A quantitative real-time PCR method for monitoring *Symbiodinium* in the water column

K. Koike¹,*  H. Yamashita¹,  A. Oh-Uchi¹, M. Tamaki¹, T. Hayashibara¹

¹School of Fisheries Sciences, Kitasato University, Sanriku, Ofunato, Iwate 022-0101, Japan
²Ishigaki Tropical Station, Seikai National Fisheries Research Institute, Fisheries Research Agency, Fukai-ôhta, Ishigaki, Okinawa 907-0451, Japan
*Present Address: K. Koike Graduate School of Biosphere Science, Hiroshima University, Kagamiyama, Higashi-Hiroshima, Hiroshima 739-8528, Japan

Abstract: A simple and rapid quantification system for zooxanthellae released from corals or existing in environmental waters was developed. Real-time PCR with a primer set designed to amplify the nuclear 18S rRNA gene of any genetic clade of *Symbiodinium* was used with intercalating dye SYBR® Green I to enable the low-cost, simple assay of *Symbiodinium* density in surrounding waters. With the combination of a simple DNA extraction method developed recently by another group that traps cells onto a filter, the utility of the qPCR system was tested by investigating both the diel release pattern of *Symbiodinium* from *Acropora digitifera* in an aquarium and the occurrence of cells in field waters. Results showed that the system was fast and could accurately monitor *Symbiodinium* densities released from coral, and could possibly be applied to the *Symbiodinium* occurring in the field water—an outcome that could never be matched by conventional microscopic counts.

Key words: Quantitative real-time PCR, qPCR, *Symbiodinium*, coral bleaching, *Acropora digitifera*

*Corresponding author: kazkoike@hiroshima-u.ac.jp

INTRODUCTION

Symbioses between zooxanthellae, as represented by the dinoflagellate genus *Symbiodinium*, and marine invertebrates including corals, are common in shallow marine environments. *Symbiodinium* contributes to host nutrition by translocating photosynthetic products and enabling the host to thrive in oligotrophic conditions (Muscatine and Porter 1977). “Coral bleaching” that results in the mass mortality of coral has occurred in tropical and subtropical seas with increased frequency over the past two decades (Hoegh-Guldberg 1999). The phenomenon has been well documented for various corals under a variety of field conditions, and the relevant environmental factors, mostly involving an increase in water temperature, are now understood (e.g., Podesta and Glynn 2001). In a strict sense, the term “coral bleaching” refers to the loss of zooxanthellae and/or loss of zooxanthellal photosynthetic pigments (Brown 1997). Among such cases, the decline of *Symbiodinium* cell numbers, or more specifically, the release of *Symbiodinium* cells from coral, is most likely a case of coral bleaching. Therefore a simple and rapid quantification method to monitor *Symbiodinium* release from coral would be of interest to researchers who are designing aquarium experiments with a view to investigating environmental factors
related to coral bleaching.

Moreover, the abundance or even the occurrence of free-living *Symbiodinium* in the environment is matter of debate. Many coral species are known to spawn aposymbiotic gametes, and *Symbiodinium* are normally horizontally acquired from the surrounding environment (Richmond and Hunter 1990). In addition to acquisition of *Symbiodinium* during corals’ ontogenic stages, the recruitment of exogenous *Symbiodinium* from the environmental pool following a bleaching event has now been well-studied as an important aspect of the coral recovery system (Lewis and Coffroth 2004). However, little is known about free-living *Symbiodinium* (Rowan 1998; Coffroth and Santos 2005). As the cells of *Symbiodinium* are small (nearly 10 μm in cellular diameter) and somewhat indistinguishable from other sediment particles or plankters, conventional microscopic counts are inappropriate for a quantitative analysis. As an alternative, fluorescent *in situ* hybridization (FISH) using specific genetic probes bound to the *Symbiodinium* DNA locus or rRNA has been employed (Yokouchi et al. 2003). However, this method requires many hybridization steps and may not be of general interest to coral researchers, whose needs may require (i) the absence of labor-intensive microscopic work, and (ii) a simple method that can be used to process numerous samples. Moreover, genetic clade identification in *Symbiodinium* would not always be of interest in the early stage of coral-bleaching studies, and we believe development of an easy-method suitable to detect whole *Symbiodinium* biomass would encourage much output of elemental and valuable data in the studies of *Symbiodinium* release from corals or the free-living cells.

