Mar Biol (2011) 158:87–100
DOI 10.1007/s00227-010-1544-z

ORIGINAL PAPER

Do corals select zooxanthellae by alternative discharge?

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Received: 6 November 2009 / Accepted: 8 September 2010 / Published online: 21 September 2010
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Abstract Loss of zooxanthellae (dinoflagellate *Symbiodinium*) from corals will sometimes lead to mass mortality of corals. To detect and quantify *Symbiodinium* released from corals, we developed a zooxanthellae "trap" and a quantitative PCR (qPCR) system with *Symbiodinium* clades A–F-specific primer sets. The trap was attached to a branch or the surface of several wild stony corals, and the water samples within the traps, including released *Symbiodinium*, were subjected to qPCR. All tested corals released clade C *Symbiodinium* at estimates of ~5,900 cells h^{-1} cm^{-2} of coral surface. Although all tested *Pocillopora eydouxi* harboured both clades C and D, some of these colonies released only clade C or released a lesser amount of clade D than that in the tissues. Our *Symbiodinium* quantification system revealed that wild hermatypic corals constantly release *Symbiodinium* to the environment. Our result suggests that some corals may discharge certain clades of *Symbiodinium* alternatively.

Communicated by C. Riginos.

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Introduction

Many marine invertebrates, including stony corals, harbour symbiotic microalgae commonly referred to as zooxanthellae within their tissues. The zooxanthellae, specifically the dinoflagellate genus *Symbiodinium*, translocate their photosynthetic products to the host corals and contribute to the vigour of corals and the coral reef ecosystem. "Coral bleaching," which results in mass mortality of corals, has occurred in many tropical and subtropical seas with increasing frequency over the past two decades (Hoegh-Guldberg 1999). As an example of the catastrophic effect of coral bleaching in coral reef ecosystems, coral reefs have decreased by 30–50% in the Caribbean Sea over the last three decades (Gardner et al. 2003) and more than 70% of corals died between 2003 and 2008 in Sekisei Lagoon (Okinawa, Japan) (National Institute for Environmental Study; http://www.nies.go.jp/topics/2008/20080910/20080910.html).

In a strict sense, the term coral bleaching refers to the loss of *Symbiodinium* and/or reduction of their pigments (Brown 1997). Thus, release of *Symbiodinium* may be directly correlated with coral bleaching. Causes of *Symbiodinium* discharge include stresses such as elevated temperature (Gates et al. 1992) or metal exposure (Jones 1997). *Symbiodinium* discharge has been investigated in a variety of aquarium experiments. Bhagooli and Hidaka (2004) showed that photosynthetically active *Symbiodinium* could be released from a coral, and Stimson and Kinzie (1991) reported that a *Pocillopora damicornis* colony in nutrient-enriched water released more *Symbiodinium* than did the control colony in ambient seawater. Perez et al. (2001) found that *Symbiodinium* expulsion increased significantly with increasing temperature in the sea anemone *Aiptasia pallida*. Although these aquarium experiments have

provided useful information, data from the field are necessary, as little is known about *Symbiodinium* release from corals in natural environments. Such information is essential to understanding coral bleaching in the field.

Because *Symbiodinium* cells are relatively small and have a featureless spherical morphology, it is difficult to enumerate them once they are released into the sea. To detect microorganisms that cannot be feasibly recognized and counted under a microscope, researchers often use quantitative real-time PCR (qPCR) (e.g., Bowers et al. 2000; Dyhrman et al. 2006; Galluzzi et al. 2004; Hosoi-Tanabe and Sako 2005; Kamikawa et al. 2006). qPCR has been applied in a number of studies involving coral-*Symbiodinium* systems (e.g., Correa et al. 2009; Loram et al. 2007; Mieog et al. 2007). Our previous attempt to quantify *Symbiodinium* by means of qPCR showed that (1) more than 2×10^6 cells h^{-1} (enumeration based on using a *Symbiodinium* culture as the quantification standard) were released from two colonies of *Acropora digitifera* with a combined total skeletal weight of 210 g and (2) more than 30,000 cells l^{-1} of *Symbiodinium* occurred in the water column around a reef (Koike et al. 2007). These data are preliminary but suggestive: The *Symbiodinium* biomass present in the water column could be a sink for cells from the nearby corals, and this might be indicative of regional coral bleaching and/or a source for cells that might be inherited by other corals.

In *Symbiodinium*, nine phylogenetically distinct clades have been defined based on rRNA genes (clades A–I) (see Coffroth and Santos 2005; Pochon and Gates 2010). Furthermore, *Symbiodinium* phylogeny is highly diverse and includes many "types" within a clade (LaJeunesse 2002; LaJeunesse et al. 2003). Phylotypes belonging to different clades can exhibit similar patterns of sensitivity to elevated temperatures but their closely related sister phylotypes can exhibit different patterns of sensitivity to that stress (Tchernov et al. 2004). This study demonstrates that rDNA genotyping may not be diagnostic of thermal sensitivity in *Symbiodinium*. However, long-term field observations demonstrated that the cladal composition of some corals shifted from clade C to clade D after bleaching events (Baker et al. 2004; Jones et al. 2008). Although *Symbiodinium* has many types within a clade, roughly speaking, some of the corals with clade D symbionts are more resistant to thermal stress than those with clade C symbionts (Glynn et al. 2001; Rowan 2004). Therefore, it is plausible that the assemblage of *Symbiodinium* clades present in a coral could confer greater environmental tolerance to the host, and indeed, some hosts seem to replace symbiont clades as a result of changing environmental conditions (e.g., Baker 2001).

Buddemeier and Fautin (1993) proposed the "Adaptive Bleaching Hypothesis (ABH)," which states that stressed corals bleach to change the symbiont clade according to environmental conditions. The hypothesis includes two plausible modes for changing clades: "switching" and "shuffling" (Baker 2003). Switching can be attributed to an uptake of exogenous symbionts, which is a phenomenon found in anemones (Kinzie et al. 2001) and soft corals (Lewis and Coffroth 2004). Shuffling can be a shift from background endogenous symbionts to a major one, which is a phenomenon that is assumed to occur in scleractinian corals (Jones et al. 2008). Recently, qPCR systems revealed that many coral species harbour a low abundance of clade D along with a major clade (Correa et al. 2009; Mieog et al. 2007). These results suggest that in many coral species, symbionts may be shuffled by environmental change, such as an increase in temperature. Regardless of the mode of change, a loss or deterioration of the former dominant symbiont clade must occur as the primary step, perhaps when it becomes unfit in the new environment. However, this phenomenon has not yet been demonstrated in any survey. Based on our previous laboratory experiment in which corals released numerous symbionts to the surrounding water (Koike et al. 2007), we hypothesize that such active release may be attributed to the primary step of the ABH process. To test this hypothesis, we developed and evaluated a new qPCR system that enabled us to enumerate *Symbiodinium* biomass by clade (A–F) and a "trap" to collect symbionts released from corals in the field. We then attempted to quantify the *Symbiodinium* released from wild stony corals using the trap and the qPCR system.

