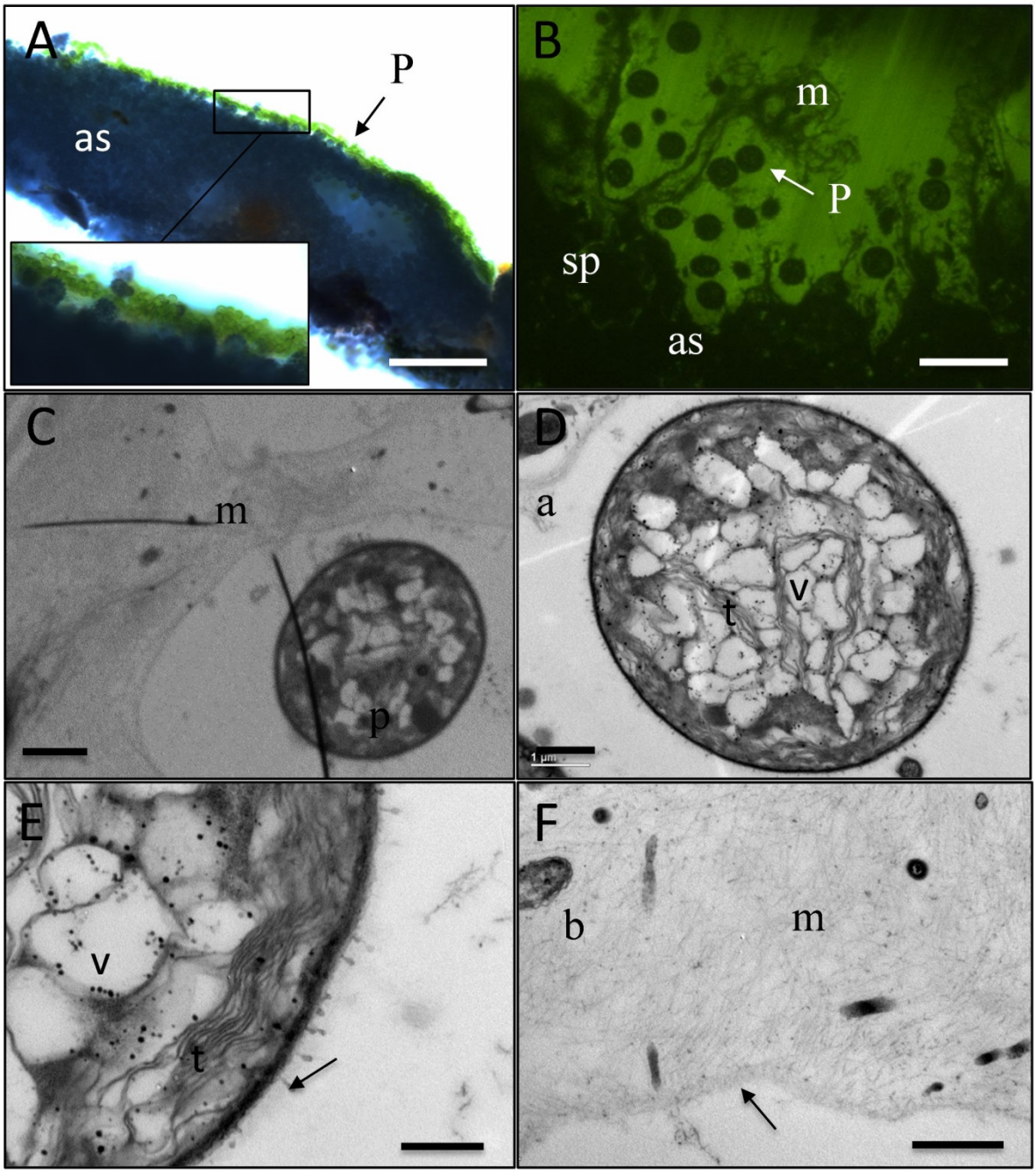
FIGURES

Figure 1:



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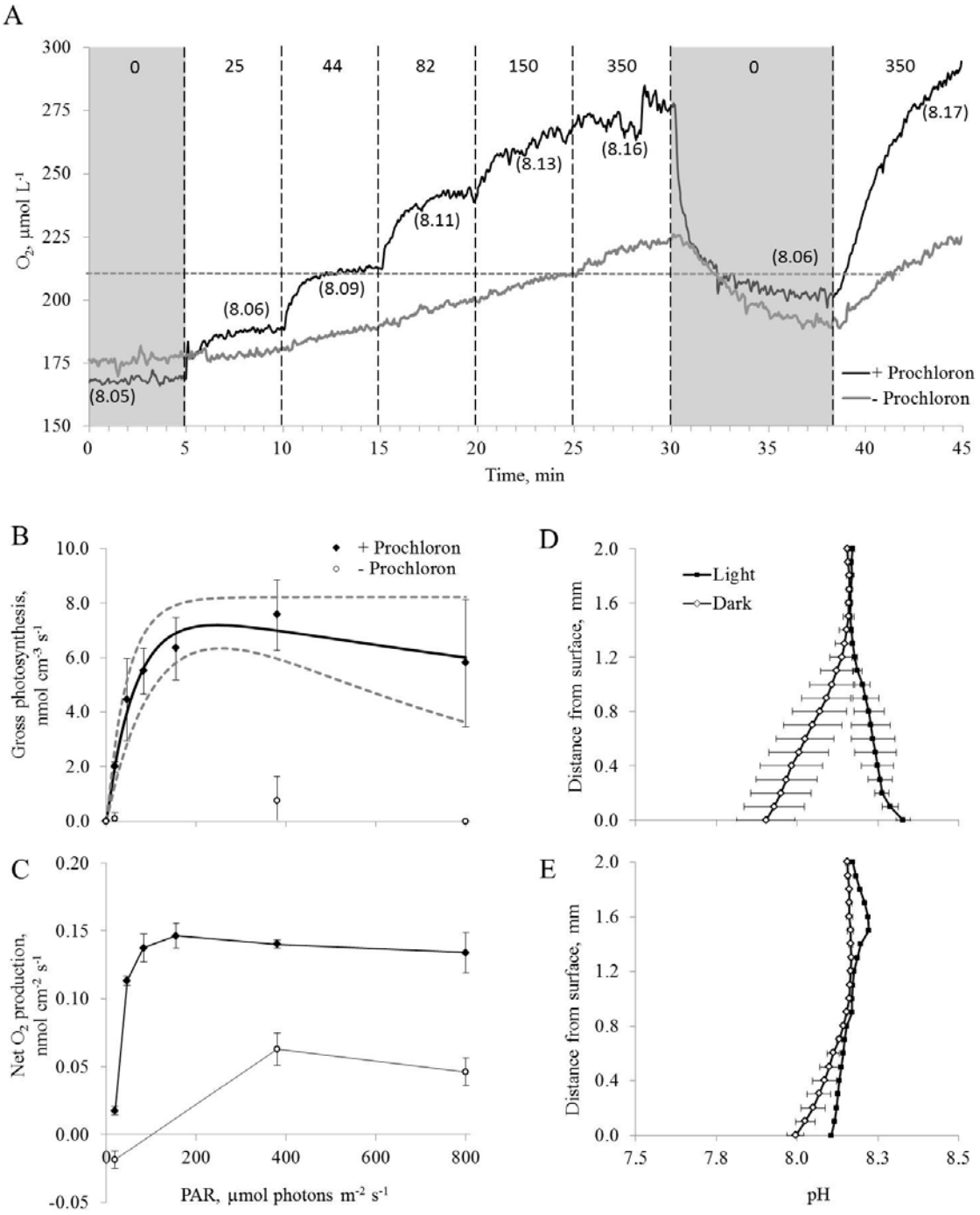

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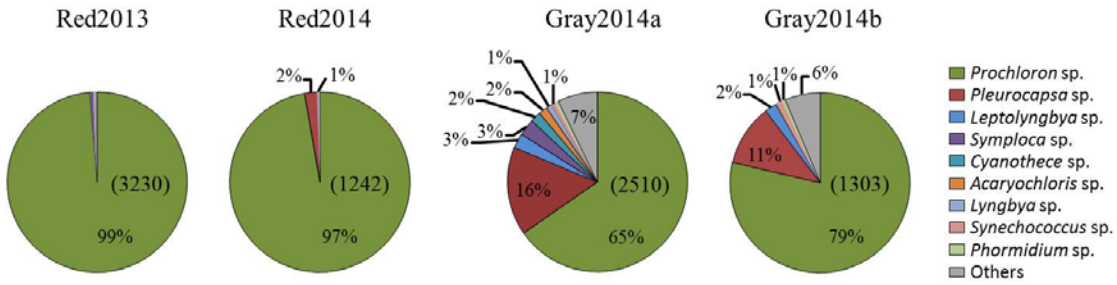