To address this, the application of a quantitative real-time PCR (qPCR) methodology for clade-universal *Symbiodinium* enumeration was developed. Quantification by qPCR relies on detection of the increase in fluorescence from exponentially amplified DNA by a polymerase-chain reaction (PCR) involving a primer set and/or a fluorochrome-labeled probe designed to bind to the desired DNA locus. The qPCR-based quantification provides a highly sensitive and specific assay for the identification of target organisms. This method has proven indispensable in molecular biological research and investigations involving hazardous microbe quantifications, and is increasingly being applied to marine microbiological studies such as the detection of harmful microalgal blooms (Bowers et al. 2000; Moorthi et al. 2006). A pioneer study involving a qPCR application for *Symbiodinium* quantification is a noteworthy report by Ulstrup and van Oppen (2003), in which the relative abundance of specific genetic clades (C and D) of *Symbiodinium* were obtained for multiple coral specimens. Our study aims to develop a low-cost system that can be utilized in a rapid and simple manner by coral researchers who want to record the occurrence of any genetic clade of *Symbiodinium* in a water column, or enumerate release from coral in an aquarium.

**MATERIALS AND METHODS**

**Designing the PCR primer**

Intercalator chemistry employing SYBR Green fluorochrome for qPCR detection was chosen in this study. PCR primer sequences were designed with reference to nucleotide sequences of nuclear 18S rRNA gene (18S rDNA) obtained from GenBank for 11 taxa of *Symbiodinium* (note that all of these taxa are nested within a lineage containing clades A ~ D based on 18S rDNA) and 22 taxa of other dinoflagellates, and two apicomplexan parasites (GenBank accession numbers are listed in Table 1). The derived sequences were aligned by Clustal W (Thompson et al. 1994) and manually edited by eye. A specific region was found that was universal to all *Symbiodinium* clades but allowed for the elimination of other dinoflagellate or apicomplexan taxa, and then the primer set was designed with Primer Express® v2.0 software (Applied Biosystems) to amplify 106 or 107 bp of the region. The nucleotide sequence of both primers were as follows: 5'-ATCTGGGCTGCCCTTGAAT-3' (Sym 18S-s1F, Tm=58) and 5'-CGCTTGCTTTGAAACACCTCTAATTTC-3' (Sym 18S-s1R, Tm=59.5). Matches with known sequences within the GenBank database were investigated using BLAST.

**PCR specificity to *Symbiodinium* on the qPCR**

qPCR using the aforementioned primers was performed against a clade A strain of *Symbiodinium* (CS-161; purchased from the Commonwealth Scientific & Industrial Research Organization), a chlorophyte *Chlamydomonas reinhardtii* (UTEX90; purchased from The Culture Collection of Algae, University of Texas), a cryptophyte *Protoecononas sulcata* (CCMP765; purchased from The Provasoli-Guillard National Center for Culture of Marine Phytoplankton), a haptophyte *Isochrysis galbana* (MBIC10554,
Table 1. Genbank accession numbers of nuclear small-subunit rRNA gene (SSU rDNA) used to design the *Symbiodinium*-specific PCR primer set.

