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Development of a protocol for specific detection and quantification of free-living and endosymbiotic *Symbiodinium* communities in coral reefs

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ABSTRACT: This study focused on the evaluation of a novel specific Taqman PCR assay to accurately determine the intercolony cell density of *Symbiodinium* in surrounding seawaters and intracolony cell density in coral tissue, which can be used to constantly monitor health status of the coral in field studies. Our Taqman method using ITS-rDNA-based specific primer–probe sets can differentiate *Symbiodinium* genotypes at the clade level. An additional primer–probe set based on the coral paired-box (*Pax*) gene was used as a universal internal reference to estimate the relative abundance of coral host cells. This assay was highly reproducible and reliable, which allowed accurate quantification of extremely low *Symbiodinium* DNA (up to 2.0 pg) in different coral DNA backgrounds with high specificity and efficiency. The Taqman PCR assay was further successfully applied for the detection and quantification of *Symbiodinium* in complex samples from multiple origins, including free-living individuals and endosymbionts within the coral *Galaxea fascicularis*.

KEY WORDS: Taqman · Coral bleaching · Primer-probe design

INTRODUCTION

Members of the genus *Symbiodinium* are essential photosynthetic endosymbionts in most corals (Lin et al. 2015). Coral bleaching results from climate or environment changes, which cause the loss of the symbiotic *Symbiodinium* (Chen et al. 2005, Rowan 2004). The endosymbiosis is essential for the survival of the host and underpins the productivity and calcification that creates habitat for the immense biodiversity that coral reefs support (Pochon et al. 2010). Meanwhile, it is proposed that the association of corals with their mutualistic *Symbiodinium* acts as a significant opportunity for corals to flexibly adapt to the changing environmental conditions. However, this view has been the subject of much controversy in the field (Manning & Gates 2008, De Palmas et al. 2015).

The term 'free-living' is defined as *Symbiodinium* that has the ability to associate with but lives outside the hosts (Hirose et al. 2008). The importance of the free-living *Symbiodinium* communities is highlighted by the fact that many corals release aposymbiotic larvae that need to acquire the symbiotic complement from environmental pools (Manning & Gates 2008). The genus *Symbiodinium* is highly diverse and is divided into 9 phylogenetically distinct clades (Clades A to I). Five of them, including A–D and F, are known to exist in corals (LaJeunesse et al. 2012). The genetic and/or physiological diversity of *Symbiodinium* may be favorable for corals that adopt a

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horizontal transmission strategy (Takabayashi et al. 2012). Implicit in the adaptive bleaching hypothesis is that free-living pools of *Symbiodinium* are available for corals to select from (Manning & Gates 2008); thus, the free-living *Symbiodinium* have been proposed to be a source for replenishing endosymbiotic communities in coral hosts to recover from bleaching (Takabayashi et al. 2012). Surprisingly, only a few studies have documented the existence of these important communities in coral reef ecosystems (Coffroth et al. 2006, Littman et al. 2008, Manning & Gates 2008). To date, the methods to detect the free-living *Symbiodinium* genus in reef waters are relatively limited.

It is essential to develop a method that can quantitatively detect the dynamic changes of endosymbiotic and free-living *Symbiodinium*, which would be useful for understanding the mechanisms driving coral community change in response to climate change. To satisfy this objective, we developed an improved detection method that allows for the consistent and quantitative detection of *Symbiodinium* diversity at the clade level (Green et al. 2014). In this study, we present a rapid and efficient Taqman PCR method to discriminate *Symbiodinium* based on the nucleotide differences in the ITS-rDNA sequences among Clades A to F in coral tissues and seawater. This assay has been successfully used to explore the diversity of *Symbiodinium* in aquarium water and in coral tissue from the sea area of Hainan, China. Our study will be useful for future work examining the spatial and temporal patterns of coral–*Symbiodinium* symbiosis.

MATERIALS AND METHODS

Coral collection and *Symbiodinium* strains

Seven healthy *Galaxea fascicularis* coral colonies were collected from 7 locations on the east and west coast of Hainan, China, in July 2015 (see Fig. S1 in the Supplement at www.int-res.com/articles/suppl/a080p001_supp.pdf). Three to 5 coral polyps were taken from each colony and frozen in liquid nitrogen for transfer to the laboratory for DNA extraction. The field studies and sample collection for these locations/activities complied with the provisions of the Department of Coral Reef Protection, Hainan Province, China. The field studies did not involve endangered or protected species. Each colony was broken into 6 to 8 fragments using a hammer and chisel, and the coral were acclimated to a common aquarium environment at 27°C with running artificial seawater (salinity: 34) under fluorescent lights (approximately 25 to 40 µE m^{-2} s^{-1}) on a 12 h light:12 h dark regime. Following a period of 6 mo acclimation, 4 small healthy and bleaching *G. fascicularis* colonies from different sites were selected from the aquarium and used in this study. Artificial seawater from the aquarium was also used to quantify the free-living *Symbiodinium* population.

Four strains representing 3 clades of *Symbiodinium* were assayed for comparisons in this study. Two Clade A strains from *Symbiodinium* sp., including ITO 10 and WZD 35, were isolated from a coral reef in Beihai, Guangxi, China, in 2014. Clade C of *Symbiodinium* sp. strain WZD 17 was isolated from a coral reef in Sanya, Hainan, China, in 2014. Clade F strain (Symka) of *S. kawagutii* was originally isolated from a Hawaiian reef ecosystem. These strains were cultured in f/2 medium at 25°C with a regime of 14 h light:10 h dark at 100 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR). In addition, the DNA extraction of 4 *Symbiodinium* strains SSG (Clade C), XMH (Clade F), SGA1 and SGC1 (Clade D) were included in this study to augment the Taqman PCR analysis.

Culture test for free-living *Symbiodinium*

The artificial seawater from the aquarium (~0.5 ml volume) was collected in plastic Petri dishes (diameter = 9 cm) and mixed with an equal volume of f/2 medium (containing 40 mg l^{-1} of ampicillin and 30 mg l^{-1} of streptomycin). The mixture was inoculated into a 96-well plate and grown under cool-white fluorescent light (100 µmol m^{-2} s^{-1}) with a 14 h light:10 dark regime at 25°C. Microscopic examinations were carried out after 7 d of cell growth on plates (Fig. S2A in the Supplement). Individual *Symbiodinium*-like swimming dinoflagellate cells were randomly picked up by micropipettes from the plates. The *Symbiodinium*-like strains were subcultured in f/2 liquid medium under the same conditions (Hirose et al. 2008).

