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REPORT

Presence of *Symbiodinium* spp. in macroalgal microhabitats from the southern Great Barrier Reef

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Abstract Coral reefs are highly dependent on the mutualistic symbiosis between reef-building corals and dinoflagellates from the genus *Symbiodinium*. These dinoflagellates spend part of their life cycle outside the coral host and in the majority of the cases have to re-infect corals each generation. While considerable insight has been gained about *Symbiodinium* in corals, little is known about the ecology and biology of *Symbiodinium* in other reef microhabitats. This study documents *Symbiodinium* associating with benthic macroalgae on the southern Great Barrier Reef, including some *Symbiodinium* that are genetically close to the symbiotic strains from reef-building corals. It is possible that some of these *Symbiodinium* were *in hospite*, associated to soritid foraminifera or ciliates; nevertheless, the presence of *Symbiodinium* C3 and C15 in macroalgal microhabitats may also suggest a potential link between communities of *Symbiodinium* associating with both coral hosts and macroalgae.

Keywords Macroalgal-associated *Symbiodinium* · *Symbiodinium* clade C · Coral–algal interactions · Coral reef macroalgae

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Introduction

Coral reefs provide goods and services that are valuable to many millions of people throughout tropical coastal areas (Moberg and Folke 1999). Reef-building corals (Order Scleractinia) are central to coral reefs, providing much of the productivity and calcification required to build these ecosystems (Muller-Parker and D'Elia 1997). These organisms form mutualistic symbioses with dinoflagellates *Symbiodinium* spp., which provide corals with abundant photosynthetic energy, enabling them to lay down copious quantities of calcium carbonate and thrive in nutrient-poor environments (Muller-Parker and D'Elia 1997).

Unfortunately, coral reefs are facing a number of serious threats and are severely in decline (Hughes et al. 2003; Hoegh-Guldberg et al. 2007). These threats are arising from declining coastal water quality and over-fishing, as well as warming and acidification of the world's oceans as a result of rising atmospheric carbon dioxide and other greenhouse gases. Rapid changes in water temperature, for example, have caused sudden breakdown of the mutualistic endosymbiosis of corals and dinoflagellates (mass coral bleaching; Hoegh-Guldberg 1999). Mass bleaching events have had a serious impact on coral reefs throughout the world since 1979 when they were first reported in the literature. Our current understanding of the temperature tolerance of

corals suggests that projected sea temperatures will soon approach and exceed the known thermal thresholds to reef-building corals, putting in doubt the future of coral-dominated reef systems (Hoegh-Guldberg 1999; Hoegh-Guldberg et al. 2007).

Due to the potentially devastating impacts of climate change on reef-building corals and coral reefs in general, considerable attention has been given to the physiology and ecology of coral–dinoflagellate symbioses, particularly on the factors and mechanisms that cause its maintenance or breakdown (see Lesser 1997; Ralph et al. 2001). Other studies have focused on the diversity, phylogeny (e.g., Carlos et al. 1999; Lajeunesse 2001; Santos et al. 2002; Rodriguez-Lanetty 2003; Coffroth and Santos 2005), biogeography, community ecology (e.g., Rodriguez-Lanetty et al. 2001; LaJeunesse et al. 2003; Sampayo et al. 2007) and the acquisition of *Symbiodinium* (e.g., Lewis and Coffroth 2004; Pasternak et al. 2006; Gomez-Cabrera et al. 2008), which forms an important base for understanding the dynamics of coral–dinoflagellates endosymbiosis. By contrast, however, studies exploring the biology and ecology of *Symbiodinium* in other reef microhabitats are rare.

Studies on the diversity of *Symbiodinium* have revealed that it is a very diverse group with at least nine distinct genetic clades, A-I (Baker 2003; Coffroth and Santos 2005; Stat et al. 2006; Pochon and Gates 2010). Clades A, B, C and D are the predominant symbionts of scleractinians, while clade E is found in sea anemones, and clades F, G and H are common in foraminifera (Baker 2003; Coffroth and Santos 2005; Stat et al. 2006). Clades C and D can also inhabit in foraminifera, while clades F and G sometimes can be found, although rarely, in scleractinians (Rodriguez-Lanetty et al. 2002; Pochon et al. 2004, 2007; Pochon and Pawlowski 2006). Clade I was recently discovered, and it establishes symbiosis with foraminifera (Pochon and Gates 2010). Clade C has a very wide range of hosts, which include a marine ciliate (Lobban et al. 2002) in addition to scleractinian and foraminiferan hosts (Pochon et al. 2001, 2004, 2007; Pochon and Pawlowski 2006).

Within each *Symbiodinium* clade, there is even more diversity grouped in subclade types (Baker 2003; Coffroth and Santos 2005; Stat et al. 2006). Clade C is the most diverse *Symbiodinium* lineage in the Pacific with more than 100 subclade types (LaJeunesse et al. 2003; Pochon et al. 2004; Sampayo et al. 2007). Some of these subclade types can be identified with several molecular markers including large subunit ribosomal DNA (LSUrDNA) and internal transcriber spacers (ITS) (LaJeunesse et al. 2003; Sampayo et al. 2009). Comprehensive reviews of *Symbiodinium* diversity can be found in LaJeunesse (2001), Baker (2003), LaJeunesse (2005), Coffroth and Santos (2005), and Stat et al. (2006).

Symbiodinium spend part of their life cycle as free-living *Gymnodinium*-like dinoflagellates and in some cases re-infect corals each generation (Gomez-Cabrera et al. 2008; Adams et al. 2009). Despite the fairly extensive information on *Symbiodinium* as coral symbionts, there are a number of key questions surrounding these organisms. For example, our understanding of the importance of other habitats that they use, their population dynamics through space and time, and how they are taken back into host corals cells remain incomplete. This said, there are a growing number of studies indicating that *Symbiodinium* are present in the seawater column (Gou et al. 2003; Coffroth et al. 2006; Manning and Gates 2008; Pochon et al. 2010), interstitial water of sands (Carlos et al. 1999; Hirose et al. 2008; Pochon et al. 2010), rocky reefs and seagrasses (Coffroth et al. 2006) and on benthic macroalgae (Porto et al. 2008). Moreover, it has been demonstrated that both pelagic and benthic *Symbiodinium* can establish symbiosis with corals (Lewis and Coffroth 2004; Coffroth et al. 2006; Adams et al. 2009), although some genotypes are apparently unable to establish symbiosis (Coffroth et al. 2006; Pochon et al. 2010).