Materials and methods

Designing the clade-specific PCR primers

To date, several clade-specific PCR primer sets for qPCR detection of *Symbiodinium* have been reported. For example, Correa et al. (2009) developed four clade-specific primer sets targeting the ITS region for clade A and 28S rRNA genes for clades B, C, and D. In the present study, we used the intercalator chemistry that employs the SYBR® Green I fluorochrome and we designed the PCR primer sets based on specific regions of the nuclear 28S rRNA gene (28S rDNA) to differentiate clades A–F; as references we used sequences obtained from GenBank for 32 taxa of *Symbiodinium* (GenBank accession numbers are listed in Table 1). Twelve taxa of another dinoflagellate lineage and one apicomplexan parasite were also referenced as negative targets. The obtained sequences were aligned using Clustal × 2.0 (Larkin et al. 2007) and manually edited by eye. A region was found that was specific to each *Symbiodinium* clade (A–F) but allowed the elimination of other clades, other dinoflagellate taxa, and the apicomplexan

Table 1 GenBank accession numbers of nuclear 28S rRNA genes used to design the clade-specific PCR primer sets

Strain or taxa	Clade	GenBank/EMBL/DDBJ accession number
Symbiodinium spp.		
FLAp#4	A	AF427453
Cx	A	AF427454
T	A	AF427455
Zs	A	AF427456
CCMP831	A	AY684263
CCMP827	A	AY684262
CCMP828	A	AY684261
HIAp	B	AF427457
Pk13	B	AF427458
Pk702	B	AF427459
PurPflex	B	AF427460
s15e10 u	B	DQ200711
CCMP1633	B	AY684266
Mp	C	AF427461
Ua#31	C	AF427463
LII160 1933x	C	AJ830930
GIII100 2224x	C	AJ830931
PsammoL	D	DQ312328
PorLarak	D	DQ312325
A024	D	AF396627
Ua#2	D	AF396628
A002	D	AF396626
CCMP421	E	AY684264
Ro42 2096x	F	AJ830926
Ro39 2093x	F	AJ830925
GIII99 2223x	F	AJ830924
1361j	F	AJ830922
LII41 1814x	F	AJ830921
E170 1338j	F	AJ830920
207j	F	AJ830919
LII162 1935x	F	AJ830918
RII40-971X	F	AJ872077
Other dinoflagellates		
Coolia monotis		AM902741
Akashiwo sanguinea		EF613348
Alexandrium tamarense		AY566189
Amphidinium klebsii		EU046328
Ceratium fusus		EF517276
Dinophysis fortii		AB355151
Gambierdiscus toxicus		EF202961
Gonyaulax spinifera		EF416284
Gymnodinium catenatum		AY916534
Gyrodinium instriatum		EF613354
Heterocapsa triquetra		EF613355
Karenia brevis		DQ847431
Apicomplexian parasites		
Plasmodium falciparum		NC004325

parasite. Each primer set then was designed with Primer Express® v2.0 (Applied Biosystems, Foster City, CA, USA) to amplify about 100 bp of the region and to have almost the same *Tm* value.

Testing for primer specificity to each *Symbiodinium* clade using PCR and qPCR

To determine expected conformity with the target clade and unexpected conformity with non-target clades of the established primer sets, a Web site (Primer-BLAST; National Center for Biotechnology Information) search for DNA sequences was conducted with an input setting of 80–200 bp for the PCR product size and including sets of "all GenBank + RefSeq Nucleotides + EMBL + DDBJ + PDB sequences." Along with these database searches, we conducted experimental confirmations with PCR using the designed primers against cultures of each *Symbiodinium* clade and other microalgae kept in our laboratory (Table 2). Samples (10 ml) of algae from the exponentially grown cultures were harvested by centrifugation (2,000*g*, 10 min), and the resultant cell pellets were subjected to total DNA extraction using a SepaGene® kit (Sanko Junyaku, Tokyo, Japan). Because *Symbiodinium* cells possess a rigid cell wall that cannot be feasibly extracted by the kit, cell pellets were initially homogenized using a glass-bead beating method (Mini-BeadBeater-8, Biospec Products, Bartlesville, OK, USA; 0.5-mm bead, operated at 3,200 rpm for 3 min). Extracted and purified total DNAs were dried, dissolved in 100 μl of TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0), and quantified by measuring the absorbance at 260/280 nm (Lightwave S2000, WPA, Cambridge, UK). A mixture of 1 ng μl^{-1} DNA, 1 pmol μl^{-1} of each forward and reverse primer, and the recommended volume of TaKaRa Ex Taq™ Hot Start Version (Takara Bio Inc., Shiga, Japan) were subjected to conventional PCR to verify the clade-specific amplicon from a designated *Symbiodinium* clade. A thermal-cycling protocol of 1 cycle at 94°C (5 min), 30 cycles at 94°C (5 s), 63°C (10 s) and 72°C (15 s), and 1 cycle at 72°C (5 min) was run in a 2720 Thermal Cycler (Applied Biosystems). The PCR amplicons were electrophoresed on 2% agarose gel and stained with ethidium bromide.

qPCR against the above-extracted DNAs from the *Symbiodinium* clades and other aligned microalgae were subsequently performed. Mixtures of 1 ng μl^{-1} DNA, 0.4 pmol μl^{-1} of each forward and reverse primer, and the recommended volume of TaKaRa SYBR® Premix Ex Taq™ II (Takara), including a ROX reference dye (as a passive reference), were analysed in an ABI PRISM 7000 (Applied Biosystems) under the PCR cycles of 1 cycle at 95°C (30 s) and 40 cycles at 95°C (5 s) and 60°C (31 s).

Table 2 *Symbiodinium* and other microalgal culture strains used to confirm the specificity of the designed primers

Strain name	Clade	Taxa
Symbiodinium spp.		
CS-161	A	
CCMP1633	B	
CCMP2466	C	
CCMP2556	D	
CCMP421	E	
CS-156	F	
Other microalgae		
AmIS-B6		*Amphidinium carterae*
PrTN-A5		*Prorocentrum concavum*
PrIS-B3		*Prorocentrum cassibicum*
PrIS-C3		*Prorocentrum emarginatum*
BM-U2-D4		*Prorocentrum lima*
ProroA4		*Prorocentrum micans*
NIES680		*Karenia mikimotoi*
OFAC9982-101		*Alexandrium catenella*
990615-A1		*Protoceratium reticulatum*
Asterionella spL		*Asterionella* sp.
MBIC10554		*Isochrysis galbana*
CCMP765		*Proteomonas sulcata*
UTEX90		*Chlamydomonas reinhardtii*

After the qPCR runs, melting profiles of the PCR product vs. temperature (dissociation curves) were obtained for each sample to check that positive amplifications of target DNA without primer–dimers had occurred.

Quantification of *Symbiodinium* cells using qPCR

Symbiodinium cell numbers in exponentially grown cultures of CS-161 (clade A; purchased from The Commonwealth Scientific & Industrial Research Organization, Australia), CCMP1633 (clade B; purchased from The Provasoli-Guillard National Center for Culture of Marine Phytoplankton, USA), CCMP2466 (clade C), CCMP2556 (clade D), CCMP421 (clade E), and CS-156 (clade F) were counted under a light microscope, and culture aliquots equivalent to 250,000, 25,000, 2,500, and 250 cells each were filter-trapped onto a polycarbonate filter (0.8-μm pore size, 47-mm Φ, Advantec-Toyo, Tokyo, Japan) under gentle vacuuming. The filters were then stored at −20°C for more than a day, thawed at room temperature for 1 h, and immersed in 500 μl TE buffer in a 1.5-ml microtube. The tubes were then placed in boiling water with intermittent vortexing (30 s, 3-min intervals) for a total of 10 min and then triplicate aliquots of 2 μl (each equivalent to 1,000, 100, 10, and 1 cell/reaction) were retrieved and subjected to qPCR using each clade-specific primer set, with H_2O as a non-template control (NTC). At the same time, to determine whether each primer set produces a signal from non-target clades, 1,000 cell-equivalent aliquots derived from other clades were also subjected to qPCR.

qPCR Application: how many *Symbiodinium* cells were released from corals in the natural environment?