Genomic DNA extraction

Fig. S2B in the Supplement shows the newly developed standard protocol for DNA extraction from coral colony, *Symbiodinium* culture and seawater sample. Components of the TIANamp Marine Animals Genomic DNA Kit (Tiangen Biotech, Beijing) were used in multiple steps of the protocol. Briefly, for a coral

colony, corallites (~5 mm^3, 0.1 to 0.2 g of tissue from verrucae and surrounding corallites, including entire polyps) frozen in liquid nitrogen were thawed in 500 µl of lysis GA buffer. For *Symbiodinium* culture, algal cells were harvested by centrifugation at 800 to 1000 × *g* for 5 min. The 500 ml seawater samples for DNA analysis were filtered on a 0.45 µm pore-size polyester filter (Meck Milliporec). The algal material or the filters were placed into 2.0 ml Eppendorf tubes with 500 µl lysis GA buffer. Then, 50 µl Proteinase K (20 mg ml^{-1}) was added to the solutions and vortexed to release DNA, then incubated at 56°C for 1 h to hydrolyze protein and improve DNA extraction. Thereafter, 500 µl of GB buffer was slowly added to the extraction mixture, and the mixture was incubated at 70°C for 10 min to lyse cells completely. Then, the protein precipitate solution was separated by centrifugation, and pellets were discarded. The supernatant containing DNA was mixed with 500 µl of cold isopropanol for 5 min to precipitate DNA. Each pooled DNA sample was further cleaned by CB3 mini spin-column purification. GD and PW buffers, 0.5 ml each, were used to wash the column. Finally, DNA was eluted in 100 µl of Buffer TE.

The quantities of DNA samples were estimated by measuring absorption at 260 nm wavelength with a NanoDrop 1000 spectrophotometer (Thermo Scientific), and purity was evaluated by examining ratios of 260:280 nm and 260:230 nm.

PCR analysis of *Pax* and ITS genes

Pax transcription-factor genes play key roles in animal embryonic development, are conserved in structure and ubiquitously present among Metazoa (Zhang & Emmons 1995), and do not exist in unicellular organisms such as *Symbiodinium*. The exon region of the *Pax* paired-box domain shows high similarity in cnidarians (Catmull et al. 1998, Miller et al. 2000); therefore, we used coral *Pax* genes available in GenBank to design PCR primers. All available *Pax* sequences for cnidarians were downloaded from NCBI GenBank and expressed sequence tag (EST) libraries of *Acropora digitifera* and *Nematostella vectensis*. Initial primers (Table 1) were designed to amplify the region containing the possible introns and exons for coral *Pax* genes.

Table 1. Primers and probes used in this study for development of the *Symbiodinium* quantification

Target	Primer or probe name	Orientation	Sequence (5′–3′)
End-point PCR			
Coral: Pax D gene nucleotide	PaxD-2F	Forward	ACG AGC TCT GCG GTT TG
sequence (product size ca. 2350 bp)	PaxD-R	Reverse	TAA ACC AGC TTG GTG GAG T
Coral: Pax D exon	PaxD-extF	Forward	AGG AAC CTT TGT AAT TTC TTG TTC C
(product size ca. 400 bp)	PaxD-extR	Reverse	TGG CAC GTT TAG GTG TGA G
Coral: Pax D intron	PaxD-intF	Forward	GGG CGT ACC TCA TAT GTG
(product size ca. 700 bp)	PaxD-intR	Reverse	GCA CTT TTG TTT GTA GTA TTG
Taqman PCR			
Coral internal reference *Pax*			
(product size 99 bp)	Coral Pax-F	Forward	TGT GAG AGC AAG YGA TAT YAG
	Coral Pax-P	Probe	ATC TTG CTC ACR CAD CCA TGA GAA A
	Coral Pax-R	Reverse	ACC AGG YTC RAT WGA GCC
Symbiodinium Clade A-specific	Clade A-2F	Forward	CAA TAG TGG AAG GTC CAA AAG G
(product size 100 bp)	Clade A-2P	Probe	CCA GAA CAT ACA CTC TGG GTG CAG C
	Clade A-2R	Reverse	CAA GTG GAA GCT ATG GGC GAG TG
Symbiodinium Clade B-specific	Clade B-2F	Forward	CAT TAT CTA CCT GTG CTT GCT TG
(product size 137 bp)	Clade B-P	Probe	TGT TGG AAA GCA AAG CAG CAG TGT
	Clade B-2R	Reverse	AAC AAC AGT ACG CTC AAG CTT
Symbiodinium Clade C-specific	Clade C-F	Forward	CAC ACT CAC CAA CCC TTG T
(product size 90 bp)	Clade C-P	Probe	CTG AAC ACG GAC CCA TGG CCA
	Clade C-R	Reverse	AGC AGC TTA GCC TTC ATT CC
Symbiodinium Clade D-specific	Clade D-F	Forward	GGC GTT TGC TAT CGG GTA T
(product size 104 bp)	Clade D-P	Probe	AAA CAA CAA CGC ACA AGC AAC GGC
	Clade D-R	Reverse	ACA GAT TGG ATC ATC AGT CTA AGG
Symbiodinium Clade F-specific	Clade F-F	Forward	GCC CAA TCT TGC GGA TAG A
(product size 100 bp)	Clade F-P	Probe	TTT CAA TGG CTC ACA GGG GCG C
	Clade F-R	Reverse	CGT CAC TCA AGA AAT ACC ATT T

The sequence of the ribosomal internal transcribed spacer (ITS-rDNA) is highly conserved but sufficiently variable among the *Symbiodinium* clades (Stat et al. 2011) and could be used to identify different clades of *Symbiodinium*. ITS-rDNA of *Symbiodinium* was amplified using primers designed from conserved fungal rDNA sequences ITS1 (TCC GTA GGT GAA CCT GCG G) and ITS4 (TCC TCC GCT TAT TGA TAT GC) (White et al. 1990).

Each amplification reaction included 25 µl of Dream Taq Green PCR Master MIX (Thermo Fisher Science), 5 µl (10 mM) of each primer, 1 µl of sample DNA template and 14 µl of double distilled H_2O to a final volume of 50 µl. The amplification program was as follows: an initial denaturation step of 4 min at 94°C, followed by 35 cycles consisting of 30 s at 94°C, 45 s at 60°C, and 1 min at 72°C, and a final elongation step of 10 min at 72°C. The amplified products of *Pax* and ITS of all samples were sequenced by Sangon, Shanghai (Sangon Biotech).