Macroalgae are the most abundant benthic component of many coral reefs (Wilkinson 2004; Diaz-Pulido 2008). Benthic macroalgae release organic substances (Khailov and Burlakova 1969; Wada et al. 2007), which may promote the establishment of microbial communities on their surfaces (Armstrong et al. 2000; Longford et al. 2007). Also, many epiphytic dinoflagellates show a distinct preference for macroalgal hosts which may release growth-stimulating algal compounds or provide large surface areas for attachment (Morton and Faust 1997; Parson and Preskitt 2007). Consequently, there is a strong possibility that macroalgae may serve as an important reservoir for *Symbiodinium* within coral reef habitats.

Porto et al. (2008) found *Symbiodinium* associated to the benthic macroalgae *Halimeda* spp., *Lobophora variegata*, *Amphiroa* spp., *Caulerpa* spp. and *Dictyota* spp. in Caribbean coral reefs. However, the potential role of macroalgae as a source of *Symbiodinium* to infect reef-building corals still remains an important unknown. This study explores the presence, identity and potential relationship of macroalgal-associated *Symbiodinium* to those associated with reef-building corals on the southern Great Barrier Reef. Specifically, this study evaluates whether the *Symbiodinium* subclades found in macroalgal microhabitats are the same as those found in reef-building corals.

Materials and methods

Sample collection

Seawater samples were collected from macroalgal microhabitats and sediments in coral reefs from the Heron and

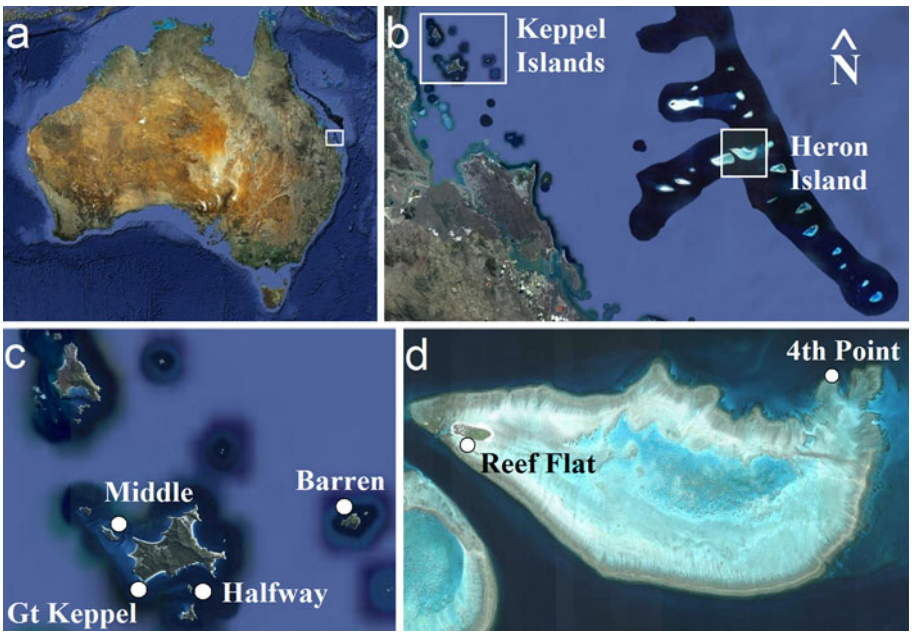

Fig. 1 Location of study sites: **a** southern Great Barrier Reef, **b** Heron Island and Keppel Islands. Specific collection sites are also shown around **c** Keppel Islands and **d** Heron Island. (*Images* modified from Google Earth©)

Keppel Islands on the southern Great Barrier Reef, Australia (Fig. 1). Four types of samples were taken:

(1) Complete sections of macroalgal thalli and cyanobacterial mats were collected with associated seawater, taking care to avoid pieces of the substrate to which the macroalgae were attached.
(2) Crustose coralline algae (CCA) and tiny algal turfs were collected with associated seawater and substrates, which were broken off from the calcareous matrix.
(3) Sediments were collected with associated seawater.
(4) Seawater from the interstitial space of sediments and from the surface of algal turfs or CCAs was collected with 50 ml syringes.

Field collection was made with 0.5-L, 1-L and 2-L plastic bags, depending on the amount of material, and some samples needed more than one 2-L bag. A total of 33 macroalgal samples (including one cyanobacterial sample and four CCA samples) and four sediment samples were collected (Table 1). Two sediment samples were collected near sand-dwelling algal turfs (one from 2 m deep in Heron Island and the another from 12 m deep in Keppel Islands), while two others were collected away from macroalgae.

Each sample was vigorously shaken after collection and filtered through a 200-µm-pore-size mesh. Samples were then filtered again through a 0.5-µm Millipore GFC using a vacuum pump (Capex 8C). The differential pressure in the filter was about 720 mbar. To preserve the potential *Symbiodinium* DNA content, each filter paper was immersed in 20% Dimethylsulfoxide (DMSO) within a dark flask (covered with aluminium foil) and transported to the Centre for Marine Studies at The University of Queensland, where the samples were stored in a freezer (−20°C) until the DNA extraction.

