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Cover image - Patch reef near the wall off Grotto Beach (photo by Lee Florea).

Patterns of Millepore-*Symbiodinium* associations at two Caribbean locations: San Salvador, The Bahamas and South Water Cay, Belize

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1. Abstract

Symbiodinium (Dinoflagellata, Chromalveolata) forms symbiotic relationships with many marine organisms, including numerous coral species. Both temperature and light appear to play important roles in the diversity of symbionts, and different *Symbiodinium* clades provide different physiological benefits to their coral hosts. This symbiosis may provide coral with a mechanism to cope with thermal stress events associated with global warming.

The vast majority of the research examining the coral-*Symbiodinium* relationship has focused on Scleractinian corals, while ignoring the important reef framework building Millepores (fire coral). The purpose of our research was to examine the diversity of the Millepore-*Symbiodinium* symbiosis at two thermally different Caribbean reef locations: San Salvador, The Bahamas and South Water Cay, Belize. Our preliminary results indicate that sea surface temperature may play an important role in diversity of symbionts residing in the Millepores. These results appear to be associated with different environmental conditions at each geographic location and may reflect that the South Water Cay location is exposed to more frequent thermal stress events.

2. Introduction

The presence of coral in a diverse range of environments has been attributed to the variability in the coral-*Symbiodinium* relationship (Baker 2003). Most hard and soft-bodied corals, among a large number of other marine organisms (sponges, jellyfish, anemones, snails, clams and flatworms), harbor symbiotic unicellular algae of the genus

Symbiodinium (Coffroth and Santos 2005). Symbiont populations can reach densities of several million per square centimeter of host tissue (LaJeunesse 2002). These photosynthetic symbionts are vital to coral health because they provide up to 95% of the coral's energy requirements (Hoegh-Guldberg 1999). Our current understanding of the genetic diversity of *Symbiodinium* includes the existence of nine clades (A-I) and as of yet, an undetermined number of subclade types (Pochon and Gates 2010). It is not uncommon for the same coral colony to host multiple symbiotic clades (Chen et al. 2005; Mieog et al. 2007). Molecular studies have shown that these symbiotic relationships are more flexible than previously thought with different hosts and symbionts showing changes in their associations in response to environmental conditions (reviewed in Coffroth and Santos 2005).

Differences in host-symbiont associations across latitude, longitude and environmental gradients have been reported (reviewed in Baker 2003). These differences appear to be related to symbionts with different tolerances to thermal stress and irradiance. Although controversial, the identification of functional differences in *Symbiodinium* physiology promoted the introduction of the adaptive bleaching hypothesis (Buddemeier and Fautin 1993), which states that thermal stress provides an opportunity for the host to be repopulated with different symbionts that are better adapted to help coral survive environmental changes. Stress events promote stress-resistant combinations through symbiont shuffling (changes in the dominant symbiont present in a coral colony) or switching (recruitment of new symbionts from the environment)

(Berkelmans and van Oppen 2006; Mieog et al. 2007; Coffroth et al. 2010). This allows corals to acquire a variety of symbiont combinations containing different physiological abilities that may help them adapt to environmental perturbations.

Other investigators have associated host-symbiont diversity with other coral adaptations. Little et al. (2004) linked specific *Symbiodinium* clades to a two to threefold difference in host coral growth rates, Berkelmans and van Oppen (2006) have shown that specific clades of *Symbiodinium* can influence seasonal coral host heat tolerance by 1-1.5°C, and Stat et al. (2008) linked variation of *Symbiodinium* to host disease susceptibility. Although it appears that symbiont flexibility is beneficial through improved holobiont (coral and symbiont) resistance, there are indications that this might not be the case for all species. Putnam et al. (2014) reported that environmentally sensitive *Acropora* corals displayed a high degree of symbiont flexibility while the environmentally resistant *Porites* corals exhibited low symbiont flexibility. Brown et al. (2002, 2015) showed that bleaching patterns in *Coelastrea aspera* are not attributed to genetic differences in *Symbiodinium* but instead appear to be due to the presence of high host levels of stress proteins and antioxidants as well as xanthophyll cycling. These findings show that holobiont resilience is more complicated than originally thought. Continued examination of coral and their symbionts over geographic ranges will allow us to define spatial and temporal limitations governing the extent to which corals are capable of responding and adjusting to future environmental stresses.

Coral reefs in the Caribbean offer an ideal setting to explore host-symbiont dynamics because the Caribbean Sea has warmed on average by 0.27°C per decade over the period from 1985-2009 (Chollett et al. 2012). Additionally, there are considerable temperature differences across the Caribbean region, with northern areas showing cooling and western areas warming (Chollett et al.