Specific primer-probe design and PCR program

Degenerate primers and probes for Taqman PCR were made for amplification of internal reference *Pax* gene based upon the coral hosts analyzed here. The available ITS sequences of clades/types-representative *Symbiodinium* were downloaded from GenBank (including Clades A1, A2, A3, A4, A5, B1, B2, B3, C1, C2, C3, C4, D1, D2, E, F and other common species) and the ITS region of rDNA gene sequence from the above identified *Symbiodinium* clades were aligned in DNAMAN v. 7 software (www.lynnon.com). The aligned nucleotide sequences were used to construct phylogenetic trees using the MEGA software package (v. 6.0) (Tamura et al. 2013). Five sets of clade-specific primers were designed to amplify the ITS corresponding to *Symbiodinium* Clades A, B, C, D, and F, respectively. The optimum Taqman probes and primers were designed using IDT Sci Tools® Web Tools (Integrated DNA Technologies) with the following criteria: GC% ≥40–50, a higher melting temperature (T_m) of 60°C, primer length = 18–28 bp with amplicon size ranging from 80 to 150 bp. The T_m for the Taqman probes was set 8°C higher than the T_m for primers (Owczarzy et al. 2008). The Taqman probes were labeled at the 5′-terminal nucleotide with FAM reporter dye and at the 3′-terminal nucleotide with TAMRA.

Assays of Taqman PCR were performed using the RG 6000 (Qiagen). Reactions of Taqman PCR were performed in a 20 µl reaction system consisting of the following reagents at the optimized concentrations: 400 nM each primer, 200 nM each probe, 10 µl AceQ qPCR Probe Master Mix (Vazyme), and 1 µl of template DNA. PCR conditions were 95°C for 5 min, 45 cycles of each 95°C for 5 s, and 60°C for 30 s. Each plate contained at least 2 negative control wells, and each sample contained at least 3 replicates. Results were analyzed using Rotor-Gene®Q software. Raw data were analyzed using the default settings of the software.

Evaluation of specificity and sensitivity of primer-probe

To test whether the internal reference *Pax* primer-probe set was universal across diverse host coral species, we used DNA extracted from corals of *G. fascicularis* and *Seriatopora hystrix* as well as from anemone *Heteractis malu*.

To ensure that the designed primer-probe sets were unique to the '*Symbiodinium* clade', an *in silico* search was performed against available microbial sequences in GenBank with automatically adjusted word size and other parameters for searching short and near exact matches. The specificity of Taqman PCR was evaluated using DNA extracted from *Symbiodinium* Clades A, C, D, and F as well as DNA prepared from a number of related microbes, including marine red tide causative algae (*Chlorella vulgaris*, *Phaeocystis globosa*, and *Chaetoceros* sp.) and coral endophyte fungi and bacteria commonly found in China (*Cladosporium* sp., *Fusarium* sp., *Engyodontium* sp., *Aeromonas trota*, *Shewanella algae*, and *Aeromonas hydrophila*).

A 10-fold serial dilution of extracted DNA from the *Symbiodinium* strains of Clades A, C, D, and F were quantified for evaluation of the sensitivity of Taqman PCR. For DNA extraction from coral tissues, the interval for the serial dilutions was 2^1 or 2^2 depending on the concentration of the target DNA (Bai et al. 2013).

Standard curves and samples assessment

A standard curve was developed using a serial dilution of *Symbiodinium* genomic DNA containing 200 ng to 2 pg DNA. Standard linear regressions ($Y = a + bX$) of the log concentration of the target DNA mass (Y) versus the mean threshold cycle number (Ct) values (X) were obtained. The Taqman PCR was used to detect the *Symbiodinium* from coral samples

(*G. fascicularis*) and seawater samples as mentioned above. Multiplex Taqman PCR for ITS and internal reference *Pax* from each sample were performed simultaneously and the results were compared. If necessary, ANOVA was performed on the means of *C*t values. SPSS Statistics v. R23.0 (IBM) was used for data ANOVA (PROC ANOVA). Treatment means were separated at the 0.05 significance levels by Tukey's test. PROC REG was used for linear regression.

RESULTS

Development of the primer-probe sets

The full-length sequence of coral *Galaxea fascicularis Pax* was amplified by PCR using the initial primers, and the sequence was 2350 bp in length. The produced fragment contained 2 introns and 3 exons. The *Pax* sequence was deposited in GenBank with accession numbers KU359953 to KU359955. A partial *Pax* exon sequence of *G. fascicularis* aligned well with *Acropora digitifera*, *Nematostella vectensis, Anthopleura japonica* and *Amphimeddon queenslandica Pax* genes, although some sequence differences were present (Fig. S3A in the Supplement). To cover universal coral species, the conserved areas of the alignment were used for the design of degenerate primers (Fig. S3B).

The sequences of ITS from *Symbiodinium* strains were amplified by PCR using universal primers ITS1 and ITS4 and submitted to GenBank (see GenBank accession no. in Fig. S3). To accurately identify the *Symbiodinium* strains, a phylogenetic tree for genus *Symbiodinium* was constructed using the maximum likelihood method (Tamura et al. 2013). A sufficient number of clades/type-representative ITS sequences of *Symbiodinium* were retrieved from the Genbank of NCBI, and *S. californium* ITS was used as the reference for the root (Fig. S4 in the Supplement). The 5 *Symbiodinium* clades were distinguished clearly in the phylogenetic tree based on the ITS phylogeny. These findings were largely in congruence with the observations of LaJeunesse (2001). The alignment results showed that positions and sizes (ranging from 580 to 620 bp) of *Symbiodinium* ITS genes differed among the clades, and areas of variation were used to produce *Symbiodinium* clade-specific Taqman PCR primers and probes (Fig. 1 & Fig. S5 in the Supplement). All primer-probe sets developed in this study are shown in Table 1.

Specificity and sensitivity of Taqman PCR

The *in silico* BLAST searches against all available microbial or phytoplankton sequence databases in NCBI did not identify any potentially competing sequences, suggesting that the sequence locus selected for primers and probes are specific to *Symbiodinium*. The specificity was further confirmed by Taqman PCR experiments from *Symbiodinium* strains (Clades A, C, D and F; Table S1 in the Supplement). As expected, the Taqman PCR using the internal reference *Pax* probe-primer combination that detected coral and anemone *pax* genes yielded negative results for all strains of *Symbiodinium* and negative controls. All sources of *Symbiodinium* A, C, D and F were tested positively with A-, C-, D- and F-specific probes, respectively. The *C*t values ranged from 20.08 to 23.79. The other marine algal, fungal and bacterial controls showed negative results using any primer-probe sets, indicating that the sequence locus selected for the *Symbiodinium* detection system was organism-specific. In addition, TaqMan PCR validation of the assay of the internal reference *Pax* primer-probe yielded positive results of coral (*G. fascicularis* and *Seriatopora hystrix*) and anemone (*Heteractis malu*) samples.