DNA extraction

In order to remove the DMSO, which is undesirable for the following procedures, each filter paper was cut in smaller pieces and rinsed in DNA-Buffer [50 mM EDTA (pH 8.0), 0.4 M NaCl]. DNA was then isolated from filter papers with the Phenol–Chloroform method, following the protocol of Vidal et al. (2002). This protocol was originally designed to improve the DNA extraction from CCA but also enhanced DNA extractions from any alga. This protocol is desirable for samples with low or indeterminate amounts of DNA. The steps involved in this protocol that were intended for cleaning up and grinding CCAs were skipped, given the different material. Consequently, the isolation of the DNA was started by transferring pieces of each filter paper to a 2-ml eppendorf tube containing 700 µl of extraction buffer (4 M Urea, 250 mM Tris–HCl (pH 8.0), 250 mM NaCl, 50 mM EDTA (pH 8.0), 5% 2-Mercaptoethanol, 2% Sodium dodecyl sulphate) and 15 µl of Proteinase K (20 mg ml^{-1}). The final dried pellet was re-suspended in 50 µl of TE buffer (10 mM Tris–HCL (pH 8.0), 1 mM EDTA). The quality and concentration of the extracted DNA was analyzed through gel electrophoresis and NanoDrop spectrophotometry (Thermo Scientific, Wilmington, USA); samples with less than 15 ng μl^{-1} of DNA were concentrated through vacuum centrifuging at 37°C for 20 min.

Table 1 Results of molecular analyses (PCRs, cloning and sequencing) for macroalgal and sediment samples

Macroalgal Phyla	Macroalgae	#S	# Plates	NSD	Isolates	Depth (m)	Sites
Heron Island							
Chlorophyta	*Chlorodesmis fastigiata*	2	1 (1)	0	Clade C	0–4	Reef flat
Chlorophyta	*Halimeda discoidea*	3	3 (4, 2, 1)	1	Clade C	0–4	Reef flat
Chlorophyta	*Halimeda opuntia*	2	1 (1)	0	Clade C	5–11	4th Point
Cyanophyta	Cyanobacteria	1	1 (0)	1	–	5–11	4th Point
Ochrophyta	*Padina* sp.	2	2 (1, 0)	0	Clade C	0–4	Reef flat
Rhodophyta	*Porolithon onkodes*	4	3 (0, 0, 0)	5	–	0–2	Reef flat, 4th Pt.
Rhodophyta	*Hypnea pannosa*	2	2 (2, 0)	1	Clade C	0–4	Reef flat
Rhodophyta	*Hypnea spinella*	1	1 (1)	0	Clade C	0–4	Reef flat
Rhodophyta	*Laurencia intricata*	1	1 (2)	2	Clade C	0–4	Reef flat
Rhodophyta	*Plocamium* sp.	1	1 (0)	0	–	5–11	4th Point
Mixed	Algal turfs	4	2 (1, 1)	2	Clade C	0–2	Reef flat
Mixed	Algal turfs	1	0	0	–	5–11	4th Point
–	Sediments	2	0	1	–	0–4	Reef flat
Keppel Islands							
Chlorophyta	*Chlorodesmis fastigiata*	1	0	0	–	1–6	Middle I
Ochrophyta	*Lobophora variegata*	3	1 (1)	2	Clade C	1–6	Middle I
Ochrophyta	*Lobophora variegata*	1	1 (1)	0	Clade C	10	Halfway I
Ochrophyta	*Lobophora variegata*	1	0	0	–	0–3	Gt Keppel I
Rhodophyta	*Asparagopsis taxiformis*	1	1 (2)	2	Clade C	10	Halfway I
Rhodophyta	*Peyssonnelia* sp.	1	0	0	–	0–3	Gt Keppel I
Mixed	Algal turfs	1	1 (0)	0	–	0–3	Gt Keppel I
–	Sediments	2	2 (0,0)	0	–	12	Barren I

#S indicates the number of samples collected for each macroalga, *# Plates* numerals outside parenthesis indicate the number of cloning plates used for each macroalga; numerals within parenthesis indicate how many *Symbiodinium* sequences were found on each plate. Among 96 clones sequenced (24 plates*4 clones/plate), 21 were *Symbiodinium.* NSD indicates the number of non-*Symbiodinium* dinoflagellates found for each macroalga

PCRs, cloning and sequencing

The variable domains D1 and D2 of 28S large subunit ribosomal DNA (28S-LSUrDNA) were used given that they provide moderate resolution of *Symbiodinium* to the subclade level (e.g., LaJeunesse et al. 2003; Sampayo et al. 2009). D1 and D2 28S-LSUrDNA of potential *Symbiodinium* were amplified using the Toha PCR primer set (see Rodriguez-Lanetty et al. 2001): forward (Toha F): 5′-CCT CAG TAA TGG GGA ATG AAC A-3′ and reverse (Toha R): 5′-CCT TGG TCC GTG TTT CAA GA-3′. All PCR contained >15 ng of template DNA, 1× PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM of each primer, 200 mM dNTP and 0.1 mM Taq polymerase platinum, and filter-sterilized water for a total volume of 20 µl. The PCR conditions involved an initial denature period of 2 min at 94°C, followed by 30 cycles of 15 s at 94°C, 15 s at 60°C, 60 s at 72°C and a final extension period of 5 min at 72°C. After the PCR, the samples were held at 4°C. The PCR products were purified with the QIAquick PCR purification kit, QIAGEN.

The different 28S-LSUrDNA fragments contained at each sample were separated and cloned in TOP TEN cells (Invitrogen, AU) by using the pGEM-T Vector System, following the manufacturer's protocol. For ligation, 32 ng of PCR products (3:1 insert:vector molar ratio) was used. Four clones per library were PCR-amplified at their 28S-LSUrDNA, purified with QUIAquick and sent to the Australian Genomic Research Facility for sequencing. Unfortunately, because of time limitations, it was not possible to sequence more clones per library.

Data analysis

Amplified and sequenced DNA was compared to *Symbiodinium* sequences using the Basic Local Alignment Search Tool (BLAST; Altschul et al. 1990) and the GenBank database. Those *Symbiodinium* sequences that showed the highest similarity scores with the resulting sequences and *E*-values $<e^{-10}$ were downloaded. 28S-LSUrDNA sequences of the *Symbiodinium* subclade types C1, C1b, C3, C15, C17, C21 and C27, described and reported by LaJeunesse et al.

