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Seasonal stability of coral–*Symbiodinium* associations in the subtropical coral habitat of St. Lucie Reef, Florida

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ABSTRACT: The coral community at St. Lucie Reef (Stuart, Florida; 27° 8′ N, 80° 8′ W) is found near the northern latitudinal range limit for Florida reefs and persists under environmental variability from freshwater discharges, summer upwelling, and thermal instability. Since aspects of coral physiology can be attributed to the composition of endosymbiotic zooxanthellae (genus *Symbiodinium*), we examined the dynamics of *Symbiodinium* strains in St. Lucie corals to gain insight into the organization of coral–algal symbioses under local stressors. Two scleractinian coral species that dominate the reef, *Montastraea cavernosa* and *Pseudodiploria clivosa*, were repeatedly sampled at 4 reef sites over 17 mo, during both wet and dry seasons. *Symbiodinium* cellular density and photosynthetic pigments differed between the 2 coral hosts, where *Pseudodiploria clivosa* had higher cell densities and chlorophyll concentrations than *Montastraea cavernosa*. Over time, these parameters varied, but were not significantly altered following freshwater discharge events. *Symbiodinium* diversity and abundances were identified using ITS2 region amplification and next-generation sequencing, which revealed remarkable stability of the relative proportions of different *Symbiodinium* genotypes throughout the sampling period. Novel associations with unique *Symbiodinium* strains observed for each coral species as well as the stability of these symbioses could indicate local adaptation of St. Lucie Reef corals to their marginal environmental conditions.

KEY WORDS: *Montastraea* · *Pseudodiploria* · *Symbiodinium* · Symbiosis · Zooxanthellae · Next-generation sequencing

INTRODUCTION

High productivity of coral ecosystems in shallow waters is primarily attributed to the symbiotic associations between corals and single-celled dinoflagellate algae. These algal symbionts are classified in the genus *Symbiodinium* (Muscatine & Porter 1977), which is subsequently subdivided into 9 clades ranging from A to I (Pochon & Gates 2010). Of these, clades A–D, F, and G are known to occur in scleractinian corals (Baker 2003). Over the past 2 decades, much research has focused on determining the diversity and organization of *Symbiodinium* spp. (Trench 1993, LaJeunesse 2002, Savage et al. 2002, LaJeunesse et al. 2004, 2010a, Pochon et al. 2006, Thornhill et al. 2007, Finney et al. 2010, LaJeunesse & Thornhill 2011). Taxonomic understanding is limited, where sub-generic clades are recognized, and

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within each clade are types (or sub-clades) that have been identified using molecular marker sequence differences and DGGE profiles (LaJeunesse 2002, 2005, Litaker et al. 2007, Loram et al. 2007, Thornhill et al. 2007, 2014, Bellantuono & Baker 2008, Sampayo et al. 2009, Pochon et al. 2012).

The current understanding of *Symbiodinium* diversity has primarily been achieved through molecular genotyping of the ribosomal internal transcribed spacer region 2 (ITS2; Litaker et al. 2007, Sampayo et al. 2009, Stat et al. 2011), but a suite of other markers including ITS1, microsatellites, the non-coding region of the plastid psbA minicircle, mitochondrial cytochrome *b*, and actin genes have been employed to characterize *Symbiodinium* variants (van Oppen et al. 2001, Sampayo et al. 2009, LaJeunesse & Thornhill 2011, Pochon et al. 2012, LaJeunesse et al. 2014, Thornhill et al. 2014). The multicopy nature of the ITS2 gene (Thornhill et al. 2007, Arif et al. 2014) and the intragenomic sequence variation detected among *Symbiodinium* within an individual coral can influence interpretations regarding the composition of the algal assemblage (LaJeunesse 2002, Thornhill et al. 2007, Sampayo et al. 2009). Increased sequence diversity estimates revealed using next-generation sequencing has identified numerous low-abundance ITS2 variants that were not previously detected with DGGE (Stat et al. 2011, Silverstein et al. 2012).

