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Symbiont Dependent Thermal Bleaching Susceptiblity in Two Reef-building Corals, *Stylophora pistillata* and *Platygyra ryukyuensis*.

Ranjeet Bhagooli
Faculty of Science,
University of Mauritius
Email: r.bhagooli@uom.ac.mu

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ABSTRACT

Coral species, one susceptible (*Stylophora pistillata*) and the other resistant (*Platygyra ryukyuensis*) to bleaching, were exposed to a sudden elevated temperature (33.5°C) under dim light (5 µmol quanta m^{-2} s^{-1}) for 10 to 720 min in time course experiments and to temperatures varying from 30°C to 40°C at 65 µmol quanta m^{-2} s^{-1} for 10 min at each temperature. Chlorophyll *a* fluorescence measurements in *Symbiodinium* of both coral species indicated that the maximum electron transport rate (ETR_{max}) and the maximum quantum yield of photosystem II (PSII) fluorescence (F_v/F_m) were sensitive to thermal stress. The non-stressed *Symbiodinium* ETR_{max} value in *S. pistillata* was halved earlier and at a lower temperature stress than in those of *P. ryukyuensis*. Denaturing gradient gel electrophoresis (DGGE) analysis of the internal transcribed spacer 2 (ITS2) region of the ribosomal DNA revealed inter-colony diversity of *Symbiodinium* in both species, though each species contained genetically distinct *Symbiodinium* types. Heat dissipation in PSII, through non-photochemical quenching (NPQ), increased in *Symbiodinium* of *P. ryukyuensis* irrespective of *Symbiodinium* genotype (C60 and C55), while in *S. pistillata* it either increased (C59) or decreased (C1) depending on genotype. Thus distinct *Symbiodinium* ITS2 types exhibit diverse photo-physiological responses to thermal stress, and may partially explain the variable bleaching susceptibilities of some hermatypic coral species.

Keywords: coral; PAM fluorometry; *Symbiodinium*; thermal stress

1. INTRODUCTION

Over the last three decades, the coral-bleaching phenomenon has been widely reported as increasing in frequency and severity on tropical coral reefs (Hoegh-Guldberg, 1999; Wilkinson, 2000). The term bleaching refers to the loss of photoautotrophic endosymbiotic dinoflagellates (*Symbiodinium* spp.) and/or their photosynthetic pigments (Glynn & D'Croz, 1990). Bleaching as a general stress response can be caused by multiple environmental factors, either acting separately or in combination (Brown, 1997; Lesser, 2004). Elevated temperature has been mostly reported as the main causative factor and sea surface temperature-based models suggest that temperatures are currently increasing at 1-2oC per century (Hoegh-Guldberg, 1999).

The underlying mechanism of coral bleaching is generally considered to be a result of the breakdown of the photosynthetic machinery, initially assessed by photosynthetic oxygen evolution, of the *Symbiodinium* (Iglesias-Prieto *et al.* 1992; but see review by Baird et al. (2009)). Recent studies, using chlorophyll fluorescence techniques (Fitt & Warner, 1995; Warner *et al.* 1996; Jones *et al.* 1998; Bhagooli & Hidaka, 2002, 2003, 2004; Abrego *et al.* 2008), have shown that damage to photosystem II (PSII) of *Symbiodinium* for several species of corals occurs at temperatures above 30oC. A detailed molecular investigation (Warner *et al.* 1999) has revealed that damage to the D1 protein of the photosystem II (PSII) reaction centers during thermal stress is a major determinant of coral bleaching. Lesser & Farrell (2004) also showed that damage to the D1 protein of *Symbiodinium* is significantly increased when corals are exposed to both thermal stress and high irradiances of solar radiation.

A study by Jones *et al.* (1998) suggested that the impairment of CO_2 fixation is the first step in heat-induced damage, which in turn led to PSII dysfunction under moderate light levels. These findings were based on the observation of decreases in maximum quantum yield of PSII and effective quantum yields accompanied by increases in the non-photochemical quenching process from induction curves using a bleaching susceptible coral species, *Stylophora pistillata*. Other studies have shown that damage to both Rubisco and PSII occur simultaneously when exposed to thermal and/or ultraviolet radiation (Lesser, 1996; Lesser & Farrell, 2004). Recent studies have shown that recovery of PS II D1 proteins are actually the site of heat damage (Takahashi *et al.* 2009). Hill and Ralph (2008) has also assessed the effect of bleaching stress on the oxygen evolving complex of *Symbiodinium*.

Differential bleaching susceptibility among scleractinian corals has been documented both in the field in Okinawa, Japan (Loya *et al.* 2001), on the Great Barrier Reef, Australia (Marshall & Baird, 2000; Baird & Marshall, 2002), and in the Indian Ocean (Spencer *et al.* 2000) and under experimental trials (Fitt & Warner, 1995; Bhagooli & Yakovleva, 2004). Bhagooli & Hidaka (2003) showed that *Symbiodinium* isolated from five different coral species exhibited different photosynthetic responses to elevated temperature and/or high light exposures. It has also been shown that those corals more capable of dissipating excess excitation energy through non-photochemical quenching are less susceptible to temperature-induced bleaching (Warner et al. 1996). Sampayo *et al.* (2009) demonstrated that host species of coral harbouring different genetic types of *Symbiodinium* explained different bleaching susceptibility in *S. pistillata*. Suggett *et al.* (2008) have assessed the production of hydrogen peroxide in *Symbiodinium* with different

thermal tolerances. However, the different tolerances of corals to elevated temperature stress due to different *Symbiodinium* types exhibiting variable capacity to dissipate excess excitation energy has yet to be thoroughly investigated.

Ample evidence at the biochemical, physiological, morphological and behavioral levels (Blank & Trench, 1985; Trench & Blank, 1987; Blank *et al.* 1988; Iglesias-Prieto & Trench, 1994; Trench, 1997; Banaszak *et al.* 2000) is suggestive of a high genetic diversity among *Symbiodinium* in coral reef sites. Molecular genetic studies have revealed distinct clades (Rowan, 1991, 1998; Rowan & Powers, 1991) using restriction fragment length polymorphism and ecotypes (LaJeunesse, 2002; LaJeunesse *et al.* 2004) employing denaturing gradient gel electrophoresis. Recent reports have compiled eight different gene regions that have so far been used to genetically characterize *Symbiodinium* (Sampayo *et al.* 2009). Moreover, Howells *et al.* (2009), and Pettay and LaJeunesse (2009) have found high genetic diversity among *Symbiodinium*.