(2003), were also downloaded. Additionally, at least two 28S-LSUrDNA sequences of the *Symbiodinium* clades A, B, C, D, E, F and H were also downloaded.

All resulting and GenBank sequences were aligned using ClustalX 2.0. Sequences that did not belong to *Symbiodinium* (according to the BLAST search) were not aligned. Phylogenetic inference analyses were performed with the aligned sequences to determine the phylogenetic position of *Symbiodinium* isolates obtained in this study (Table 2). Sequences that belonged to *Symbiodinium*, according to the BLAST analyses but had too much baseline noise, were excluded from the phylogenetic analyses. The phylogenetic analyses were then performed with the Maximum-Parsimony and Maximum-Likelihood methods, using the best fit model of evolution (TrN + G) according to jModeltest (Posada 2008). These analyses were run using the beta version of the PAUP 4.0 software (Sinauer Associates, Massachusetts, USA). Gaps were treated as a fifth character state, starting trees were obtained via stepwise addition, sequence addition was simple, and it used the tree-bisection-reconnection (TBR) branch-swapping algorithm to find the best tree(s). If more than one tree was found, then the 50% Majority-Rule Consensus Tree was calculated. Five hundred bootstrap replicates were performed for each analysis.

Results

The 28S-LSUrDNA sequences from a total of 25 samples were amplified. Of these, 24 were cloned while the remaining sediment sample was directly sequenced and identified as a non-symbiotic dinoflagellate. Among 96 clones that were sequenced (four clones per library), 21 were identified as belonging to *Symbiodinium* ($\sim$22% of clones sequenced) and 16 as non-symbiotic dinoflagellates. The remaining 59 clones were either of poor-quality or had sequences similar to platyhelminthes or non-symbiotic ciliates (e.g., *Paramecium*). *Symbiodinium* were associated with samples from the green macroalgae *Chlorodesmis fastigiata, Halimeda opuntia* and *H. discoidea*, the red macroalgae *Hypnea spinella* and *H. pannosa*, *Laurencia intricata* and *Asparagopsis taxiformis*, the brown macroalgae *Padina* sp. and *Lobophora variegata*, and algal turfs (see Fig. 2, Tables 1 and 2).

Non-symbiotic dinoflagellates (e.g., *Gyrodinium*, *Karlodinium* and *Prorocentrum*) were associated with samples from *Porolithon onkodes* (CCA), cyanobacterial mats, sediments and also *Halimeda discoidea*, *Hypnea pannosa*, *Laurencia intricata, Asparagopsis taxiformis, Lobophora variegata* and algal turfs. All *Symbiodinium* found in macroalgal microhabitats belonged to the clade C and were similar to strains of *Symbiodinium* that establish symbiosis with corals, according to the BLAST search (score >920; *E*-value = 0.0; similarity >98%) (Table 3). Only Dc11 from *Lobophora variegata* (score = 720) showed a score <920 in the BLAST search.

The phylogenetic analyses revealed that all *Symbiodinium* isolated from macroalgal microhabitats belonged to the clade C (Fig. 3). Interestingly, however, they are spread between at least two subclades. Strain Dc58, isolated from *Hypnea pannosa*, grouped with *Symbiodinium* C15 (a strain that normally associates with the coral *Porites;* LaJeunesse et al. 2003); strains Dc25, Dc54, Dc60, Dc70, Dc72 grouped with *Symbiodinium* C3 (a strain associating with *Acropora*, *Favia* and a large number of other coral genera; LaJeunesse et al. 2003). Dc30 grouped with *Symbiodinium* C17 (a strain that associates with *Montipora*; LaJeunesse et al. 2003) but with a very low bootstrap support (<50%). Strains Dc1, Dc2, Dc3, Dc4, Dc6, Dc8, Dc15, Dc21, Dc27, Dc68, Dc82, Dc88 did not clearly fall within any subclade. Sequences Dc36 and Dc11 were not included in the phylogenetic analyses because they had too much baseline noise. Phylogenetic positions of macroalgal-associated *Symbiodinium* did not vary much among trees generated by Maximum-Parsimony and Maximum-Likelihood. The tree inferred by Maximum-Parsimony (data not shown) was a consensus (50% majority rule) of ten trees. Subclades C3 and C15 were supported with bootstrap values of 96 and 88%, respectively.

Discussion

The present study has found evidence that macroalgae may serve as potential microhabitats for *Symbiodinium* spp. when they are outside their cnidarian hosts. *Symbiodinium* were found associated with macroalgae from a wide variety of depths and sites (Heron-reef flat, Heron-4th point, Keppel-Middle Island, Keppel-Halfway Island) on the southern Great Barrier Reef. *Symbiodinium* were associated with a wide variety of macroalgal forms that included foliose (*Padina* sp., *Lobophora variegata*), turfing filamentous (mixed algal turfs), non-turfing filamentous (*Chlorodesmis fastigiata*), calcareous articulated (*Halimeda* spp.) and corticated macrophytes (*Laurencia intricata*, *Hypnea* spp., *Asparagopsis taxiformis*). *Symbiodinium* also associated with a range of macroalgal phyla including Chlorophyta (green algae: *Chlorodesmis fastigiata*, *Halimeda* spp.), Ochrophyta (Phaeophyceae, brown algae: *Lobophora variegata, Padina* sp.) and Rhodophyta (red algae: *Asparagopsis taxiformis*, *Hypnea* spp., *Laurencia intricata*).