Symbiodinium clade distribution within a reef location is believed to be primarily determined by reef host species (LaJeunesse et al. 2010b, Davies et al. 2014), biogeography (Rodriguez-Lanetty et al. 2003, LaJeunesse 2005, Finney et al. 2010, LaJeunesse et al. 2010a, Davies et al. 2014, Thornhill et al. 2014) and environmental conditions that affect photophysiology, most notably light and temperature (Rowan & Knowlton 1995, Warner et al. 1996, LaJeunesse & Trench 2000, LaJeunesse 2002, Baker 2003, Berkelmans & van Oppen 2006, Sampayo et al. 2007, Thornhill et al. 2008, Finney et al. 2010). It is likely that each *Symbiodinium* strain has particular environmental thresholds (LaJeunesse & Trench 2000, LaJeunesse 2002), and genetic differentiation can arise under the demands of unique environments. One reef locale or region can contain an ecologically dominant type or an assortment of coexisting types (LaJeunesse 2002, Baker 2003, Goulet 2006, LaJeunesse et al. 2010a). Furthermore, the particular coral–*Symbiodinium* combination can influence how the holobiont functions as an ecological unit and responds to environmental changes (Berkelmans & van Oppen 2006).

Maintenance of *Symbiodinium* assemblages under fluctuating environments is important for the persistence of reef-building corals. The coral–algal symbiosis can persist, breakdown (as observed during coral bleaching), undergo advantageous modifications, or evolve adaptive traits (Buddemeier & Fautin 1993, Baker 2003, Chen et al. 2005, Berkelmans & van Oppen 2006, Thornhill et al. 2006a, LaJeunesse et al. 2010b). Generally, stable or monotypic *Symbiodinium* assemblages are maintained under steady environmental conditions (LaJeunesse 2002, 2005, LaJeunesse et al. 2004, Thornhill et al. 2006a, Bellantuono et al. 2012). Unless resident *Symbiodinium* are broadly tolerant to the local environment, advantageous responses to environmental change must occur with the necessary scale and pace to allow for acclimatization (Hoegh-Guldberg et al. 2007, McGinley et al. 2012, Putnam et al. 2012). To overcome or recover from stress events, adaptive mechanisms such as symbiont 'switching' (symbiont acquisition from external environmental sources) or 'shuffling' (differential growth of resident background populations) can promote endosymbionts that are physiologically tolerant to thermal stress (Baker et al. 2004). Broadly, there is evidence that persistent *Symbiodinium* assemblages can confer increased host survival (Berkelmans & van Oppen 2006, Jones et al. 2008). Symbiont switching and shuffling are depicted as a short-term benefit (Mieog 2009, Mieog et al. 2009, Putnam et al. 2012), with ecological consequences such as reduced growth (Jones & Berkelmans 2010) or increased intraspecific competition. If the coral–algal holobiont association can remain stable under environmental fluctuations, low symbiontic flexibility may be the result of a fixed trait (Thornhill et al. 2006b, Bellantuono et al. 2012, McGinley et al. 2012, Kenkel et al. 2013) or evidence of physiological flexibility by the particular holobiont (Bellantuono et al. 2012).

Many coastal coral reef habitats persist despite high thermal variability and intermittent eutrophic conditions. Elucidating the mechanisms of coral resilience has become a priority of coral reef ecology (ISRS 2004, NOAA Coral Reef Conservation Program 2009). Numerous studies have documented coral–algal responses to episodic and seasonal temperature fluctuations (Gates et al. 1992, Jones 1997, Brown et al. 1999). However, the mechanisms that allow corals to persist in subtropical habitats influenced by both coastal runoff stressors (fluctuating light, salinity, nutrients, and water clarity; Fabricius 2005) and strong seasonal environmental changes are less understood. Locally adapted *Symbiodinium* assemblages could exhibit top-down control on responses to changing environmental factors, or the environment may im-

pose bottom-up influences that ultimately dictate coral–*Symbiodinium* strain composition.

St. Lucie Reef (SLR) is a shallow reef habitat within the St. Lucie Inlet State Park in Stuart, Florida, lying 2.3 km south of the St. Lucie Inlet and subject to episodic freshwater discharge events from regulated canals (including Lake Okeechobee) and unregulated freshwater runoff from the broader St. Lucie Estuary watershed (Beal et al. 2012). During the wet season (June to November), discharges and runoff can result in nutrient over-enrichment and harmful algal blooms in the St. Lucie Estuary (Graves et al. 2004, Lapointe et al. 2012), as well as migration of turbid, low salinity water over SLR. During this study, 2 scheduled discharge events occurred, one during July 2013 where flow rates reached 84 m^3 s^{-1} and lasted 4 wk, and the other during October 2013 where flow increased to 43 m^3 s^{-1} and lasted 2 wk. In addition to freshwater discharge, summer upwelling events can lower temperatures from an average of 23 to 31°C down to 17°C (Beal et al. 2012). Light limitation, variable water temperatures and salinity synergistically reduce the growing season at reef habitats experiencing upwelling (Glynn 1977) in addition to terrestrial runoff. Corals at SLR encrust the substrate, likely to maximize light reception and avoid displacement (Beal et al. 2012). Although subject to variable water conditions, SLR exhibits coral species richness that is 5× greater than in reef habitats north of St. Lucie Inlet along the Florida coast (Beal et al. 2012). The relatively low diversity of coral communities north of the reef suggests that the corals at SLR are at the northernmost limit for many Caribbean corals along the Florida coastline (Reed 1982).