However, not many of the studies so far have dealt with molecular genetic and photo-physiological examinations to heat stress simultaneously. Studies examining the links between the genetic and photo-physiological responses to thermal stress are limited (Rowan, 2004; Tchernov *et al.* 2004; Sampayo *et al.* 2008; Abrego *et al.* 2008). The main objective of this study was to characterize the differences in photo-physiological responses, using the maximum quantum yield PSII fluorescence, maximum photosynthetic electron transport rate, and non-photochemical quenching determinations measured by chlorophyll fluorescence signals, for *in hospite Symbiodinium* from two species of coral, one that is susceptible, and one that is resistant to bleaching. Another objective was to match the photo-physiological responses to the molecular genetic types of *Symbiodinium* present in the respective coral species. The results are then discussed in relation to the different levels of heat-induced damage in genetically distinct *Symbiodinium*, and their potential for predicting the susceptibility to thermal coral bleaching.

2. MATERIALS AND METHODS

2.1 Collection and maintenance of organisms

Four colonies of each of the scleractinian corals *Stylophora pistillata* Esper, 1797 and *Platygyra ryukyuensis* Yabe and Sugiyama, 1935 were randomly collected from 0.5 m depth at low tide from Okinawa Island, Japan. The colonies were taken from well-lit shallow areas and assumed to be exposed to similar prevailing environmental conditions. Coral fragments, about 2 cm long for *S. pistillata* and approximately 2cm x 2cm for *P. ryukyuensis*, were mounted on glass slides and kept in running sea water with continuously supplied aeration and under neutral density cloth until they were used for experiments. Four healthy-looking fragments, one per colony, for each test coral species were used for each treatment in each experiment. Thus, four replicates (n=4), one from each colony, were used.

2.2 Experimental protocols

To assess the photo-physiological responses to thermal stress two sets of experiments were undertaken. A time course experiment was carried out. Coral fragments (n=4) were exposed to 33.5°C in dim light (~5 µmol quanta m^{-2} s^{-1}) for various lengths of time: *Stylophora pistillat*a - 10, 30, 45, 60, 120, 240 and 360 min; *Platygyra ryukyuensis* - 60, 120, 240, 360, 480, 720 min. Chlorophyll a fluoresecence parameters were measured initially (prior to exposures) and after respective duration of elevated temperature treatments. Samples from respective colonies were kept at 26°C under similar light levels over 720 min as controls. In an additional experiment, coral fragments of both *S. pistillata* and *P. ryukyuensis* were treated at 26, 30, 32, 34, 36, 38 and 40°C under 65 µmol quanta m^{-2} s^{-1} for 10 min and the chlorophyll a fluorescence parameters were recorded before and after respective thermal stresses. These treatments were sudden shock exposures and not acclimatization experiments. Chlorophyll a fluorescence parameters, F_v/F_m, ETR_{max} and NPQ, were recorded before and after each treatment.

2.3 Chlorophyll *a* fluorescence measurements

Chlorophyll *a* fluorescence parameters were measured using a pulse-amplitude-modulated (PAM) fluorometer (MINI-PAM Walz, Germany) on *in hospite Symbiodinium*, that is, *Symbiodinium* within the tissues of coral fragments held in seawater in a custom-made black box. The initial (background) fluorescence, F_o, was measured by applying a weak pulsed light (<1 µmol quanta m^{-2} s^{-1} PAR), and a saturating pulse (8000 µmol quanta m^{-2} s^{-1}) was used to determine the maximum fluorescence, F_m, on dark acclimated samples The change in fluorescence (F_v) caused by the saturating pulse, in relation to the maximal fluorescence yield (F_m), that is F_v/F_m, has been shown to be a good measure of maximum quantum yield of PSII fluorescence (Genty *et al.* 1989).

In an illuminated sample, the F_o and F_m values change based on the proportion of open and closed reaction centers which provide new fluorescent values, F and F_m', respectively. Steady-state quantum yields $(F_m'-F)/ F_m'$, are lower compared to F_v/F_m due to partial closure of the reaction centers and a relative increase in non-radiative energy dissipation. The new ratio is a measure of the effective quantum yield of PSII ($\Delta F / F_m'$) in an illuminated sample. Since electrons leading to CO_2 reduction in the dark reactions of photosynthesis are derived from the splitting of water in PSII, photosynthetic electron transport rate (ETR) may be estimated from the effective quantum yield. Thus, ETR is expressed as the effective quantum yield x PFD, where PFD is the photosynthetic flux density of photosynthetically active radiation (PAR, 400-700 nm, µmol quanta m^{-2} s^{-1}). Non-photochemical quenching (NPQ) was determined using the formula $(F_m - F_m')/ F_m'$, with F_m determined after the dark adaptation and in samples before any respective trials to allow reliable comparison between treatments possible.

In the present study, all mounted corals were placed in darkness (dark-adapted) for 20 min (sufficient for relaxation of reaction centers - Jones & Hoegh-Guldberg, 2001) using a custom-made black box, before measurement of F_v/F_m. Rapid light curves (RLC), which are similar to photosynthesis versus irradiance (P-I) curves, for in hospite Symbiodinium were also measured.

The RLC was measured by illuminating the samples for 20 s before each $\Delta F / F_m'$ measurement at each of a series of eight irradiances increasing in steps from 0 to 1285 µmol quanta m^{-2} s^{-1}. A 5 min light-acclimation (ca. 130 µmol quanta m^{-2} s^{-1}) using a Coldspot lighting system (Model PUDC24Z4, 400-700 nm) followed by a 30 s dark period was employed prior to the automatic series of measurements and initiated using the internal actinic light source. The actual actinic light levels that reached the measured coral surface were determined for each species using a LICOR lightmeter (Model, LI-250). The custom-made black box was used to guide the optic fibre head of the fluorometer on the specimens ensuring the same position on the coral was measured before and after stress treatments. ETR_{max} was then determined from the RLCs.
The changes in the non-photochemical quenching (NPQ) in the colonies with the identified respective *Symbiodinium* ITS2 types were also examined either separately or with the data pooled together for each coral species under investigation. This was undertaken to demonstrate the variability in dissipating heat as a thermal response of different genotypes of *Symbiodinium* in the studied corals.