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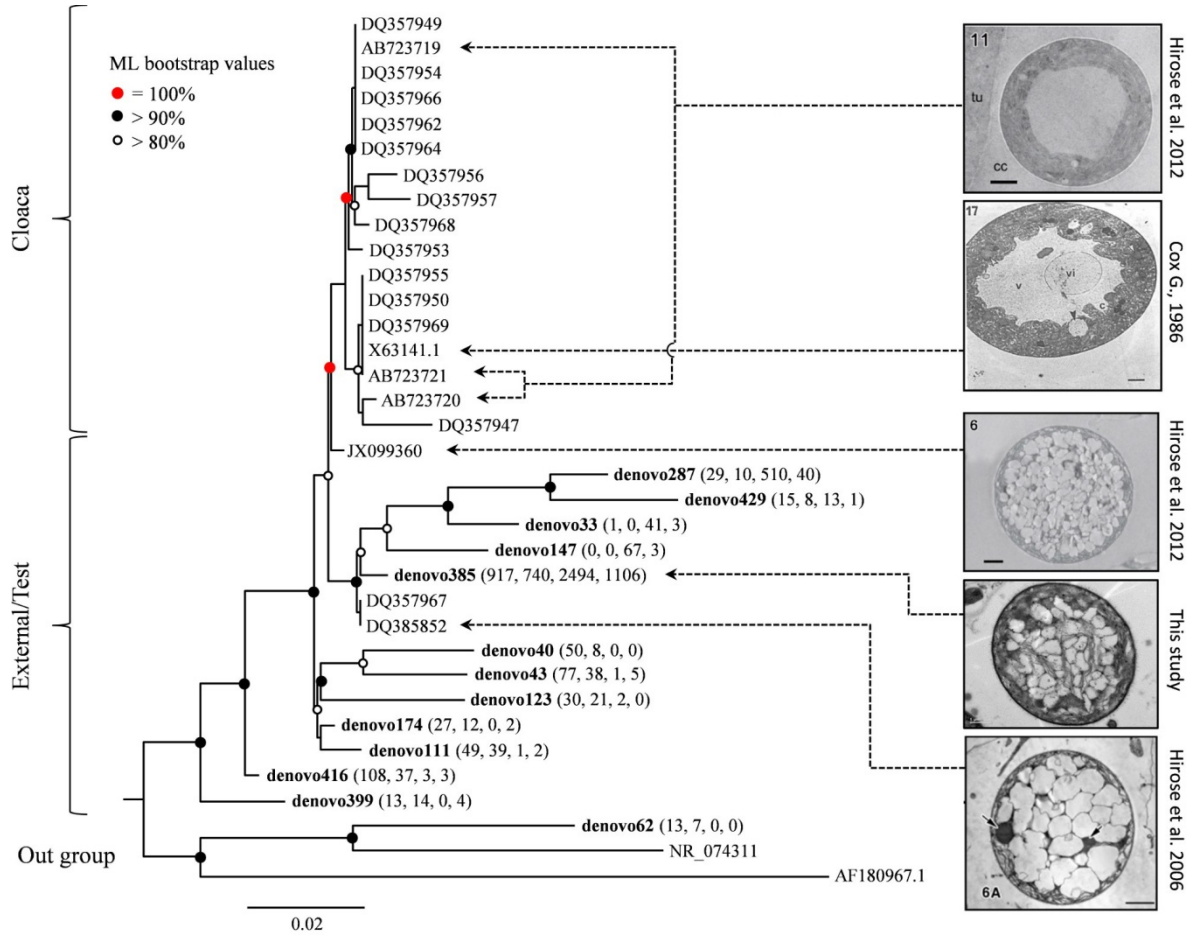

