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Loss of *Symbiodinium* from bleached soft corals *Sarcophyton ehrenbergi*, *Sinularia* sp. and *Xenia* sp.

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Abstract

The deleterious effects of temperature-induced coral bleaching, a process by which corals lose their endosymbiotic algae (zooxanthellae; genus *Symbiodinium*) primarily at temperatures above mean yearly maximums, has not been well described for alcyonacean soft corals (Coelenterata, Octocorallia). The study of *Symbiodinium* cells lost from *Sarcophyton ehrenbergi*, *Sinularia* sp., and *Xenia* sp., which have not been compared in bleaching studies, indicate that the soft coral *S. ehrenbergi* released the greatest number of symbiont cells, however, it was less susceptible to heat stress surviving temperatures of 34 °C for >39 h. *Sinularia* sp. showed intermediate levels of bleaching tolerance to elevated temperatures, surviving prolonged exposures at 32 °C, but dying within 24 h at 34 °C. *Xenia* sp., however, was the most vulnerable to high heat stress maximally releasing *Symbiodinium* at temperatures ≤30 °C. This evidence indicates that *Xenia* sp. is even more susceptible to elevated temperatures than *Acropora* spp., previously reported to be the most vulnerable coral species to elevated temperature-induced bleaching.

Molecular analysis showed that the more resistant soft coral species (*S. ehrenbergi*) had the same type of *Symbiodinium* (clade C) as less resistant soft corals (*Xenia* sp.). In comparison to scleractinian corals collected from the same region that show similar bleaching resistance to high temperatures (e.g. *Porities solida*—more robust; *Favites complanata*—moderate resistance; *Acropora hyacinthus*—less robust), all scleractinian corals were symbiotic with *Symbiodinium* from clade C. *A. hyacinthus*, however, was found to possess multiple symbionts (clades B and C), and this represents a first report of Clade B in any *Acropora* species.

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Keywords: Alcyonacea; Bleaching; Octocorallia; Soft corals; *Symbiodinium*; Zooxanthellae

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1. Introduction

Coral bleaching, a process by which corals lose their symbiotic algae called *Symbiodinium*, is primarily a heat-stress response to water temperatures above their maximum tolerance levels (Brown, 1990). Studies indicate that *Symbiodinium* associated with scleractinian corals exposed to elevated temperatures $>30\text{ }^{\circ}\text{C}$ (or above their mean maximum seasonal temperature) may also have reduced chlorophyll concentrations (Warner et al., 1996; Jones et al., 1998) and/or reduced densities (Brown and Ogden, 1993; Salih et al., 1997). Most scleractinian corals, however, appear to be unaffected by short-term exposures to temperatures below $30\text{ }^{\circ}\text{C}$ and probably acclimate to the heat stress (i.e. thermal acclimatization). Fitt and Warner (1995) found that *Montastrea annularis* and *M. cavernosa* did not bleach at $30\text{ }^{\circ}\text{C}$. Warner et al. (1996) also found that *M. annularis*, *Agaricia lamarki*, *A. agaricites*, and *Siderastrea radians* exposed to temperatures $\leq 30\text{ }^{\circ}\text{C}$ were not adversely affected if the duration was relatively short. Further, different temperature requirements for the growth of different *Symbiodinium* have been proposed. Fitt et al. (2000), who monitored growth differences between various *Symbiodinium* species, observed that some zooxanthellae grew better between 26 and $32\text{ }^{\circ}\text{C}$ while others did not grow well above $26\text{ }^{\circ}\text{C}$. Kinzie et al. (2001) also describes growth variations between different *Symbiodinium* isolates at 27 , 29 , and $31\text{ }^{\circ}\text{C}$, where some symbionts display poor growth rates with increasing temperature, some show no change of growth rate regardless of temperature, and some depict higher growth rates at $31\text{ }^{\circ}\text{C}$. Warner et al. (1996) suggested that short-term tolerance at $30\text{ }^{\circ}\text{C}$ does not rule out the possibility that prolonged exposures over 2 to 3 weeks may reduce coral health. In many countries, few corals survive prolonged exposure to water temperatures at $\geq 32\text{ }^{\circ}\text{C}$ suggesting that $32\text{ }^{\circ}\text{C}$ is the highest tolerable temperature for most corals (Jokiel and Coles, 1990; Iglesias-Prieto et al., 1992; Berkelmans and Willis, 1999); exceptions, for example, include corals of the Persian Gulf exposed to temperatures of 36 to $40\text{ }^{\circ}\text{C}$ (Kinsman, 1964). Bleaching is not confined to scleractinian corals, however, and has also been observed in sea anemones, zoanthids, sponges, foraminifera, tridac-

nids and soft corals (Gomez and Mingo-Lucuanan, 1998; Fromont and Garson, 1999).

Information regarding temperature-induced bleaching of alcyonacean soft corals is very limited. Wilkinson's (1998) report of bleaching events help to identify some of the soft corals affected by bleaching. These include *Sinularia* spp., which bleach at temperatures between 30 and $34\text{ }^{\circ}\text{C}$ (observations from Maldives, Sri Lanka, Indonesia, Philippines, and Australia) and *Sarcophyton* spp., which were not affected at $30\text{ }^{\circ}\text{C}$, but showed symptoms of bleaching at $32\text{ }^{\circ}\text{C}$. Further, soft corals located near Sri Lanka bleached much less than scleractinian corals, which reached levels of 80% bleached, indicating that some soft corals may tolerate heat-induced bleaching better than some scleractinian corals. Observations of soft corals bleaching from various reefs world-wide (e.g. *Sarcophyton* spp., *Sinularia* spp., *Dendronephthya* spp. and *Lobophyton* spp.; see Obura et al., 2000; Rajasuriya et al., 2000a,b; Smith et al., 2000; Wilkinson, 2000) that display symptoms of bleaching are reported to recover quickly, rapidly colonizing local reefs, as opposed to scleractinian corals, which appear to recover slowly. Marshall and Baird (2000), however, observed bleaching of *Acropora* spp. (severe), *Sinularia* spp. (high), *Sarcophyton* spp., *Favites* spp. and *Porites* spp. (moderate) at water temperatures $>31\text{ }^{\circ}\text{C}$ and a slow recovery.

On the Australian Great Barrier Reef (GBR), soft coral abundance and diversity can equal or exceed that of scleractinian corals (Dinesen, 1983; Alderslade and Shirwaiker, 1991). *Sinularia* spp. and *Sarcophyton* spp., for example, cover 42 and 55% (respectively) of 14 mid- and outer-shelf reefs on the GBR (Fabricius, 1995). Despite reports of high abundances, there is a lack of information describing soft coral bleaching, which suggests that the effect of heat stress on reef organisms other than scleractinian corals (e.g. soft corals) may be underestimated. *Xenia* spp., for example, is commonly found on many reefs and can be the most abundant soft coral species (Mergner and Schuhmacher, 1981; Dinesen, 1983; Benayahu, 1985), however, there are no descriptions of bleaching in this genus.

In order to better understand the bleaching process in alcyonacean soft corals, we compared temperature sensitivities of dominant soft coral species in the GBR located in central Queensland,

Australia. Our primary aim was to determine if soft corals resisted elevated temperature stress (bleaching) better than scleractinian corals. In addition, some authors suggest that different *Symbiodinium* have different temperature susceptibilities (Fitt et al., 2000; Kinzie et al., 2001), which may contribute to better host resistance to bleaching. Thus, we attempted to determine if stress resistance was an attribute of the host or the specific type of *Symbiodinium* clade (i.e. A, B or C) associated with the host.

The designation of clades A, B, C (e.g. Rowan and Powers, 1991; Lobban et al., 2002), D (Baker, 1999), E (Toller et al., 2001a,b), F (La Jeunesse, 2001) and G (Pochon et al., 2001) indicate that as many as seven clades exist in organisms that harbour *Symbiodinium*. Here, we chose not to focus on molecular evolution; we did not examine clades (D–G), but rather, focused on the well described 18S rDNA clades A, B and C, consisting of >1500 nucleotides commonly used from scleractinian corals as a method to generalize *Symbiodinium* clade associations amongst bleaching coral in this study. We did not examine multiple sub-clade designations of *Symbiodinium* associated with coral since that finite degree of resolution requires the use of other methodologies (e.g. ITS analysis).

2. Materials and methods

2.1. Bleaching—the coral host

2.1.1. Coral collection, storage and acclimation

Sarcophyton ehrenbergi (von Marenzeller, 1886), *Simularia* sp. (Alcyoniidae, Lamouroux, 1816) and *Xenia* sp. (Xeniidae; Sprung and Delbeek, 1997) were collected at 7–10 m depths from the fringing reefs Barren Island (23°10'S, 151°55'E) and Outer Rocks (23°4.3'S, 151°57.2'E), located on the GBR, along the east coast of the central Queensland, Australia. All corals were maintained at the Central Queensland University laboratory (as described previously Strychar et al., 2004b).

2.1.2. Experimental design and collection of *Symbiodinium* cells

Corals whose wet weight (W_w) was ~100 g were subjected to filtered sea water (FSW) at 30, 32 or 34

°C and compared to a control at 28 °C. Corals were stressed via elevated temperatures over 48 h using the experimental design described in Strychar et al. (2004a).

Symbiodinium lost from the corals were sampled using a non-invasive method by collecting 250 mL from the excurrent pipe of each incubation chamber every 3 h over a 48 h time period (see Strychar et al., 2004a). We chose this non-invasive method since homogenization of host tissues (see Suharsono and Brown, 1992) used to collect *Symbiodinium* have been reported to yield inaccurate mitotic measurements. Further, Strychar (2002) also speculated that the invasive method using water piks to extract symbionts from host tissue may contribute to false-positives of live and dead *Symbiodinium* cells, triggering different processes of cell death (e.g. apoptosis and necrosis; see Dunn et al., 2002; Franklin et al., 2004; Strychar et al., 2004b).