<table>
<thead>
<tr>
<th>Species</th>
<th>Genbank accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dinoflagellates</strong></td>
<td></td>
</tr>
<tr>
<td>Akashiwo sanguinea</td>
<td>U41085</td>
</tr>
<tr>
<td>Alexandrium tamarense</td>
<td>AF022191</td>
</tr>
<tr>
<td>Amphidinium sp.</td>
<td>AF069516</td>
</tr>
<tr>
<td>Amphidinium carterae</td>
<td>AF099217</td>
</tr>
<tr>
<td>Ceratium tenue</td>
<td>AF022192</td>
</tr>
<tr>
<td>Dinophysis fortii</td>
<td>AB073118</td>
</tr>
<tr>
<td>Gonyaulax spinifera</td>
<td>AF022155</td>
</tr>
<tr>
<td>Gymnodinium catenatum</td>
<td>AF022193</td>
</tr>
<tr>
<td>Gym. fuscum</td>
<td>AF022194</td>
</tr>
<tr>
<td>Gyrodinium helveticum</td>
<td>AB120004</td>
</tr>
<tr>
<td>Gyr. impudicum</td>
<td>AF022197</td>
</tr>
<tr>
<td>Gyr. instriatum</td>
<td>DQ847433</td>
</tr>
<tr>
<td>Gyr. spirale</td>
<td>AB120001</td>
</tr>
<tr>
<td>Heterocapsa triqueta</td>
<td>AF022198</td>
</tr>
<tr>
<td>Karenia brevis</td>
<td>AF172714</td>
</tr>
<tr>
<td>K. mikimotoi</td>
<td>AF022195</td>
</tr>
<tr>
<td>Karlodinium micrum</td>
<td>AF172712</td>
</tr>
<tr>
<td>Noctiluca scintillans</td>
<td>AF022200</td>
</tr>
<tr>
<td>Peridinium balticum</td>
<td>AF231803</td>
</tr>
<tr>
<td>Pfiesteria piscicida</td>
<td>AF077055</td>
</tr>
<tr>
<td>Prorocentrum concavum</td>
<td>Y16237</td>
</tr>
<tr>
<td>Pyrocystis noctiluca</td>
<td>AF022156</td>
</tr>
<tr>
<td>Symbiodinium sp. (strain not specified)(^a)</td>
<td>M88509</td>
</tr>
<tr>
<td>Symbiodinium sp. (strain PSP1-05)(^b)</td>
<td>AB016578</td>
</tr>
<tr>
<td>Symbiodinium sp. (strain CS-156)(^b)</td>
<td>AB016594</td>
</tr>
<tr>
<td>Symbiodinium sp. (strain CS-163)(^b)</td>
<td>AB016581</td>
</tr>
<tr>
<td>Symbiodinium sp. (cloned DNA from wild specimens)(^b)</td>
<td>AB016539</td>
</tr>
<tr>
<td>Symbiodinium sp. (cloned DNA from wild specimens)(^b)</td>
<td>AB016580</td>
</tr>
<tr>
<td>Symbiodinium sp. (strain JCUCS-1)(^b)</td>
<td>AB016722</td>
</tr>
<tr>
<td>Symbiodinium sp. (strain JCUSG-1)(^b)</td>
<td>AB016723</td>
</tr>
<tr>
<td>Symbiodinium sp. (strain JCUZ-1)(^b)</td>
<td>AB016724</td>
</tr>
<tr>
<td>Symbiodinium sp. (strain PHSC HH1B)(^b)</td>
<td>AB016596</td>
</tr>
<tr>
<td>Symbiodinium corculorum (strain 350)(^b)</td>
<td>L13717</td>
</tr>
<tr>
<td><strong>Apicomplexian parasites</strong></td>
<td></td>
</tr>
<tr>
<td>Perkinus sp.</td>
<td>L07375</td>
</tr>
<tr>
<td>Toxoplasma gondii</td>
<td>X65508</td>
</tr>
</tbody>
</table>