2012). These warming trends are sufficient to push corals outside of their range of thermal tolerance. This is a particular concern in the Caribbean, which is considered a disease “hotspot” (Harvell et al. 2007).

Symbiont diversity and flexibility has received much attention in Caribbean scleractinian corals (stony corals), but almost no attention has been paid to the important reef-framework building millepore corals (fire corals) (reviewed in Davy et al. 2012). The calcareous hydrozoan coral, *Millepora*, is one of the most common skeleton-forming animals on Caribbean reefs, second only to their scleractinian cousins (Lewis 2006). The morphology of the Millepores is variable and demonstrates a high degree of phenotypic plasticity (Stearn and Riding 1973; Lewis 2006). Current taxonomic classification of the Millepores is based on morphological characters, and the Caribbean Millepores are represented by two species, *Millepora alcicornis* and *Millepora complanata* (Cairns et al. 1999). The various growth forms of Caribbean Millepores range from thinly encrusting sheets and dendroid branches for *M. alcicornis* to thicker, rigid bladed forms for *M. complanata* (Stearn and Riding 1973). However, there is a wide range of intermediate growth forms (Figure 1) whose species status remains unclear (Tepper et al. 2012).

The goal of our study is to improve our understanding of Millepore-*Symbiodinium* diversity across different latitudes of the Caribbean. We used a molecular genetics approach to identify the *Symbiodinium* clades present in Millepore colonies located on reefs surrounding San Salvador, The Bahamas (24.1°N) and South Water Cay, Belize (16.8°N). San Salvador, The Bahamas has a yearly ocean temperature range of 22-28°C and South Water Cay, Belize has a yearly ocean temperature range of 26-30°C (www.nodc.noaa.gov/General/temperature.html). We hope to provide a baseline of pre-bleaching symbiont diversity in millepore corals located in the western and eastern Caribbean for

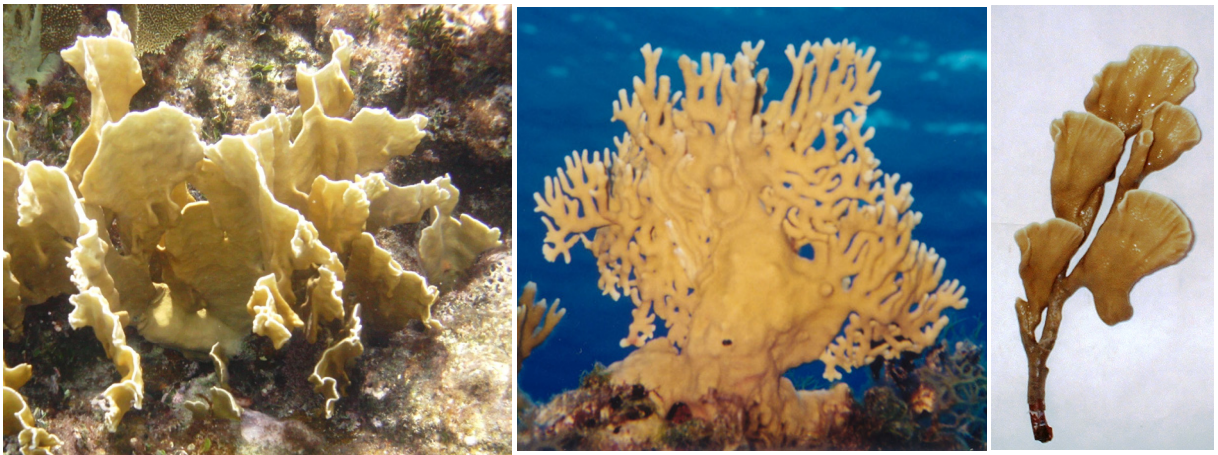


Figure 1. Typical growth forms of the recognized species of *Millepora* found in the Caribbean. *Millepora complanata* (Left) *Millepora alcicornis* (Center) and an intermediate growth form (Right).

assessing future changes in coral-dinoflagellate symbioses with on-going climate change.