SLR is a unique reef that has been relatively unstudied until recently. To establish baseline observations of the coral–algal symbiosis at SLR and assess the potential influences of human-induced and seasonal environmental variability, the *Symbiodinium* assemblages within 2 host coral species were analyzed. *Symbiodinium* cellular density (cells per unit area) and chlorophyll concentrations were assessed in the 2 dominant host coral species (*Montastraea cavernosa* and *Pseudodiploria clivosa*) at SLR. To complement these measures, next-generation sequencing of the ITS2 region of ribosomal DNA was used to detect *Symbiodinium* genetic variants and determine if proportions change over time. If freshwater runoff during the wet season correlates with changes in these parameters, it can be inferred that external stressors modify the performance of the symbiotic association, and may have implications for long-term functioning under stressful conditions. Conversely, if the coral–*Symbiodinium* association remains relatively stable through time, this relationship may have been conditioned to tolerate the environmental conditions present.

MATERIALS AND METHODS

Study site and coral collection

Coral fragments were collected at SLR (~2–8 m in depth) within the St. Lucie Inlet State Park from 4 sites of increasing distance from the St. Lucie Inlet (Fig. 1). Individually mapped monitored *Montastraea cavernosa* (not present at North site) and *Pseudodiploria clivosa* colonies were sampled during spring (12 June 2012 and 12 June 2013), summer (8 and 14 August 2013), fall (18 October and 13 November 2013), and winter (24 January and 21 February 2013). Five to six host colonies of each species at each site were GPS marked or mapped to ensure subsequent collection. Coral fragments (11.77 ± 0.66 cm^2) were chiseled from the perimeter of each coral colony, stored in ambient seawater, and transported to the laboratory on ice (<6 h), and frozen at −20°C. Five sampling events yielded a total of 100 samples of *P. clivosa* (5 colonies × 4 sites × 5 sampling events), while only 67 samples of *M. cavernosa* (~5 colonies × 3 sites × 5 sampling events) were collected. Resampling of some *M. cavernosa* colonies was not possible due to small colony sizes and permit regulations requiring that the sampled corals not be consumed entirely, resulting in 8 fewer *M. cavernosa* samples than projected in the study design.

Symbiodinium density and chlorophyll analysis

Tissue was removed from coral fragments with a Waterpik containing 35 psu seawater (Instant Ocean). The resulting slurry was concentrated twice through centrifugation (Eppendorf Centrifuge 5430) and filtered to remove skeletal material. Samples were resuspended and transferred to 15 ml centrifuge tubes, and coral tissue was then removed. *Symbiodinium* were resuspended in 35 psu seawater (Instant Ocean) to yield a final 10 ml cell suspension. For each sample, 3 individual aliquots of *Symbiodinium* suspensions were used for subsequent analysis. One aliquot was preserved in 0.01% formalin for *Symbiodinium* density determination. The second was used to determine chl *a* and chl *c* concentrations. The final aliquot was resuspended, centrifuged, and