To evaluate the sensitivity of primer-probe sets, serial dilutions (~10^{-1} to 10^{-6}) of DNA extracted from the strain ITO 10 (Clade A), strain WZD 17 (Clade C), strain SGA1 (Clade D) and strain Symka (Clade F) were used (Fig. 2). Taqman PCR detected *Symbiodi-*

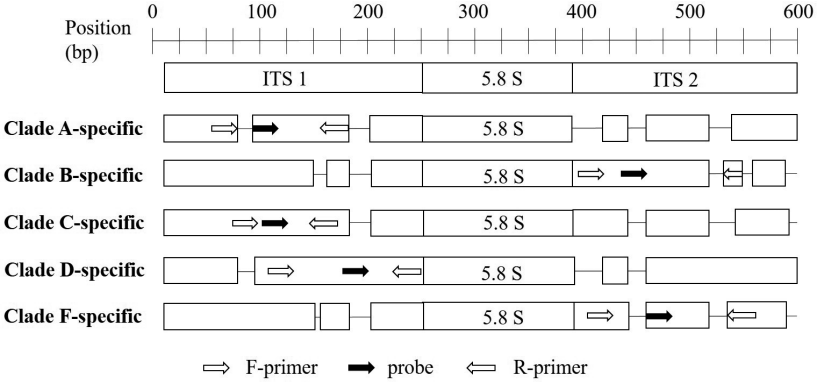

Fig. 1. Overview of the internal transcribed spacer-1 and -2 regions and 5.8S genes of *Symbiodinium* (Clades A to F). Top line gives position in bp from start of the alignment. ▭: present; —: absent. Arrows show the specific primer–probe combinations given in Table 1

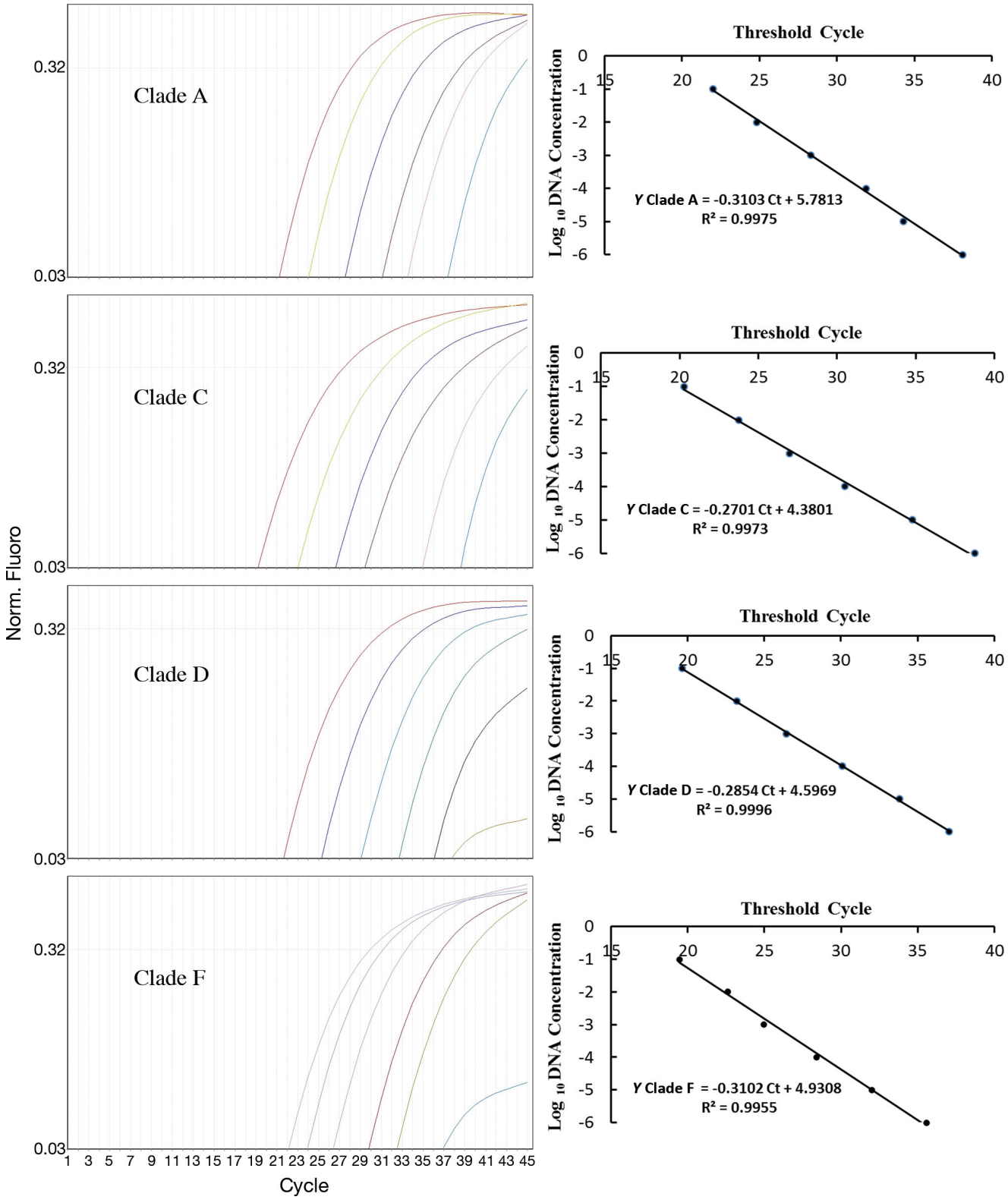

Fig. 2. Sensitivity of the clade-specific Taqman PCR for detection of *Symbiodinium* using serial dilutions of pure *Symbiodinium* DNA extracted from strains of Clade A, C, D and F. Their regression analysis equations were obtained in Taqman PCR with the clade-specific primer–probe sets using serial dilutions of pure *Symbiodinium* DNA. Templates were serial dilutions (~10^{-1} to 10^{-6}) of *Symbiodinium* DNA. *Y*: the log concentration of the target DNA mass; *C*t: threshold cycle number

nium in 1 µl of the 10^{-6} dilution of DNA (2.0 pg, 200 ng $\mu l^{-1} \times 10^{-5} \times 1$ µl). These results showed that the detection limit of Taqman PCR was 2.0 pg genomic DNA input of *Symbiodinium*.