3. Methods

3.1 Collection and preparation of *Millepora* samples

Millepores used in this study were collected in February of 2014 and June 2015 from reefs located on the west side of San Salvador, The Bahamas (24.1°N, 74.4°E), and in February of 2013 and 2015 from reefs located along the barrier on the South Water Cay Marine Reserve (SWCMR) close to South Water Cay (16.8°N, 88.1°E) and Carrie Bow Cay, Belize (Figure 2). San Salvador is located on the eastern edge of The Bahamas Island Archipelago. Millepore collection sites were shallow patch reefs (1-5m) located at Lindsay Reef, Rocky Point Reef, Grotto and French Bay. SWCMR is the largest marine reserve in Belize covering an area of 117,878 acres. It is located approximately 15 miles southeast of Dangriga. Millepore collection sites were Whales Shoal, Aquarium, Angel's Reef, and Carrie Bow Fore Reef. All SWCMR reef collection sites were shallow (1-5 m).

Millepore samples were randomly collected from each of the aforementioned reefs by removing a small piece, approximately

4 sq. cm in size. Samples of *M. alcicornis*, *M. complanata*, and intermediate growth forms were transported from collection sites to the lab in buckets containing seawater and held for no more than two days prior to DNA isolation.

3.2 *Symbiodinium* DNA isolation

Symbiodinium DNA was isolated using a procedure modified from Rowan and Powers (1991), Lopez et al. (1999) and Tepper et al. (2012). Coral tissue was removed by repeatedly blasting the skeleton with a 50 cc syringe containing L buffer (100 mM EDTA, 10 mM Tris, pH 7.6). Coral tissue was centrifuged at 3,500 rpm for 10 minutes; the resulting pellet was washed in 10 mL of L buffer and re-centrifuged. The tissue pellet was resuspended in 900 μ L of L buffer and macerated manually with a tissue homogenizer. The homogenate was centrifuged at 13,000 rpm for 10 minutes and the pellet was resuspended in 500 μ L of L buffer. Following the addition of 1% (w/v) SDS to the supernatant, the lysate was incubated at 65 °C for 30-60 minutes. Pro K (0.5 mg/mL) was added and the lysate was incubated at 37 °C for at least 6 hours. NaCl (0.8 M) and CTAB (1% w/v) were added and samples were incubated at 65 °C for 30 minutes. Nucleic acids were precipitated twice in 70% (v/v) ethanol and 3 M sodium acetate (pH

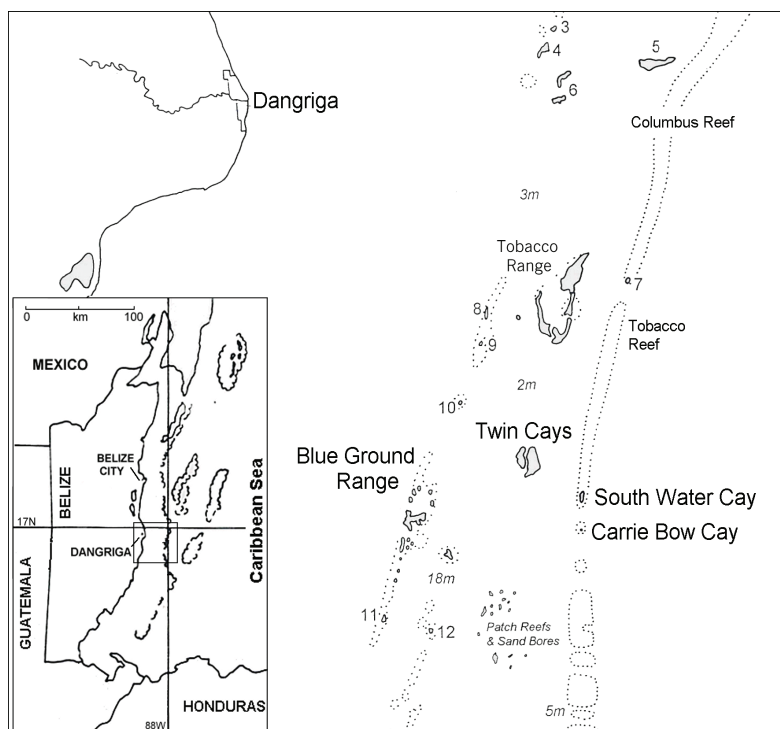
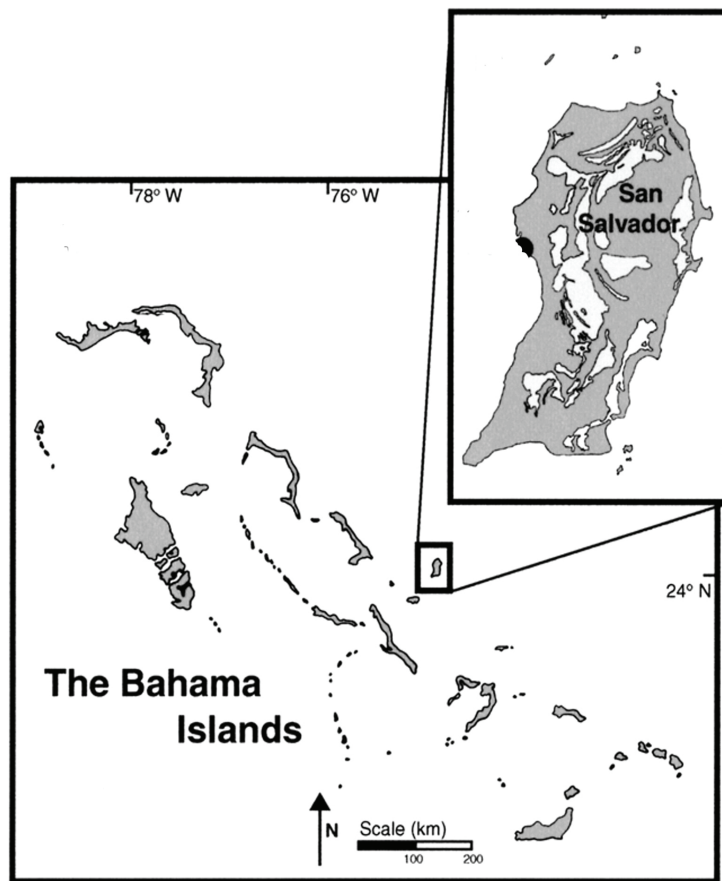