Sampling locations and coral specimens

Field sampling was performed in Urasoko Bay, Ishigaki Island, Okinawa, Japan (Fig. 1). Two distinct locations were sampled: Point 1, where substantially healthy corals cover the reef slope, and Point 2, which is shallow (~1 m) and is exposed to turbid and stagnant water, and thus experiences high temperatures. On 27–28 August 2007, two colonies of *Acropora muricata* and four colonies of *Pocillopora eydouxi* were selected at Point 1 and two colonies of *Porites lutea* were selected at Point 2. Additionally, a single colony each of *Acropora* cf. *grandis*, *Favites abdita*, and *Cyphastrea serailia* was selected at Point 2 on 26 November 2007, and two colonies of *Poc. eydouxi* at Point 1 were selected on 9 October 2008. Finally, on 29–31 August 2009, three colonies of *A. muricata* were selected at Point 1, and a single colony of *A. muricata* and one of *Acropora nobilis* were selected at Point 2. The subsurface seawater temperatures on these sampling dates were ~28°C (Point 1) and 28°C (Point 2) on 27–28 August 2007; 24°C (Point 2) on 26 November 2007; 26°C (Point 1) on 9 October 2008; and 29°C (Point 1) and 30°C (Point 2) on 29–31 August 2009. At each sampling time point, a coral colour chart (distributed from CoralWatch Project from Project

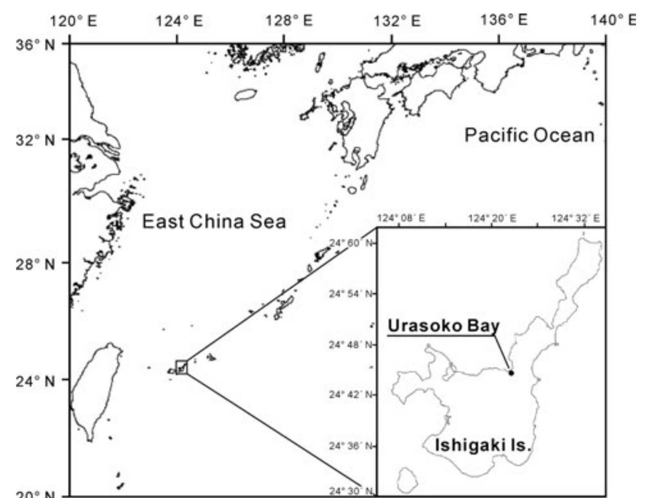

Fig. 1 Map showing the locations sampled in the present study. Urasoko Bay is located at the northern part of Ishigaki Island, Okinawa Prefecture. The *solid black circle* indicates Ishigaki Tropical Station of the Seikai National Fisheries Research Institute, Fisheries Research Agency

AWARE; http://www.projectaware.org) was used to categorize the bleaching index for the tested corals.

Sampling procedures

SCUBA divers attached a cylindrical trap for collecting released *Symbiodinium* from corals to a branch or the surface of the selected corals. The trap was composed of transparent PVC material, a cylindrical bottle (97 mm inner diameter, 167 mm depth, 1 l volume), a screw-top cap with a circular opening (73 mm inner diameter), and a textile pouch (with the bottom cut). The bottom-cut pouch was fitted to the circular opening of the cap by pinching it between the cap and the bottle mouth. The trap mouth was closed by squeezing a cord of the pouch after a branch of coral was inserted into the bottle (Fig. 2). To attain neutral buoyancy, a small lead sinker and a floater were tied to the trap. To set the trap on a non-branching encrusting coral (i.e., *Por. lutea*, *F. adbita*, or *C. serailia*), the textile part of the trap was folded up and the bottle opening was placed directly onto the coral's surface. The trap itself was strung to a coral with a rubber band. At the time of trap setting, the divers were careful not to rub the coral surface with the textile pouch or bottle. Our previous aquarium experiment (Koike et al. 2007) using *Acropora digitifera* indicated that the release of *Symbiodinium* from the coral could exhibit daily rhythmicity, with a peak at noon. For this reason, we set the traps for ~3 h between 10:00 and 15:00. At the end of the experiment, the trap was carefully removed from the coral and the mouth was instantly closed by squeezing the pouch cord. The coral branches that had been covered by the trap were marked by twisting a PVC-coated wire next to the trap mouth, and then the branch was snapped off from the colony. For the encrusting corals, it was difficult to retrieve the exact coral area over which the trap mouth had been placed; thus, another easily removable part near the trap was broken off. Each coral sample was placed immediately into an individual plastic bag without seawater, and within 1 h, these samples were placed in a −20°C freezer.

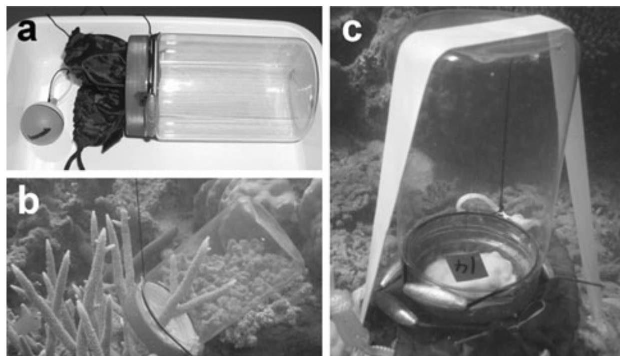

Fig. 2 The trap used to collect *Symbiodinium* released from corals. **a** The trap consists of a cylindrical bottle and a textile pouch, and it maintains neutral buoyancy with a small lead sinker and floater. **b** Deployment of the trap around a coral branch. The branch is inserted into the bottle, and the trap mouth is closed by squeezing a cord of the pouch. **c** Deployment of the trap around an encrusting coral. The trap is strung to the coral surface with a rubber band

On the boat, water samples from the traps were subjected to dissolved oxygen (DO) and temperature measurements using a portable DO meter (LDO-HQ10, HACH, Loveland, CO, USA); these measurements were used to detect possible stresses (e.g., DO decrease due to respiration, photosynthetic deterioration of *Symbiodinium*). The samples then were transferred to another bottle and kept in an ice-chilled cooler box. They were subjected to filtrations of coral-released *Symbiodinium* within 6 h, as described below. For the blank of *Symbiodinium* in seawater, ambient waters were also collected.

To make sure that the trapping of the coral did not cause stress-induced *Symbiodinium* release, at point 2 the traps were set on each of two branches of *A. muricata* (total two colonies) and *A. nobilis* (one colony). Five hours later, the trap-covered branches and adjacent free branches (three from each colony) were snapped off, and the *Symbiodinium* densities per surface area were determined and compared according to the method described below.

DNA extraction from the Symbiodinium in coral tissue

Frozen corals were thawed at room temperature, *Symbiodinium* cells were removed from the coral surface by spraying with 0.2-μm-filtered seawater containing 5 mM EDTA with an airbrush, and the subsequent cell suspension was collected in a plastic bag. The suspension was diluted to 50 ml with filtered seawater containing 5 mM EDTA, and the *Symbiodinium* cell number was counted under a light microscope. To remove animal tissue or cells, Triton X-100 was added to 10 ml of the suspension to give a final concentration of 0.01%. This sample then was vortexed for 30 s and allowed stand for 5 min at room temperature. After centrifugation at 1,450*g* for 3 min, the supernatant was removed and the filtered seawater containing 5 mM EDTA was added to the cell pellet. These washing steps were repeated three times (LaJeunesse 2002). Finally, each *Symbiodinium* pellet was subjected to bead beating homogenization and DNA extraction with a kit according to the aforementioned method, and the resultant DNA was dissolved in TE buffer.