\(^a\)Data obtained by Rowan and Powers (1992)
\(^b\)Data obtained by Carlos et al. (1999)
\(^c\)Data obtained by McNally et al. (1994)

purchased from the Marine Biotechnology Institute Culture Collection, a diatom *Asterionella* sp. (*Asterionella* spL), and the five dinoflagellates *Karenia mikimotoi* (NIES680, purchased from the National Institute for Environmental Studies), *Prorocentrum micans* (ProroA4), *Prorocentrum lima* (BM-U2-D4), *Protoceratium reticulatum* (990615-A1) and *Alexandrium catenella* (OFAC9982-101). Samples comprising 10 mL of exponentially grown cultures were harvested by centrifugation (2,000 x g, 10 min), and the resultant cell pellets subjected to total DNA extraction using a SepaGene\(^\circledast\) kit (Sanko Junyaku, Tokyo, Japan). The extracted DNA was vacuum-dried,
dissolved in 100 μL of TE buffer, quantified by measuring the absorbance at 260/280 nm (GeneQuant Pro, GE Healthcare, Buckinghamshire, England), and then used for qPCR. Aliquots of 200 pg DNA, 0.5 μL of forward and reverse primers (10 μM), and the recommended volume of SYBR® Premix Ex Taq™ (TaKaRa Bio, Shiga, Japan) were mixed and analyzed in an ABI PRISM® 7000 (Applied Biosystems) under the following thermal-cycling protocol: 1 cycle at 95°C (10 sec) and 40 cycles at 95°C (5 sec) and 60°C (31 sec). For a passive reference, ROX™ reference dye enclosed in the kit was used with the recommended volume. Following completion of the cycle, melting profiles of the PCR products vs. temperature (dissociation curve) were obtained. Non-template controls (NTC) were prepared by mixing with an equal volume of H₂O in lieu of a DNA template. Repeated analyses (more than five times) were performed in an effort to check the variation among experimental batches.

qPCR against several Symbiodinium clades

Symbiodinium culture strains CS-161 (clade A), CCMP1633 (clade B), Sin (clade C), a strain originally isolated by Dr. S. R. Santos, Auburn University, and provided to us from Dr. M. A. Coffroth, State University of New York at Buffalo), CCMP2556 (clade D), CCMP421 (clade E) and CS-156 (clade F) were used for the analysis. Note that all of these strains are within a lineage containing clades A ~ D based on 18S rDNA, although CCMP 421 and CS-156 have been classified into the more diverse clades of E and F based on chloroplast 23S rDNA (Santos et al. 2002) or 5.8S-ITS rDNA (Lajeunesse 2001). Hereafter, the classification of clades A ~ F is adopted in this report. DNA was obtained by the aforementioned method, and accurately quantified by a fluorescence method using SYBR® Green I (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Each 500 pg of total DNA (in triplicate) was subjected to qPCR in the same way.

Quantitative collection and extraction of Symbiodinium DNA

Since the goal of this study was to collect Symbiodinium cells quantitatively from ambient waters and then subject numerous samples to qPCR analysis, a simple DNA extraction method was chosen that involved a boiling method for DNA extraction from filter-collected cells, rather than using conventional DNA extraction protocols. Briefly, cells were collected onto a polycarbonate filter by vacuum filtration, and then the filter with collected cells was boiled in TE buffer to extract the DNA. The original method of extracting DNA from filters was first introduced by Fuhrman et al. (1988), and has been modified several times (e.g., Riemann and Winding 2001). Recently, a group from Kyoto University developed a simple method of collecting dinoflagellate cells onto a polycarbonate filter and performing an extraction with TE in boiling water (Shiroishi et al. unpublised). Cell numbers in exponentially grown CS-161 and Sin cultures were counted under a light microscope, and 250,000, 25,000, 2,500 and 250 cells were filtered by vacuum onto polycarbonate filters (0.2-μm pore size, 47-mm ø, K020A047A, Advantec-Toyo, Tokyo, Japan). 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 of TE buffer in a 1.5-mL micro-tube. The tubes were then placed in boiling water with intermittent vortexing (for 30 sec, 3-min interval) for a total of 10 min. Aliquots of 2 μL (equivalent to 1,000, 100, 10 and 1 cell) were retrieved from the DNA extracts in TE, and subjected to qPCR (in triplicate) according to the aforementioned protocol.