Figure 2. Map locations of collection sites: (Top) San Salvador, The Bahamas (<http://palaios.sepmonline.org/content/16/4/372/F1.large.jpg>) and (Bottom) South Water Cay and Carrie Bow Cay, Belize (<http://cbc.riocean.com/location.htm>).

5.2) and immediately centrifuged. Following resuspension of the pellet in dH₂O, the DNA was briefly centrifuged and the supernatant was retained.

3.3 Quantitative PCR

Four clade-specific *Symbiodinium* primer pairs (Table 1) targeting ITS1-5.8S-ITS-2 rDNA of clade A (Correa et al. 2009), domain 2 of LSU (28S rDNA) of clade B (Correa et al. 2009), and ITS1 of clades C and D (Ulstrup and van Oppen 2003) were used. The 20 µl qPCR reaction contained 10 µl of Power SYBR Green Mastermix (Applied Biosystems), 200 nM clade-specific primer pairs (Table 1), and 200 ng *Symbiodinium* DNA. Amplifications were run and analyzed on an Applied Biosystems StepOnePlus Real-Time PCR System. After an initial 10 minute 95 °C pre-heating step to activate the polymerase, the qPCR profile consisted of 40 three-step cycles of 95 °C, 60 °C and 72 °C, each for 30 seconds. In order to determine if amplification signals represented the amplicon or primer dimers, at the end of each run a melt curve was generated starting at 55 °C and increasing the temperature by 0.5 C° each 5 seconds until a temperature of 95 °C was reached.

The cycle-threshold (C_T) was set at a fixed value (0.1) in order to allow comparison of C_T values between runs. Duplicate reactions were run for each sample along with negative (no-

template) controls. C_T values for each duplicate reaction were averaged and the relative abundance of each clade was determined using the following equation: dominant clade DNA to background clade DNA = $(2^{C_T(Y)-C_T(X)})$, where $C_T(X)$ is the threshold of the background clade and $C_T(Y)$ is the threshold of the dominant clade (Correa et al. 2009). Background refers to all clades within a coral colony that are at a lower abundance when compared to the dominant clade. A Fisher's Exact Test was used to determine if there was a significant correlation between symbiont clade presence and geographic location, reef site, and colony growth form.

4. Results

A total of 57 Millepore colonies were examined for the presence of symbionts. The samples included 28 colonies collected from reefs on the west side of San Salvador, The Bahamas (*M. complanata* N=16, *M. alvicornis* N=9, and intermediate growth forms N=3) and 29 colonies collected from reefs located on the SWCMR (*M. complanata* N=16, *M. alvicornis* N=6, and intermediate growth forms N=7). An example of a *Symbiodinium* qPCR profile for a colony of *M. complanata* collected at Rocky Point in The Bahamas is shown in Figure 3. Clade B is the dominant *Symbiodinium* clade present with A and C representing background clades. The clade relative abundance ratio for

Table 1. Clade-specific qPCR primer pairs used for amplifying portions of multi-copy rDNA in *Symbiodinium* clades A, B, C, and D.