Feasibility of Taqman PCR detection

To investigate the influence of coral DNA or exogenous DNA (i.e. endogenous fungus and bacterium) on the accuracy of amplification and quantification of target DNA, the total DNA (50 ng μl^{-1}) extracted from coral tissue that contain *Symbiodinium* DNA was serially diluted with water. Taqman PCR reactions for each DNA dilution series were performed independently. The mean *C*t values inversely correlated with increasing dilution of the mixed DNA as expected (Table 2). In addition, when multiplex Taqman PCR was applied for internal reference *Pax* and *Symbiodinium* clade-specific ITS-rDNA, we found a proportional serial dilution vs. Δ*C*t (*C*t *Pax* – *C*t clade-specific ITS) change. Therefore, use of the Taqman PCR with total genomic DNA extracted from coral tissue samples demonstrated that background DNA or other substances from coral colony did not affect the results of this assay. Collectively, the efficiency and reproducibility of Taqman PCR assays of coral *Pax* or *Symbiodinium* clade-specific ITS were not influenced by the presence of the background DNA.

Calculation of free-living *Symbiodinium* population

Four standard curves were prepared from *Symbiodinium* (Clades A, C, D and F) DNA samples in this study. For example, a standard curve was developed using a serial dilution of *Symbiodinium* C genomic DNA containing 200 ng to 2 pg of DNA along with a regression analysis. The log concentrations of the target DNA mass (Y) in the serial dilutions of the DNA samples were estimated based on the following standard curve:

$$Y \text{ Clade C} = -0.2701\ C\text{t} + 4.3801 \quad (1)$$

The DNA mass (pg) of *Symbiodinium* in each reaction (well) could be calculated using the following:

$$[\text{DNA mass}] = 2 \times 10^5 \times 10^{4.3801 - 0.2701\ C\text{t}} \quad (2)$$

Table 2. Influence of coral DNA backgrounds from different dilution on multiplex Taqman PCR assays for the relative abundance of *Symbiodinium*. *Symbiodinium* genomic DNA was extracted following the standard procedure showed in Fig. S2 in the Supplement. The pure *Symbiodinium* genomic DNA in a total DNA extract (50 ng μl^{-1}) from coral tissue, then serial dilution by water was used. Correlation between ITS and *Pax*: a linear regression *t*-test was conducted to determine the significance of the correlation

Dilution factor	*C*t value		Δ*C*t	Correlation coefficient between ITS and *Pax*
	Clade A ITS	Coral *Pax*	*C*t *Pax*– *C*t Clade A	$R^2 = 0.996$
$\times 2^0$	20.24 ± 0.32	30.53 ± 0.05	10.27 ± 0.32[a]	
$\times 2^2$	22.41 ± 0.03	32.61 ± 0.31	10.20 ± 0.31	
$\times 2^4$	24.96 ± 0.28	35.30 ± 0.15	10.29 ± 0.16	
$\times 2^6$	27.39 ± 0.18	37.55 ± 0.35	10.15 ± 0.18	
	Clade C ITS	Coral *Pax*	*C*t *Pax*– *C*t Clade C	$R^2 = 0.996$
$\times 2^0$	20.43 ± 0.03	32.52 ± 0.26	12.09 ± 0.23	
$\times 2^2$	22.57 ± 0.14	34.64 ± 0.29	12.07 ± 0.15	
$\times 2^4$	24.91 ± 0.04	37.18 ± 0.15	12.08 ± 0.49	
$\times 2^6$	27.25 ± 0.15	39.52 ± 0.34	11.99 ± 0.18	
	Clade D ITS	Coral *Pax*	*C*t *Pax*– *C*t Clade D	$R^2 = 0.992$
$\times 2^0$	20.31 ± 0.05	31.41 ± 0.57	11.09 ± 0.52	
$\times 2^2$	22.32 ± 0.23	33.42 ± 0.37	11.09 ± 0.14	
$\times 2^4$	24.78 ± 0.24	36.03 ± 0.36	11.25 ± 0.42	
$\times 2^6$	27.09 ± 0.63	38.12 ± 0.97	11.08 ± 0.40	
	Clade F ITS	Coral *Pax*	*C*t *Pax*– *C*t Clade F	$R^2 = 0.965$
$\times 2^0$	22.54 ± 0.09	33.09 ± 0.28	10.55 ± 0.26	
$\times 2^2$	24.81 ± 0.09	35.58 ± 0.19	10.76 ± 0.07	
$\times 2^4$	27.42 ± 0.12	37.63 ± 0.16	10.52 ± 0.28	
$\times 2^6$	29.46 ± 0.08	39.95 ± 0.18	10.60 ± 0.20	

[a]Average of 3 replicates ± SD

Furthermore, the total *Symbiodinium* (Clade C) genome DNA mass could be expressed as genome DNA size using LaJeunesse's flow cytometric analysis of fluorescence (3.0 pg cell^{-1}) (LaJeunesse et al. 2005). The *Symbiodinium* population calculation as cell numbers is:

$$[Symbiodinium\ \text{cells}] = \frac{[\text{DNA mass}]}{3.0} \quad (3)$$

Considering sample volume and dilution factors, the *Symbiodinium* population in seawater could be expressed as:

$$[Symbiodinium\ \text{cells}]/\text{ml seawater} = [Symbiodinium\ \text{cells}] \times \frac{V}{v} \div M \quad (4)$$

where V is the total volume of template DNA (amount of DNA in elution buffer), v is the volume of template DNA per reaction (well), and M is the total volume of seawater (ml) used for DNA extraction.

The *Symbiodinium* population calculation follows the equation below when Ct = 23.62:

$$Symbiodinium\ \text{cells/ml seawater} = \frac{2 \times 10^5 \times 10^{4.3801-0.2701 \times 23.62}}{3.0} \times \frac{100}{1.0} \div 500 = 133.34\ \text{cells/ml seawater} \quad (5)$$

where 500 ml of seawater was used for DNA extraction, total DNA elution was 100 µl, and 1.0 µl of template DNA was used per reaction (well). The results are 133.34 × 10^3 *Symbiodinium* cells per liter of seawater when Ct = 23.62.