qPCR trial to monitor Symbiodinium release from coral

The experiment was conducted in the Ishigaki Tropical Station of the Seikai National Fisheries Research Institute (24° 27' 16" N, 124° 13' 25" E). Two colonies (a total of 210 g of skeletal weight) of scleractinian coral Acropora digitifera were collected from the reef, and kept for four weeks in an outdoor FRP tank. Prior to the experiment, colonies were transferred to an indoor 10-l aquarium and acclimatized for 40 h. Ultra-filtered seawater obtained through a MEMCOR Ultra-filtration unit (0.2-μm pore size membrane module; JFE Engineering, Tokyo, Japan) was supplied with an overflow rate of 1 l min⁻¹. The water temperature was 27.3±28.1 °C and the aerated aquarium was positioned beside a north-side window (Fig. 1a: 6:54 sunrise - 19:30 sunset at the day of experiment in June 7, 2006). Light intensities measured in the aquarium water are indicated in Fig. 1c. Water samples were collected according to the time schedule shown in Fig. 1b. Briefly, the water flow was stopped during the periods 08:40 to 09:40, 12:40 to 13:40, 16:40 to 17:40, 20:40 to 21:40, 04:40 to 05:40, and again from
08:40 to 09:40: aquarium water samples were collected at the beginning and end of these periods. Thus obtained seawater samples were first sieved through mesh possessing a pore size of 20 μm to remove particles larger than a *Symbiodinium* cell, and then 1 l of each sample was filtered using the polycarbonate filter, and analyzed by the method described above. Additionally, PCR inhibition was initially checked by the addition of internal controls to each sample: DNA standard solutions (equivalent to 100 and 10 cells of CS-161) were added to extracted samples (internal controls) and signal increases, which could be equivalent to the optional DNA additions, were checked. After confirming that PCR inhibition did not occur, triplicate analyses were again performed for each sample. For an external qPCR standard, quantitative DNA obtained from the aforementioned method (equivalent to 1,000, 100, 10 and 1 cell: triplicate) from vacuum filtered *Symbiodinium* strain ‘Sin (clade C)’ was used since the coral was revealed to predominantly harbor clade C *Symbiodinium*, by RFLP analysis according to the method of Rowan and Powers (1991). The amount of *Symbiodinium* released hourly from the coral was estimated by subtracting the result obtained at the beginning from that obtained at the end of above water-stop period.

**qPCR trial to field-collected waters**

Seawater samples were collected (12 June 2006) from Ulusoko Bay (c.a. 2 km offshore; 24°
28° 23′ N, 124° 12′ 24″ E) at the surface and bottom (5 m) of the coral reef slope of Tomino Reef (24° 27′ 38″ N, 124° 12′ 23″ E) off Ishigaki Island, Okinawa, Japan. Collection times were between 09:55 and 10:05. Collected water samples were subjected to sieving and filtering, and then to qPCR analysis as described above. Also, PCR inhibition was initially checked by adding internal control as described above. After confirming that PCR inhibition did not occur, triplicate analyses were again performed for each sample. For an external qPCR standard, quantitative DNA obtained from the aforementioned method (equivalent to 1,000, 100, 10, and 1 cell; triplicate) from vacuum filtered CS-161 was used since this strain is known to produce an intermediate signal:DNA ratio among the tested strains.

RESULTS

PCR specificity for Symbiodinium with qPCR

Analysis of DNA extracted from Symbiodinium strain (CS-161) revealed that delta Rn values (fluorescence normalized with the ROX™ passive reference dye and with base-line fluorescence substraction) from SYBR Green intercalated into double-strand DNA increased exponentially at the early stage of the PCR cycle, while those on non-Symbiodinium microalgae and NTC (Non-template controls) only increased after 30 cycles, indicating that the designed primers specifically amplified Symbiodinium 18S rDNA (Fig. 2). A BLAST search revealed that the forward primer did not match any known sequences within the GenBank database. However, the reverse primer partially matched sequences from dinoflagellate species belonging to Gymnodiniales, including Gyrodinium instriatum (GenBank acc. no. AY443015), G. simplex (DQ888466), G. uncatenatum (DQ888457) and G. dorsum (AF274261). This is not surprising given that Symbiodinium had once been classified into this class, although it is now placed within the Suessiales group. In order to eliminate the possibility that the primers unexpectedly amplified these dinoflagellate genes, qPCR analysis against gymnodinioid cells, in addition to those of Karenia mikimotoi in the previous experiment, was then performed. Since cultures of the aforementioned Gyrodinium species were not available, cells of Akashiwo sanguineus, Gymnodinium abbreviatum and Gyrodinium spirale were collected from the field (field information previously described in Koike et al. 2001) and 10 cells from each species were directly applied to qPCR wells and analyzed. No positive fluorescence increases were obtained in these cases (data not shown), suggesting that the pair of primers worked well only against Symbiodinium DNA.