| Clade | Primer Pair | Target | Reference |
|-------|--|----------------|----------------------------|
| A | 5'-CCTCTTGGACCTTCCACAAC-3' 5-'GCATGCAGCAACACTGCTC-3' | ITS1-5.8S-ITS2 | Correa et al. 2009 |
| B | 5'-GTCTTTGTGAGCCTTGAGC-3' 5'-GCACACTAACAAGTGTACCATG-3' | LSU-28S | Correa et al. 2009 |
| C | 5'-AAGGAGAAGTCGTAACAAGGTTTCC-3' 5'-AAGCATCCCTCACAGCCAAA-3' | ITS1 | Ulstrup and van Oppen 2003 |
| D | 5'-AAGGAGAAGTCGTAACAAGGTTTCC-3' 5'-CACCGTAGTGGTTCACGTGTAATAG-3' | ITS1 | Ulstrup and van Oppen 2003 |

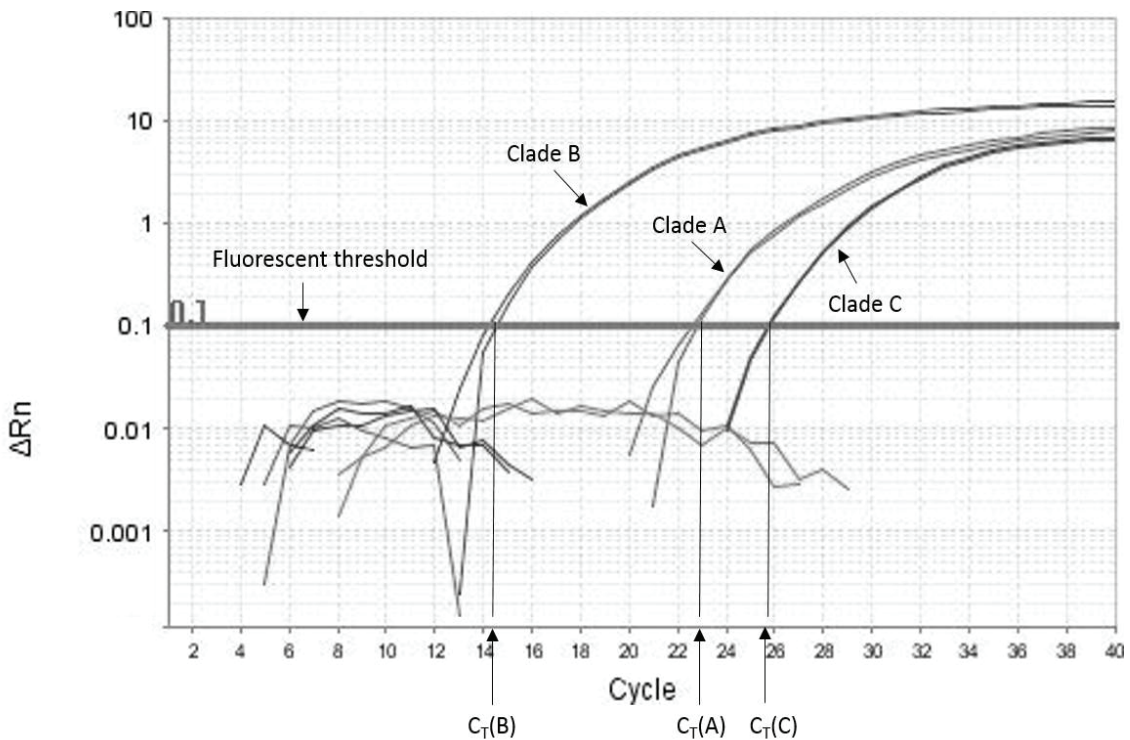


Figure 3. A typical example of a qPCR amplification profile for *Symbiodinium* detection in a *Millepora complanata* coral sample collected at Rocky Point in San Salvador, The Bahamas. *Symbiodinium* clade B ($C_T(B)=14.2$) was the dominant endosymbiont in this sample with background levels of A ($C_T(A)=22.7$) and C ($C_T(C)=25.8$). Cycle (X axis) represents the qPCR cycle number and ΔRn (Y axis) is the fluorescence of the reporter dye divided by the fluorescence of a passive reference dye minus the baseline.

the colony is B:A-362:1, B:C-3,104:1, and A:C-9:1.