The gain setting on the fluorometer was set to two and maintained throughout the experiment. Relative F_v/F_m, ETR_{max} and NPQ representing ratios of respective F_v/F_m, ETR_{max} and NPQ after treatment to before experimentation for each specimen from each coral species, were employed. Relative values of F_v/F_m, ETR_{max} and NPQ are independent of the topography of the coral species and thus confer reliable comparison to be made among treatments and species.

2.4 DNA extraction from *Symbiodinium*

The coral samples for each of the four colonies (one per colony) belonging to the two species of corals under investigation were water picked and the blastate were homogenized in MFSW (0.45 µm). Filtration through 350 µm nylon mesh was used to get rid of the mucus. After three washes at 1000, 2000, and 3000 rpm for 5, 10 and 15 min respectively, the samples were preserved in CHAOS solution (4M guanidine thiocyanate, 0.5% salcosyl, 2.5 mM Tris (pH, 8.0), 0.1 M 2-mercaptoethanol and distilled water).

DNA extraction and purification from *Symbiodinium* was done using the Wizard Genomic Purification kit (Promega USA, A7170). The algal cells preserved in the CHAOS were centrifuged at 14,000 rpm for 10 min. To wash the excess CHAOS 500 µl 10x Tris-EDTA (TE) was used. Centrifugation (Model, 5415C) and washing with TE was done and the remaining fluid was dried using a vacuum suction system. 20-40 mg (20-40 µl) of the pellet was placed in a new 1.5 ml eppendorf tube containing 250 µl glass beads (0.5 mm) and 600 µl nuclei lysis buffer. These tubes were then placed in a mini-beadbeater (Biospec products, UF-80A12H) and beating was done for 3 min at maximum speed. They were then placed in water bath at 65°C for 5-10 min.

Then 3µl of (20 mg ml^{-1}) proteinase K was added and vortexed for 2-3 s. They were then incubated at 65°C for at least 1 h with 2-3 s vortexing every 15-20 min. 1 µl RNAse (4 mg l^{-1}) was added and the extracts were incubated at 37°C for 15-20 min. 250 µl protein precipitation solution was added to each sample and vortexed for 5 s. The samples were placed on ice for 15 min followed by centrifugation at 14,000 rpm for 10 min.

Six hundred micro-liter of clear supernatant was placed in a new 1.5 ml eppendorf tube containing 700 µl isopropanol (100%) and 50 µl sodium acetate (3M). After gently mixing the contents, they were placed on ice for 10 min and then centrifuged at 14,000 rpm for 10 min. The isopropanol was aspirated and 500 µl of 70% ethanol was added and mixed followed by a 5 min centrifugation at 14,000 rpm. The ethanol was carefully aspirated and the samples dried in a fume hood overnight.

2.5 ITS amplification, denaturing gradient gel electrophoresis analysis and sequencing

The ITS2 region (330-360 bp) primers (modified from LaJeunesse and Trench, 2000) were used for PCR amplification. An internal primer "ITSintfor2" (5′ GAATTGCAGA ACTCCGTG-3′) annealing to a conserved region of the 5.8S rDNA and paired with the conserved 3′ flanking primer, ITS reverse, was used. The end primer, called the "ITS2CLAMP" with a 39 bp GC clamp (underlined) (5′ CGCCCGCCGC GCCCCGCGCC CGTCCCGCCG CCCCCGCCC GGGATCCATA TGCTTAAGTT CAGCGGGT-3′) was employed. Reactions were carried out on a BioRad Thermal Cycler using a "touchdown" amplification protocol with annealing conditions (LaJeunesse, 2002) 10°C above the final annealing temperature of 52°C. A 1°C increase in annealing temperature was programmed to occur every two cycles. Following 20 cycles the 52°C annealing temperature was maintained for an additional 15-18 cycles.

The amplicons from the different specimens were run on a gradient gel. All products were loaded with a 2% Ficoll loading buffer (2% Ficoll-400, 1.0 mM tris-HCl pH 7.8, 1 mM EDTA, 1% bromophenol) onto a 45-80% polyacrylamide denaturing gel (gradient of 3.15 M urea/18% deionized formamide to 5.6 M urea/37% deionized formamide). Separation by electrophoresis was carried out at 160 V for 9.5 h at a constant temperature of 60°C. The gel was stained with Syber Green (Molecular Probes, Eugene,Ore.) for 25 min in darkness and was visualized with UV transillumination (BioRad SyncMaster 753DF).

To identify the *Symbiodinium* ITS2 types the brightly stained bands were excised from denaturing gels as in LaJeunesse *et al.* (2004). Briefly, eluted DNA was re-amplified by using the same set of primers without the GC-clamp and the PCR thermal cycle profile with an annealing temperature of 52°C for 40 cycles. The sequencing was performed and analysed as in LaJeunesse (2002) and LaJeunesse *et al.* (2004).

2.6 Statistical analyses

The relative values of F_v/F_m, ETR_{max} and NPQ for the time and temperature course experiments were compared to controls (26°C under respective light regimes) using the non-parametric Kruskal-Wallis test for analysis of variance by ranks as no significant changes within the controls were detected. Significance was determined using $P \leq 0.05$. Initial and after experimentation values for F_v/F_m, and ETR_{max} were also statistically compared using Kruskal-Wallis test.