A typical run for 1 seawater sample from the aquarium including calculations is presented in Fig. 3 and Table S2 in the Supplement. *Symbiodinium* Clades C and D were the dominant symbionts in our aquarium

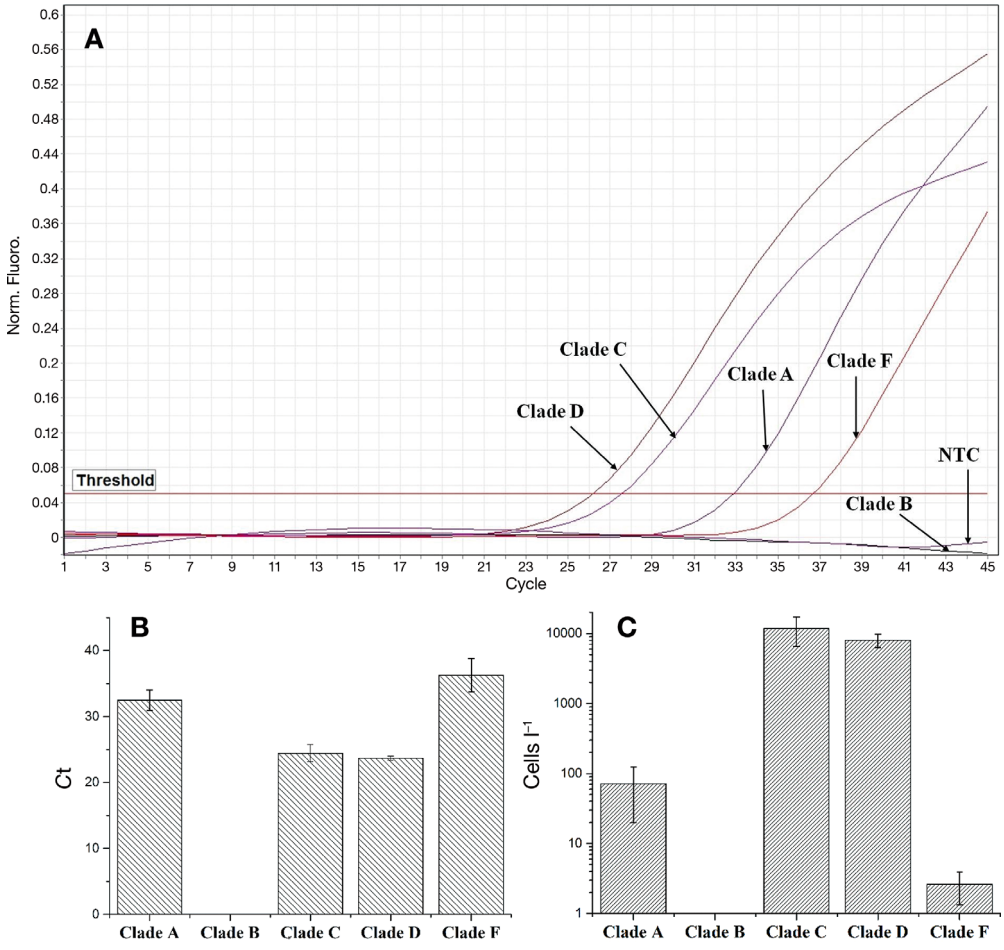

Fig. 3. A typical Taqman PCR profile for the detection of the free-living *Symbiodinium* from aquarium. (A) Template was the DNA extracted from seawater sample; NTC: no template control. (B) Cycle threshold (Ct) value of multiple *Symbiodinium* clades. (C) Estimation of symbiont cell numbers. n = 6; error bars are ±SE

water samples, with average amounts of 117 891.71 ± 52 808.22 and 80 520.13 ± 17005.64 cells l^{-1} seawater (mean ± SE), respectively. Clades A and F were significantly lower in aquarium seawater, ~719.32 ± 520.80 and 26.26 ± 13.07 cells l^{-1} seawater, respectively. We did not find Clade B in the aquarium seawater. This pattern is possibly because the water samples were collected from different depths and distances in the aquarium, and the amount of variation for free-living *Symbiodinium* quantification may reflect a patchy distribution pattern in the water environment (Littman et al. 2008).

To further confirm that the free-living *Symbiodinium* strains exist in aquaria, a culture test and ITS-PCR identification of free-living *Symbiodinium* strains from seawater were performed. *Symbiodinium* C and D free-living strains were isolated from seawater. These findings were largely in congruence with the detection results of our Taqman PCR method.

Relative abundance of *Symbiodinium* endosymbiosis in corals

To avoid variability caused by sample preparation, the relative abundance of *Symbiodinium* ITS was compared with the abundance of internal reference *Pax* DNA.

$$\Delta Ct = Ct_{Pax} - Ct_{Clade\text{-}specific\ ITS} \quad (6)$$

A larger Δ*C*t represents more *Symbiodinium* abundance endosymbiont in coral tissue. With this method, errors resulting from different sample preparations and dilutions can be canceled out by comparison with the internal reference gene. Each unit increase at Δ*C*t value means a 2-fold increase in the copy number of *Pax* genes (Livak & Schmittgen 2001). The relationship between copy number of ITS and *Pax* is:

$$[\text{ITS copies}] = [\text{Pax copies}] \times 2^{\Delta Ct} \quad (7)$$

Multiplex Taqman PCR analysis was used to identify and estimate relative abundance of different *Symbiodinium* clades present in the healthy and bleaching *G. fascicularis* colonies from the field and aquarium acclimatization conditions. All the healthy *G. fascicularis* samples harbored *Symbiodinium* Clades C, which is the dominant symbiont. Most samples contained *Symbiodinium* D except those collected from the eastern points. Statistical analysis of the mean relative abundance showed that the amounts of Clade C were significantly different between the east and west sampling locations (Table 3). The healthy corals from the west points, east points and the aquarium acclimatization generally had significantly higher Clade C abundance compared with bleaching corals ($p < 0.05$). However, it should be noted that Clade D had become dominant in the bleaching colonies (Fig. 4).

Table 3. Multiplex Taqman PCR assays for the relative abundance of *Symbiodinium* in extracts of the field and acclimatized corals. *C*t ≥ 40 is considered not detectable. Each *C*t value is the average of 3 replicates. Dates given as d/mo/yr

Sample	*C*t value		Relative abundance of Clade C	*C*t value		Relative abundance of Clade D
	Clade C ITS	Coral *Pax*		Clade D ITS	Coral *Pax*	
Field sample harvested on east reefs of Hainan island, 19/7/2015						
E1	24.59	37	12.41	ND	37	–
E2	25.97	39.07	13.1	ND	39.07	–
E3	23.04	38.03	14.99	ND	38.03	–
E4	22.63	32.16	9.53	30.79	34.69	3.9
Field sample harvested on west reefs of Hainan island, 21/7/2015						
W1	19.7	39.82	20.125	35.68	39.82	4.14
W2	19.97	36.21	16.24	35.08	36.21	1.13
W3	19.98	41.03	21.05	36.68	41.03	4.35
Healthy corals harvested after a 6 mo period of acclimation in an aquarium, 12/1/2016						
H1	20.66	31.87	11.21	28.49	34.63	6.14
H2	20.75	32.73	11.98	34.13	34.09	−0.04
H3	22.45	32.89	10.44	38.98	34.3	−4.68
H4	21.43	31.87	10.44	25.14	31.04	5.9
Bleached-looking corals harvested after a 6 mo period of acclimation in an aquarium, 12/1/2016						
B1	32.6	38.39	5.79	20.02	39.79	19.77
B2	31.59	38.24	6.65	18.98	38.22	19.24
B3	31.62	35.32	3.7	19.81	35.6	15.79
B4	32.07	35.12	3.05	20.04	31.41	11.37