While the present study did not find any *Symbiodinium* associated with Cyanobacteria, or the red macroalgae *Porolithon onkodes*, *Plocamium* sp. and *Peyssonnelia* sp., the finding of other dinoflagellates that were associated with *Porolithon onkodes* and Cyanobacteria suggest that it

Table 2 List of *Symbiodinium* 28S-LSUrDNA sequences used in this study for phylogenetic tree reconstruction

Strain	Source	Clade	Origin	Accession	Reference
Cx	*Cassiopea xamachana*	A	Jamaica	AF427454	Santos et al. (2002)
Unspecified	*Acropora longicyathus*	A	GBR, OT	DQ981495	Gomez-Cabrera et al. (2008)
B1	*Nephthea* sp.	B	GBR, HI	AF060892	LaJeunesse et al. (2003)
Pk13	*Plexaura kuna*	B	FK	AF427458	Santos et al. (2002)
PurPflex	*Plexaura flexuosa*	B	FK	AF427460	Santos et al. (2002)
CG60	*Pavona divaricata*	C	Guam	AJ308889	Pochon et al. (2001)
Ap7	*Acropora palifera*	C	Oki	EU159441	Schonberg et al. (2008)
Gas 1.1	*Goniastrea aspera*	C	MB	AY906989	Unpublished
Gt8	*Goniopora tenuidens*	C	GBR, OT	DQ060732	Loh et al. (1998)
aga1	*Agaricia* sp.	C	Bermuda	AY074938	Savage et al. (2002)
Am8	*Acropora millepora*	C	GBR, OT	DQ060730	Loh et al. (1998)
Ag7	*Acropora glauca*	C	MB	EF420766	Unpublished
Ag10	*Acropora glauca*	C	MB	EF420765	Unpublished
Mc39	*Montastrea curta*	C	MB	EF420771	Unpublished
KPC1	*Porites cylindrica*	C	Kenya	AY588439	Visram and Douglas (2006)
C1	Hard corals, anemones	C	GBR, HI	AY239384	LaJeunesse et al. (2003)
C1b	*Pavona varians*	C	GBR, HI	AY239385	LaJeunesse et al. (2003)
C3	Hard corals, soft corals	C	GBR, HI	AY239386	LaJeunesse et al. (2003)
C15	Hard corals, hydrozoa	C	GBR, HI	AY239387	LaJeunesse et al. (2003)
C17	*Montipora* spp.	C	GBR, HI	AY239383	LaJeunesse et al. (2003)
C21	Hard corals	C	GBR, HI	AY239382	LaJeunesse et al. (2003)
C27	*Pavona varians*	C	GBR, HI	AY239381	LaJeunesse et al. (2003)
D1a	Hard corals, soft corals	D	GBR, HI	AF170149	LaJeunesse et al. (2003)
A024	*Acropora brueggemanni*	D	Oki	AF396627	Santos et al. (2002)
P.di 1	*Pavona divaricata*	D	Oki	AB248878	Unpublished
CCMP421	Free Living	E	New Zealand	AY684264	Santos et al. (2002)
CCMP423	Free Living	E	Falmouth MA	EF205004	Moestrup and Daugbjerg (2007)
LII41_1814x	*Sorites* sp.	F	GBR, LI	AJ830921	Garcia-Cuetos et al. (2004)
LII162_1935x	*Amphisorus hemprichii*	F	GBR, LI	AJ830918	Garcia-Cuetos et al. (2004)
FL34_1286x	*Sorites* sp.	H	FK	AJ621148	Coffroth and Santos (2005)
LII141_1914x	*Sorites* sp.	H	GBR, LI	AJ830907	Garcia-Cuetos et al. (2004)
Dc1	*Halimeda discoidea*	C	GBR, HI	FJ851413	This study
Dc2	*Halimeda discoidea*	C	GBR, HI	FJ851408	This study
Dc3	*Halimeda discoidea*	C	GBR, HI	FJ851412	This study
Dc4	*Halimeda discoidea*	C	GBR, HI	FJ851405	This study
Dc6	*Halimeda discoidea*	C	GBR, HI	FJ851407	This study
Dc8	*Halimeda discoidea*	C	GBR, HI	FJ851409	This study
Dc15	*Lobophora variegata*	C	GBR, KI	FJ851411	This study
Dc21	*Padina* sp.	C	GBR, HI	FJ851415	This study
Dc25	*Laurencia intricata*	C	GBR, HI	FJ851416	This study
Dc27	*Laurencia intricata*	C	GBR, HI	FJ851410	This study
Dc30	Algal turfs	C	GBR, HI	FJ851414	This study
Dc54	Algal turfs	C	GBR, HI	FJ851418	This study
Dc58	*Hypnea pannosa*	C	GBR, HI	FJ851421	This study
Dc60	*Hypnea pannosa*	C	GBR, HI	FJ851417	This study
Dc68	*Hypnea spinella*	C	GBR, HI	FJ851403	This study
Dc70	*Asparagopsis taxiformis*	C	GBR, KI	FJ851419	This study
Dc72	*Asparagopsis taxiformis*	C	GBR, KI	FJ851420	This study

Table 2 continued

Strain	Source	Clade	Origin	Accession	Reference
Dc82	*Chlorodesmis fastigiata*	C	GBR, HI	FJ851406	This study
Dc88	*Halimeda opuntia*	C	GBR, HI	FJ851404	This study

GBR Great Barrier Reef, Australia, *HI* Heron Island, *LI* Lizard Island, *KI* Keppel Islands, *OT* One Tree Island, *MB* Moreton Bay, Australia, *FK* Florida Keys, USA, *Oki* Okinawa Island, Japan, *MA* Massachusetts, USA