3. RESULTS

3.1 Changes in quantum yield of PSII and maximum electron transport rate under thermal stress

Initial values for F_v/F_m and ETR_{max} were 0.6-0.7 and 85-101, respectively, for coral species tested. Relative values for chlorophyll a fluorescence parameters are shown in Figs. 1 and 2 for the time course and temperature experiments, respectively. When results for relative F_v/F_m and

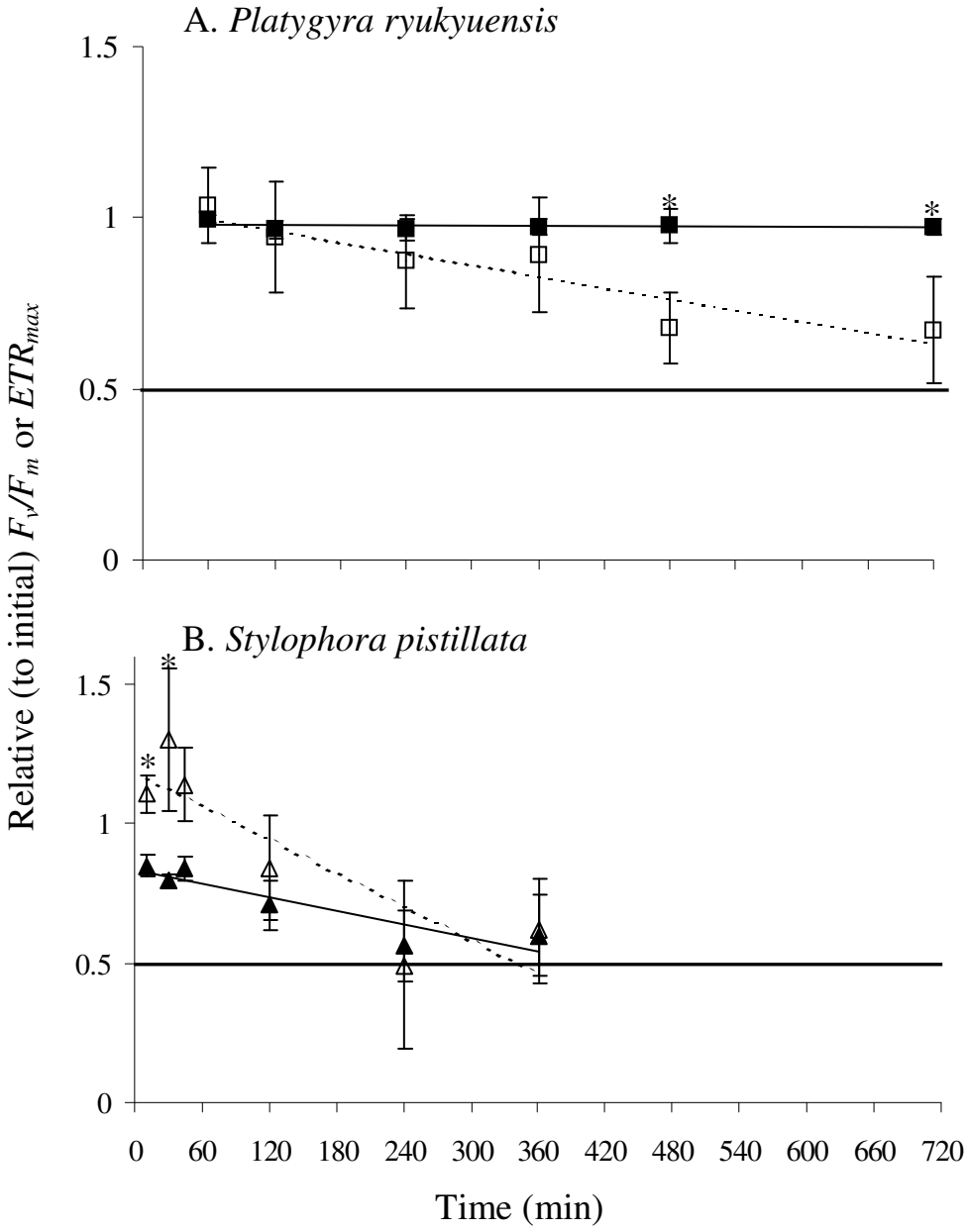

Figure 1. Time course photo-physiological responses: Maximum quantum yield of PSII (F_v/F_m) (filled squares and triangles, solid lines) and maximum electron transport rate (ETR_{max}) (empty squares and triangles, dotted lines) of *in hospite Symbiodinium* of *Platygyra ryukyuensis* (A) and

Stylophora pistillata (B) exposed to elevated temperature (33.5°C) under dim light (5 µmol quanta m^{-2} s^{-1}). Curves were linearly fitted ($r^2 > 0.77$). Horizontal block line with y-intercept 0.5 marks 50% reduction of the chlorophyll *a* fluorescence parameters. Data points represent mean ± SD (n = 4). Asterisk indicates significant differences (Kruskal-Wallis test) when the relative values of ETR_{max} at the 33.5°C were compared to the 26°C (control, which did not change significantly over time) ones at each time point. * $P < 0.05$.

ETR_{max} of the time series experiments (Fig. 1) are examined, it is clear that the *Symbiodinium* in the two corals species responded differently. While the F_v/F_m ratios of *Symbiodinium* in *Platygyra ryukyuensis* did not change significantly (Kruskal-Wallis test, $P > 0.05$) over the 12 h exposure period (Fig. 1A), those of *Stylophora pistillata* declined significantly (Kruskal-Wallis test, $P < 0.05$) after 10 min treatment and onwards at 33.5°C and 5 µmol quanta m^{-2} s^{-1} (Fig. 1B). *Symbiodinium* in *S. pistillata* exhibited a 50% reduction in both their F_v/F_m ratios and ETR_{max} after 4 h (240 min) under the experimental thermal stress. However, the ETR_{max} increased during the first 30 min for *Symbiodinium* of *S. pistillata*. On the other hand, the ETR_{max} of *Symbiodinium* in *P. ryukyuensis* declined significantly (Kruskal-Wallis test, $P < 0.05$) after 8 h (480 min) without significant alterations in F_v/F_m (Table 1) and a 50% decline, using linear fittings to the data, was not observed even after 12 h exposure to the same heat stress.

Table 1. Summary of statistics comparing initials and after experimentation values of F_v/F_m and ETR_{max} for both time and temperature course experiments. Asterisks *, ** and *** represent $P <$ *0.05*, *0.01* and *0.001* for Kruskal-Wallis test, respectively. NS represents no significant difference ($P > 0.05$, Kruskal-Wallis test). Hyphen (-) indicates no data has been collected.