The dominant symbiont clades detected in both Bahamas and Belize Millepore colonies are represented in Figure 4. In all Millepore colonies collected in The Bahamas ($N=28$), clade B was the single dominant clade represented. However, in Belize Millepores ($N=29$), clade A was dominant in 69% ($N=20$) and clade B was dominant in 31% ($N=9$) of the collected colonies. Although Millepore taxonomy is poorly defined because of the high degree of phenotypic plasticity in the complex (Stearn and Riding 1973; Tepper et al. 2012), symbiont clade dominance was not significantly correlated with growth form in the Belize Millepores.

The majority of Millepore colonies contained multiple clades of symbionts (Figure 5). In The Bahamas, 71.4% (20/28) of the colonies hosted multiple clades of symbionts

while 72.4% (21/29) of the Belize colonies hosted multiple clades. Of the clade B dominant Bahamas Millepore colonies, 28.6% (8/28) had no background, 46.4% (13/28) had only clade A background, 3.6% (1/28) had only clade C background and 21.4% (6/28) had background of both clades A and C. Of the Clade B dominated Belize Millepores, 22.2% (2/9) contained no background clades, 33.3% (3/9) contained only clade A background and 44.4% (4/9) contained backgrounds of clades A and C. Of the clade A dominated Belize Millepores, 30% (6/20) contained no background clades, 55% (11/20) contained clade B background, 10% (2/20) contained clades B and C background and 5% (1/20) contained a clade D background. As far as we are aware, this is the first report of a Caribbean Millepore containing clade D. Statistical analysis revealed no correlation between geographic collection location (Bahamas or Belize) and background symbiont

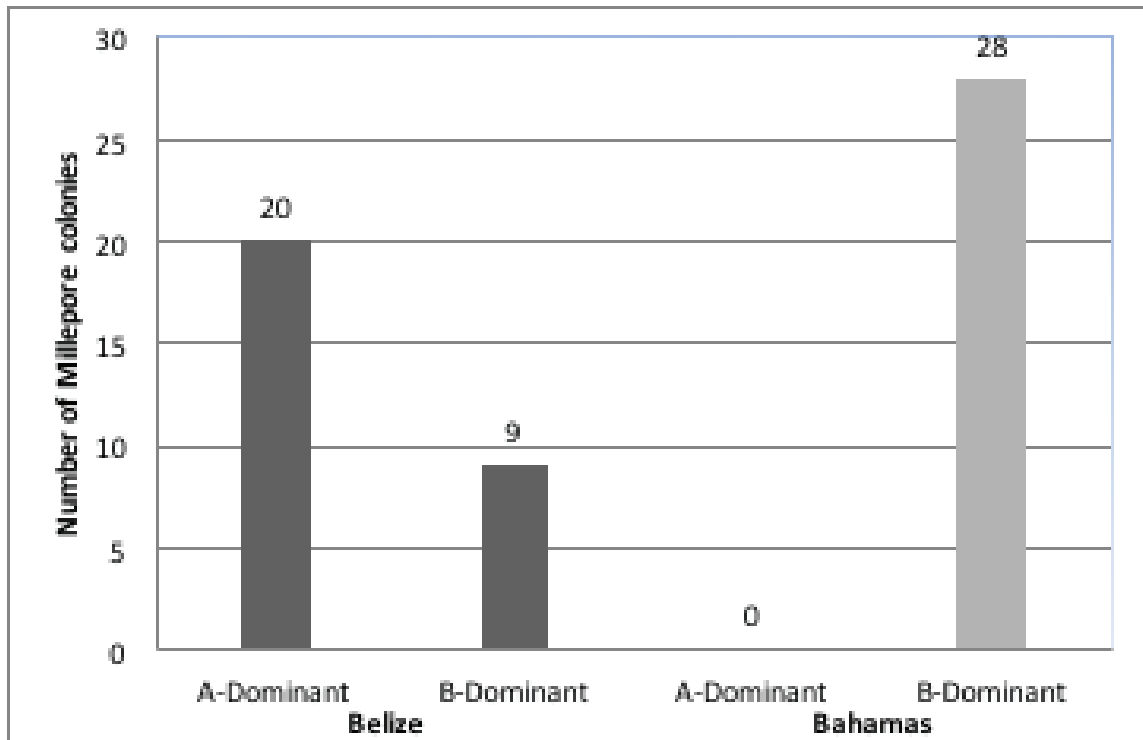


Figure 4. Comparison of the dominant *Symbiodinium* clades present in Millepores collected from reefs around San Salvador, The Bahamas and along reefs on SWCMR surrounding South Water Cay, Belize. The graph shows the actual number of Millepore colonies collected that were either *Symbiodinium* clade A or B dominant.

clades. Additionally, background clades were not correlated with reef location nor was there a correlation between background clade and Millepore growth form.