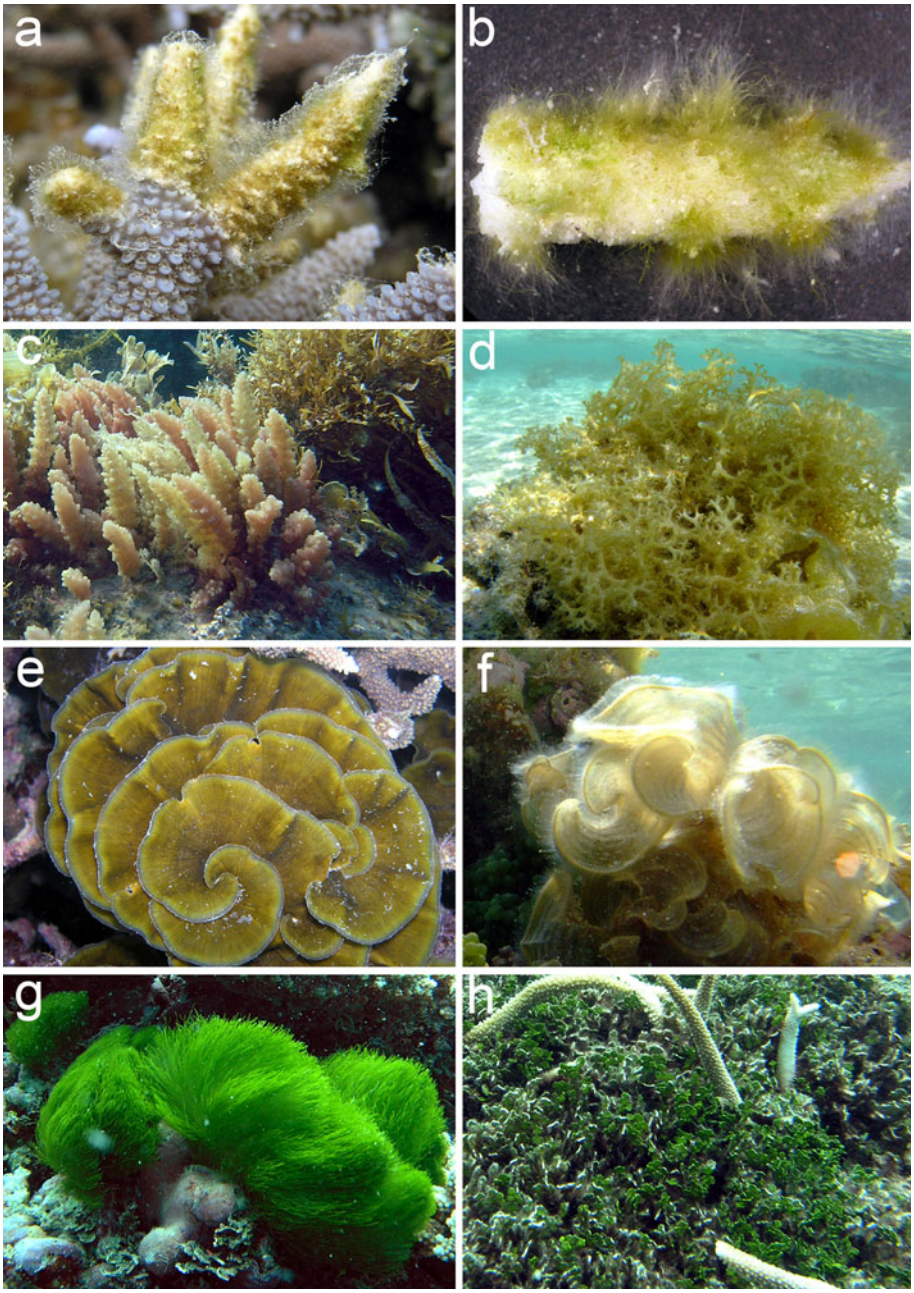

Fig. 2 Representative examples of macroalgae that were shown to harbor *Symbiodinium* spp. **a** and **b** Algal turfs from Heron Island, **c** *Asparagopsis taxiformis* from Keppel Islands, **d** *Hypnea pannosa* from Heron Island, **e** *Lobophora variegata* from Keppel Islands, **f** *Padina* sp. from Heron Island, **g** *Chlorodesmis fastigiata* from Heron Island, **h** *Halimeda opuntia* from Heron Island

would be too early to rule out these macroalgae as potential microhabitats for *Symbiodinium*. Moreover, the presence of *Symbiodinium* associated to *Plocamium* sp. and *Peyssonnelia* sp. was evaluated on few samples per species (see Table 1), thereby limiting the chance of finding *Symbiodinium*.

Symbiodinium was not found to be associated with sediments on the southern Great Barrier Reef. The presence

Table 3 List of *Symbiodinium* isolated from macroalgal microhabitats and their subclades (according to the phylogenetic analysis)