DISCUSSION

Coral reefs are among the most biodiverse ecosystems on Earth and play important roles in ocean biogeochemical cycles. The great success of huge productivity within the reef ecosystem is due to the interactions with endosymbiotic *Symbiodinium* spp. (Schwarz et al. 2008). For most corals, *Symbiodinium* are acquired from the environment by horizontal transmission and reside in the endodermal cells of coral hosts as an endosymbiont (Little et al. 2004). Coral bleaching results in symbionts being expelled from the host (Kemp et al. 2014). Rapid and efficient detection of the *Symbiodinium* community dynamics is therefore important to establish a protection and recovery strategy of the coral reefs. It requires sensitive and reliable diagnostic methods for early detection rather than symptom-based diagnosis. However, it has been difficult to quantify and distinguish the titer of the *Symbiodinium* spp. in host or in seawater because molecular genetic studies have revealed that the genus *Symbiodinium* is a highly diverse group of dinoflagellates (Coffroth & Santos 2005). Some of these dinoflagellates are difficult to isolate and culture in artificial media (Ishikura et al. 2004). These potential problems demonstrate that knowledge of the symbioses of *Symbiodinium*–coral during a bleaching event or in response to environmental change is still in its nascent stage. One of the main objectives of our study was to practice a cost-viable technology for field studies that would allow better understanding of the dynamics of the symbiont population shuttling from the host.

Conventional microscopic counts are inappropriate for quantitative analysis when measuring *Symbiodinium* density in the water because the density of cells is generally too low. Another high throughput method, the automated particle counter (FlowCAM), is more efficient in detecting cells in the water where densities are low (Littman et al. 2008). Both methods are based on morphology, which is not useful for distinguishing different species and characterizing the

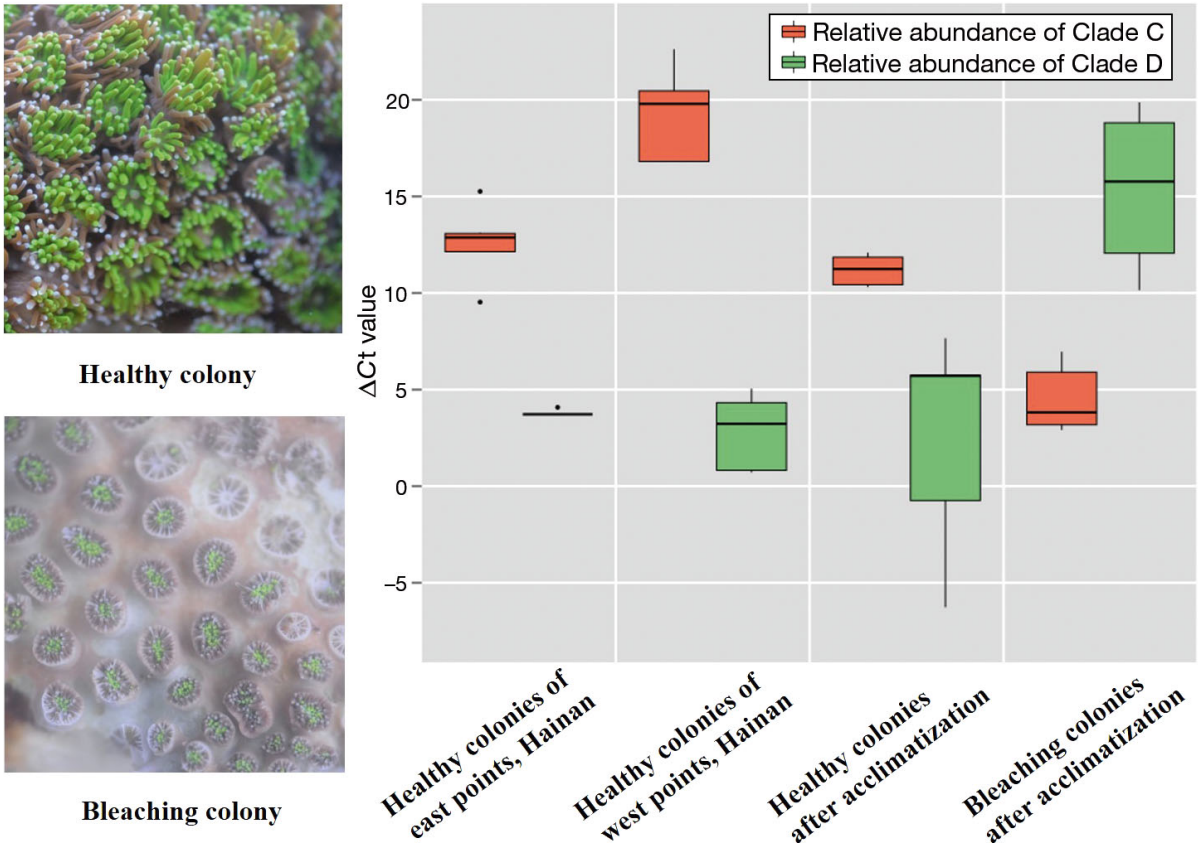

Fig. 4. Mean relative abundance of *Symbiodinium* Clades C and D in healthy *Galaxea fascicularis* colonies of east points (n = 4) and west points (n = 3), and the healthy (n = 4) and bleached (n = 4) *G. fascicularis* after acclimatization. Boxes show the median values (central lines), lower outline and upper outline, and whiskers. The whiskers are set at 1.5 times the interquartile range above upper outline and 1.5 times the interquartile range below lower outline. If the maximum or minimum values are outside this range, they are shown as outliers (•)

diversity of *Symbiodinium*. Also, the swimming velocity of *Symbiodinium* small cells is somewhat indistinguishable from other sediment particles or plankters (Koike et al. 2007). Fortunately, real-time qPCR (RT-qPCR) has become a mainstream method for quantification of microbes (Livak & Schmittgen 2001, Lin et al. 2014). To the best of our knowledge, only 3 studies have developed RT-qPCR protocols for the identification or quantification of the endosymbiotic *Symbiodinium* spp. (Koike et al. 2007, Correa et al. 2009, Mieog et al. 2009). Although the specific PCR method using SYBR Green is available for detecting extremely low *Symbiodinium* DNA input described in the literature, false positives commonly occur by the cross-species reactions. The Taqman method utilizes 2 primers and an additional probe and therefore is generally more specific than the intercalator method (Bowers et al. 2000). Furthermore, the Taqman fluorescence signal is not affected by many common contaminants (Kriger et al. 2006) and can offer higher specificity when host DNA is present in the reaction. In this study, the average assay sensitivity for pure DNA samples of *Symbiodinium* is comparable to that of the qPCR primer sets developed by Correa et al. (2009) and Mieog et al. (2009). Thus, we support that the use of an additional and more reliable technique may improve the ability to detect and quantification of the *Symbiodinium* biomass in complex samples from multiple origins, including naturally endosymbiotic samples and environmental waters.