Coral Species	**Parameters**	**Time course experiment (mins at either 26°C or 33.5°C)**									**Temperature course experiment (°C for 10 mins)**						
		10	**30**	**45**	**60**	**120**	**240**	**360**	**480**	**720**	**26**	**30**	**32**	**34**	**36**	**38**	**40**
P. ryukyuensis	**26°C**																
	F_v/F_m	-	-	-	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	***
	ETR_{max}	-	-	-	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	**	***
	33.5°C																
	F_v/F_m	-	-	-	NS	NS	NS	NS	NS	NS							
	ETR_{max}	-	-	-	NS	NS	NS	NS	*	**							
S. pistillata	**26°C**																
	F_v/F_m	NS	NS	NS	NS	NS	NS	NS	-	-	NS	NS	*	-	-	**	***
	ETR_{max}	NS	NS	NS	NS	NS	NS	NS	-	-	NS	*	NS	-	-	**	***
	33.5°C																
	F_v/F_m	*	*	*	**	***	**	**	-	-							
	ETR_{max}	*	*	NS	NS	**	**	**	-	-							

For the temperature series experiments, polynomial fits of third order were employed to determine the 50% reduction in the chlorophyll *a* fluorescence parameters recorded in this investigation (Fig. 2). Although significant declines in F_v/F_m ratios of *Symbiodinium* in *S.pistillata* could be observed, 50% reduction could not be estimated using the experimental thermal stresses employed in this study (Fig. 2B). However, *Symbiodinium* in *P. ryukyuensis*

exhibited 50% reduction in F_v/F_m ratio at 39.5°C (Fig. 2A). The ETR_{max} declined to 50% in both species at 37.8 and 38.9°C for *Symbiodinium* in *S. pistillata* and *P. ryukyuensis*, respectively.

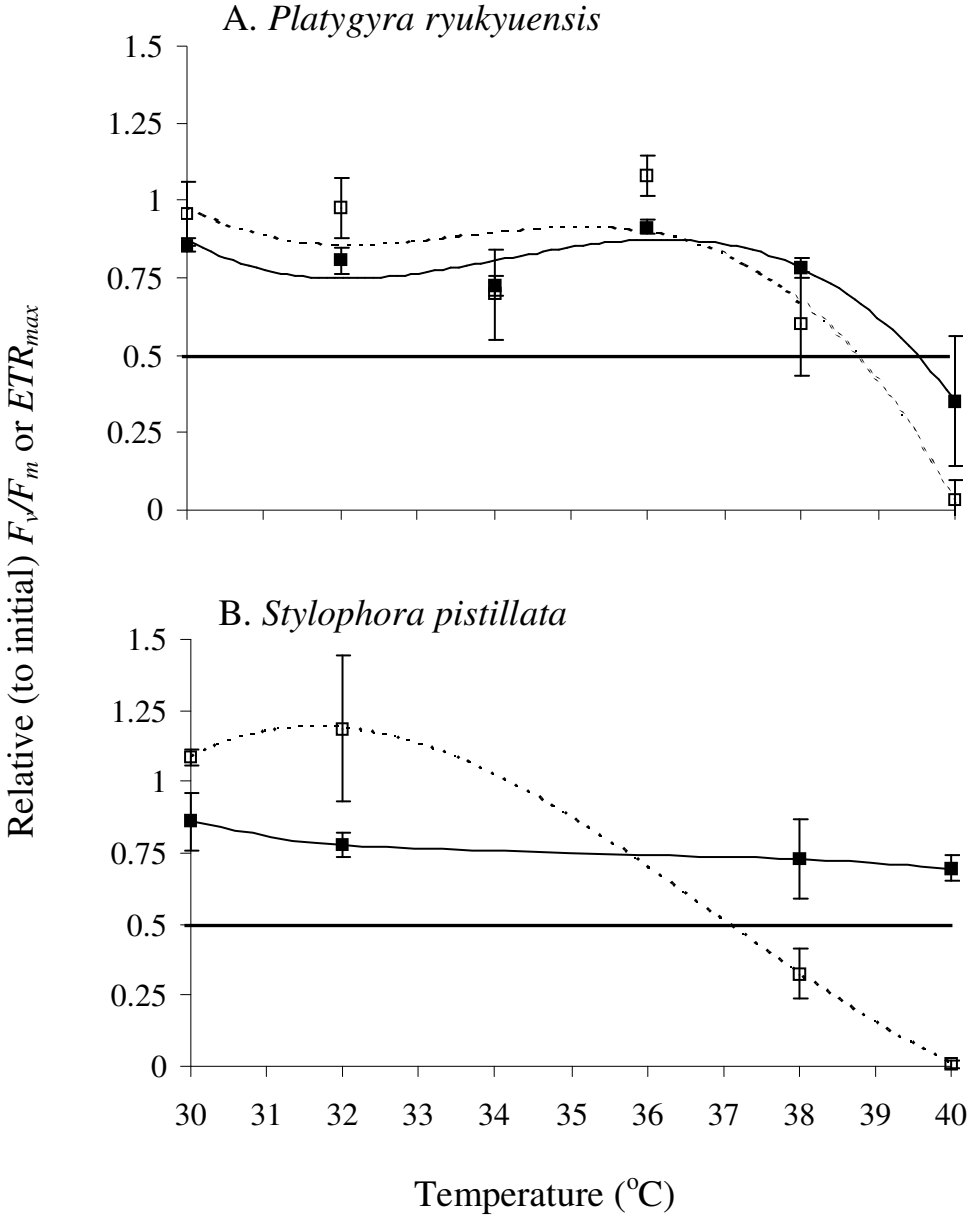

Figure 2. Temperature course photo-physiological responses: maximum quantum yield of PSII (F_v/F_m) (filled squares, solid lines) and maximum electron transport rate (ETR_{max}) (empty

squares, dotted lines) of *in hospite Symbiodinium* of *Platygyra ryukyuensis* (A) and *Stylophora pistillata* (B) exposed to temperatures ranging from 30 to 40°C under 65 µmol quanta m^{-2} s^{-1} for 10 min at each respective temperatures. Curves were fitted with polynomials of third order (r^2 > 0.67). Horizontal block line with y-intercept 0.5 represents 50% reduction of the chlorophyll fluorescence parameters. Data points represent mean ± SD (n = 4).

3.2 Analyses of DGGE profiles and sequences

Fig. 3 depicts the DGGE profiles for the ITS2 typing of the *Symbiodinium* from the two coral species under investigation. A single distinctive band occasionally accompanied by faint "background" bands typically characterized the DGGE profiles for the isolated *Symbiodinium* from the corals. The profiles revealed two ITS2 types of *Symbiodinium* in both *S. pistillata* (from three colonies) and *P. ryukyuensis* (from two colonies). Sequence analyses suggested presence of *Symbiodinium* ITS2 types C1 and C59 in *S. pistillata*, and C60 and C55 in *P. ryukyuensis*.