Strain	Macroalgal host	Subclade	Site	Potential coral host
Dc1	*Halimeda discoidea*	Undetermined	HI, RF	*Goniopora**, *Pavona** (DQ060732, AJ308889)
Dc2	*Halimeda discoidea*	Undetermined	HI, RF	*Pavona** (AJ308889)
Dc3	*Halimeda discoidea*	Undetermined	HI, RF	*Pavona** (AJ308889)
Dc4	*Halimeda discoidea*	Undetermined	HI, RF	*Pavona** (AJ308889)
Dc6	*Halimeda discoidea*	Undetermined	HI, RF	*Pavona** (AJ308889)
Dc8	*Halimeda discoidea*	Undetermined	HI, RF	*Pavona** (AJ308889)
Dc11	*Lobophora variegata*	–	KI, M	*Montastrea**, *Acropora** (EF420771, EF420766)
Dc15	*Lobophora variegata*	Undetermined	KI, Hw	*Goniastrea**, *Pavona** (AY906989,AJ308889)
Dc21	*Padina* sp.	Undetermined	HI, RF	*Goniopora** (DQ060732)
Dc25	*Laurencia intricata*	C3	HI, RF	*Acropora**, *Echinopora*, *Favia*, *Goniastrea*, *Leptoria*, *Montastrea*, *Platygyra*, *Hydnophora*, *Merulina*, *Acanthastrea*, *Lobophyllia*, *Symphyllia*, *Seriatopora*, *Isis* (EF420766)
Dc27	*Laurencia intricata*	Undetermined	HI, RF	*Pavona** (AJ308889)
Dc30	Algal turfs	C17	HI, RF	*Montipora*, *Goniopora** (DQ060732)
Dc36	*Halimeda discoidea*	–	HI, RF	*Acropora**, *Pavona**, *Heliopora**, *Ctenactis** (EU159441, AJ308889, AJ308888, AJ308887)
Dc54	Algal turfs	C3	HI, RF	*Acropora**, *Echinopora*, *Favia*, *Goniastrea*, *Leptoria*, *Montastrea*, *Platygyra*, *Hydnophora*, *Merulina*, *Acanthastrea*, *Lobophyllia*, *Symphyllia*, *Seriatopora*, *Isis* (EF420766)
Dc58	*Hypnea pannosa*	C15	HI, RF	*Porites**, *Montipora* (AY588439)
Dc60	*Hypnea pannosa*	C3	HI, RF	*Acropora**, *Echinopora*, *Favia*, *Goniastrea*, *Leptoria*, *Montastrea*, *Platygyra*, *Hydnophora*, *Merulina*, *Acanthastrea*, *Lobophyllia*, *Symphyllia*, *Seriatopora*, *Isis* (EF420765)
Dc68	*Hypnea spinella*	Undetermined	HI, RF	*Acropora**, *Heliofungia** (EU159441, DQ060761)
Dc70	*Asparagopsis taxiformis*	C3	KI, Hw	*Acropora**, *Echinopora*, *Favia*, *Goniastrea*, *Leptoria*, *Montastrea*, *Platygyra*, *Hydnophora*, *Merulina*, *Acanthastrea*, *Lobophyllia*, *Symphyllia*, *Seriatopora*, *Isis* (DQ060730)
Dc72	*Asparagopsis taxiformis*	C3	KI, Hw	*Acropora**, *Echinopora*, *Favia*, *Goniastrea*, *Leptoria*, *Montastrea*, *Platygyra*, *Hydnophora*, *Merulina*, *Acanthastrea*, *Lobophyllia*, *Symphyllia*, *Seriatopora*, *Isis* (DQ060730)
Dc82	*Chlorodesmis fastigiata*	Undetermined	HI, RF	*Pavona** (AJ308889)
Dc88	*Halimeda opuntia*	Undetermined	HI, 4th pt	*Pavona** (AJ308889)

Potential coral hosts are those of the most similar sequences found in the BLAST search (*) and those on which LaJeunesse et al. (2003) found the same subclades (underlined). Accession numbers of similar sequences isolated from potential coral hosts are shown. Samples Dc11 and Dc36 were not used in the phylogenetic analysis. HI: Heron Island; RF: Reef flat; KI: Keppel Islands. M: Middle Island; Hw: Halfway Island

of *Symbiodinium* in reef sediments, however, has been demonstrated by previous studies in the Caribbean Sea (Porto et al. 2008) and Pacific Ocean (Carlos et al. 1999; Hirose et al. 2008; Adams et al. 2009; Pochon et al. 2010). Previous studies have emphasized the limitations of directly extracting DNA from sediments, especially the action of sediment-associated substances interfering with the DNA extraction process (Steffan et al. 1988; Lovell and Piceno 1994; Gray and Herwig 1996). For that reason, studies reporting sediment-associated *Symbiodinium* have generally used direct isolation and culture of potential candidates before molecular identification (e.g., Carlos et al. 1999; Hirose et al. 2008). Given that the present study did not use direct isolation and culture, it would be premature to rule out the presence of *Symbiodinium* in sediments on the southern Great Barrier Reef.

Previous studies of free-living *Symbiodinium* have shown contrasting effectiveness at recovering *Symbiodinium* sequences from clone libraries. Littman et al. (2008) were 1% efficient (four *Symbiodinium* among 319 clones) using 18S rDNA, while Manning and Gates (2008) were >90% efficient using a hypervariable region of 23S (see Table 2 of Manning and Gates 2008). A rapid comparison of this study with these studies suggests that the recovery

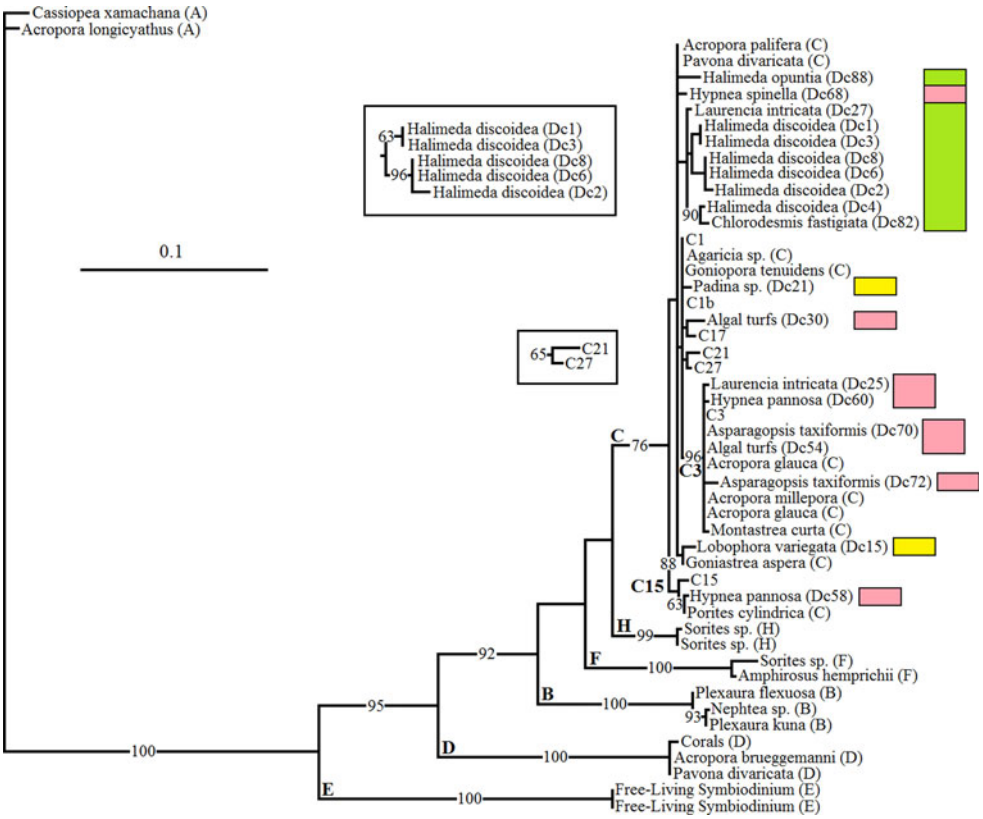

Fig. 3 Phylogenetic tree inferred by Maximum-Likelihood (ML) from the 28S-LSUrDNA of 19 macroalgal-associated *Symbiodinium* and 31 *Symbiodinium* reference sequences, downloaded from the GenBank. Clades, subclades and ML bootstrap values >50% are indicated at their respective branches. Names of strains indicate their host/environmental origin. *Colors* next to macroalgal-associated strains indicate their macroalgal phyla (green macroalgae, red macroalgae or brown macroalgae)