qPCR against several Symbiodinium clades

Delta Rn values increased exponentially for all tested Symbiodinium clades (A to F), and were significantly different from values measured for non-template controls (NTC; Fig. 3).

![Fig. 2. Delta Rn (°SYBR® Green I fluorescence normalized with the ROX™ passive reference dye with base-line fluorescence substraction) curves versus PCR cycle in qPCR against extracted DNA (200 μg) from a Symbiodinium strain (CS-161; clade A) and various microalgae. NTC represents a non-template control, in which H2O was used in lieu of a DNA template.](image-url)
This clearly showed that the primers amplified all *Symbiodinium* clades, as expected: however, the start of amplification varied significantly between strains, regardless of the equal concentration of total DNA loaded. This might be attributed to the copy number of rDNA among the clade, although this has not been verified to date. An *in situ* application of this method must therefore express quantification results on the basis of the specific culture strain used as an external standard.

In the dissociation curves (Fig. 4), where the amplified product could be briefly identified based on the melting temperature (*Tm*), the clades were categorized into three groups: (i) *Tm* = 80 (clade A), (ii) *Tm* = 84 (clade E) and (iii) *Tm* = 81–82 (clades B, C, D and F).

**Fig. 3.** Delta Rn (â€œSYBR® Green I fluorescence normalized with the ROX™ passive reference dye with base-line fluorescence subtracted) curves versus PCR cycle in qPCR against extracted DNA (600 pg) from various *Symbiodinium* strains. Note that all of these strains are within a lineage containing clades A ~ D based on 18S rDNA, although CCMP 421 and CS-156 have been classified into clades E and F using the more diverse loci of chloroplast 23 S rDNA (Santos *et al.*, 2002) or 5.8S-ITS rDNA (Ladenmeesse 2001). NTC represents a non-template control, in which H2O was used in lieu of a DNA template.

**Fig. 4.** Dissociation curves obtained following the qPCR shown in Fig. 3. Each curve shows the derivative of the amplicon during the temperature increase.
Quantification for filter-trapped *Symbiodinium*

Delta Rn values began to increase exponentially according to the applied DNA content, equivalent to 1,000, 100, 10 and 1 cell for vacuum filtered CS-161 and Sin (data not shown). The results of delta-Rn vs. PCR cycle allowed a threshold line to be set in the region associated with an exponential increase in delta Rn, and corresponding PCR cycles (Ct value) were determined. When plotting Ct values against cell number (Fig. 5) for CS-161 and Sin, linear regression curves were obtained between logarithmic cell number against the corresponding Ct value, indicating that the quantitative relation of qPCR results vs. vacuum filtered and boiled extracted *Symbiodinium* cells was satisfied. The regression between cell number and Ct values showed a tight correlation \( R^2 = 0.986 \) for CS-161, and 0.983 for Sin) up to 1 cell at a minimum, although results for this number varied and depended on the experimental batch. It could therefore be concluded that the detection limit of this method was > 10 cells per reaction.

**qPCR trial to monitor *Symbiodinium* release from coral**

The aquarium experiment revealed that *A. digitifera* released significant numbers of *Symbiodinium* into the ambient water (Fig. 6). The release seemed to follow a diel pattern: from morning to early afternoon (08:40 to 13:40), two coral colonies released ca. 1.4 ~ 2.2 x 10^6 cells of *Symbiodinium* (equivalent to the external standard Sin) per hour; the release remained at a low level (ca. 0.4 ~ 1.3 x 10^5 cells h^-1) from early evening to early morning (16:40 to 05:40), and rose to a higher level (1.5 x 10^5 cells h^-1) the following morning (08:40 to 9:40).