Although both Bahamas and Belize populations contained clade B dominant symbionts, abundance results showed that the relative B:A abundance for the two populations was different. The relative abundance of The Bahamas population ranged from a low 17:1 to a high of 251,150:1 while the B:A relative abundance in Belize ranged from a low of 12:1 to a high of 22:1. This indicates that clade B-dominated Belize Millepores contained a much higher ratio of clade A symbionts, and that clade B is more prevalent in the Bahamas population. Additionally, the B-dominated Bahamas Millepores had a relative abundance range of B:C that spanned from 156:1 to 268,227:1 while the clade B dominant Belize

Millepores ranged from 2.6:1 to 22,026:1 indicating clade C might be more abundant in Belize.

5. Discussion

Symbiont distribution may provide a mechanism for coral hosts to tolerate thermal stress events because different clades of symbionts do not appear to be functionally equivalent. This diversity is manifested in symbiont variation in photosynthetic response to irradiance and thermal tolerance (Goulet et al 2005; Stat et al. 2008). Global latitudinal and longitudinal patterns of symbiont diversity have been studied extensively in anthozoans (corals and sea anemones), but little information is available for the hydrozoan Millepores (Burnett 2002; Savage et al. 2002; LaJeunesse et al. 2004). The key result of our

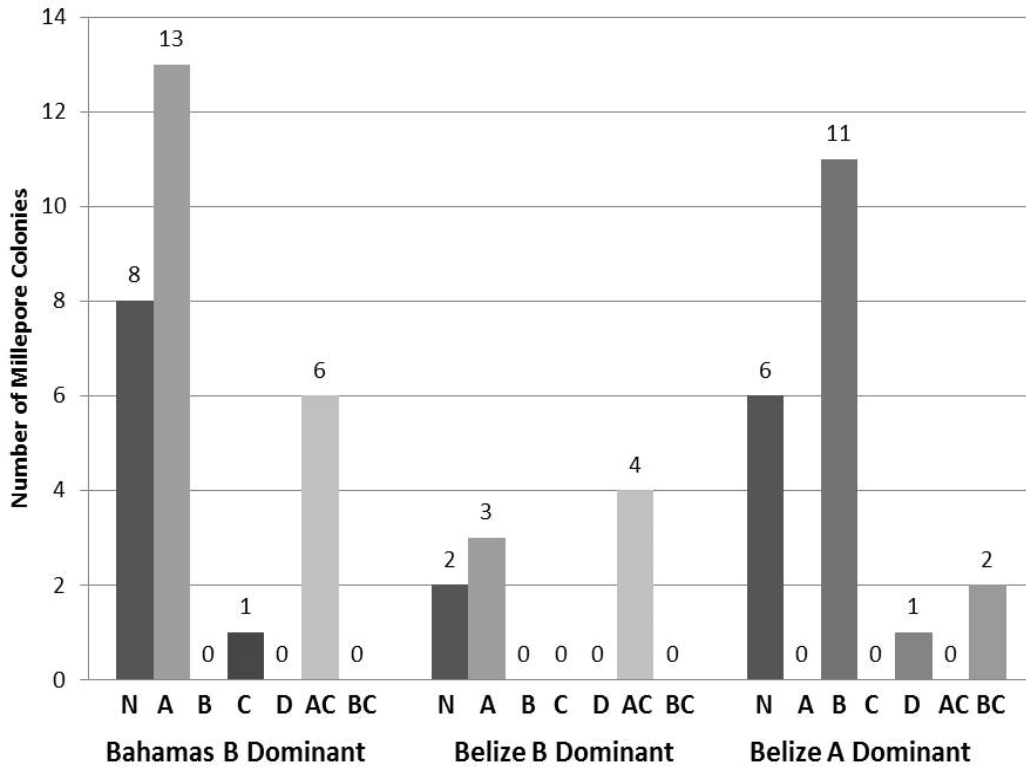


Figure 5. Comparison of the actual number of Millepore colonies that contained background *Symbiodinium* clades in either a clade B or A dominant host collected from reefs around San Salvador, The Bahamas and along reefs on SWCMR surrounding South Water Cay, Belize. N = no background, A = clade A background only, B = clade B background only, C = clade C background only, D = clade D background only, AC = clades A and C background, and BC = clades B and C background.

preliminary study is that symbiont diversity in *Millepora* differs between higher latitude Caribbean coral reef sites of San Salvador, The Bahamas (24.1° N) and lower latitude coral reef sites of SWCMR, Belize (16.8° N). B was the dominant *Symbiodinium* clade present in all Millepore samples collected in The Bahamas while clades A (69%, 20/29) and B (31%, 9/29) were dominant in Belize. Although clade B was dominant in some Belize Millepores, the relative abundance ratio range of 12:1-22:1 (B:A) demonstrates that clade A is still prevalent in these coral colonies. In contrast, the relative abundance ratio of dominant B clade to background A clade in the Bahamas ranged from 17:1-251,150:1.