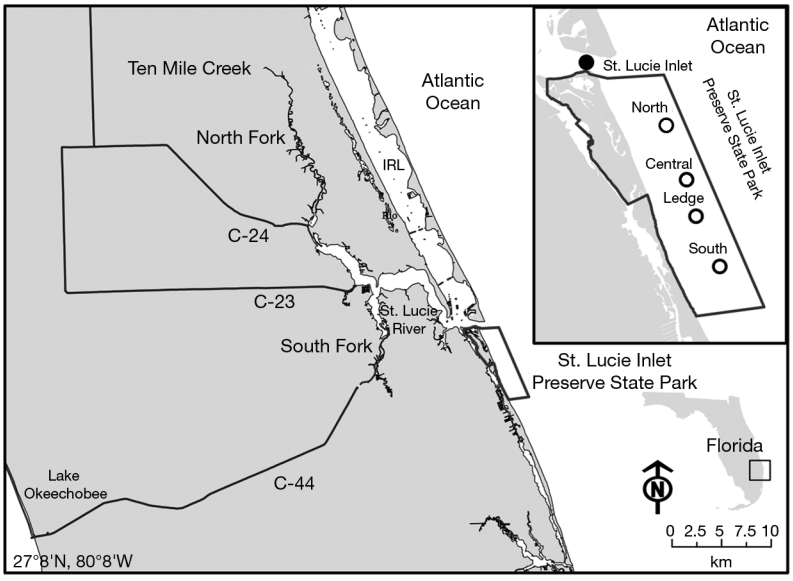

Fig. 1. St. Lucie Estuary and Reef with study sites indicated by circles. The black circle in St. Lucie Inlet indicates location where additional water quality loggers were installed. Left panel shows water routes of canals entering St. Lucie estuary

then preserved in 20% DMSO-EDTA for molecular phylotyping.

Formalin-preserved *Symbiodinium* were enumerated from 5 scaled replicate haemocytometer (1 mm^2) counts that were photographed using the digital program Coral Point Count with Excel extensions (Kohler & Gill 2006). The surface area of coral fragments was determined using the foil method (Marsh 1970), where each fragment was analyzed in triplicate. Cell counts were normalized to slurry volume, then to coral fragment surface area to obtain cell densities. To generate preliminary data on variation in *Symbiodinium* cell sizes, cell volumes ($\mu m^3 = 1/6\pi d^3$) were calculated in the final sampling event of this study (summer 2013) by measuring diameters (d) of 50 haphazardly selected cells for each coral sample using CPCe. Mean cell volume (50 cells $sample^{-1}$) was used as a proxy for total cellular volume of *Symbiodinium* within each sample.

Approximately 0.5 to 1 ml of chlorophyll stock samples was filtered in triplicate through a 25 mm GF/F filter (Whatman) and frozen at –80°C overnight. Filters were homogenized in 10 ml of 90% acetone on ice using a tissue homogenizer (GLH), after which chlorophyll was extracted with acetone in the dark for 20 to 24 h at 4°C. Absorbances of the solution at 750, 663, and 630 nm were determined on a Spectronic 601 spectrophotometer (Milton Roy). Cellular chlorophyll content was calculated using the equations from Jeffrey & Humphrey (1975), and then standardized to density (number of cells ml^{-1}). *Symbiodinium* density as well as chl *a* and *c* were standardized to coral fragment surface area. Chlorophyll content data from summer 2013 samples were also standardized to *Symbiodinium* cell volume.

DNA extraction

Symbiodinium DNA was extracted from *M. cavernosa* and *P. clivosa* using a modified DNA extraction protocol (Promega Wizard Genomic; LaJeunesse et al. 2010b). An aliquot of 20 to 40 mg of isolated *Symbiodinium* cells was washed with 10X Tris-EDTA buffer. For cell lysation, 500 µl of 0.5 mm glass beads and 600 µl of nuclei lysis buffer (Promega) were added and bead beaten for 140 s at 6.5 m s^{-1} in a FastPrep-24 (MoBio). Lysates were incubated with 3 µl of 20 mg ml^{-1} proteinase K and mixed at 1200 rpm every 15 min at 65°C for a total of 90 min. After cooling, 250 µl of protein precipitation buffer (Promega) was added and samples were chilled on ice for 20 min. Following centrifugation at 14 167 × *g* (Eppendorf Centrifuge 5430) for 5 min, 400 µl of supernatant was removed and transferred to a new 1.5 ml microcentrifuge tube containing 700 µl of chilled 100% isopropanol and 25 µl of sodium acetate (3M, pH 5.6). Precipitated DNA extracts were stored overnight at –20°C and centrifuged afterwards to pellet nucleic acids. The pellet was washed twice with chilled 70% ethanol, air dried for 30 min, and rehydrated in 80 µl of resuspension solution (Promega) at 65°C for 60 min. DNA extracts were stored at –80°C.