Briefly, the collected sample in this study was centrifuged at $700\times g$ for 5 min at room temperature (R_t). A 1 mL aliquot of supernatant was removed and the remaining supernatant discarded. The pellet containing *Symbiodinium* cells was then resuspended in the 1 mL aliquot and centrifuged at $700\times g$ for 5 min at R_t . Finally, 800 μ L of the supernatant was removed and 400 μ L of 0.2% trypan blue (TB) was added to the remaining 200 μ L. The microfuge tubes (M_T) were then gently vortexed for 10 sec and incubated at R_t for 5 min to allow absorption of TB into the dead cells. Replicate counts ($n=10$) of live, dead and mitotic *Symbiodinium* cells, using a light microscope and a haemo-cytometer, were characterized as: 1) live cells not mitotic (did not stain with TB); 2) dead cells not mitotic (stained blue with TB) and; 3) mitotic cells. As a positive control for the TB staining of live vs. dead cells, transmission electron microscopy (TEM) was used (Fig. 1); Dunn et al. (2002) and Franklin et al. (2004), who used a variety of other tests to examine *Symbiodinium* cells from bleached cnidarians, also used TEM to confirm the presence or absences of symbiont cell death. Mitotic cells, recognized by the presence of paired nuclei or two mitotic figures per cell (Brown and Zamani, 1992), included both live and dead cells since it was not known if these cells died in situ or upon exposure to the environment outside of the host.

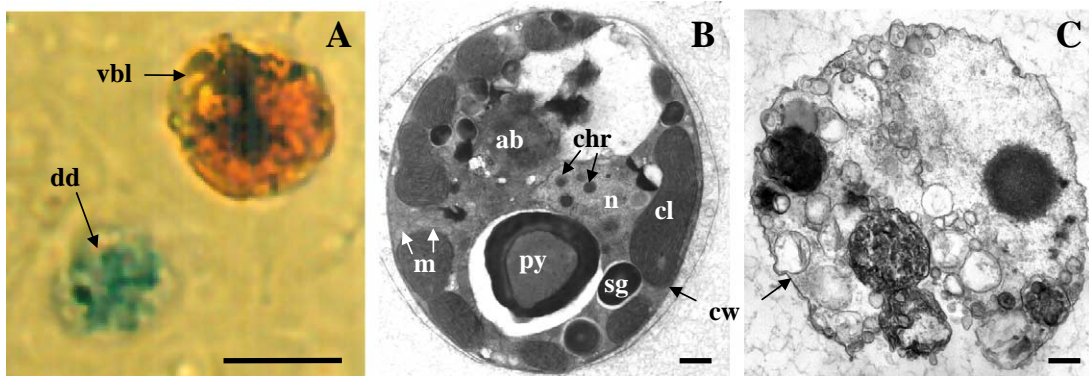


Fig. 1. Light micrograph (A) of viable and dead *Symbiodinium* cells using trypan blue (TB) staining methods when viewed under a light microscope. Legend: dd=dead cell, vbl=viable cell. Transmission electron micrographs (TEM) of viable (B) and dead (C) *Symbiodinium* cells. Legend: ab=accumulation body, chr=chromosome, cl=chloroplast, cw=cell wall, m=mitochondria, n=nucleus, py=pyrenoid, sg=starch grain. Scale bars: A=10 μm , B=1 μm , C=500 nm.

2.1.3. Indices of live, dead, and mitotic *Symbiodinium* cells

At the beginning of each experimental period, the number of in situ live, dead and mitotic *Symbiodinium* cells at 28 °C was determined for each replicate coral (Table 1). Standing stock was measured as the total number (live+dead+mitotic) of *Symbiodinium* per unit volume of extracted coral tissue (1 g in 1 mL FSW).

Cell state indices were graphed through time using both instantaneous and cumulative data. The former depict the host-specific time-course for *Symbiodinium*; the latter reveal host-specific patterns of temperature susceptibility. The latter illustrates how temperature affected the overall depletion of a coral's *Symbiodinium* complement over a 48 h period.

Rates of loss of *Symbiodinium* standing stock cells (live, dead and mitotic) were measured for each host at each temperature over 48 h. The specific expulsion rate (S_{ER} day⁻¹; Eq. (1); Hoegh-Guldberg et al., 1987), where N is the standing stock of *Symbiodinium*

at 28 °C and ΔE is the total number of *Symbiodinium* lost over 2 days (Δt), was determined at each temperature. Calculations of in situ specific growth rate (S_{GR} day⁻¹; Eq. (2)) were based on the fraction of cells in mitosis and the duration of the division (Hoegh-Guldberg et al., 1987). This helped to establish which symbionts from each host could maintain high in situ growth rates to compensate for increased expulsion rates associated with elevated temperatures, where t_d is the duration of *Symbiodinium* cytokinesis day⁻¹ and f is the fraction of mitotic cells (Strychar et al., 2004a). Mitotic index (MI%; Eq. (3)) is the percentage of observed *Symbiodinium* cells in situ undergoing mitosis (Wilkinson et al., 1983). The number of mitotic (MN) *Symbiodinium* cells (live+dead) in situ after 48 h at each temperature provides an estimate of the effect of temperature on symbiont cell division. The 'total' number of *Symbiodinium* cells at 28 °C (live+dead) was used as a best estimate of the maximum number of cells immediately before elevated heat stresses (Strychar et al., 2004a). Thus, the rate of expulsion in Eq. (1) and the rate of growth in Eq. (2) are both relative to the total number of cells present at the beginning of the experiment. The $S_{\text{ER}}/S_{\text{GR}}$ ratio (Eq. (4)) determines if the loss of *Symbiodinium* can be compensated by increased symbiont growth rates (Hoegh-Guldberg, 1989).

$$S_{\text{ER}} = (1/N) \times (\Delta E/\Delta t) \quad (1)$$

$$S_{\text{GR}} = (1/t_d) \times \ln(1 + f) \quad (2)$$

Table 1

A summary of the standing stock means g⁻¹, total *Symbiodinium* cells (live+dead+mitotic) lost from each coral host day⁻¹, and the percentage of standing stock expelled day⁻¹ at the control temperature 28 °C

Host	Standing stock ($\times 10^7$)	Expulsion (loss) (cells day ⁻¹ $\times 10^5$)	Expulsion (%) day ⁻¹
<i>Sarcophyton ehrenbergi</i>	13.03	3	0.23
<i>Simularia</i> sp.	4.34	3	0.69
<i>Xenia</i> sp.	5.49	0.1	0.02

$$MI\% = (MN/\text{total}) \times 100 \quad (3)$$

$$S_{ER}/S_{GR}(\%) = \frac{\text{Specific expulsion rate } (S_{ER})}{\text{Specific growth rate } (S_{GR})} \times 100 \quad (4)$$

2.1.4. Statistical analyses

Data were checked for normality and heteroscedasticity of sample variances (Zar, 2000). Heteroscedasticity data were log-transformed prior to analysis to normalize the data. Data were analysed by standard parametric tests using model I ANOVAs, Tukey's a-posteriori tests, multiple comparisons tests, and linear regression analyses. Only significant results are discussed.

2.2. Bleaching—the *Symbiodinium* gene

2.2.1. Extraction, amplification and sequencing of 18S rDNA

Symbiodinium were obtained from the soft corals by sectioning ~100 mg of capitulum and stalk tissue ~1 mm below the surface. *Symbiodinium* from scleractinian corals collected from the same location, for

comparative purposes, were processed by pulverizing a 100 mg tissue sample and sampling the supernatant. Each tissue type was homogenized in separate 1.5 mL M_T containing 1 mL isolation buffer [2% CTAB, 1.4 M NaCl, 100 mM Tris-HCl (pH 8.0), 20 mM EDTA, 1% PVP-40 and 0.2% β -mercaptoethanol; Strychar (2002)]. It was then heated at 60 °C for 30 min, followed by incubation at 39 °C in the presence of proteinase K (0.5 mg ml⁻¹ final conc.). The slurry was then centrifuged for 3 min at 5000×g and the supernatant transferred to new 2 mL M_T . The lysate was then extracted 3× with chloroform and isoamyl-alcohol (24:1), incubated for 3 h in ice-cold isopropanol and centrifuged for ~7 min at 15,400×g. The DNA pellet was washed 3× with 1 mL of 70% ethanol and finally resuspended in 50 μ L of Milli-Q water.