Quantification of Symbiodinium in the coral tissues

To quantify *Symbiodinium* in the coral tissue using qPCR, the DNA extracts could not be used with precision because there was an expected loss of the cells during the Triton X-100 treatment, centrifugation, and/or the extraction steps. Therefore, we estimated cell numbers by classic

microscopic counts, as described above, but we estimated relative clade abundances (%) among the total *Symbiodinium* cell numbers using qPCR. Prior to qPCR, the first screening of the existent clades was performed by conventional PCR. This was necessary to reduce the effort and cost in qPCR and to confirm the presence or absence of minor background clades. Therefore, the minimum detection level (both absolute value and relative % among the major clades) of the minor *Symbiodinium* clades was determined by conventional PCR. In our primary estimation using each clade of *Symbiodinium* cultures, amplicons from the DNA extract at a minimum of 0.1 cell (clades B and E) or 1 cell (clades A, C, D, and F) in one PCR volume could be detected reliably on an agarose gel by ethidium bromide staining. These detection levels were confirmed to be invariable, even when the sample was mixed with a 1,000 cell-equivalent of non-target clade cells. Therefore, the presence of minor background clades in the DNA extract obtained from coral tissues can be detected at a level of 0.01% for clades B and E and 0.1% for clades A, C, D, and F. In other words, clade presence below these levels was excluded in the remaining discussion.

The conventional PCR-based screening revealed that all coral specimens possessed only clade C and/or D. Therefore, qPCR for these two clades was performed next. Mixtures of 1 ng μl^{-1} DNA extract, 0.4 pmol μl^{-1} of each forward and reverse primer, and the recommended volume of TaKaRa SYBR® Premix Ex Taq™ II, with the included ROX reference dye, were analysed with the ABI PRISM 7000 as described above. For use as quantification standards, DNA extracts from *Symbiodinium* cultures CCMP2466 (clade C) and CCMP2556 (clade D), each corresponding to 1, 10, 100, and 1,000 cells/reaction, were loaded at every run (all performed in triplicate). Based on these culture-based standardizations, the relative percentages of each clade were estimated. To determine whether these standard cultures were representative of wild *Symbiodinium* in coral tissues, 10^6 cell-equivalent DNA extracts (without Triton X-100 treatment) comprising only wild clade C obtained from two colonies of *Porites lutea* were diluted to a final concentration of 100 cells/reaction (three times replicate of DNA extractions from each colony) and subjected to qPCR together with the 100 cell-equivalent DNA extract from CCMP2466 (clade C). The same comparison was performed for CCMP2556 (clade D) with wild clade D obtained from *Favites adbita* (four times replicate of DNA extractions from single colony). We did not encounter cases of clades A, B, E, and F in the field (see Results).

Quantification of Symbiodinium released into the trap

The trapped seawater was passed through a 20-µm sieve, and 500 ml of the filtrate was filtered again with a 0.8-µm polycarbonate filter (Advantec-Toyo). Particles smaller than 20 µm were thus trapped onto the filter, and DNA from the particles was extracted using the TE boiling method described above (Koike et al. 2007). This method has advantages over other DNA extraction protocols because even a single cell of target specimen on the filter is never lost. The extracted DNAs were initially subjected to conventional PCR using all of the clade-specific primers, and *Symbiodinium* clade compositions in the traps were qualitatively estimated. However, if the trap water contained a relatively small number of *Symbiodinium* cells, coexisting background clades occurring in small numbers might escape detection. Therefore, for the samples obtained from multi-cladal colonies (in our case, clades C and D), nested-PCR was employed. Here, a partial 28S rRNA gene region covering the clade-specific regions was primarily amplified using a clade-universal primer set of Sym-28Sf (5′-TAGCATGAAGTCARACAAG-3′) and ls1.3 M (5′-ACCGATTTGCACGTCAGTA-3′; modified from Wilcox 1998), and then the primary PCR amplicons were subjected to secondary amplification following the above-mentioned clade-specific runs. The primary PCR cycle was 1 cycle at 94°C (5 min), 10–12 cycles at 94°C (30 s), 55°C (30 s), 72°C (1 min), and 1 cycle at 72°C (7 min). qPCR was then employed using the primers for the clades detected with nested and/or conventional PCR, using culture strain-based standards. To test for possible interference due to coral exudates or other extracts (e.g., organic acids or polysaccharides in coral mucus or in other materials) from filter-trapped particles in the qPCR, 10 or 100 cell-equivalent DNAs from the culture strains were initially added to every sample as internal standards, and the extra signal increases in qPCR corresponding to these additional DNAs were monitored. When interference was suspected, the samples were diluted sufficiently to diminish interference. All quantifications from qPCR were measured in triplicate.

Estimation of surface area of the corals

To express *Symbiodinium* cell density on a coral surface, estimations of coral surface areas were needed. The detached coral skeletons were soaked in melted 1.5% agarose three times, which caused the surface to become coated with a thin layer of agarose. Next, 0.2% methylene blue with ethanol was poured onto the coral and allowed to drip off. The coated layer was then re-melted in 500 ml of hot water, and the resultant methylene blue solution was measured for 660 nm absorbance (maximum absorbance of methylene blue at neutral pH) using a spectrophotometer (Lightwave S2000, WPA, Cambridge, UK). As a standard linear regression, correlations between the 660 nm absorbance and the surface area were estimated using several pieces of equally treated polystyrene foam with known

surface areas. In the case of encrusting corals, we did not collect the exact portions where the traps were laid; instead, the area of the trap opening (41.8 cm^2) was used.

Results

PCR specificity and quantification of six clades of *Symbiodinium*

Table 3 lists sequences and *Tm* values of the qPCR primer sets designed to amplify each 28S rRNA gene of *Symbiodinium* clades A–F. The desired amplifications against each *Symbiodinium* clade were simulated on the Primer-BLAST Web site (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) using the settings described in the Materials and Methods. Mismatched regions were sometimes found in all of the searches. For example, clade A *Symbiodinium* in *Anemonia rustica* (AY074976) had a four-base difference (3 in the forward primer and 1 in the reverse) with our clade A primers, clade B in *Montastraea annularis* (DQ200701) had a five-base difference (all 5 in the forward primer) with our clade B primers, clade C in *Amphisorus hemprichii* (AJ308894) had a three-base difference (all 3 in the forward primer) with our clade C primers, clade D in *Haliclona koremella* (AJ308899) had a 10-base difference (6 in the forward primer and 4 in the reverse primer) with our clade D primers, and clade F of the strain Z3 culture (DQ174742) had a one-base difference (in the reverse primer) with our clade F primers. In addition, our clade B primer may inadvertently amplify *Symbiodinium* clades H or F, since they have only five-base substitutions at the priming site. While our primers were not perfect in all instances, they matched the designated clades in the database. Moreover, experimental confirmations were rigorously undertaken for positive PCR amplifications of a designated *Symbiodinium* clade and negative amplification of other clades using *Symbiodinium* from established cultures, and other non-*Symbiodinium* algae were also checked using conventional PCR and qPCR.