Symbiodinium genotyping

To elucidate *Symbiodinium* diversity for each of the 167 samples, we used an Illumina-based amplicon sequencing method (Quigley et al. 2014) to investigate diversity at the ITS2 locus. The ITS2 region was amplified via touch-down PCR (Don et al. 1991), using the forward primer its-dino (GTGAATTGCAGAACTCCGTG) and the reverse primer its2rev2 (CCTCCGCTTACTTATATGCTT; Pochon et al. 2001). Each 30 µl reaction consisted of 20 ng of template DNA, 3 µl of *ExTaq* HS 10x *ExTaq* Buffer (Takara

Biotechnology), 0.7 µl of 10 mM dNTPs, 1 µl of an ITS2 10 µM forward and 10 µM reverse primer mix, 0.4 U *ExTaq* HS polymerase (Takara Biotechnology), and 0.2 U Pfu polymerase (Agilent Technologies); milli-Q water was added to achieve final reaction volume. To effectively quantify Ct threshold values, qPCR amplifications (Cycle-check) were run on a thermal cycler (Tetrad 2 Peltier, Bio-Rad), using reaction parameters employed by LaJeunesse & Trench (2000). An initial melting temperature of 94°C was set for 5 min, followed by 19 cycles of 94°C for 40 s, 59°C for 2 min, and 72°C for 1 min, then a final extension of 72°C for 10 min. Any sample that did not represent equal band intensity on a 1% agarose gel following 19 cycles had 1 to 12 cycles added until a faint band was visible on the gel to avoid over/underrepresentation of product amplicons (Kenkel et al. 2013).

Each PCR product was cleaned using a PCR purification kit (GeneJET, Fermentas Life Sciences), quantified, and diluted to 10 ng μl^{-1}. These templates were subject to a second PCR reaction to ligate unique dual barcoded primers and adaptors to each sample. Since the identity of each sample is preserved through the barcode sequence, samples can be pooled for sequencing. The adaptor designs were as follows: **Rapid primer** + **unique barcode** + its-dino and its2rev2 primer, respectively (**TCGTCGGCAGCGTC** + **AGATGTGTATAAGAGACAG** + GTGAATTGCAGAACTCCGTG, **GTCTCGTGGGCTCGG** + **AGATGTGTATAAGAGACAG** + CCTCCGCTTACTTATATGCTT; see Green et al. 2014, Quigley et al. 2014). The reaction profile was identical to ITS2 amplification but only 4 cycles were needed to incorporate barcode primers. Once all samples were verified on a 1% agarose gel, relative band intensity was visually inspected as an indicator to pool PCR products into a final sample (N = 14 to 16 samples per pooled tube). The 6 final pooled samples were cleaned using a PCR purification kit (GeneJET). Samples were eluted in 20 µl milli-Q water and run on a 1% agarose gel stained with SYBR green to achieve a single product band at ~500 bp, ensuring that all pooled PCR products were of appropriate length. Bands were excised and products were extracted using a commercial gel extraction kit. The resulting samples were submitted for Illumina MiSeq sequencing at the Genomic Sequencing and Analysis Facility at the University of Texas (Austin, TX).

Following trimming and quality filtering of individual reads to remove adaptors, barcodes, and primers, 2 757 308 reads remained across 167 samples. The program CD-HIT-OTU (Li & Godzit 2006) was used to cluster concatenated reads from all samples into identical groups at 97% similarity for identification of true operational taxonomic units (OTUs). After mapping sequence reads from all samples to each OTU and calculating the total number of mapped reads per sample, the proportion of reads mapped to each cluster relative to the total reads mapped per sample was calculated. Nineteen OTU sequences were then aligned in the publicly available Cyberinfrastructure for Phylogenetic Research (CIPRES) gateway using 2 multiple sequence alignment programs for nucleotide sequences: Multiple Alignment using Fast Fourier Transform (MAFFT; Katoh et al. 2002) and ClustalX (Larkin et al. 2007). A BLASTn search was run on the sequences identified against the GenBank (NCBI) nucleotide reference collection.

The divergence among the 19 OTUs was determined by constructing a haplotype network using the program Network 4.6.1.2 (Fluxus Technology). Aligned reference sequences were identical in length and included gaps. An additional haplotype network was constructed between clade-identified OTUs and *Symbiodinium* ITS2 sequences known to occur in both host corals within the wider Caribbean.