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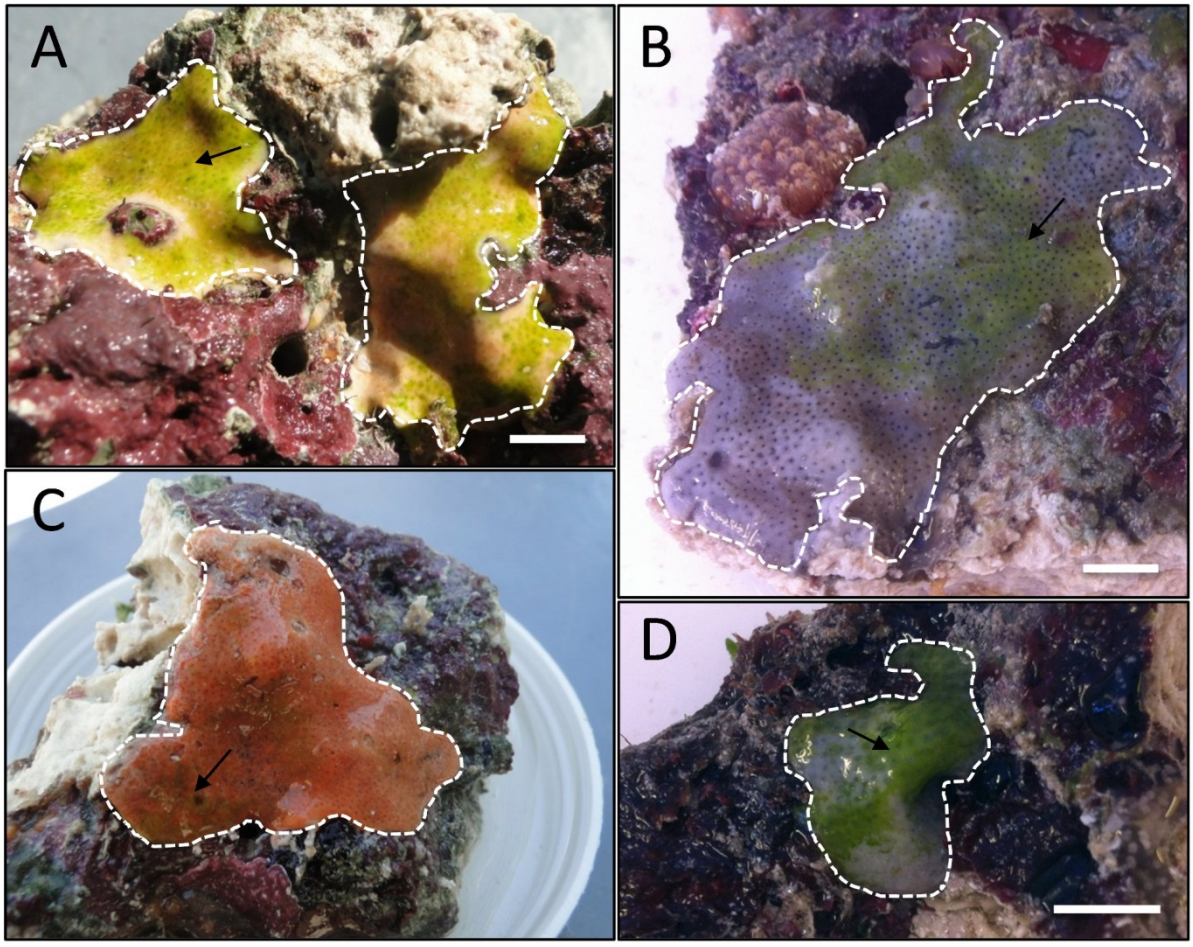
SUPPLEMENTARY FIGURES

Figure S1:



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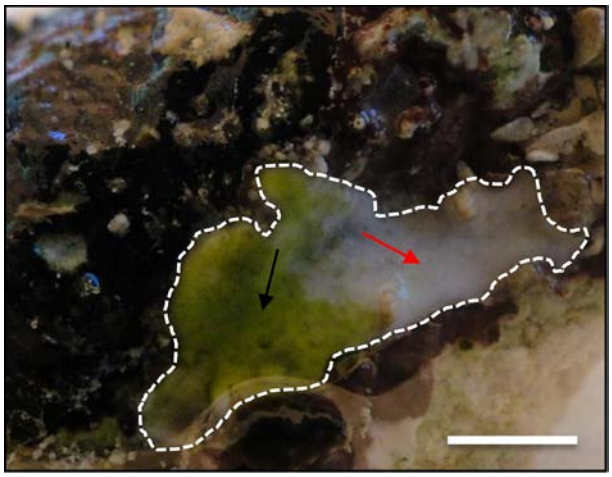