Figure 3 summarizes the quantification of the delta Rn curves of clades A–F with the different primer sets. In the graphs for clades A-, C-, D-, E-, and F-specific primers, the fluorescence equivalent to even one cell/reaction of the designated clade increased significantly in comparison with other 1,000 cell-equivalents of non-target clades or NTC. However, for the clade B primer, the signals from one cell/reaction were not easily differentiated from other 1,000 cell clades. Based on these results, the determination limits were ~1 cell/reaction for clades A, C, D, E, and F and ~10 cells/reaction for clade B. The relationship between Ct values (PCR cycles at which a certain delta Rn value is obtained) and a logarithmic plot of cell numbers of each *Symbiodinium* clade (1, 10, 100, 1,000 cells/reaction) yielded good linear correlations (Fig. 4). In these runs, linear regression fits between cell numbers of standard clades (X) and the corresponding Ct values (Y) were calculated as follows: $Y = -0.777 \ln X + 29.503$ (clade A, $r^2 = 0.961$); $Y = -1.038 \ln X + 32.006$ (clade B, $r^2 = 0.999$); $Y = -1.249 \ln X + 27.811$ (clade C, $r^2 = 0.995$); $Y = -1.111 \ln X + 27.108$ (clade D, $r^2 = 0.999$); $Y = -1.310 \ln X + 27.382$ (clade E, $r^2 = 0.992$); and $Y = -0.958 \ln X + 31.049$ (clade F, $r^2 = 0.981$).

qPCR application: how many *Symbiodinium* cells were released from corals in the natural environment?

Trap setting

Using our qPCR system, we enumerated *Symbiodinium* cells released from the corals. For this purpose, the original trap (Fig. 2) was set on the branches of *A. muricata,*

Table 3 Sequences, *Tm* values, and amplicon sizes of clade-specific primer sets

Clade	Name	Sequence	*Tm* (°C)	Amplicon size (bp)
A	SymA28S-1F	5′-GAT TGT GGC CTT TAG ACA TAC TAC C-3′	56.3	126
	SymA28S-1R	5′-CTC TGA GAG CAA GTA CCG TGC-3′	56.5	
B	SymB28S-1F	5′-CAC ATG TCG TGC TGA GAT TGC-3′	58.9	108
	SymB28S-1R	5′-CTC GCA TGC TGA GAA ACA CTG-3′	58.3	
C	SymC28S-1F	5′-TTG CTG AGA TTG CTG TAG GCT -3′	56.5	124
	SymC28S-1R	5′-TCC TCA AAC AGG TGT GGC -3′	53.5	
D	SymD28S-1F	5′-AAT GCT TGT GAG CCC TGG TC-3′	58.7	114
	SymD28S-1R	5′-AAG GCA ATC CTC ATG CGT ATG-3′	58.7	
E	SymE28S-1F	5′-CGA GTT TTC ACT AGC CTT GTG TG-3′	57.8	99
	SymE28S-1R	5′-AGC GTT GCA GCT GAC GAG-3′	57.4	
F	SymF28S-1F	5′-ACA GAT CTT GCT GAG ATT GCT GTG-3′	59.6	143
	SymF28S-1R	5′-GAA GGC CGT CCT CAA ACA GAC-3′	59.7	

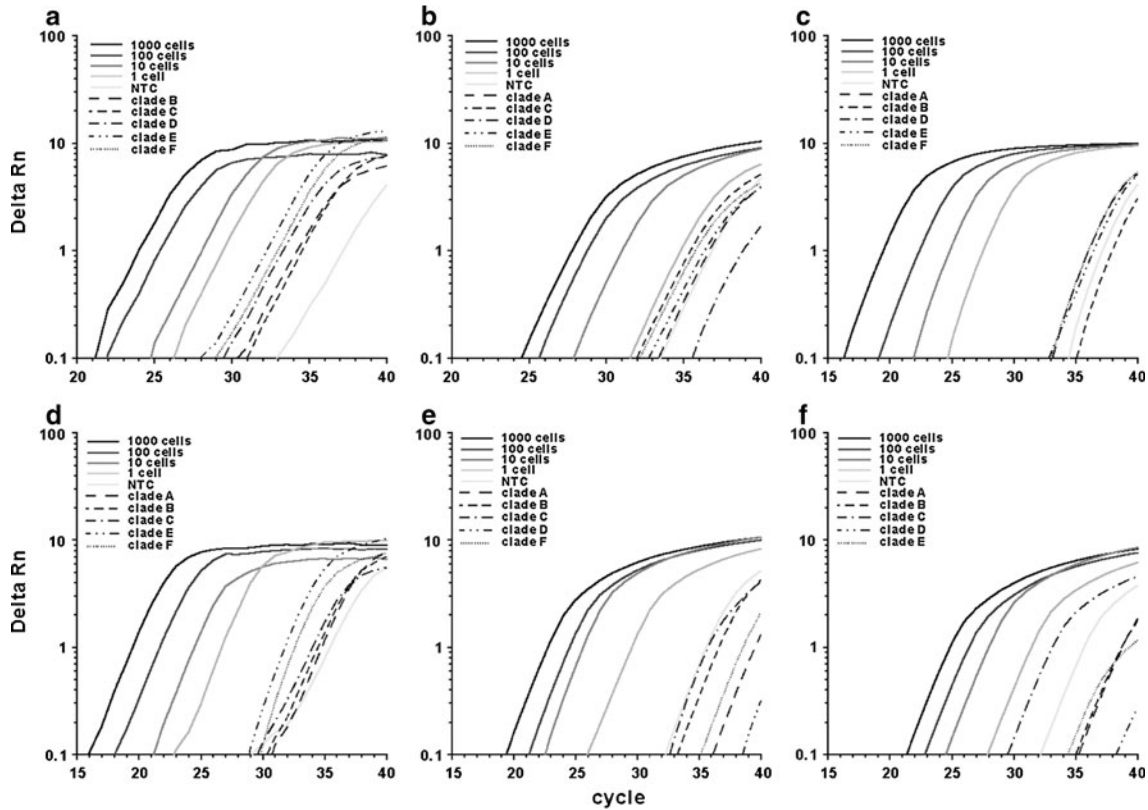

Fig. 3 Delta Rn curves versus PCR cycle in qPCR using clade-specific primer sets (clades A–F) against target clades equivalent to 1, 10, 100, and 1,000 cells/PCR and non-target clades of 1,000 cells/PCR. NTC represents a non-template control in which H_2O was used. For clade A-, C-, D-, E-, and F-specific primers, the fluorescence equivalent to one cell/reaction of the target clade increased significantly in comparison with other 1,000 cell-equivalents of non-target clades. For the clade B-specific primer, the signals from one cell/reaction were not reliably differentiated from the other 1,000 cells of non-target clades. **a**–**f** Represent data for specific primers for clades A–F, respectively