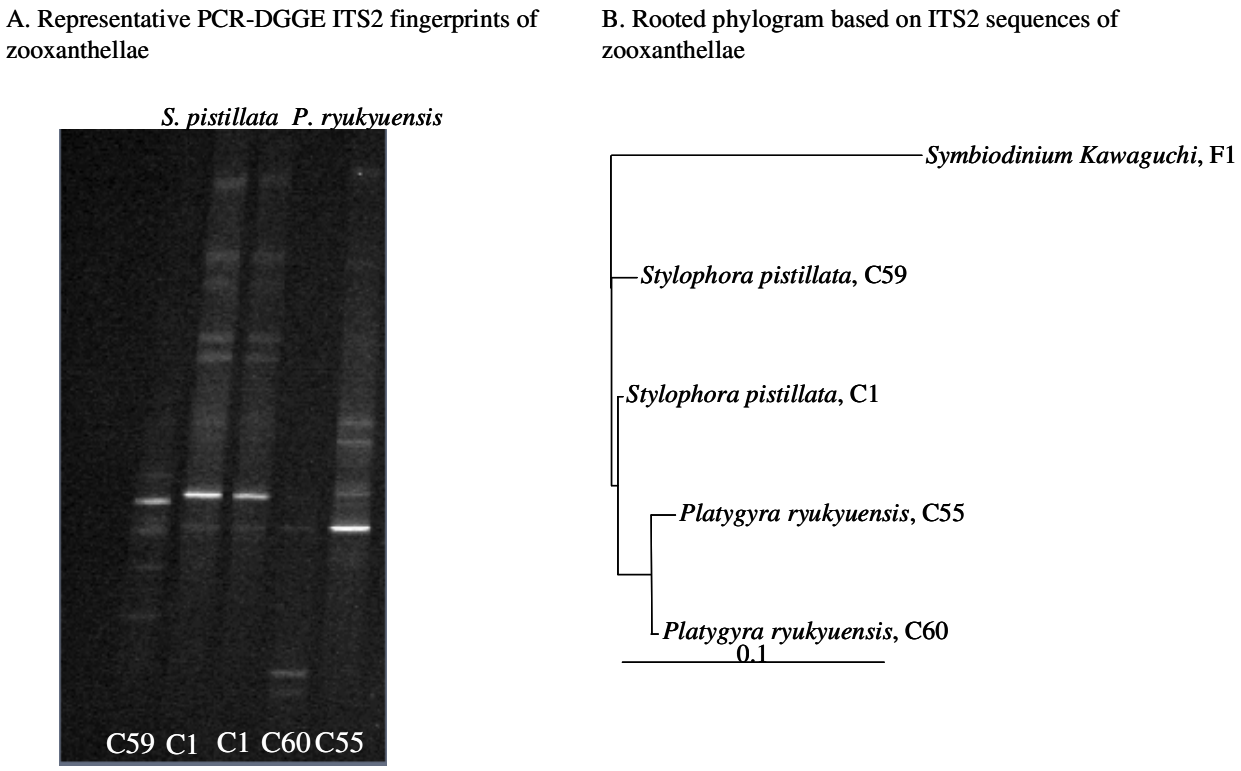

Fig. 3. A. DGGE profiles of representative ITS2 types of *Symbiodinium* of *Stylophora pistillata* and *Platygyra ryukyuensis* . B. Sequence analyses of the four identified distinct ITS2 types of *Symbiodinium* – C59, C1, C60 and C55. *Symbiodinium kawaguchi*, F1 was used as an outgroup to produce a rooted phylogram.

3.3 Changes in non-photochemical quenching (NPQ) under thermal stress

The changes in NPQ of *in hospite Symbiodinium* for the time course experiments are shown in Fig. 4. For the *Symbiodinium* in *P. ryukyuensis*, NPQ increased over the exposed time course both when the data was pooled and when kept separate for each colony (Fig. 4A) though *P. ryukyuensis* colonies 2 and 4 harbored different *Symbiodinium* ITS2 types, that is, C60 and C55, respectively (Fig. 3). The pooled data for *P. ryukyuensis* revealed a significant increase in NPQ only after 6 h exposure to high temperature (Fig. 4A). In *S. pistillata* significant increases in NPQ of *in hospite Symbiodinium* were observed as early as 0.17 h but beyond 0.75 h it seemed to return to normal level (Fig. 4B). However, when the colonies were examined separately, based on the ITS2 types, the NPQ tended to either increase (colony 2 with *Symbiodinium* C59) or decrease (colonies 3 and 4 with *Symbiodinium* C1), especially beyond 0.75 h of exposure (Fig. 4B).