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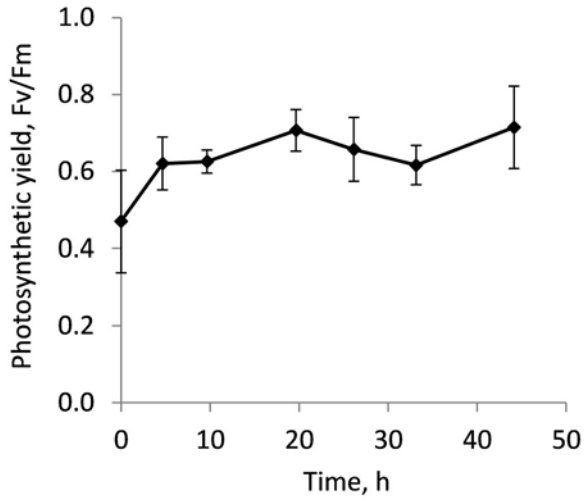

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696 Table S4. *Prochloron* sp. sequences included in16S rRNA gene-based phylogeny (Fig. 6).
697 Information about each sequence was retrieved from their respective publication and the
698 GenBank database.

Genbank accession #	**Original Sample ID**	**Host species**	**Location of symbionts**	**Sample origin**	**Reference**
X63141.1		*Lissoclinum patella*	Cloaca	Kamori Channel, Koror, Palau	Urbach et al., (1992)
DQ385852	BO-136	*Tridemnum miniatum*	In tunic	Bise,Okinawajima Island, Japan	Hirose et al., (2006)
DQ357947	HI-3	*Diplosoma* sp.	Cloaca	Heron Island, Queensland, Australia	Munchhoff et al., (2007)
DQ357949	HW-6	*Diplosoma simile*	Cloaca	Coconut Island, Hawaii, USA	Munchhoff et al., (2007)
DQ357950	HI-10	*Lissoclinum patella*	Cloaca	Heron Island, Queensland, Australia	Munchhoff et al., (2007)
DQ357953	AK-122	*Lissoclinum bistratum*	Cloaca	Maehama, Akajima, Japan	Munchhoff et al., (2007)
DQ357954	AK-121	*Diplosoma* sp.	Cloaca	Maehama, Akajima, Japan	Munchhoff et al., (2007)
DQ357955	AO-100	*Tridemnum cyclops*	Cloaca	Amami Oh-shima, Japan	Munchhoff et al., (2007)
DQ357956	AO-103	*Diplosoma virens*	Cloaca	Amami Oh-shima, Japan	Munchhoff et al., (2007)
DQ357957	AO-104	*Diplosoma*	Cloaca	Amami Oh-shima, Japan	Munchhoff et al., (2007)

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DQ357962	TA-113	*Diplosoma virens*	Cloaca	Oh-gomori, Takarajima, Japan	Munchhoff et al., (2007)
DQ357964	LI-86	*Diplosoma simile*	Cloaca	Blue Lagoon, Lizard Island, Queensland, Australia	Munchhoff et al., (2007)
DQ357966	NA-107	*Diplosoma simile*	Cloaca	Yoriki, Nakanoshima, Japan	Munchhoff et al., (2007)
DQ357967	HA-96	*Tridemnum miniatum*	In tunic	Haterumajima, Japan	Munchhoff et al., (2007)
DQ357968	AO-101	*Lissoclinum* sp. (cf. *Lissoclinum bistratum*)	Cloaca	Ayamaru, Amami Ohshima, Japan	Munchhoff et al., (2007)
DQ357969	LI-88	*Tridemnum paracyclops*	Cloaca	Blue Lagoon, Lizard Island, Australia	Munchhoff et al., (2007)
AB723719	A127	*Diplosoma simile*	Cloaca	Okinawa, Okinawajima Is., Teniya, Japan	Hirose et al., (2012)
AB723720		*Diplosoma simile*	Cloaca	Bocas del Toro, Crawl Key, Panama	Hirose et al., (2012)
AB723721		*Diplosoma simile*	Cloaca	Bocas del Toro, Isla Cristobal, Panama	Hirose et al., (2012)
JX099360	PLV2	*Lissoclinum verrilli*	Test surface	Panama, Bocas del Toro, Isla Cristobal	Hirose et al., (2012)

699

700