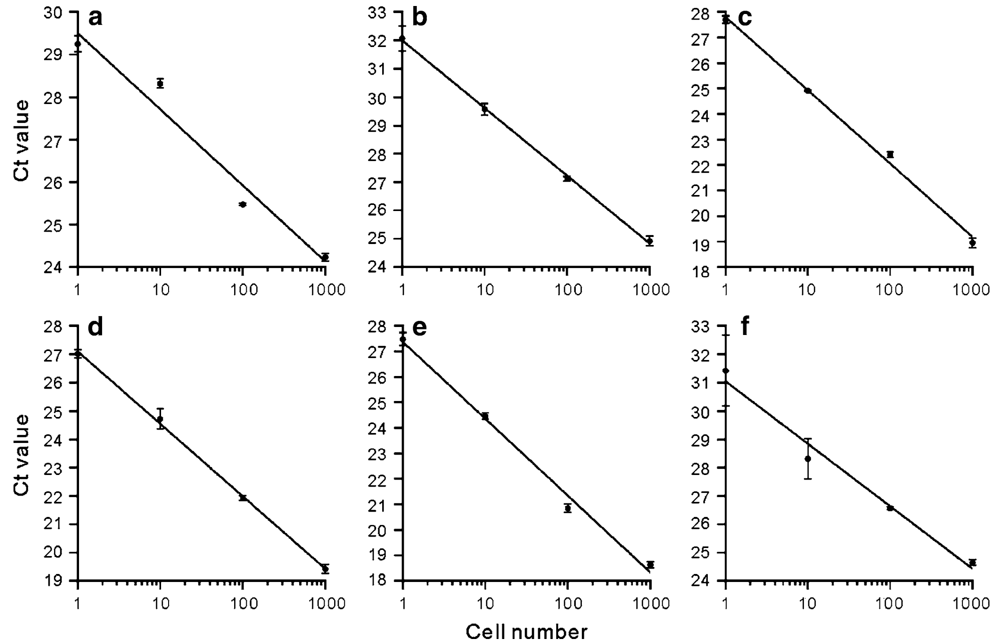

Fig. 4 Linear regression curves of Ct values (PCR cycle at which a certain delta Rn value was obtained) versus logarithmic cell numbers of each *Symbiodinium* clade. The standard DNA solutions equivalent to 1, 10, 100, and 1,000 cells/reaction were analysed by qPCR using clade-specific primer sets. Data are shown as the mean (*circle*) and standard deviations (*bars*) for triplicate measurements. Each *Symbiodinium* clade-specific primer set showed strong linear correlations. **a** CS-161 clade A, $r^2 = 0.961$, **b** CCMP1633 clade B, $r^2 = 0.999$, **c** CCMP2466 clade C, $r^2 = 0.995$, **d** CCMP2556 clade D, $r^2 = 0.999$, **e** CCMP421 clade E, $r^2 = 0.992$, **f** CS-156 clade F, $r^2 = 0.957$

A. cf. *grandis*, *A. nobilis*, and *Poc. eydouxi* or to the surfaces of *Por. lutea*, *F. abdita*, and *C. serailia*. To be sure that the trap would not lead to stress-induced *Symbiodinium* escape from the corals, the water inside the traps was monitored for DO concentrations and NH_4-N concentrations because DO is expected to decrease and NH_4-N is expected

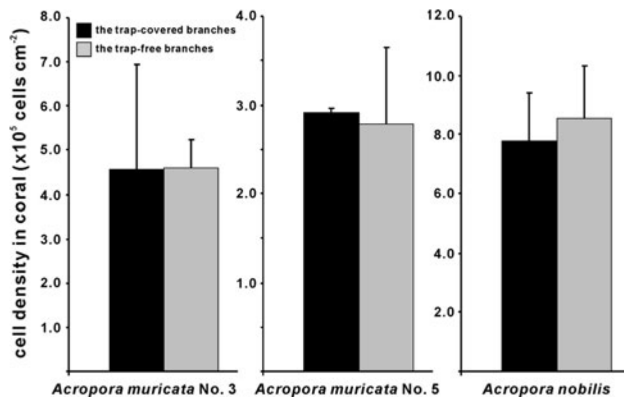

Fig. 5 Comparisons of *Symbiodinium* cell densities between the trap-covered and the trap-free branches in *Acropora muricata* and *Acropora nobilis* colonies. None of the tested corals showed significant differences in cell density between trap-covered and trap-free branches ($P > 0.05$, Student's *t*-test). The bars indicate standard error among the branches

to increase when corals are under stress. We found significantly higher DO or lower NH_4-N in all test cases compared to ambient water. For example, during the survey conducted on 27–28 August 2007, the trap waters from one colony of *A. muricata*, *Poc. eydouxi*, and *Por. lutea* contained 7.9 mg l^{-1}, 8.7 mg l^{-1}, and 9.4 mg l^{-1} of DO, respectively, all of which were above ambient DO concentrations (6.5 mg l^{-1} where *A. muricata* and *Poc. eydouxi* were collected and 8.5 mg l^{-1} for *Por. lutea*). The NH_4-N concentrations in the trap waters around *A. muricata, Poc. eydouxi*, and *Por. lutea* were 0.62 μmol l^{-1}, 0.08 μmol l^{-1}, and under the detection level (0.05 μmol l^{-1}), respectively; these values were lower than the concentration in ambient water (0.8 μmol l^{-1}). Furthermore, comparison of the *Symbiodinium* densities between the trap-covered and the trap-free branches (Fig. 5) illustrated that the trap-covered branches did not show any conspicuous decrease in *Symbiodinium* densities ($P > 0.05$, Student's *t*-test). From these results, we concluded that a maximum of 3 h of trap setting would not result in stress-induced *Symbiodinium* release from the corals, which is consistent with our previous study (Koike et al. 2007), in which we found that aquarium corals that seemingly did not suffer from stress released *Symbiodinium* periodically. It should also be noted that water temperature inside the traps was always equal to ambient temperature.

Quantification of released and retained Symbiodinium from/in corals

In our field studies, corals bearing only clades C and D were found. To determine whether these "wild" clades C and D were quantifiable by qPCR based on the culture-based standardizations, we compared Ct values (PCR cycles at which a certain delta Rn value is obtained) of both wild cells extracted from corals and cultures of the same clade. The mean Ct values (± SD) from 100 cells of CCMP2466 (clade C) and wild cells from *Por. lutea* (harbouring only clade C) were 22.8 ± 0.3 ($n = 3$ extracts × 3 measurements) and 22.5 ± 0.7 ($n = 2$ colonies × 3 extracts × 3 measurements), respectively. These were significantly congruent with each other ($P > 0.05$, Student's *t*-test). In the same manner, mean Ct values from 100 cells of CCMP2556 (clade D) and wild cells from *F. adbita* (composed only of clade D) were 22.0 ± 0.3 ($n = 3$ extracts × 3 measurements) and 21.7 ± 0.3 ($n = 4$ extracts × 3 measurements), respectively. These results suggested that culture-based quantification could be applied to wild *Symbiodinium* cells, at least for clades C and D. Because we encountered several instances wherein small numbers of clade D coexisted with clade C, a sample containing virtually one cell from the clade D culture (CCMP2556) and 1,000 cells from the clade C culture (CCMP2466) was prepared and subjected to clade D quantification. As a result, a Ct value (mean ± SD) of 22.7 ± 0.1 ($n = 5$) was obtained for the single clade D cell, and the value was congruent with that obtained from independent clade D analyses (Ct = 22.6 ± 0.3, $n = 5$). Based on these independent quantifications of clades C and D using culture-based standardizations, each co-existing percentage could be determined.

Table 4 lists the released cell numbers and clade compositions of *Symbiodinium* released to the traps and the retained cell density and clade compositions in the coral tissues. The former are represented as released cell numbers/cm^2 of coral surface covered by the trap divided by the number of hours of trap setting. For the *Symbiodinium* densities retained in the corals, a minimum value of 1.0×10^5 cells cm^{-2} in *Poc. eydouxi* sample No. 3 (August 2007) and a maximum value of 1.3×10^6 cells cm^{-2} in *Por. lutea* sample No. 1 (August 2007) were recorded. Roughly speaking, indexes estimated from the coral colour chart corresponded to the cell densities of *Symbiodinium*. It should be noted that corals suffering from bleaching (as in the cases shown by low colour indexes of 2 or 3) still retained significant numbers of *Symbiodinium*.