Symbiodinium 18S rDNA genes were amplified using a Corbett FTS 960 thermal cycler using *Symbiodinium* specific primers (SS5-Fuz, SS3-Ruz; Table 2; Strychar, 2002). Each PCR contained PCR buffer (Amersham Pharmacia Biotech), dNTPs (200 nmol), 1 nmol of each primer, 1 unit of *Taq* DNA polymerase (GibcoBRL Life Technologies), and 50–100 ng of DNA template, in a final volume of 50 μ L (see Strychar, 2002). The PCR products were charac-

Table 2
Primers used to amplify and sequence zooxanthellae 18S SSU rDNA from coral hosts

Primer	Primer sequence (5'–3')	Primer site ^a	Citation
SS5-FU _Z	GGTTGATCCTGCCAGTAGTCATATGCTTG	1–29	Rowan and Powers, 1992
ST7 Plasmid Primer	TAATACGACTCACTATAGGG	–	GIBCO, BRL manufactures
SS5-F _Z	Equal mixture of oligonucleotides (5'-AGTTATAATTTATTTGATGGTCACTGCTAC-3' and 5'-GCAGTTATAGTTTATTTGATGGTTGCTGCTAC-3')	100–132	Rowan and Powers, 1992
373-F _Z	GATCCGGAGAGGGAGCCTGA	372–392	Weekers et al., 1994
570-F _Z	GTAATTCAGCTCCAATAGC	570–589	Weekers et al., 1994
GH690-F _Z	YAGAGGTGAAATTCT	892–906	Hinkle, pers. comm., 1999
GH958-F _Z	AATTTGACTCAACACGGG	1178–1195	Hinkle, pers. comm., 1999
1200-F _Z	CAGGTCTGTGATGCCC	1422–1438	Weekers et al., 1994
SP6 Plasmid Primer	TATTTAGGTGACACTATAG	–	GIBCO, BRL manufactures
373-R _Z	TCAGGCTCCCTCTCCGGAATC	372–392	Weekers et al., 1994
570-R _Z	GCTATTGGAGCTGGAATTAC	570–589	Weekers et al., 1994
GH690-R _Z	AGAATTTACCTCTG	892–906	Hinkle, pers. comm., 1999
W1173-R _Z	GTGCCCTCCGTCAT	1138–1153	Weekers et al., 1994
1200-R _Z	GGGCATCACAGACCTG	1623–1638	Hinkle, pers. comm., 1999
SS3-R _Z	AGCACTGCGTCAGTCCGAATAATTCACCGG	1664–1693	Rowan and Powers, 1991
SS3-RU _Z	GATCCTTCCGCAGGTTACCTACGGAAACC	1739–1769	Rowan and Powers, 1991

Primers are listed as: "FU_Z" Forward *Symbiodinium* Universal Primer; "F_Z" Forward *Symbiodinium* specific primer; "RU_Z" Reverse *Symbiodinium* Universal Primer; "R_Z" Reverse *Symbiodinium* specific primer.

^a Primers were located according to the DNA sequence of the 18S rRNA gene from *Symbiodinium microadriaticum* (Rowan and Powers, 1992).

Table 3

List of 18S SSU rRNA gene sequences *Symbiodinium* spp. taxa used in this study consisting of >1500 nucleotides

Accession #	Symbiont	Host	Strain	Origin	Nucleotides (bp)	Citation
<i>Scleractinia</i>						
AJ271761	<i>Symbiodinium</i> sp.	<i>Fungia paumotensis</i>	FP 2	Tahiti	1574	Darius et al., 1998
AJ271760	<i>S. sp.</i>	<i>F. paumotensis</i>	FP 3	Tahiti	1574	Darius et al., 1998
AJ271762	<i>S. sp.</i>	<i>F. paumotensis</i>	FP 7	Tahiti	1574	Darius et al., 1998
AJ271763	<i>S. sp.</i>	<i>F. paumotensis</i>	FP 8	Tahiti	1574	Darius et al., 1998
AJ271765	<i>S. sp.</i>	<i>F. scutaria</i>	FS 1	Tahiti	1574	Darius et al., 1998
AJ271766	<i>S. sp.</i>	<i>F. scutaria</i>	FS 2	Tahiti	1573	Darius et al., 1998
AJ271764	<i>S. sp.</i>	<i>F. scutaria</i>	FS 8	Tahiti	1574	Darius et al., 1998
AJ271770	<i>S. sp.</i>	<i>Montastraea curta</i>	MC 1	Tahiti	1574	Darius et al., 1998
AJ271771	<i>S. sp.</i>	<i>M. curta</i>	MC 2	Tahiti	1574	Darius et al., 1998
AJ271772	<i>S. sp.</i>	<i>M. curta</i>	MC 6	Tahiti	1574	Darius et al., 1998
M88509	<i>S. sp.</i>	<i>Aiptasia pulchella</i>	SYM18SRRN	Hawaii	1792	Rowan and Powers, 1992
AJ271754	<i>S. sp.</i>	<i>Acropora formosa</i>	AF 5	Tahiti	1572	Darius et al., 1998
AJ271481	<i>S. sp.</i>	<i>A. formosa</i>	AF 6	Tahiti	1569	Darius et al., 1998
AF290918	<i>S. sp.</i>	<i>A. hyacinthus</i>	Acro 21	Australia	1594	This Study
AY937258	<i>S. sp.</i>	<i>A. hyacinthus</i>	Acro 33	Australia	1590	This Study
AJ271767	<i>S. sp.</i>	<i>Leptastrea transversa</i>	LT 2	Tahiti	1576	Darius et al., 1998
AJ271768	<i>S. sp.</i>	<i>L. transversa</i>	LT 3	Tahiti	1574	Darius et al., 1998
AJ271769	<i>S. sp.</i>	<i>L. transversa</i>	LT 4	Tahiti	1375	Darius et al., 1998
AJ271776	<i>S. sp.</i>	<i>Pocillopora verrucosa</i>	PV 2	Tahiti	1572	Darius et al., 1998
AJ271777	<i>S. sp.</i>	<i>P. verrucosa</i>	PV 9	Tahiti	1573	Darius et al., 1998
AJ271773	<i>S. sp.</i>	<i>Pavona cactus</i>	PC 3	Tahiti	1574	Darius et al., 1998
AJ271774	<i>S. sp.</i>	<i>P. cactus</i>	PC 5	Tahiti	1575	Darius et al., 1998
AJ271775	<i>S. sp.</i>	<i>P. cactus</i>	PC 9	Tahiti	1574	Darius et al., 1998
AJ271755	<i>S. sp.</i>	<i>Galaxea fascicularis</i>	GF 4	Tahiti	1572	Darius et al., 1998
AJ271756	<i>S. sp.</i>	<i>G. fascicularis</i>	GF 6	Tahiti	1572	Darius et al., 1998
AJ271757	<i>S. sp.</i>	<i>G. fascicularis</i>	GF 14	Tahiti	1572	Darius et al., 1998
AJ271758	<i>S. sp.</i>	<i>G. fascicularis</i>	GF 32	Tahiti	1572	Darius et al., 1998
AJ271759	<i>S. sp.</i>	<i>G. fascicularis</i>	GF 46	Tahiti	1572	Darius et al., 1998
L13718	<i>S. meandrinae</i>	<i>Meandrina meandrites</i>	SYMRGSSA strain 130	Jamaica	1796	McNally et al., 1994
X62650	<i>S. pilosum</i>	<i>Zoanthus sociatus</i>	–	Jamaica	1797	Sadler et al., 1992
AB016724	<i>S. sp.</i>	<i>Zoanthus</i> sp.	JCUZS-1	Australia	1792	Carlos et al., 1999
AB016594	<i>S. sp.</i>	<i>Montipora verrucosa</i>	CSIRO156	Hawaii	1792	Carlos et al., 1999
AF290917	<i>S. sp.</i>	<i>Porites solida</i>	BC	Australia	1594	This Study
AF260260	<i>S. sp.</i>	<i>Favites complanata</i>	MMFV	Australia	1594	This Study
<i>Alcyonacea</i>						
AB016723	<i>S. sp.</i>	<i>Sarcophyton glaucum</i>	JCUSG-1	Australia	1790	Carlos et al., 1999
AF271291	<i>S. sp.</i>	<i>S. ehrenbergi</i>	Sarco-1	Australia	1573	This Study
AB030646	<i>S. sp.</i>	<i>Sinularia lochmodes</i>	–	Japan	1595	Jimbo et al. (unpubl)*
AF255737	<i>S. sp.</i>	<i>Sinularia</i> sp.	Sin 11	Australia	1596	This Study
AF271292	<i>S. sp.</i>	<i>Xenia</i> sp.	Xen 02	Australia	1602	This Study
<i>Bivalvia</i>						
AB016538	<i>S. sp.</i>	<i>Corculum cardissa</i>	PLCC-1	Palau	1790	Carlos et al., 1999
AB016573	<i>S. sp.</i>	<i>C. monstrosum</i>	PLCM-1	Palau	1790	Carlos et al., 1999
AB016539	<i>S. sp.</i>	<i>Fragum unedo</i>	Cloned DNA	Palau	1726	Carlos et al., 1999
AB016574	<i>S. sp.</i>	<i>F. unedo</i>	PLFU-1	Palau	1790	Carlos et al., 1999
AB016580	<i>S. sp.</i>	<i>F. fragum</i>	Cloned DNA	Palau	1787	Carlos et al., 1999
AB016575	<i>S. sp.</i>	<i>Tridacna squamosa</i>	PLTS-1	Palau	1790	Carlos et al., 1999

Table 3 (continued)