Both geographic locations contained background clades. In the Bahamas, single clade backgrounds were found in 50% (14/28)

of the Millepore colonies and 21% (6/28) of the colonies contained multiple background clades (Figure 5). In contrast, 52% (15/29) of the Belize Millepores contained a single clade background and 21% (6/29) contained multiple clade backgrounds. Background levels of clade C were present in 25% (7/28) of Millepores collected in The Bahamas and 21% (6/29) of the Belize Millepores, but their relative abundance was low at both sites. Rowan and Knowlton (1995) showed that the Caribbean coral *Montastraea annularis* was dominated by clades A and B in shallow, well illuminated reef sites and by clade C in deep and poorly illuminated locations. Since our samples were collected from shallow reef locations, this could explain the low abundance of clade C in all samples at both locations. This explanation can easily be tested once we collect and analyze

Millepores from deeper locations.

There was no significant correlation found between the presence of background clades and geographical location, reef site or Millepore growth form. Interestingly, one Belize Millepore (*M. complanata*) contained background levels of clade D which has not been previously reported to be present in Caribbean Millepores even though clade D has been implicated in the prevention of bleaching in scleractinian corals (Baker et al. 2004). This particular colony was A dominant and the relative abundance ratio of clade A:D was 26,607:1.

Caribbean Millepores appear to be dominated by clade B at the higher latitude Bahamas sites where sea surface temperatures are slightly cooler, and clade A dominates at lower latitude Belize sites where sea surface temperatures are slightly warmer. However, it is important to determine if variation in symbiont diversity is seasonal. The tropical sea anemone, *Condylactis gigantean*, showed seasonal variation in *Symbiodinium* dominance. Venn et al. (2008) reported an increase in prevalence of clade A in summer months and clade B in winter months. Since our collection times were both February and June in The Bahamas and clade B was the only dominant clade present, seasonal changes do not appear to alter the dominant clade present. However, collections in Belize were only made in February and seasonal variation remains unknown.

Venn et al. (2008) have shown that at thermally variable inshore sites clade A predominates in *C. gigantea*, but at sites that are cooler and more thermally uniform, clade B dominates. In laboratory analyses, they demonstrated that tentacles containing clade B, but not clade A, bleached at elevated temperatures (32°C) suggesting that thermal tolerance might be responsible for the higher prevalence of clade A at thermally stressed sites. This is consistent with our data showing that clade A is dominant in Belize Millepores, which have likely experienced

more thermal stress events than Millepores residing on reefs in The Bahamas. However, clade B symbionts appear to be prevalent in Caribbean scleractinian corals regardless of latitude (Baker and Rowan 1997; Loh et al. 1998). One possible explanation is clade B's variation in photosynthetic output in response to temperature. At elevated temperatures, clade B fixed carbon at a lower rate, but translocated higher proportions of photosynthetic product to the *C. gigantea* host than clade A (Loram et al. 2007). However, they also showed that *C. gigantea* containing clade B were more susceptible to bleaching and symbiont expulsion at elevated sea temperatures. It is possible that clade B, present in Millepores at both geographic locations, provides the host with more photosynthetic material, but the higher prevalence of background clade A in Belize allows Millepores to successfully tolerate isolated thermal stress events.

The effects of elevated temperature on symbiosis is of considerable interest at present because increased sea temperatures due to global warming have been associated with bleaching (reviewed in Coles and Brown 2003) and coral disease prevalence (Frierler et al. 2013). Seawater temperatures have increased by 0.74°C during the 20th century and a further rise of 1.8-4°C is predicted for this century (Hoegh-Guldberg et al. 2007). An increase in water temperature of only 1 to 2°C for 3-4 weeks is sufficient to induce bleaching (Hoegh-Guldberg et al. 2007). Our analysis demonstrates that symbiosis is complicated, highly dynamic and that understanding *Symbiodinium* diversity may provide insights into how corals cope with global thermal stress.

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