In the analyses of clade compositions retained within the corals and estimated by qPCR, every colony of *Poc. eydouxi* (Nos. 1–6) and one colony of *F. adbita* showed multi-cladal (C and D) presence of *Symbiodinium*. However, the relative abundance (%) of clade D was low and ranged from traces (under the determination level of qPCR but feasibly detectable at ~0.1% by conventional PCR) to 2.2% in *Poc. eydouxi* and 1.1% in *F. abdita*. In these multi-cladal cases, *Poc. eydouxi* No. 4 exhibited trace levels of clade D release just below the confidence level using qPCR (estimated as 0.5 cell/reaction, which was below the minimum standard of 1 cell/reaction) and roughly calculated to

Table 4 Released cell numbers, clade compositions of released *Symbiodinium* into the traps, retained cell density, and clade compositions within the coral host

Coral samples		Released cell number and clade		Cell density and clade in coral		Ratio (%)[b]
Coral species	Coral chart[a]	Cell number (cells cm^{-2} h^{-1})	Clade (%)	Cell density (cells cm^{-2})	Clade (%)	
August, 2007						
Acropora muricata (No.1)	5	48.2 ± 0.9	C 100	8.2×10^5	C 100	0.006
A. muricata (No.2)	2	31.4 ± 0.8	C 100	2.9×10^5	C 100	0.011
Pocillopora eydouxi (No.1)	5	88.4 ± 1.1	C 100	4.3×10^5	C 99.8 D 0.2	0.021
Poc. eydouxi (No.2)	3	11.9 ± 1.0	C 100	1.2×10^5	C 97.9 D 2.1	0.010
Poc. eydouxi (No.3)	3	8.7 ± 0.5	≈C 100[c]	1.0×10^5	C 97.8 D 2.2	0.009
Poc. eydouxi (No.4)	5	5906.3 ± 179.2	C 99.98 D 0.02[d]	5.0×10^5	C 99.9 D 0.1	1.2
Porites lutea (No.1)	4	654.9 ± 57.5	C 100	1.3×10^6	C 100	0.050
Por. lutea (No.2)	5	191.0 ± 9.7	C 100	1.0×10^6	C 100	0.019
November, 2007						
Acropora cf. *grandis*	5	209.8 ± 15.8	C 100	6.9×10^5	C 100	0.030
Favites adbita	2	65.8 ± 4.8	≈C 100[c]	5.4×10^5	C 98.9 D 1.1	0.012
Cyphastrea serailia	2	24.5 ± 4.1	C 100	8.2×10^5	C 100	0.003
October, 2008						
Poc. eydouxi (No.5)	4	11.4 ± 1.5	≈C 100[c]	1.4×10^5	C 99.1 D 0.9	0.008
Poc. eydouxi (No.6)	4	35.3 ± 1.6	≈C 100[c]	2.0×10^5	≈ C 100 D < 0.1	0.018
August, 2009						
A. muricata (No.3)	4	34.6 ± 4.8	C 100	2.1×10^5	C 100	0.016
A. muricata (No.4)	4	4.1 ± 1.2	C 100	7.3×10^5	C 100	0.0006
A. muricata (No.5)	4	7.9 ± 0.3	C 100	2.9×10^5	C 100	0.003
A. muricata (No.6)	5	5.9 ± 0.6	C 100	9.8×10^5	C 100	0.0006
Acropora nobilis	4	23.1 ± 5.6	C 100	9.4×10^5	C 100	0.002

[a] The bleaching index is based on a coral colour chart (distributed by the CoralWatch Project from Project AWARE; http://www.project-aware.org). A lower index value corresponds to extensive bleaching

[b] Calculated losses of *Symbiodinium* cells from coral tissues per cm^{-2} per hour

[c] Clade D was detected by nested-PCR but was not quantified

[d] Clade D was detected by qPCR but was just below the confidence level (below the minimum standard of 1 cell/reaction)

be 1.4 cells cm^{-2} h^{-1}. It was then estimated at 0.02% of the total release (the remaining 99.98% was clade C), making it much lower than that present in the tissues (clade D = 0.1%). Quantifications by qPCR failed for other multi-cladal colonies, although *Poc. eydouxi* Nos. 3, 5, and 6 and *F. adbita* showed cryptic occurrences of clade D in the trap waters after nested-PCR amplifications, whereas *Poc. eydouxi* Nos. 1 and 2 did not. The estimated releases reached a maximum of ~5,900 cells cm^{-2} h^{-1} from *Poc. eydouxi* No. 4 (August 2007) and a minimum of ~4 cells cm^{-2} h^{-1} from *A. muricata* No. 4 (August 2009). The ratios of released *Symbiodinium* to retained *Symbiodinium* within the corals were estimated at 0.0006–1.2%. We also collected ambient water to estimate background *Symbiodinium* occurrence in the water column. However, we could not detect any signals from ambient sea water. Thus, background *Symbiodinium* in the water column did not have deleterious effects on the estimation of *Symbiodinium* cell numbers in the traps.

Discussion

Properties of the qPCR developed in the present study

In the present study, we developed PCR primers to amplify and differentiate DNAs from *Symbiodinium* clades A–F and examined their specificities and applications using database searches and corroboration with conventional PCR and qPCR. The database searches revealed some *Symbiodinium* types that had sequence differences from our designated clade primers. Although most of these cases involved symbionts in sea anemones or foraminiferans and thus may be immaterial for coral applications, we did find unconformities

such as clade B in the coral *Montastraea annularis* having five bases different from our clade B primers. Moreover, our clade D primer may not work on a peculiar type of clade D symbiont (type D1: Pochon et al. 2006; Takabayashi et al. 2004) (e.g., in *Haliclona koremella*) because of the 10-base difference between primers. Nevertheless, at least in our field study, coral-borne clades C and D were reliably quantified using our primers.

Another particular concern about our qPCR system is the possibility that some non-specific signal increases might have been recognized in later PCR cycles (usually above 33 cycles) due to properties of the SYBR® Green chemistry used in qPCR. Such signal increases likely would result from the trace formation of primer–dimers, which cannot be eliminated. However, in the cases of clades A, C, D, E, and F, even 1 cell/reaction was enough to be discriminated from 1,000 times higher contaminants of other clades, and we concluded that minimum detection levels for these clades were above 1 cell/reaction and at least 0.1% relative abundance among other clades. For clade B, SYBR® Green signals around such small amounts of DNA (one cell/reaction) could not be discriminated from high contaminants of other non-target DNAs, probably due to the formation of primer–dimers or the non-specific amplification of DNA. Therefore, a minimum of 10 cells/reaction and at least 1% relative abundance among other clades would be the limit for quantification. Above such ranges, and with appropriate dilutions covering the standard ranges, the quantitative results obtained by our qPCR analyses were adequately rigorous, as linear relationships between a logarithmic plot of cell numbers and Ct values were obtained for all clades (Fig. 4). It should be repeated here that prior to the qPCR analyses, we performed conventional PCR to determine the presence or absence of the clades. This was necessary to reduce the effort and cost of qPCR and to confirm the quantification result in qPCR for background clades. In this conventional PCR analysis, detection levels of background clades were set to be equal to or much more sensitive than qPCR; this means that the first screening did not exclude the qPCR-detectable clades.

qPCR trial to enumerate *Symbiodinium* cells released from corals in the field

To determine how many and what clades of *Symbiodinium* are released from corals, we needed to develop novel methodologies beyond the qPCR systems. Thus, we first developed a trap to collect *Symbiodinium* discharged from corals. Trapped waters were monitored for concentrations of DO and ammonium ions after collection: these samples had higher DO and lower NH_4-N than ambient levels. Moreover, there were no significant differences in the *Symbiodinium* densities between the branches with and without the trap. From these results, we assumed that the setting of the trap did not negatively affect or promote *Symbiodinium* discharge.