Accession #	Symbiont	Host	Strain	Origin	Nucleotides (bp)	Citation
AB016576	<i>S. sp.</i>	<i>T. maxima</i>	PLTM-1	Palau	1790	Carlos et al., 1999
AB016577	<i>S. sp.</i>	<i>T. derasa</i>	PLTD-1	Palau	1790	Carlos et al., 1999
AB016581	<i>S. sp.</i>	<i>T. gigas</i>	CSIRO163	–	1790	Carlos et al., 1999
AB016593	<i>S. sp.</i>	<i>T. derasa</i>	CSIRO161	–	1790	Carlos et al., 1999
AB016595	<i>S. sp.</i>	<i>T. gigas</i>	Cloned DNA	–	1792	Carlos et al., 1999
AB016597	<i>S. sp.</i>	<i>T. crocea</i>	PHQU TCK	Philippines	1790	Carlos et al., 1999
AB016596	<i>S. sp.</i>	<i>Hippopus hippopus</i>	PHSC HH1B	Philippines	1792	Carlos et al., 1999
<i>Foraminifera</i>						
AB016579	<i>S. sp.</i>	<i>Amphisorus hemprichii</i>	PO82-2	Palau	1790	Carlos et al., 1999
U10892	<i>S. sp.</i>	<i>A. hemprichii</i>	–	Red Sea	1788	Lee et al., 1995
AF182822	<i>S. sp.</i>	<i>Sorites orbiculus</i>	–	Polynesia	1783	Langer and Lipps, 1994
U10893	<i>S. sp.</i>	<i>Marginopora kudakajimensis</i>	–	Japan	1800	Lee et al., 1995
U52911	<i>S. sp.</i>	<i>Colonial radiolarian</i>	BBSR 159	Bermuda	1802	Gast and Caron, 1996
U52352	<i>S. sp.</i>	<i>Colonial radiolarian</i>	BBSR 153	Bermuda	1802	Gast and Caron, 1996
U52353	<i>S. sp.</i>	<i>Colonial radiolarian</i>	BBSR 332	Bermuda	1802	Gast and Caron, 1996
U52354	<i>S. sp.</i>	<i>Collozoum caudatum</i>	–	Bermuda	1802	Gast and Caron, 1996
U52355	<i>S. sp.</i>	<i>Spongostaurus sp.</i>	–	Bermuda	1802	Gast and Caron, 1996
U52356	<i>S. sp.</i>	<i>Thalassicolla nucleata</i>	BBSR 323	Bermuda	1802	Gast and Caron, 1996
<i>Porifera</i>						
AB016578	<i>S. sp.</i>	<i>Haliclona koremella</i>	PSP1-05	Palau	1792	Carlos et al., 1999
<i>Hydrozoa</i>						
M88521	<i>S. microadriaticum</i>	<i>Cassiopea xamachana</i>	–	Jamaica	1790	Rowan and Powers, 1992
AB016722	<i>S. sp.</i>	<i>Cassiopea sp.</i>	JCUCS-1	Australia	1792	Carlos et al., 1999
AF225965	<i>Symbiodinium californium</i>	<i>Anthopleura elegantissima</i>	–	California	1777	LaJeunesse and Trench, 2000
<i>Other</i>						
L13717	<i>S. corcolorum</i>	–	Strain 350	Palau	1793	McNally et al., 1994
AB016572	<i>S. sp.</i>	Free Living (Subtidal Sands)	HA3-5	Hawaii	1790	Carlos et al., 1999
GSU41086	<i>Gymnodinium simplex</i>	–	CCMP 419	Bermuda	1797	Gast and Caron, 1996
AF077055	<i>Pfiesteria piscicidia</i>	Cultured	–	Cultured	1800	Oldach et al., 2000
Y16233	<i>Prorocentrum panamensis</i>	–	–	Japan	1790	Grzebyk et al., 1998

Accession numbers are related to GenBank coding. Corals analysed in this study are given in bold. Some authors (*) listed under 'Citation' submitted gene sequences to GenBank which may not be published.

terised by electrophoresis on 1% agarose gels. Sequencing was performed using an ABI Prism Big Dye Terminator Cycle sequencing kit with AmpliTaq DNA polymerase FS following the manufacturer's recommendations (sequencing primers; Table 2).

2.2.2. Molecular statistical analysis

Alignments of *Symbiodinium* 18S rDNA sequences were done using a multiple alignment program called CLUSTAL W (Thompson et al., 1994). Data

sets were then constructed using aligned nucleotides based on sequences with a minimum length of 1500 nucleotides (Table 3). Maximum likelihood (ML) phylogenetic analyses were performed using parsimony (PAUP v4.0b4a; Swofford, 1998), the quartet-puzzling algorithm, 1000 puzzling steps and the Felsenstein 2-parameter model variant for unequal base frequencies. Statistical support for ML methods was estimated from 1000 bootstrap replicate sampled data sets (Felsenstein, 1985).

3. Results

3.1. Light and transmission electron microscopy (TEM) of live and dead *Symbiodinium* cells

Viable *Symbiodinium* cells visible with light microscopy did not absorb TB when stained (Fig. 1A) and appeared to possess evenly distributed protoplasts, however, the nuclei were difficult to detect. Dead symbiont cells observed via light microscopy stained blue in the presence of TB (Fig. 1A). Generally, however, dead cells did not have recognizable organelles, or they possessed intact cell membranes with fragmented cytoplasmic organelles.

Viable symbiont cells observed with TEM were smoothly coccoid in shape with distinct cellular components such as multi-lobed elongated chloroplasts with distinct lamellae, usually a spherical nucleus with well-developed chromosomes revealing the fibrillar material, well-developed chloroplasts, and pyrenoid bodies (Fig. 1B). Analyses of live *Symbiodinium* cells using TEM in this study resembled ‘normal’ symbiont cells described by Kevin et al. (1969). Dead *Symbiodinium* cells, however, had very few organelles that were recognizable (Fig. 1C). Dead symbiont cells possessed numerous ruptures of the cell membrane and cytoplasmic organelles that appeared ruptured.

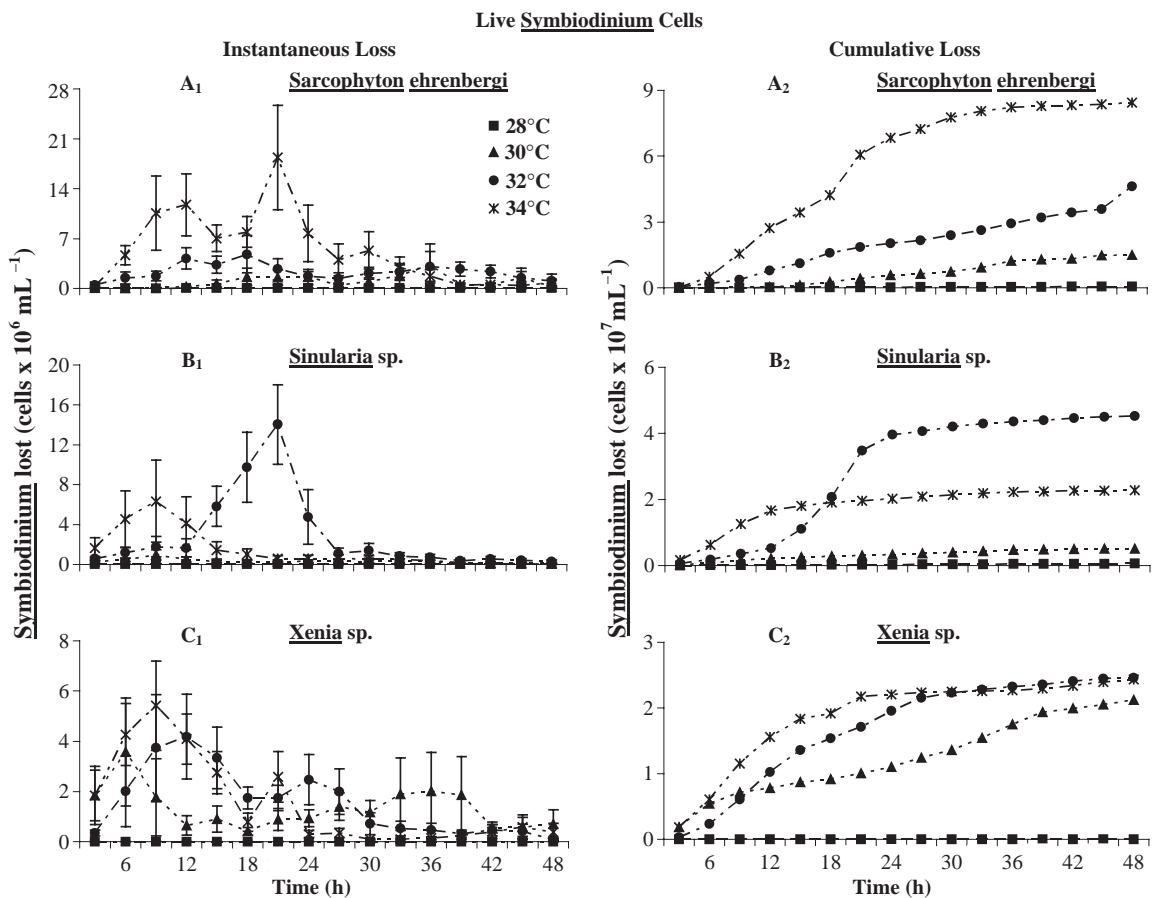


Fig. 2. Instantaneous (1) vs. cumulative (2) loss of live *Symbiodinium* in soft corals from *Sarcophyton ehrenbergi* (A₁, A₂), *Sinularia* sp. (B₁, B₂) and *Xenia* sp. (C₁, C₂) at 30 °C (▲), 32 °C (●) and 34 °C (*) sampled every 3 h over a 48 h time period. Control temperature=28 °C (■). Mean±S.E.M. shown; n_i=10. Comparisons of temporal *Symbiodinium* cells lost (×10⁶ mL⁻¹; A₁, B₁ and C₁) vs. cumulative *Symbiodinium* cells lost (×10⁷ mL⁻¹; A₂, B₂ and C₂) are shown. Note how cumulative graphs (A₂, B₂ and C₂) illustrate the effect of temperature on overall depletion of a coral's *Symbiodinium* complement over 48 h.

3.2. Bleaching—the coral host

3.2.1. Instantaneous loss of live, dead and mitotic *Symbiodinium* cells through time

Corals used in this study lost relatively few numbers of live, dead and mitotic *Symbiodinium* cells at 28 °C (Table 1). Regarding *Symbiodinium* cell states in situ at 28 °C, *S. ehrenbergi* had significantly different numbers of live ($p<0.001$), mitotic ($p<0.01$) and standing stock ($p<0.01$) symbiont cells compared to those in the other corals at 28 °C.