of *Symbiodinium* from clone libraries was moderately efficient here (22%; 21 *Symbiodinium* among 96 clones). The very low efficiency found by Littman et al. (2008) may be explained by the fact that their genetic analyses were employed on sediments samples where molecular analyses have limitations as explained above.

Most Pacific corals harbor *Symbiodinium* clade C, although most Caribbean corals, on the other hand, are associated with *Symbiodinium* clades B, with significant numbers of Caribbean corals also having clades A and C (Baker et al. 1997; LaJeunesse et al. 2003). The same biogeographic distribution was also found in *Symbiodinium* from the water column by Manning and Gates (2008). Interestingly, the present study only found *Symbiodinium* clade C to be associated with macroalgae in the southern Great Barrier Reef, while Porto et al. (2008) found a large range of *Symbiodinium* clades (A, B and C) associated to benthic macroalgae in Caribbean coral reefs. Nevertheless, it is likely that the diversity of macroalgal-associated *Symbiodinium* was underestimated in this study as it explored as many macroalgal microhabitats as possible but the number of sequenced clones per library was low. It is possible that more *Symbiodinium* clades/subclades appeared if more clones per library were sequenced.

Subclades Dc25, Dc54, Dc60, Dc70 and Dc72 from the present study grouped with *Symbiodinium* C3 (96% bootstrap support; Figs. 3, Table 3). This is interesting given that C3 is a generalist subclade which establishes symbioses with many coral species in Pacific and Caribbean coral reefs (LaJeunesse et al. 2003; Table 3) as well as some foraminifera (Pochon et al. 2007). The occurrence of this strain in several samples illustrates the potential importance of macroalgae as a source of *Symbiodinium* for a huge number of coral species. Strain Dc58 grouped with *Symbiodinium* C15 (88% bootstrap support), which associates with thermally tolerant corals (*Porites* spp. and *Montipora digitata*; LaJeunesse et al. 2003) and foraminifera (Pochon et al. 2004). It is believed that thermal tolerance of those hosts is given by *Symbiodinium* C15. Red macroalgae were the only macroalgal phylum that grouped with symbiotic strains of *Symbiodinium* (i.e., C3 and C15), while green and brown macroalgae did not clearly associate to any subclade. Given the fact that this pattern may change if more clones per library were sequenced, it will require verification in follow-up studies.

It is possible that some macroalgal-associated *Symbiodinium* found in the present study were *in hospite*, associated to soritid foraminifera or macroscopic ciliates. The latter have been reported to harbor *Symbiodinium* clade C and inhabit macroalgal microhabitats (Pochon et al. 2004, 2007; Lobban et al. 2002, Pochon and Pawlowski 2006). It is also possible that some *Symbiodinium* came from symbiotic larvae; however, the collection of samples occurred in August, which is 2 months before the coral spawning in

the Great Barrier Reef (Willis et al. 1985; Babcock et al. 1986). *Symbiodinium* C15 and C3 have been previously isolated from both corals and foraminifera (Pochon et al. 2004, 2007; Pochon and Pawlowski 2006); thus, it is uncertain whether the macroalgal-associated *Symbiodinium* C15 and C3 found here were free-living or foraminifera-associated (although careful stereomicroscopic examination of the filter papers with the samples did not find soritid foraminifera). Nevertheless, the presence of *Symbiodinium* C3 and C15 in Pacific macroalgal microhabitats may also suggest, with limitations, a potential close link between coral zooxanthellae and macroalgal-associated *Symbiodinium* populations/communities.

It is interesting to consider why *Symbiodinium* may associate with macroalgae. Porto et al. (2008) suggest that *Symbiodinium* associate to benthic macroalgae because their intricate branching networks with high surface-to-volume ratios provide substrate, light attenuation and refuge for *Symbiodinium*. On the other hand, studies on other epiphytic dinoflagellates suggest that they associate with some benthic macroalgae primarily because of the large amount of surface area upon which to attach (Parson and Preskitt 2007) and possibly because they release organic substances that stimulate the growth and provide other resources for epiphytic dinoflagellates (Morton and Faust 1997). Given that the water column associated with coral reefs tends to be nutrient poor, organic substances along with inorganic nutrients released by benthic macroalgae (Khailov and Burlakova 1969; Wada et al. 2007) may play a critical role in the survival of *Symbiodinium* outside their coral hosts. This idea requires further exploration to establish the relative importance of these macroalgal organic and inorganic compounds for the survival of *Symbiodinium*.

In conclusion, the present study has shown that *Symbiodinium* are spread among several macroalgal taxa and functional groups on the southern Great Barrier Reef. Some of these *Symbiodinium* may be *in hospite* within foraminifera or ciliates; however, the presence of *Symbiodinium* C3 and C15 in macroalgal microhabitats may also suggest, with limitations, a potential close link between coral zooxanthellae and macroalgal-associated *Symbiodinium* communities, and a continuum between symbiotic and environmental *Symbiodinium* populations. This study has implications for the role of other reef organisms and habitats as potential reservoirs for the symbionts that inhabit reef-building corals. A more complete description of these potential reservoirs is important if we are to continue to improve our understanding of the all-important mutualistic symbiosis between corals and dinoflagellates.

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