Statistical analyses

Statistical analyses were conducted using R 3.0.2 (R Developmental Core Team 2013). Mean *Symbiodinium* densities, cellular chlorophyll, and spatial chlorophyll were calculated for each host species at each site. Tests for normality were performed using the Shapiro-Wilk test. A MANOVA collectively assessed the effects of species, site, and time (season) on the 5 response variables (*Symbiodinium* cell density, chl *a* $cell^{-1}$, chl *c* $cell^{-1}$, chl *a* per unit coral area, chl *c* per unit coral area) to account for correlations among these responses. Tests were run separately for each species since the site × species interaction was significant due to the absence of *M. cavernosa* at the North site. Additionally, individual ANOVA tests were run for cell density and chl *a* and *c* concentrations, with season, species, and site as factors. Response variables were tested against each other for correlations. Mean *Symbiodinium* cell volumes and chl *a* and *c* content per cell volume from summer 2013 collections were compared between *M. cavernosa* and *P. clivosa* using a Welch's 2-sample *t*-test.

OTUs that contained low read counts (<0.1% of all samples) were removed from the dataset. The differences in representation of raw OTU counts within each species and among sites and seasons were estimated using the MCMC.OTU package (Green et al.

2014), which is a modified Bayesian Markov Chain Monte Carlo method. Since sample numbers between *M. cavernosa* and *P. clivosa* were unbalanced, analysis had to be run separately. Pairwise comparisons were carried out using Poisson-lognormal generalized linear mixed models using fixed effects of species, site, season, species × site, species × season, site × season, and species × site × season. Further analysis in MCMC.OTU adjusted the model to retain those OTUs that were modeled reliably, delimited by autocorrelations, meeting confidence parameter estimates, and de-emphasizing the absences of OTUs.

RESULTS

Symbiodinium density and chlorophyll content

An initial MANOVA examined the 3 independent variables (species, season, site) against *Symbiodinium* density, and chl *a* and *c* per cell and per cm^2 of coral. The Wilk's Lambda test indicated significant associations between species (Wilk's $\lambda = 0.59$, $F_{5,128} = 17.55$, $p < 0.001$), season (Wilk's $\lambda = 0.36$, $F_{20,425} = 7.73$, $p < 0.001$), and the interaction between species and season (Wilk's $\lambda = 0.69$, $F_{20,425} = 2.57$, $p < 0.001$). Univariate analyses indicated that there were fewer *Symbiodinium* cells cm^{-2} in *Montastraea cavernosa* than in *Pseudodiploria clivosa* ($F_{1,165} = 68.92$, $p < 0.001$), with densities in *P. clivosa* being twice those observed in *M. cavernosa* (Fig. 2). *Symbiodinium* cells from *M. cavernosa* were larger than those from *P. clivosa* (mean cell volumes = 752.17 ± 97.10 μm^3 vs. 422.81 ± 68.70 μm^3). Total mean cell volume per coral tissue area were 2.07 (±0.13) × 10^5 μm^3 cm^{-2} and 2.29 (±0.09) × 10^5 μm^3 cm^{-2} for *M. cavernosa* and *P. clivosa*, respectively. *Symbiodinium* densities did not vary significantly among sampling locations ($F_{3,164} = 2.22$, $p = 0.09$). Densities changed significantly over time ($F_{4,163} = 9.65$, $p < 0.001$) for both *P. clivosa* and *M. cavernosa* (Fig. 2). All interaction terms were not significant ($p > 0.05$).

Symbiodinium in *M. cavernosa* had significantly higher cellular chl *a* and *c* content than *Symbiodinium* associated with *P. clivosa* ($F_{1,166} = 34.20$, $p < 0.001$ and $F_{1,166} = 36.39$, $p < 0.001$ for chl *a* and *c* respectively). When chlorophyll content was normalized to *Symbiodinium* cell volume, *Symbiodinium* cells from *M. cavernosa* contained less chlorophyll (chl *a* = 0.0066 ± 0.0028 pg μm^{-3} and chl *c* = 0.0026 ± 0.0011 pg μm^{-3}) than those from *P. clivosa* (chl *a* = 0.0096 ± 0.0049 pg μm^{-3} and chl *c* = 0.0037 ± 0.0020 pg μm^{-3}) (chl *a*: $T = -2.30$, $p < 0.05$; and chl *c*: $T = -2.20$, $p < 0.05$). Cellular chlorophyll content was negatively correlated with *Symbiodinium* density (chl *a*: Pearson's $r = -0.43$, $p = < 0.001$; chl *c*: $r = -0.45$, $p < 0.001$), and chlorophyll content per unit coral sur-