The number of live *Symbiodinium* cells lost from these corals at 28 or 30 °C over 48 h was low (Fig.

2A₁–C₁). Losses of live *Symbiodinium* cells from *Xenia* sp. at 30 °C occurred within 6 h and between 33 and 39 h (Fig. 2C₁). Further temperature increases from 30 to 32 °C resulted in a greater loss of *Symbiodinium* in *Xenia* sp., exhibiting a bimodal distribution through time, peaking at 12 and 24 h (Fig. 2C₁). Live symbiont cells lost from *Sinularia* sp. at 32 °C did not occur until 21 h elapsed (Fig. 2B₁), but, at 34 °C, it occurred within 9 h (Fig. 2B₁). *S. ehrenbergi* (Fig. 2A₁) and *Xenia* sp. (Fig. 2C₁) showed a bimodal loss of live symbiont cells at 34 °C. The first peak loss of live symbiont cells from *S. ehrenbergi* occurred at 9–12 h, followed by a larger

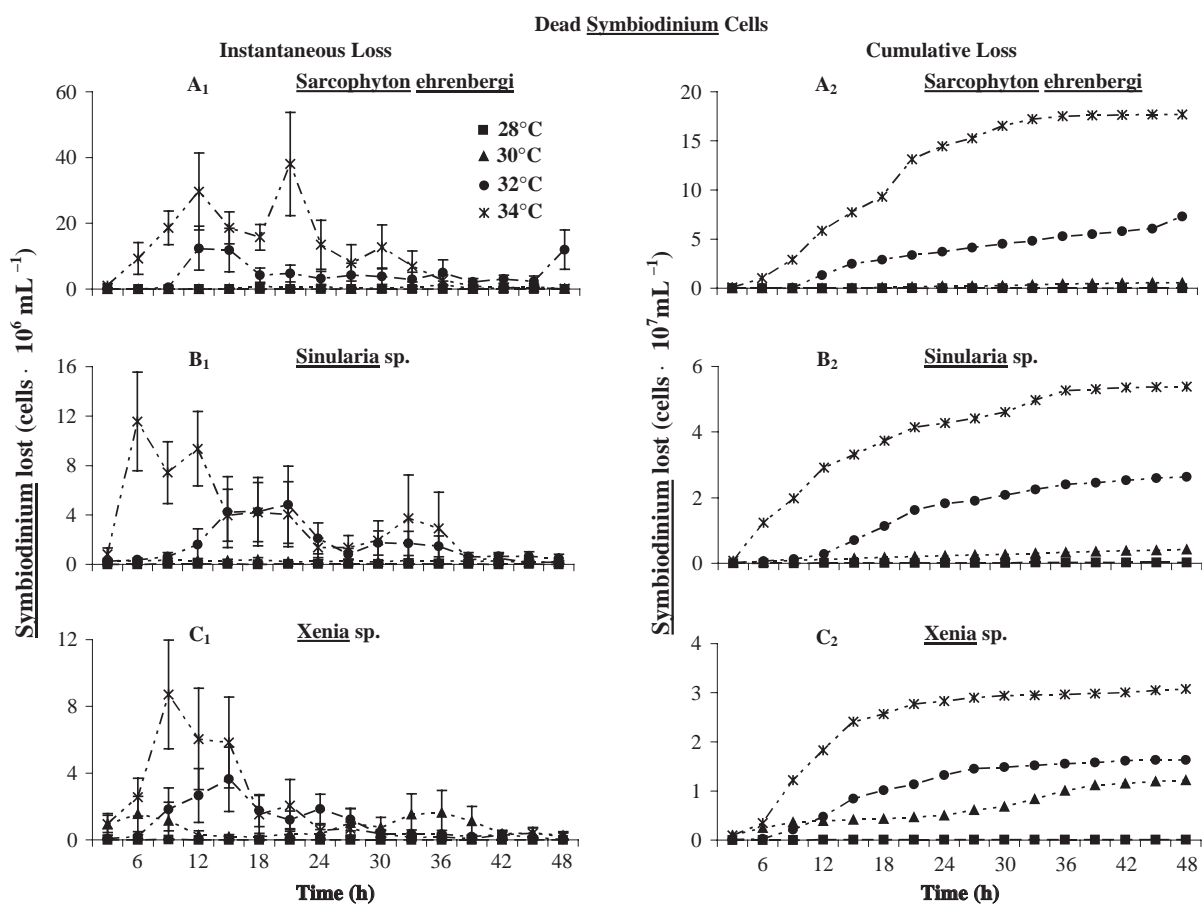


Fig. 3. Instantaneous (1) vs. cumulative (2) loss of dead *Symbiodinium* in soft corals from *Sarcophyton ehrenbergi* (A₁, A₂), *Sinularia* sp. (B₁, B₂) and *Xenia* sp. (C₁, C₂) at 30 °C (▲), 32 °C (●) and 34 °C (*) sampled every 3 h over a 48 h time period. Control temperature=28 °C (■). Mean±S.E.M. shown; $n_i=10$. Comparisons of temporal *Symbiodinium* cells lost ($\times 10^6$ mL⁻¹; A₁, B₁ and C₁) vs. cumulative *Symbiodinium* cells lost ($\times 10^7$ mL⁻¹; A₂, B₂ and C₂) are shown. Note how cumulative graphs (A₂, B₂ and C₂) illustrate the effect of temperature on overall depletion of a coral's *Symbiodinium* complement over 48 h.

loss at 21 h (Fig. 2A₁). Live *Symbiodinium* cell loss from *Xenia* sp. at 34 °C was bimodal, with peak losses occurring at 9 and 21 h (Fig. 2C₁).

Dead *Symbiodinium* cells lost from *S. ehrenbergi* were maximum at 32 °C occurring between 12 and 15 h. At 34 °C, we observed a loss at 21 and 30 h (Fig. 3A₁). The loss of dead symbiont cells from *Sinularia* sp. was multi-modal, with maximal losses occurring at 18 and 33 h at 32 °C, and 6, 12, and 33 h at 34 °C, respectively (Fig. 3B₁). The peak in loss of symbionts from *Xenia* sp. occurred at 15 h at 32 °C, and 9 h at 34 °C (Fig. 3C₂).

At <30 °C, mitotic cells were not released relatively continually throughout the course of the

experiment for all of the corals tested here (Fig. 4A₁–C₁). At 34 °C, however, evidence of heat stress was apparent in all three corals, with peak losses occurring within 9 h (Fig. 4A₁–C₁).

3.2.2. Cumulative loss of live, dead and mitotic *Symbiodinium* cells through time

Comparative analyses of live *Symbiodinium* cells lost between each host at 28 °C reflect insignificant differences between these corals (Fig. 2A₂–C₂; $p>0.05$). At 30 °C, *S. ehrenbergi* ($\sim 1.5 \times 10^7$ cells lost $g^{-1} W_w$; Fig. 2A₂) and *Xenia* sp. ($\sim 2 \times 10^7$ cells lost $g^{-1} W_w$; Fig. 2C₂) lost fewer cells than *Sinularia* sp. ($\sim 2.1 \times 10^7$ cells lost $g^{-1} W_w$; Fig. 2B₂). At 32 °C,