The basic subdivision of the genus *Symbiodinium* into 9 phylogenetic clades is well established (designated as Clades A to I) (LaJeunesse et al. 2012). The sequence of the ITS-rDNA is highly conserved but sufficiently variable among clades of *Symbiodinium* to provide accurate and rapid detection at clade level (LaJeunesse 2001, Little et al. 2004, Mieog et al. 2007, Ulstrup & Van Oppen 2003). In this study, the specific PCR primers and probes were designed based on SNPs present in the ITS region (Fig. 1). It is possible that coral sometimes contains multiple clades of *Symbiodinium* and that *Symbiodinium* ITS rDNA can show diversity even within a single clade. To address this concern, alignment with a sufficient number of clade-representatives and additional type sequences retrieved from Genbank of NCBI is necessary, which fundamentally governs the specificity and sensitivity of the Taqman assay. Certainly, the current Taqman method will be improved by designing the degeneracy/conservation of primer/probe sets derived from a larger number of representative sequences in future. Overall, use of the Taqman specific primers/probes presented here showed that they can correctly detect pure culture of *Symbiodinium* species and detect free-living *Symbiodinium* clades in water samples. The results obtained from Taqman PCR detection were also confirmed by the culture test for the free-living *Symbiodinium* strains. Furthermore, no cross-reaction effect on Taqman PCR occurred when applying the method to the DNA samples containing other related marine algae, fungi and bacteria. Therefore, the suite of specific Taqman PCR protocol presented in this paper provided an improved tool for *Symbiodinium* community and diversity research in coral hosts and environmental waters at various geographic locations.

This paper also established a method to estimate relative abundance of symbionts in coral colonies using a relative *C*t value by comparing the reference animal paired-box containing genes DNA (*Pax*) *C*t with the target *Symbiodinium* ITS-rDNA *C*t. The Δ*C*t concept makes the comparison among different samples more accurate because the sample size, extraction efficiency, and dilution factors are no longer an issue (Bai et al. 2013, Li et al. 2006). The design of the internal reference *Pax*-based primer–probe set has proven very useful in analyses because it can be amplified in distantly related coral species with the same degenerate primer pairs which target the conserved exon regions. Therefore, it could be used as an internal coral DNA control to validate the coral extracts and exclude false-negative results. Meanwhile, this primer pair was also useful to assess the quality of coral host cells. Our multiplex assays with dilutions of pure *Symbiodinium* DNA in total coral DNA samples demonstrated that non-target DNA did not inhibit the amplification of *Symbiodinium* ITS-rDNA and coral internal reference *Pax* and therefore did not affect the results of the Taqman PCR detection.

The distance between east and west sampling pools of Hainan was ~200 km. *Symbiodinium* from *G. fascicularis* of these 2 pools showed an interesting distribution pattern. Clade C was more abundant than Clade D from corals in these reefs. The lack of Clade D at some of the eastern points correlated with previous speculation that zooxanthellae may occur in a patchy spatial distribution across regional distances (van Oppen et al. 2001). The Taqman assay showed the variety and abundance of symbionts between west and east sites, which is consistent with that of the previous transcriptome profiling study (Lin et al. 2017). This pattern is reasonable because the 2 sampling sites experience different nutrition regimes of disturbed coastal environments and chronic eutrophic disturbances in the South China Sea.

The primer–probe combinations used in our Taqman PCR assay were very sensitive, with the detection limit of 2.0 pg μl^{-1} *Symbiodinium* DNA (approximately the DNA content of a *Symbiodinium* cell input) (LaJeunesse et al. 2005). It allowed us to readily detect up to 4 clades of free-living *Symbiodinium* (Clades A, C, D and F) in seawater from aquaria with high accuracy, ranging from 20×10^3 to 117×10^3 cells l^{-1} seawater. This cell density is very close to that of the report of Littman who estimated up to 80×10^3 cells l^{-1} in the water column at Great Barrier Reef by automated particle counter (Littman et al. 2008). Although the free-living *Symbiodinium* Clades A, C, D and F were detected in a lab coral culture system, yet we did not detect Clades A or F in the hosts we collected directly from the reef. It is possible that Clades A and F were facultative symbionts for *G. fascicularis* and able to persist outside the host in a free-living state (Baker 2003, Lesser et al. 2013). Changing conditions from effects of either abiotic or biotic factors could affect the dynamics between symbionts and host. The diversity of *Symbiodinium* in surrounding waters theoretically allows corals to maximize the symbioses fitness to suit to the prevailing environmental conditions (Lesser et al. 2013). Rowan proposed that *Symbiodinium* D is a high-temperature specialist. Adaptation of *Symbiodinium* D to higher temperature may improve the resistance of coral host to warm-water bleaching (Rowan 2004). Distinct shuffling in dominance of C and D was observed between healthy and bleaching corals, although we have yet to determine the detailed processes of these symbionts shifts in corals. The changes of dominant symbionts are a common feature of severe bleaching and mortality events. Some corals have the shuffling capacity because of the high symbiont flexibility (Baker et al. 2013, Thornhill et al. 2006), and these adaptive shifts are hypothesized to increase the resistance of these corals to bleaching (Baker et al. 2001). However, this hypothesis still needs further evidence (Little et al. 2004, Mieog et al. 2007, Rosenberg & Zilber Rosenberg 2011).

CONCLUSION

In conclusion, our study developed a *Symbiodinium* clade-specific Taqman PCR detection method. We present an effective and accurate protocol for estimating free-living *Symbiodinium* densities in seawater and also discuss the importance of using a relative *C*t value by comparing the internal reference coral *Pax* with the clade-specific ITS-rDNA loci to quantify the *Symbiodinium* abundances in coral tissue. This Taqman assay could be used on a broad-scale survey of spatial distribution of *Symbiodinium* communities to evaluate the prevalence of background symbionts in reefs and will be helpful for understanding the flexibility of coral-symbionts associations.

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