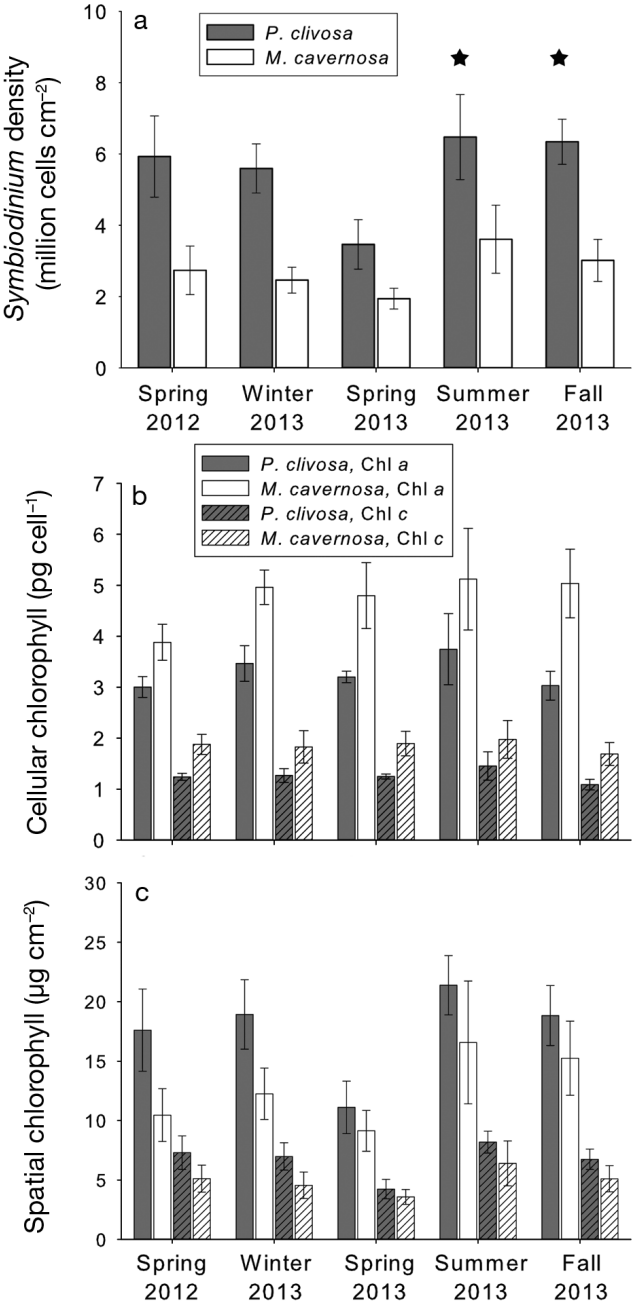

Fig. 2. (a) Density of *Symbiodinium* (million cells cm^{-2}) in relation to sampling event season. (b) Chl *a* and *c* content per cell of *Symbiodinium*, based on zooxanthellae density data. (c) Spatial chlorophyll, based on cellular chlorophyll scaled to coral fragment surface area. Samples from the 4 sites were averaged for each coral species. Error bars: SEs; (★) the 2 scheduled discharge events during summer and fall 2013

face area was positively correlated with *Symbiodinium* density (chl *a*: r = 0.77, p < 0.001; chl *c*: r = 0.77, p < 0.001). Chl *a* and *c* per coral cm^2 changed with season ($F_{4,163}$ = 10.32, p < 0.001 and $F_{4,163}$ = 9.47, p = < 0.001, respectively), where post-hoc analysis indicated that spring 2013 samples had the lowest chlorophyll coral^{-1} cm^2 compared to all other sampling seasons, except for chl *a* during summer 2013. Neither site nor interaction terms between all factors demonstrated significant effects on both cellular chlorophyll and chlorophyll per coral tissue area.

Symbiont genotyping

Cluster analysis yielded 945 730 (72%) unique sequences that were mapped to 19 reference OTUs. Of the 19 OTUs, average base pair length was 321 and ranged from 304 (OTU 15) to 361 bases (OTU 1). The number of sequences per sample mapped to each OTU ranged from 0 to 17 787 sequences (mean: 5663). The most frequent OTU (OTU 1) represented 69% of all mapped reads, where the least assigned OTU (OTU 16) represented only 1.06 × 10^{-6}% of all sample reads. Within *M. cavernosa* and *P. clivosa*, 6 of the 19 OTUs (*M. cavernosa*: OTU 1, 2, 4, 6, 9, 11 and *P. clivosa*: OTU 1, 2, 3, 5, 7, 8) in each coral species accounted for more than half of all samples (median count exceeding one), and were analyzed further. The most dominant OTU sequenced from *M. cavernosa* was OTU 2 (91%; Fig. 3), followed by OTU 4 (4%), OTU 1 (2%), and OTU 6 (2%). The dominant OTU detected in *P. clivosa* was OTU 1 (96%), followed by small proportions of OTU 3 (3%).