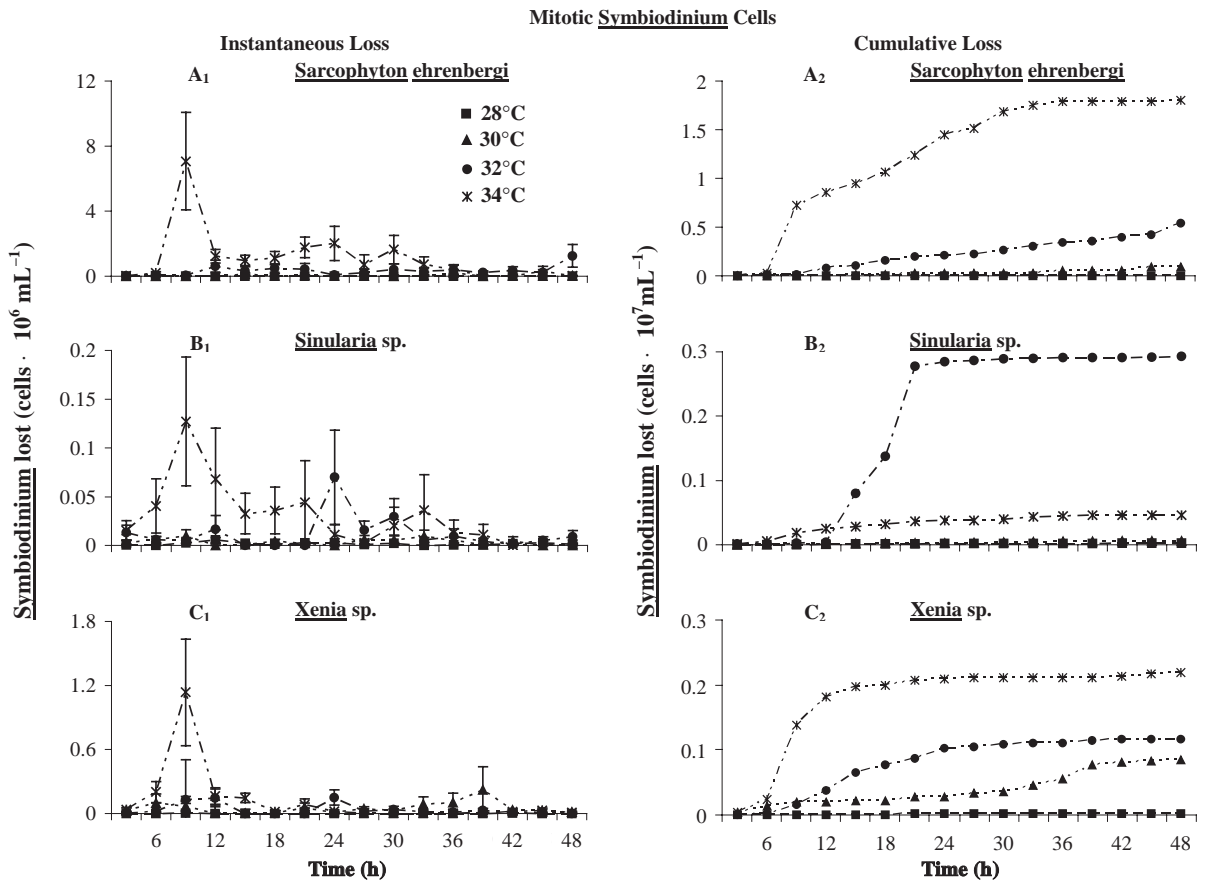


Fig. 4. Instantaneous (1) vs. cumulative (2) loss of mitotic *Symbiodinium* in soft corals from *Symbiodinium* cells lost from *Sarcophyton ehrenbergi* (A₁, A₂), *Sinularia* sp. (B₁, B₂) and *Xenia* sp. (C₁, C₂) at 30 °C (▲), 32 °C (●) and 34 °C (*) sampled every 3 h over a 48 h time period. Control temperature=28 °C (■). Mean±S.E.M. shown; $n_i=10$. Comparisons of temporal *Symbiodinium* cells lost ($\times 10^6 mL^{-1}$; A₁, B₁ and C₁) vs. cumulative *Symbiodinium* cells lost ($\times 10^7 mL^{-1}$; A₂, B₂ and C₂) are shown. Note how cumulative graphs (A₂, B₂ and C₂) illustrate the effect of temperature on overall depletion of a coral's *Symbiodinium* complement over 48 h.

however, *Sinularia* sp. lost more live *Symbiodinium* than at 34 °C (Fig. 2B₂). Losses from *Xenia* sp. reflected those at 34 °C (Fig. 2C₂). *S. ehrenbergi* (Fig. 2A₂) lost more cells at 34 °C than the other corals, but also appeared to survive longer than the other corals.

There were no significant differences in the losses of dead *Symbiodinium* between any of the corals at <30 °C ($p>0.05$). *Symbiodinium ehrenbergi* (Fig. 3A₂) was the only coral that had significantly different losses of dead *Symbiodinium* cells when compared to the other corals at 32 °C ($p<0.01$) and 34 °C ($p<0.01$).

Comparative analysis of mitotic *Symbiodinium* cells show that at 30 °C, *S. ehrenbergi* and *Xenia* sp. had significantly greater losses of symbiont cells

than *Sinularia* sp. (Fig. 4A₂–C₂; $p<0.01$). At 32 and 34 °C, *S. ehrenbergi* lost significantly more mitotic symbiont cells than the other corals tested ($p<0.01$).

3.2.3. In situ MI% of *Symbiodinium* cells after 48 h

Mitotic indices of heat-stressed *Symbiodinium* did not display any significant differences between 28 and 30 °C, except for *Xenia* sp. ($p<0.01$), or 30–32 °C, except for *Sinularia* sp. ($p<0.05$). At 32 °C, *Sinularia* sp. and *Xenia* sp. ($p<0.001$ for both species) had significant increases in MI% (Fig. 5A₁). *S. ehrenbergi* and *Xenia* sp. had significant increases in MI% at 34 °C ($p<0.001$ for both species; Fig. 5A₁). Linear

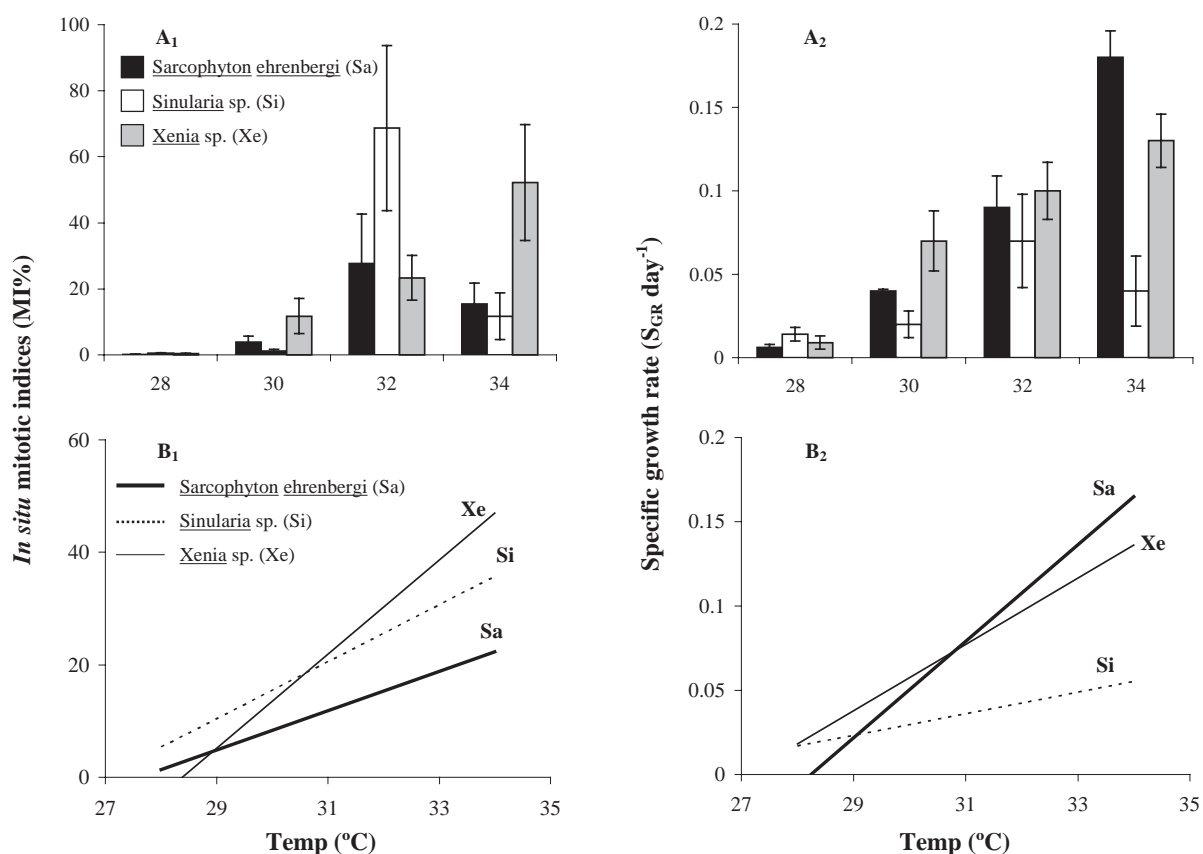


Fig. 5. *Symbiodinium* cells lost from *Sarcophyton ehrenbergi* (Sa), *Sinularia* sp. (Si) and *Xenia* sp. (Xe) at different temperatures: 30, 32, and 34 °C. Samples taken every 3 h over a 48 h time period. Control temperature=28 °C; Error bars are mean S.E.M.; $n_i=10$. A₁) In situ mitotic indices (MI%); i.e. the percentage of mitotic *Symbiodinium* cells for the three corals; B₁) *S. ehrenbergi* ($R^2=0.5231$, $p<0.002$, $y=3.492x-96.432$, least squares linear regression analysis); *Sinularia* sp. ($R^2=0.1617$, $p>0.347$, $y=5.065x-136.47$, regression); *Xenia* sp. ($R^2=0.9395$, $p<0.0001$, $y=8.3535x-237.04$, regression); A₂) Specific growth rate (S_{GR} day⁻¹) of *Symbiodinium* cells in situ; B₂) *S. ehrenbergi* ($R^2=0.9527$, $p<0.0001$, $y=0.0286x-0.8076$, regression); *Sinularia* sp. ($R^2=0.4285$, $p>0.201$, $y=0.0064x-0.1624$, regression); *Xenia* sp. ($R^2=0.964$, $p<0.0001$, $y=0.0197x-0.5319$, regression).