**qPCR trial for field-collected waters**

Figure 7 shows the occurrence of free-living *Symbiodinium* cells (clade compositions were not known, and the cell numbers were expressed as

![Graph](image.png)

*Fig. 5. Linear regression curves of Ct values (PCR cycle at which a certain delta Rn value was obtained) versus logarithmic cell numbers, accommodated in a PCR well. The microscopically counted cells were initially collected on polycarbonate filters following vacuum filtration, and then boiled in 500 μl of TE buffer for 10 min. Aliquots of 2 μl (equivalent to 1,000, 100, 10 and 1 cell) were then applied to qPCR. Data are shown as averages (square) and standard deviations (bars) for triplicate measurements. a CS-161 (clade A), and b Sin (clade C).*
Fig. 6. qPCR-determined *Symbiodinium* release from scleractinian coral *Acropora digitifera*. Two colonies of the coral (total 210 g) were kept in an indoor 10-l aquarium (under natural light) with an overflow of ultra-filtered seawater at a rate of 1 l min⁻¹ (see also Fig. 1a). The water temperature was 27.3–28.1°C. Water samples were collected at the beginning and end of intermittent water-stop periods (Fig. 1b), and the amount of *Symbiodinium* released hourly from the corals was estimated by subtracting the qPCR result (average of triplicate measurements) obtained at the beginning from that obtained at the end of water-stop. *Symbiodinium* strain Sin (clade C) was used as the external standard since the corals have been shown to predominantly harbor clade C, by RFLP analysis according to the method of Rowan and Powers (1991).

Fig. 7. qPCR-determined cell density of free-living *Symbiodinium* cells in field waters collected from Urasoko Bay (ca. 2 km offshore; 24° 28’ 23” N, 124° 12’ 24” E) at the surface and bottom (5 m) of the coral reef slope of Tomino Reef off Ishigaki Island (24° 27’ 38” N, 124° 12’ 23” E), Okinawa, Japan. Cell numbers are expressed as equivalents to the external standard CS-161. Data represent averages and standard deviations (bars) of triplicate measurements.
the external standard CS-161; therefore, the densities might not adequately express true biomass but relative occurrences) in field waters around Ishigaki Island. As expected, the occurrence of *Symbiodinium* sp. in offshore waters (c.a. 9 x 10^5 cells 1^-1^) was much lower than that of the coral reef. Furthermore, they were more abundant in the surface layer of reef waters (c.a. 5 x 10^5 cells 1^-1^) in comparison to the bottom layer (c.a. 3 x 10^4 cells 1^-1^). The dissociation curves of these samples led to estimated Tm values of 81.5, indicating that these *Symbiodinium* cells were probably not members of clade A or E.

**DISCUSSION**

Since the goal of this investigation was to develop an easy and reliable system for use in a variety of coral studies, the intercalator method using SYBR® Green I was chosen in preference to the probe detection method of TaqMan® probe. Roughly speaking, the intercalator method is less specific than the probe method since the signal is dependent on the specificity of the primer, while the signal associated with the probe method can be dependent on both the primers and probe. However, the former method is superior in cost, rapidity and ease to the later one. Most notably, excluding the filtering procedure, the addition of TE, boiling, and the application of DNA extracts and the SYBR® mix (+ primers) to qPCR wells can be conducted within 2 to 3 h, and qPCR analysis can be finished within 2 h if there are less than 30 samples (batch triplicate measurements can therefore be performed in an ordinary 96-well plate).