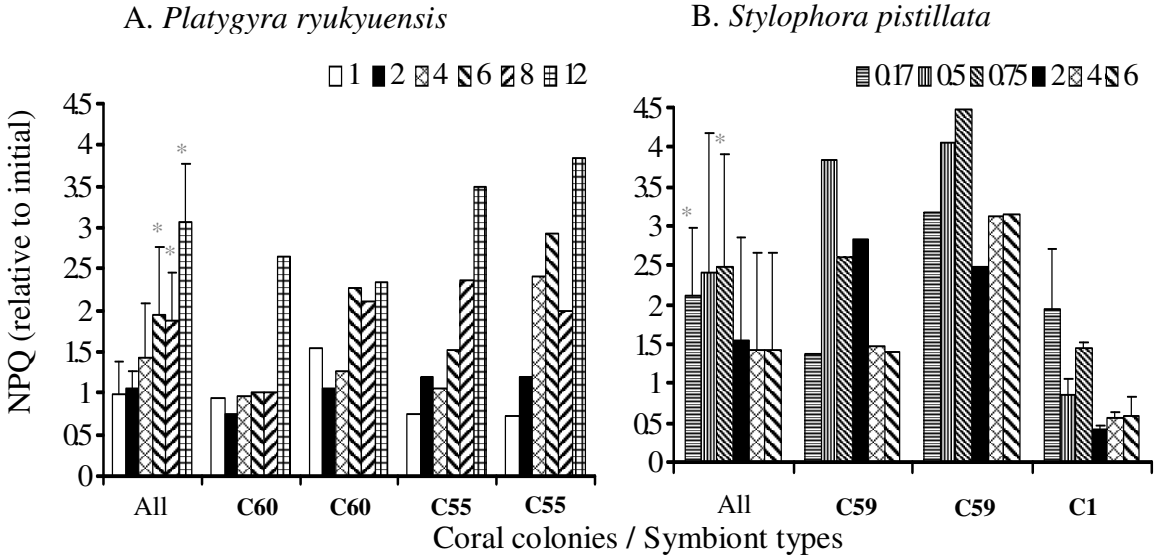

Fig. 4. Non-photochemical quenching (NPQ) of *in hospite Symbiodinium* from time course experiments for *Platygyra ryukyuensis* (A) and *Stylophora pistillata* (B) exposed to 33.5°C under 5 µmol quanta m^{-2} s^{-1} for a time range of 0.17 to 12 hours. Asterisk indicates significant difference (Kruskal-Wallis test) when compared to controls maintained at 26°C under similar light conditions. * $P < 0.05$. All, data pooled for all colonies; C1, C55, C59 and C60 represent ITS2 types of *Symbiodinium*. In case of *Stylophora pistillata*, data was pooled for C1 *Symbiodinium* ITS2 type.

3.3 Changes in non-photochemical quenching (NPQ) under thermal stress

The changes in NPQ of *in hospite Symbiodinium* of the two studied corals in the temperature course experiments are shown in Fig. 5. The data could be pooled for all colonies as they exhibited similar trends in both coral species investigated. In case of *Symbiodinium* of *P. ryukyuensis*, the NPQ increased at all treated temperatures (Fig. 5A) while in the case of those of

S. pistillata the increase was observed at 38 and 40oC treatments (Fig. 5B). Generally, significant increases in NPQ of *in hospite Symbiodinium* were evident in both studied corals.

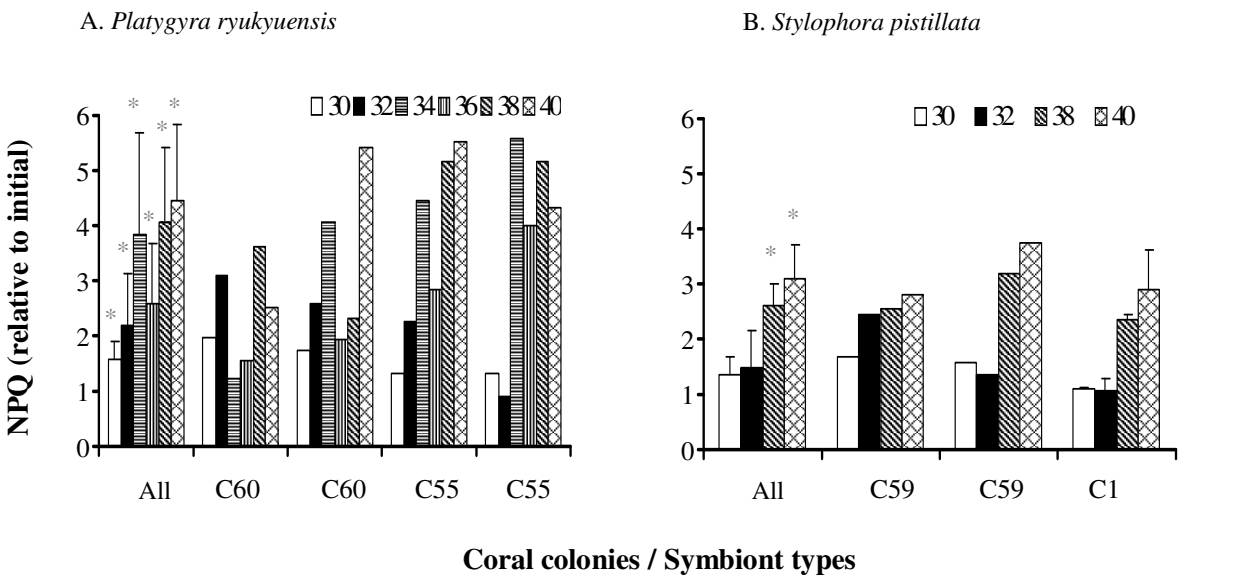

Fig. 5. Non-photochemical quenching (NPQ) of *in hospite Symbiodinium* from temperature course experiments for *Platygyra ryukyuensis* (A) and *Stylophora pistillata* (B) exposed to temperatures varying from 30 to 40°C under 65 µmol quanta m^{-2} s^{-1} for 10 min at each temperature. Asterisk indicates significant difference (Kruskal-Wallis test) when compared to controls maintained at 26°C under similar light conditions for 10 min. * $P < 0.05$. All, data pooled for all colonies; C1, C55, C59 and C60 represent ITS2 types of *Symbiodinium*. In case of *Stylophora pistillata*, data was pooled for C1 *Symbiodinium* ITS2 type.

4. DISCUSSION

This study for the first time experimentally demonstrates that distinct ITS2 types of endosymbiotic dinoflagellates from a bleaching susceptible and a bleaching resistant coral show differential photoinhibition of PSII and electron transport rate in *in hospite Symbiodinium* when exposed to thermal stress. Previous studies (Fitt & Warner, 1995; Warner *et al.* 1996; Bhagooli & Hidaka, 2003, 2004; Bhagooli & Yakovleva, 2004) have also reported differences in photo-physiological responses to thermal stress among several coral species without genetically

characterizing the endosymbiont types. Rowan (2004) documented *Symbiodinium* clade level differences in thermal responses with clade D being more tolerant than clade C members. However, both Tchernov *et al.* (2004) and Sampayo *et al.* (2008) argued that clades might not be representative of thermal tolerance in *Symbiodinium*.

4.1 Photoinhibition of PSII and changes in electron transport rate in *Symbiodinium* under thermal stress

The decline in F_v/F_m in a dark-acclimated sample, which reflects photoinhibition of PSII fluorescence, indicates a decrease in the capability of the reaction centers to convert absorbed light to photochemical energy since F_v/F_m is proportional to the fraction of functional PSII centers (Krause & Weis, 1991). In *Stylophora pistillata*, F_v/F_m decreased after 10 min exposure at an elevated temperature of 33.5°C but the ETR_{max} tended to increase. Similar changes in F_v/F_m and ETR_{max} have been reported in corals based on a diel photosynthetic responses study in the field (Hoegh-Guldberg & Jones, 1999) and this phenomenon was attributed to as an increase in either enzyme activity related to the increased expression or activation state of enzymes, specifically Rubisco, associated with carbon fixation or the Mehler-Ascorbate Peroxidase (MAP) cycle pathway, in which oxygen is the final electron acceptor. This observation is not unexpected as a minimum of 60% of maximum quantum yield of PSII is required to maintain ETR_{max} in *Symbiodinium* of the coral *Pachyseris rugosa* (Bhagooli, 2004; Bhagooli & Hidaka, 2006).