The second methodology we developed was a technique to estimate coral surface area. This was necessary to determine the *Symbiodinium* density per area. A method using aluminium foil (Marsh 1970) has often been used to determine coral surface area. Hoegh-Guldberg (1988) proposed an alternative method that is more applicable for pitted surfaces and that involves coating surfaces with water-based polyurethane and methylene blue dye. In our newly developed method, we coated the coral skeleton with melted agarose gel instead of polyurethane and then stained it with methylene blue dye. Subsequent analysis for methylene blue adhesion is quite easy and involves re-melting the agarose coating in hot water. The primer coating using agar can be removed from any unwanted area for surface determination of trap-covered branch (e.g., broken parts). The amount of dye on the agarose layer of the coral skeleton then can be determined easily by spectrophotometry after liquefying the agar. Using this approach, we obtained a rather constant value of *Symbiodinium* density of 10^5 cells cm^{-2} in all corals except *Porites.* Although *Symbiodinium* cell density can fluctuate with season (e.g., Fagoonee et al. 1999; Fitt et al. 2000), our result was apparently lower than the previous observations made by several authors. This may be because our test corals had undergone intensive bleaching events.

The next issue that we needed to address was how to determine absolute cell numbers or relative percentages of cell presence for each clade, all of which were based on the qPCR results. Expressing this parameter as a “copy number of rRNA gene” would be more accurate but less attractive for ecological purposes. Thus, we calculated cell numbers using the formula obtained from Ct value versus cell numbers of “standard cells.” For standard cells, we used a culture strain for each clade; however, we are not certain that such strains were good representatives of wild *Symbiodinium* occurring in the field or of the intragenomic variations of copies of the rRNA gene. Comparisons of Ct values between the clade C standard (CCMP2466) or clade D standard (CCMP2556) and the equivalent cell numbers of wild clades C or D from corals were not significantly different. Moreover, in our qPCR system, each qPCR-based cell number was determined based on each independent cladal standard. Therefore, our culture-based standardizations should give independent levels of detection and should not be affected by differences in the rRNA gene copy numbers among the clades. Mieog et al. (2007) reported the cell average for ITS1 copies as 984 ± 109 copy $cell^{-1}$ (clade C, mean ± SE from two colonies of *Acropora millepora*, each single colony of *Acropora tenuis* and *Pocillopora damicornis*) and 3181 ± 69 copy $cell^{-1}$ (clade D, mean ± SE from

two colonies of *A. millepora*) for wild specimens. This maximum 10% error does not seem to present an obstacle to the determination of cell number based on rRNA gene copies.

We next tried to estimate how many cells and what clades are released from corals in the field. The tested species (*A. muricata, Poc. eydouxi* Nos. 1, 2, *Por. lutea, A.* cf. *grandis, C. serailia*, and *A. nobilis*) discharged only clade C *Symbiodinium* at an estimated 4–655 cells h^{-1} cm^{-2} of coral surface. In the case of *Poc. eydouxi* No. 4, clade D in the trap water was preliminarily quantified and calculated to be 0.02% of the total discharged biomass. This value is still lower than that observed in the tissues (0.1%). Although quantifications of clade D cell numbers were not successful, *Poc. eydouxi* Nos. 3, 5, and 6 and *F. adbita* seemed to release clade D. Nonetheless, it is noteworthy that *Poc. eydouxi* Nos. 1, 2, and 4 apparently released clade C prior to clade D. The highest discharge was recorded in a coral specimen of *Poc. eydouxi* (No. 4) at ~5,900 cells cm^{-2} h^{-1}; the rates of hourly discharges were calculated to be 0.0006–1.2%. During the sampling in August 2007, at which time an enormous bleaching event occurred due to extraordinarily high temperature, a high discharge rate, such as in the case of *Poc. eydouxi* No. 4, might possibly have led to bleaching. Titlyanov et al. (1996) reported that most of discharged *Symbiodinium* cells from aquarium corals were degraded. We still do not know whether the released *Symbiodinium* cells from corals in the field were damaged or not.

In August 2007, the low-*Symbiodinium* bearing colonies of *Poc. eydouxi* (Nos. 2 and 3; $1.0–1.2 \times 10^5$ cells cm^{-2}) had higher clade D ratios to the *Symbiodinium* cells (2.1 and 2.2%) in their tissues, whereas other colonies (Nos. 1 and 4; $4.3–5.0 \times 10^5$ cells cm^{-2}) contained only 0.2 and 0.1% of clade D, respectively. The results were not obtained from long-term observation of a single colony, but the relative increase in clade D retention would be likely if the preferential discharge of clade C was prolonged. This is consistent with the findings of other studies (e.g., that *Symbiodinium* clade D content can increase after a coral bleaching event) (Baker et al. 2004; Jones et al. 2008).

Symbiodinium clades or types often differ among individuals of the same species and in hosts occurring in different habitats. For example, coral specimens of *Montastraea annularis* and *Montastraea faveolata* from shallow water harboured clade A or B, whereas specimens from deep water harboured clade C (Rowan and Knowlton 1995). Furthermore, the cladal composition in these corals differed even though they occurred within a single colony (Rowan et al. 1997). In addition, multi-clades in certain corals have been reported to shift in some instances (e.g., Berkelmans and van Oppen 2006; Thornhill et al. 2006). Two possible mechanistic explanations of this phenomenon exist: symbiont switching and symbiont shuffling (Baker 2003). Symbiont switching is attributed to uptake of exogenous *Symbiodinium* and has been reported in sea anemones and soft corals (Kinzie et al. 2001; Lewis and Coffroth 2004). Symbiont shuffling is the propagation of "background symbionts" that occur at levels lower than the detection level. Symbiont shuffling is considered to incur a risk of death during the period of shuffling (Mieog et al. 2007). Jones et al. (2008) reported that a thermally sensitive subclade, C2, initially predominated in corals of the Great Barrier Reef, but after bleaching events, including a massive case in 2006, cladal composition shifted to clade D by symbiont shuffling. Although some of the corals with clade D symbionts are thought to be more resistant to thermal stress than those with clade C symbionts (Glynn et al. 2001; Rowan 2004), clade D *Symbiodinium* can be found in high latitude coral communities (Lien et al. 2007), and the deep water colonies of *Montastraea franksi* harboured clade D symbionts (Toller et al. 2001). Recent studies have indicated that there are several types that exist within clade D (LaJeunesse et al. 2004; Pochon et al. 2006), and when exposed to elevated temperatures, most closely related sister "types" exhibited significantly different responses (Tchernov et al. 2004). Therefore, we cannot confidently generalize that all clade D *Symbiodinium* show a high-light high-temperature tolerance. However, recent studies that used highly sensitive qPCR to examine coral-*Symbiodinium* systems have revealed that many coral hosts harbour background clade D *Symbiodinium* in low numbers side by side with a major clade (Correa et al. 2009; Mieog et al. 2007). These corals may therefore have the potential for symbiont shuffling. In any case, swapping of *Symbiodinium* clades seems to be one strategy for corals to survive environmental stress. In this study, we observed a trend of selective retention of clade D and release of clade C in *Poc. eydouxi* colonies. This might be interpreted as an indicator of ongoing symbiont shifting.

Acknowledgments This work was supported by a Grant-in-Aid (No. 21310011) for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan to KK. The authors express their sincere thanks to Akiko Yura, Tomo Matsuoka, and Yohei Takaya, Faculty of Applied Biological Science, Hiroshima University, for their support during this investigation. Thanks are also given to Dr. Lawrence M. Liao, Hiroshima University, for editing the manuscript.

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