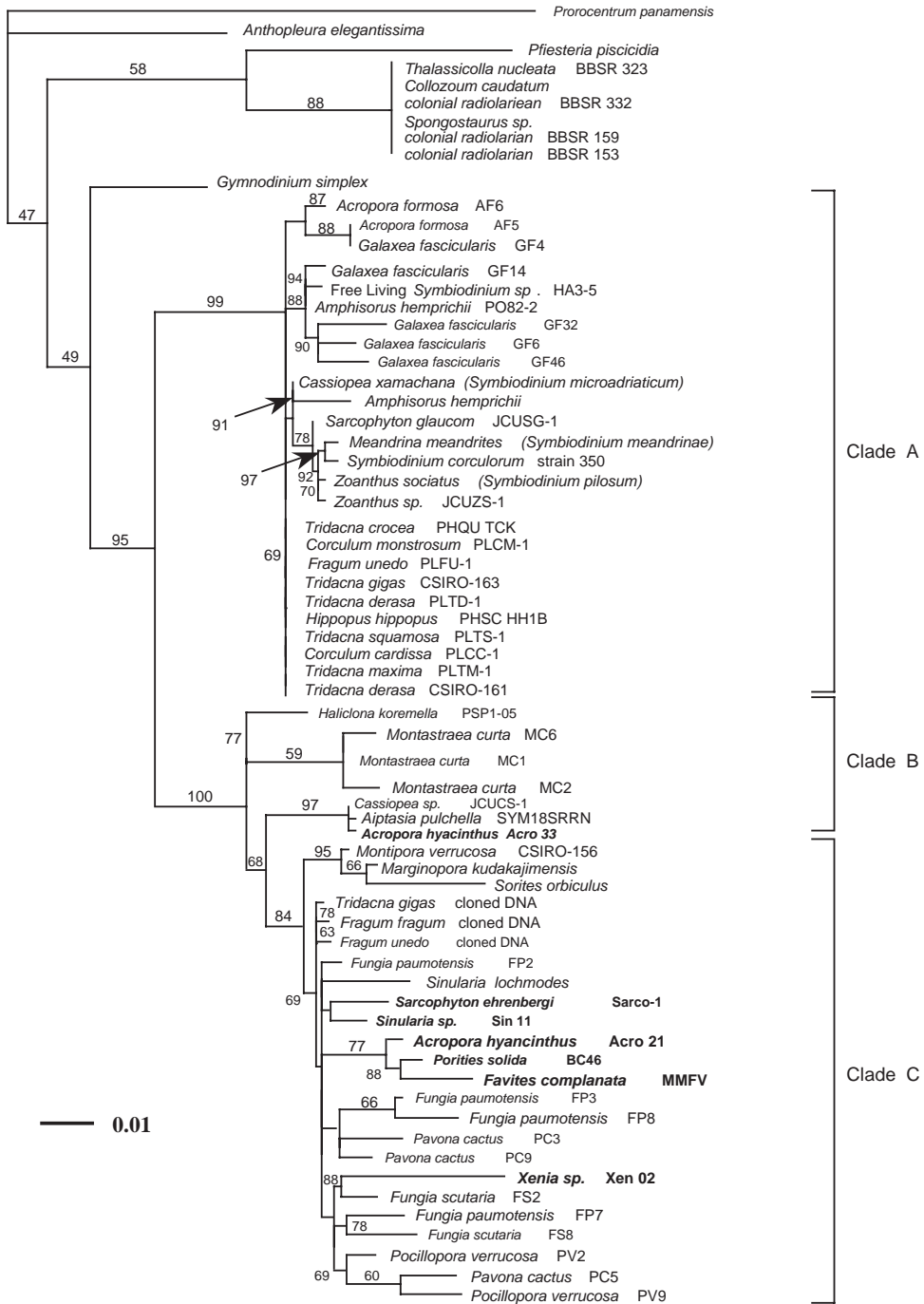


Fig. 6. Maximum likelihood (ML) trees derived from the analysis of edited 18S rDNA gene sequences (>1550 nucleotides) from zooxanthellar isolates. Out-group species include *Prorocentrum panamensis*, *Pfiesteria piscicida* and *Gymnodinium simplex*. Symbiont isolates from the scleractinian coral *Montastraea annularis* known to belong to clades A, B, and C were used to identify the major clade paradigm A, B and C. Numbers at nodes and arrows pointing to specific nodes indicate percentage bootstrap support derived from 1000 replicates. Strain sequences determined in this study are given in boldface. Scale bar represents percent divergence. Bootstrap values <50% are not shown (see Strychar, 2002 for greater detail).

regression analyses showed that significant increases of MI% in situ occur as temperatures increased with all but one coral—*Sinularia* sp. (Fig. 5B₁). This is because the in situ MI% for this species is greater at 32 than at 34 °C (Fig. 5A₁).

3.2.4. Specific expulsion (S_{ER}) and growth (S_{GR}) rate of *Symbiodinium* cells lost per 48 h period

Linear regression analyses indicated that S_{ER} varied significantly and positively with temperature/changes in all corals: *S. ehrenbergi* $y=0.0985x-2.8552$, $p<0.001$, $R^2=0.4627$; *Sinularia* sp. $y=0.0325x-0.9365$, $p<0.037$, $R^2=0.1184$; *Xenia* sp. $y=0.0189x-0.5327$, $p<0.001$, $R^2=0.3321$.

Linear regression analyses of S_{GR} in *Symbiodinium* cells in situ were significant in all corals ($p<0.001$), except for *Sinularia* sp. ($p>0.05$) indicating that temperature influenced the rate of symbiont growth (Fig. 5B₂). The S_{GR} of *Symbiodinium* from *Sinularia* sp. was not statistically significant because S_{GR} is greater at 32 °C than at 34 °C (Fig. 5A₂).

3.2.5. Ratio of S_{ER} to S_{GR} (S_{ER}/S_{GR})

At 28 °C, S_{ER}/S_{GR} ratios indicate that the replacement rate of symbiont cells within each host was considerably greater than the rate of *Symbiodinium* cell loss. As temperature increased, so did the S_{ER}/S_{GR} ratio, indicating that growth rate was less than that of replacement, eventually resulting in bleaching. *Symbiodinium* from *Xenia* sp., however, exhibited the lowest S_{ER}/S_{GR} ratio, followed by *S. ehrenbergi* and then *Sinularia* sp. at 30, 32 and 34 °C.

3.3. Bleaching—sequencing patterns of *Symbiodinium*

Phylogenetic analyses of *Symbiodinium* 18S rRNA gene sequences from *S. ehrenbergi*, *Sinularia* sp. and *Xenia* sp. indicated that the zooxanthellar group considered here belonged to clade C (Fig. 6). *Symbiodinium* isolated from the scleractinian corals *Acropora hyacinthus*, *Porites solida* and *Favites complanata* (the most abundant corals observed in close proximity to our sampled hosts) also possessed sequences consistent with clade C, with one exception. Clade B *Symbiodinium* were found in *A. hyacinthus* (Acro 33; Fig. 6). This is the first report for the association of clade B with this host.

4. Discussion

4.1. Instantaneous and cumulative patterns of heat stress (bleaching) in loss of *Symbiodinium* cells

The total loss of *Symbiodinium* cells from scleractinian corals under normal physiological conditions is $\sim 0.1\text{--}1\%$ day⁻¹ of the standing stock (Hoegh-Guldberg et al., 1987). In this study, *Xenia* sp. lost a lower number of its *Symbiodinium* than did the other soft corals. It is not known why *Xenia* sp. lost such small numbers of symbiont cells since the standing stock is similar to that of *Sinularia* sp. We speculate that the loss of *Symbiodinium* from *Xenia* sp. may be related to thermal acclimation. At low temperatures (≤ 30 °C) and short exposure intervals, Warner et al. (1996) suggests that corals appear to adapt to heat-induced stress. Muscatine et al. (1979) and Wang and Douglas (1979), however, suggest that the mechanism of control reducing the number of lost *Symbiodinium* cells may be ingestion—a means of nutrient supplementation (Muscatine et al., 1979; Wang and Douglas, 1997) and regulation of *Symbiodinium* population abundance in situ (Muscatine et al., 1986; Stimson, 1997). Shimek (1998) suggests that *Xenia* sp. does not acquire nutrition through feeding from the water column, as evidenced by a reduced gut size; rather, it may phagocytize *Symbiodinium* cells lost from host endodermal tissues when ‘excess’ nutrition is required. If these hypotheses are correct (i.e. temperature acclimation and/or ingestion), this would help to explain why at lower temperatures the loss of *Symbiodinium* cells from *Xenia* sp. was much lower when compared to the losses from *S. ehrenbergi* and *Sinularia* sp.

For some corals the number of live, dead, and reproducing *Symbiodinium* cells lost at 30 °C did not increase significantly over those lost at 28 °C. *S. ehrenbergi* and *Sinularia* sp. lost equal numbers of live cells indicating these corals were not stressed at 30 °C. Fitt and Warner (1995) obtained similar results when they examined the effect of temperature on *M. annularis* and *M. cavernosa*. Warner et al. (1996) also found that temperatures <30 °C do not adversely affect corals if the duration of the temperature stress is relatively short. Studying *M. annularis*, *A. lamarki*, *A. agaricites* and *S. radians*, these authors did not observe bleaching at 30 °C. They emphasize, however, that lack

of effect over short exposures at 30 °C does not rule out the possibility that prolonged exposures, over 2 to 3 weeks for example, may reduce coral health (Sammarco et al., work in progress).

At 30 °C, *Xenia* sp. lost more live *Symbiodinium* cells than at 28 °C; further, *Xenia* sp. lost more dead *Symbiodinium* cells at the elevated temperature than any of the other corals. Although *Xenia* sp. did not show symptoms of bleaching stress for a minimum of 12 h, the elevated loss of cells at higher temperatures (compared to the control temperature and compared to the number of *Symbiodinium* cells lost from the other corals) indicated that *Xenia* sp. may be sensitive to relatively small temperature increases. This is consistent with other investigators who report that bleaching of *Acropora* spp. also results from small temperature increases (Williams and Bunkley-Williams, 1988, 1990; Marshall and Baird, 2000). Hoegh-Guldberg (1994), in a study of bleaching scleractinian corals from French Polynesia, similarly observed bleaching at 29–30 °C where *Acropora* spp. exhibited the greatest susceptibility to bleaching (89–100% completely bleached). Marshall and Baird (2000) recorded similar observations on the GBR at 30–31 °C. They found that *A. palifera*, *A. hyacinthus* and *A. cytherea* suffered high mortality, and that <8% of some species remained alive (Marshall and Baird, 2000).

Gleason (1993) and Hoegh-Guldberg (1994) reported that *Acropora* spp. are usually the first corals to succumb to elevated sea temperatures. We propose that the alcyonacean soft coral *Xenia* sp. may be more sensitive to elevated temperatures than *A. hyacinthus*. The number of *Symbiodinium* cells lost from *Xenia* sp. was high and was not significantly different at 30, 32 and 34 °C over 48 h, indicating that this host achieved a maximum loss of its symbiont complement at temperatures ≥ 30 °C. Further, massive bleaching of *Xenia* sp. in this study occurred within 12 h at temperatures ≥ 30 °C as opposed to *A. hyacinthus* (collected from the same location as *Xenia* sp.; Strychar et al., 2004a), which did not bleach until 36 h had elapsed at 32 °C.