The reaction centers of *Symbiodinium* in *P. ryukyuensis* were not damaged over the 12 h exposure to high temperature suggesting that their PSII reaction center is tolerant to the level of thermal stress used in this study. However, their ETR_{max} exhibited a significant decline after 8 h exposure to elevated temperatures. This could be indicative of damage to the photosynthetic apparatus at sites either other than the PSII or including PS II. Damage to the Calvin-cycle enzymes due to high temperature, as has been reported in higher plants (Feller *et al.* 1998) and *Symbiodinium* (Lesser, 1996), might be a possible explanation for the observed decreases in ETR_{max}. Similarly, significant decline in ETR_{max} has also been observed in the *Symbiodinium* of *Pachyseris rugosa* exposed to thermal stress (Bhagooli, 2004; Bhagooli & Hidaka, 2006).

In the field, massive corals have been shown to take more time to bleach and remain bleached longer than branching coral species (Baird & Marshall, 2002). Loya *et al.* (2001) documented *S. pistillata* to be among the losers and *P. ryukyuensis* among the winners in Okinawa after the bleaching events. Our findings corroborate these field studies in that 50% reduction in ETR_{max} was achieved earlier and at lower temperature in the branching and susceptible coral *S. pistillata* when compared to the massive and resistant coral *P. ryukyuensis*.

4.2 *Symbiodinium* diversity at the ITS2 level

Previous studies have shown that hosts maintain symbioses with *Symbiodinium* spp. from one or more clades (e.g. A, B, C, and others) (Rowan & Knowlton, 1995; Baker & Rowan, 1997). LaJeunesse (2001, 2002) using DGGE reported that *Symbiodinium* are far more diverse and has put forward a finer classification scale (A1, A2, B1, B2, C1, C2, etc.) at the ITS level. Four distinctive *Symbiodinium* ITS2 types (C59, C1, C60 and C55) distinguished by DGGE profiles, were identified from the two host coral species used in this study. Each host species harbored

two different ITS2 types of *Symbiodinium*. Based on ITS2 sequence analyzes, *Stylophora pistillata* and *Platygyra sp.* have so far been identified to harbor clade C *Symbiodinium* (LaJeunesse *et al.* 2004). This study also highlights the variation in thermal responses of the photosynthetic machinery of *Symbiodinium* within clade C.

4.3 Photo-physiological responses to thermal stress and *Symbiodinium* ITS2 types

Although biochemical, physiological, morphological, behavioral and molecular studies have revealed high diversity of *Symbiodinium* (Blank & Trench, 1985; Trench & Blank, 1987; Blank *et al.* 1988; Rowan & Powers, 1991; Iglesias-Prieto & Trench, 1994; Trench, 1997; Banaszak *et al.* 2000; LaJeunesse, 2002), the photo-physiological responses to stress have yet to be correlated with the molecular genetic identity of these algae. The photo-physiological responses to elevated temperature of the *in hospite Symbiodinium* of *S. pistillata* and those of *P. ryukyuensis* indicated that the former are more sensitive than the latter. This is reflected by the two distinct ITS2 groups of *Symbiodinium* found in the two coral species studied. These results support the hypothesis that different *Symbiodinium* ITS2 genotypes might reflect different thermal tolerances.

Tolerances to thermal stress in *Symbiodinium* of different coral species have been attributed to different capacities to dissipate heat in PSII as non-photochemical quenching in PSII (Warner *et al.* 1996). In this study all *Symbiodinium* ITS2 types identified in *P.ryukyuensis* (C60 and C55) exhibited no significant changes in their maximum quantum yield of PSII concomitant with increases in NPQ. However, in *Symbiodinium* of *S. pistillata* decreases in maximum quantum yield of PSII were accompanied by either increase (*Symbiodinium* types C59) or decrease (*Symbiodinium* types C1) in NPQ. These findings are suggestive of *Symbiodinium* harbored by the resistant coral, *P. ryukyuensis*, possessing better heat dissipation capacities than some types residing in the susceptible coral, *S. pistillata*. Buxton *et al.* (2009) linked NPQ response to availability of inorganic carbon (C_i) to *Symbiodinium*. However, in this study it is not possible to conclusively comment on how the coral host is regulating the C_i availability to its *Symbiodinium*.

The present study focused on chlorophyll fluorescence parameters indicative of heat damage to the *Symbiodinium* photosynthetic apparatus in corals with field-observed differential bleaching/mortality susceptibilities rather than mimicking exactly the ecological field conditions. Although a sudden exposure to elevated temperature might be unlikely to happen in the field, a short-term exposure to 33.5^{o}C is not totally unrealistic as sea surface temperature at Sesoko Station, Okinawa witnessed an average temp of 30^{o}C over a month with temperature peaking at 32.5^{o}C during the 1998 and 2001 bleaching events. On the Great Barrier Reefs, temperature as high as 34^{o}C has been reported (Jones *et al.* 1998). Coral specimens in this study were exposed to high temperature similar to Jones *et al.* (1998) and Ralph *et al.* (2001). Further studies as proposed by Fitt *et al.* (2001) are necessary to explore how processes induced by experimental laboratory protocols entailing sudden and large alteration in temperatures could actually relate to the major mechanism of widespread coral bleaching susceptibilities.

5. CONCLUSION

The photo-physiological thermal-stress responses, investigated using chlorophyll *a* fluorescence technique to assess photosynthesis, in the two coral species studied were different, with the endosymbionts of *Stylophora pistillata* being more susceptible than those of *Platygyra ryukyuensis*. The endosymbiont ITS2 typing revealed differences within and between the coral species investigated. The results of the present study suggest that different groups of ITS2 types of *Symbiodinium*, with different tolerances to temperature stresses, may partly explain differential bleaching susceptibility among some hermatypic coral species.

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