In this study, delta Rn values increased even with non-template controls, indicating that very minor, but not negligible, primer dimers could be formed and thus produce fluorescence increases. This noise signal might not be expected when utilizing TaqMan® chemistry. However, a negative fluorescence increase began later, even for the 1 cell sample. It was therefore concluded that the system possessed sufficient performance, and when considering that DNA levels equivalent to more than 10 cells per reaction always yielded a significant Ct value, the detection system was valid for > 2,500 cells on a filter (which could represent a DNA level equivalent to 10 cells per reaction, when boiled in 500 µL of TE and 2 µL of the portion was applied to a qPCR tube).

However, most obstinate limitation of the present system is that the quantification may vary among the clade. We initially believed the difference might be come from length and/or denaturation properties of the amplified locus among the clade; though, the difference is too large to be thought so. Rather, it might be due to difference of copy number of rDNA among the clade, although this has not been conclusively determined so far. Therefore at the present system, it may not be appropriate to express as “cell number” on the basis of one certain clade as an external standard, when the samples contain multiple clades or a clade being different from the standard. More accurately, expression of “copy number of the target locus” should be more preferable. However in the preliminary application in this study, we still use the “cell number” based on the standards CS-161 or Sin, which can give mean signals among the tested strains (Fig. 3), since we tried to imagine “How many cells released from the corals or existing in the environmental water?” in these first attempts. Nevertheless, accurate quantification based on the known copy number of the DNA, and more preferably, development of PCR primers distinguishable each *Symbiodinium* clade should be further performed.

Even though, it is intriguing that free-living *Symbiodinium* was more abundant in field waters and in much higher density than expected, and that *A. digitifera* seemed to exhibit a diel pattern for *Symbiodinium* release, as previously demonstrated in *Acropora formosa* (Jones 1997). Since the addition of internal control DNA (representing DNA levels equivalent to 10 and 100 cells of CS-161) to these field DNA samples resulted in increased Ct values, the possibility of any PCR inhibition could be eliminated, and the quantification obtained in these trials could be regarded as being accurate as far as on the basis of *Symbiodinium* strain used as external standard. These trials are still very preliminary, though: the method offers a powerful and easy-to-use tool for studies of coral bleaching and symbiont acquisition by corals.

**ACKNOWLEDGEMENTS**

The authors express their sincere thanks to Dr. M. A. Coffroth, State University of New York at Buffalo, and Dr. S. R. Santos, Department of Biological Sciences & Cell and Molecular Biosciences Peak Program, Auburn University, for providing a *Symbiodinium* culture.
REFERENCES


(Received: 14 March 2007/Accepted: 7 June 2007)
環境水中の *Symbiodinium* 細胞をモニターするための定量 PCR 手法

小池一彦 1, 2, 3, 山下 洋 1, 大内 歩 1, 秀邦 均 1, 林原 慎 1, 2

1 北里大学水産学部，〒022-0101 岩手県大船渡市三陸町
2 西海区水産研究所石垣支所，〒907-0451 沖縄県石垣市浮海大田
3 現所属（小池）：広島大学大学院 生物圈科学研究科，〒739-8528 広島県東広島市観山1-4-4；
Tel 082-424-7996；Fax 082-424-7916

定量 PCR (real-time PCR) 手法を応用し、環境水中に存在する褐藻類の定量を可能にするシステムを開発した。全クレードの *Symbiodinium* の核 18S rRNA 遺伝子をターゲットとする PCR プライマーと、SYBR® Green 1 を用いたインターカラーエーター法により、安価で簡単・迅速なアッセイシステムを構築した。フィルター上にトラップした *Symbiodinium* 細胞からの簡便な DNA 抽出方法とのコンピネーションにより、水槽で飼育した *Acropora digitifera* による *Symbiodinium* 細胞排出の日周期性や、天然海水中に出現する *Symbiodinium* 細胞の定量を試みた。*Symbiodinium* は細胞が小さく、形態的な特徴に乏しいために、従来の顕微鏡観察ではその同定・計数がほぼ不可能であったが、上記の定量 PCR 法により、環境水中的 *Symbiodinium* 細胞の出現密度が簡便にモニターでき、また、その応用によって新たな知見が得られることが期待された。