At 34 °C, most corals completely lose their live *Symbiodinium* complement within 24 h. *S. ehrenbergi*, however, lost most of its *Symbiodinium* in two large ‘bursts’ over a 48 h period. These results suggest that some soft corals may have “layers” of symbiont

cells. ‘Layered cells’ was a concept proposed by Dubinsky et al. (1984) who described *Symbiodinium* density differences within scleractinian corals. As the *Symbiodinium* population increases within the upper layers of a soft coral, self-shading (Dubinsky et al., 1984) reduces the light available to resident cells located deeper within the host tissues. As a result, *Symbiodinium* cells located at the uppermost portions of a host may be better adapted to resist thermal and UV fluctuations than those cells located within deeper tissues (Hoegh-Guldberg and Smith, 1989). As temperatures exceed the tolerance limit of *Symbiodinium* cells located near the surface, and they are lost, those cells located within the deeper tissues become exposed to different temperatures and UV levels and eventually bleach or dissociate from the host. This concept is consistent with the two large ‘bursts’ of *Symbiodinium* loss observed in *S. ehrenbergi*. The tolerance of *S. ehrenbergi* at 34 °C for 36–39 h suggests that this soft coral species has a similar threshold level for heat-stress as observed with our earlier results (Strychar et al., 2004a) concerning *P. solida*, which did not bleach after 48 h at 34 °C.

4.1.1. Loss of mitotic *Symbiodinium* cells

The fact that *S. ehrenbergi* lost more mitotic *Symbiodinium* cells than the other corals at higher temperatures (32 and 34 °C) suggest that temperature may play a role in stimulating reproduction of symbiont cells. This finding supports the speculation that variable rates of reproduction at elevated temperatures may be *Symbiodinium* (taxon) specific (see Fitt et al., 2000; Kinzie et al., 2001). Another possibility, however, is that a surplus of nutrients is made available to the remaining *Symbiodinium* cells when others are lost from a host. McCloskey et al. (1996) reported that *Symbiodinium* cells are generally nutrient-limited, or perhaps host-limited; if so, an excess in available nutrients may cause an increase in the number of mitotic *Symbiodinium* cells. A third possibility is that a surplus of nutrients may cause a mitotic block where temperature exacerbates *Symbiodinium* growth.

4.1.2. Mitotic index (MI%) of *Symbiodinium* cells in situ

S. ehrenbergi MI% values at 28 °C were similar to other published values indicating ‘normal’

amounts of mitosis at a low temperature. Wilkerson et al. (1988) recorded a MI% for *Acropora* spp. ranging from 3.3% to 7.6% and *Porites* spp. from 2.2% to 6.3%, with other corals exhibiting a range of 2.0–12.3%. Here, the values observed from *S. ehrenbergi* (0.19%), *Sinularia* sp. (0.53%) and *Xenia* sp. (0.34%) at 28 °C were lower than those scleractinians, but similar to values published for the alcyonaceans *Heteroxenia fuscescens* (0.8%) and *X. heterocrypta* (avg.=1.2%; Hoegh-Guldberg et al., 1987). We believe the observed discrepancies of in situ MI% may be related to ambient seawater temperatures. Hoegh-Guldberg et al. (1987) measured in situ MI% of *X. heterocrypta* and *H. fuscescens* at 25 °C whilst the ambient seawater temperatures of corals collected by Wilkerson et al. (1988) were not published. The discrepancy in MI% may also be related to invasive (i.e. tissue homogenization) vs. non-invasive methods to obtain *Symbiodinium* from a host. Suharsono and Brown (1992), who compared mitotic indices of *Symbiodinium* isolates using tissue homogenates vs. serial sections, observed significant MI% differences. They suggest that the standard (invasive) method of tissue homogenate preparations are influenced by extra- and intracellular *Symbiodinium* contained within the coelenteron.

The in situ MI% of all the symbionts in the corals studied here increased with temperature. This contrasts with the results of Fitt et al. (2000) who observed increased densities of *Symbiodinium* (although not MI) in scleractinian corals as the temperature decreased from summer-time maximums. The values reported by Fitt et al. (2000), however, are related to in vitro cells vs. the in situ results reported here. Hence, the observations of Fitt et al. (2000) may not be directly comparable to the results reported here.

4.1.3. Specific expulsion (S_{ER} day⁻¹) and growth (S_{GR} day⁻¹) rates

The S_{ER} of *Symbiodinium* cells was found to be directly proportional to the temperature experienced by the host and the symbiont. The S_{ER} reported here for *Xenia* sp. was similar for that reported for *X. heterocrypta* by Hoegh-Guldberg et al. (1987). Other values of S_{ER} reported by Hoegh-Guldberg et al. (1987) for *H. fuscescens*, *Millipora dichotoma*, and *Stylophora pistillata* at 25 °C suggested that a S_{ER}

value of 9.6×10^{-4} day⁻¹ represented a loss of <1 per 1000 alga from the standing stock; thus, at 28 °C, the losses observed here were comparatively low. The S_{ER} of symbiont cells from *S. ehrenbergi*, however, varied between 28 and 34 °C indicating that, although S_{ER} of *Symbiodinium* did not increase at the lower temperatures (30 and 32 °C), the rates of cell loss were affected by heat stress.

Increased temperatures may have elevated the *Symbiodinium* S_{GR} of the corals studied here. In this study, the S_{GR} s calculated from in situ mitotic indices (MI%) measured in the soft corals are consistent with those reported by Falkowski et al. (1984) for the scleractinian *S. pistillata* from the Gulf of Elat, *S. hystrix* and *S. pistillata* reported by Hoegh-Guldberg et al. (1987), and for *S. pistillata* in the Red Sea (Wilkerson et al., 1983; Muscatine et al., 1985). Low S_{GR} s at low temperatures, however, may indicate that some unknown mechanism(s) is present that limits the rate of growth of *Symbiodinium* associated with soft corals. Evidence from molecular studies of cells in situ indicate that processes of cell development, reproduction, growth and death are genetically determined and are not under the exclusive control of extrinsic factors like temperature (Fitt and Cook, 2001a,b). The loss of increasing growth rates at higher temperatures, however, is a molecular process which few, if any studies, have addressed.

4.1.4. Ratio of S_{ER}/S_{GR}

The fact that, at 28 °C, the S_{ER}/S_{GR} values from each coral were between 0.26% and 1.42% indicated that the soft corals in this study were producing more *Symbiodinium* than they required. These are comparable to those of Hoegh-Guldberg et al. (1987) who observed ratios of 1.6–4% at 25 °C. As the temperature increased from 28 to 30 °C here, the S_{ER}/S_{GR} values increased, and at 32 and 34 °C, all coral hosts had elevated S_{ER}/S_{GR} ratios. The relatively low value observed within *Xenia* sp. at 34 °C suggests that its *Symbiodinium* were being replaced faster than they were being lost. A possible explanation for rapid death in *Xenia* sp. may be related to rapid phytopigment deterioration within *Symbiodinium*, rapid deterioration of organelles (e.g. mitochondria) within these cells leading to death in situ, and/or deterioration of host tissues.

4.2. Bleaching—sequencing patterns of *Symbiodinium*

Symbiodinium within soft corals in this study belong to clade C. There were no multiple symbioses between different soft coral tissues (e.g. stalk and capitulum) as observed in other reef organisms (e.g. Rowan and Knowlton, 1995; Rowan, 1998). Differences in *Symbiodinium* genotypes within soft corals, however, may exist and may be related to depth. Carlos et al. (1999) described the analysis of a soft coral sample taken from the northern GBR. They found that the soft coral *Sarcophyton glaucum* was symbiotic with clade A.

Clade B *Symbiodinium* within *A. hyacinthus*, contrasting with other reports that suggest *Acropora* spp. is symbiotic only with clades A and C (Loh et al., 1997), was also found to be present within *Acropora* at deeper depths than previously reported (Rowan et al., 1997). The presence of clade B *Symbiodinium* within *A. hyacinthus* supports the concept that multiple symbiotic associations of *Symbiodinium* species occur in the same species of coral (Buddemeier and Fautin, 1993; Rowan, 1998).

5. Conclusion: bleaching of soft vs. scleractinian corals

Both soft and scleractinian corals are equally susceptible to heat stress. Some soft corals are more resistant than other soft corals and/or some scleractinian corals. These two taxonomic groups (i.e. alcyonacean vs. scleractinian) contain both species of high tolerance [e.g. *S. ehrenbergi* (this study) and *P. solida* (Strychar et al., 2004a)] and low tolerance [e.g. *Xenia* sp. (this study) and *A. hyacinthus* (Strychar et al., 2004a)]. *Xenia* sp. is more susceptible to elevated temperatures than the species previously reported to be the most susceptible—*Acropora* spp. (e.g. Hoegh-Guldberg, 1994; Marshall and Baird, 2000). Since the soft-bodied *Xenia* spp. disintegrate when they die, it is difficult to document the history of their bleaching and death in the field. Thus, the results of this study imply that the impact of elevated temperatures on this group may be much greater than previously suspected. Further, some soft corals may have bleaching resistance comparable to the scleractinian *Porites* spp. Many

individuals of *S. ehrenbergi* at 34 °C reflected similar resistance to *P. solida* where (1) bleaching did not occur within the first 36 h, or (2) prolonged exposures exceeded 48 h (Strychar, 2002). This is the first report of different temperature tolerances among soft corals.

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