OTU sequence queries against the NCBI GenBank nucleotide library (blastn; Table S1 in the Supplement at www.int-res.com/articles/suppl/m532p137_supp.pdf) matched the 19 OTUs to the following *Symbiodinium* types: B1 and B224, A4 (*Symbiodinium linucheae*, LaJeunesse 2001), uncultured clade A, clade C3 variants from *Agaricia* sp. (hereafter designated as the first unknown C type, CX.1) and *M. cavernosa* (designated as type CX.2), and clade C isolates from an aeolid nudibranch, *Pteraeolidia ianthina*, collected in the Philippines (called type CX.3) and from *Stylocoeniella guentheri* (called type CX.4). The clade C OTUs are identified by the known clade to which they are most related, followed by an X. and an arbitrary number.

OTUs identified to a particular clade were clustered together in the haplotype network (Fig. 4a). Although the CD-HIT-OTU program identified OTUs 1 and 3, the median-joining network algorithm did not differentiate these 2 OTUs and grouped them into a single haplotype (B1). OTUs designated as clade A and C comprised 97.48% of sequence reads originating from *M. cavernosa*. Clade A and C clusters are more distantly related to clade B, which constituted 99.92% of the sequences derived from *P. clivosa*. Examining OTU sequences identified as CX types with reference ITS2 sequences known to occur in *M. cavernosa* from the wider Caribbean (LaJeunesse 2002, Savage et al. 2002, Pochon et al. 2006, Finney et al. 2010), OTU 2 (CX.1) was grouped with *Symbiodinium* C3 as the same haplotype following network construction. GenBank (NCBI) alignment of OTU 2 had 100% identity with clade C3, but at a lower ranked hit (E value = 7 e^{-113}). All clade C OTUs diverged from a basal C type, and OTUs 11 (CX.1), 9 (CX.4), and 6 (CX.2) were distantly related to the remaining haplotypes. OTU 11 (CX.1) sequence divergence from OTU 2 (CX.1) and similar identity matching via BLAST suggest intragenomic variation of OTU 2. OTU 6 (CX.2) grouped with type C3.N26 (Bongaerts et al. 2015), and are distinct out-

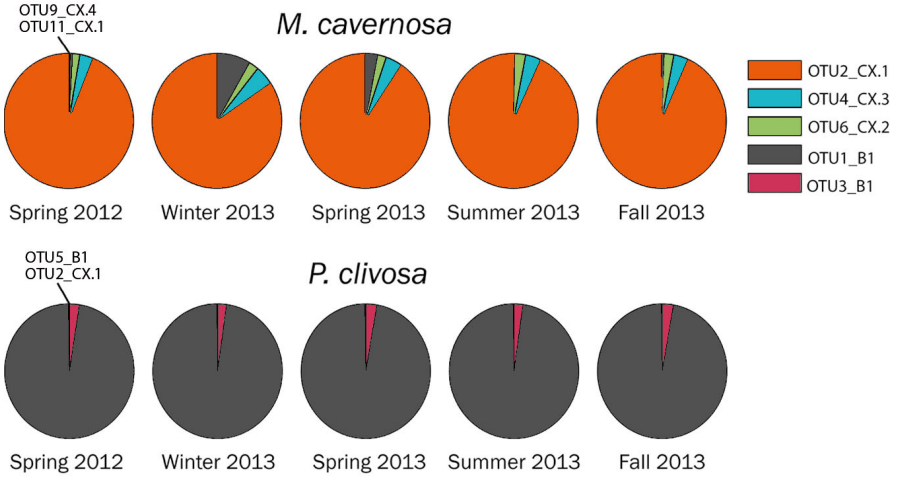

Fig. 3. Proportion of *Symbiodinium* operational taxonomic units (OTUs) present in more than half of all samples from each coral host. Pie charts are split by coral host and separated by sampling event season. OTUs representing <0.5% of the total assemblage for each coral host (OTU5_B1, OTU7_B1, OTU9_CX.4, and OTU10_B1) are not shown

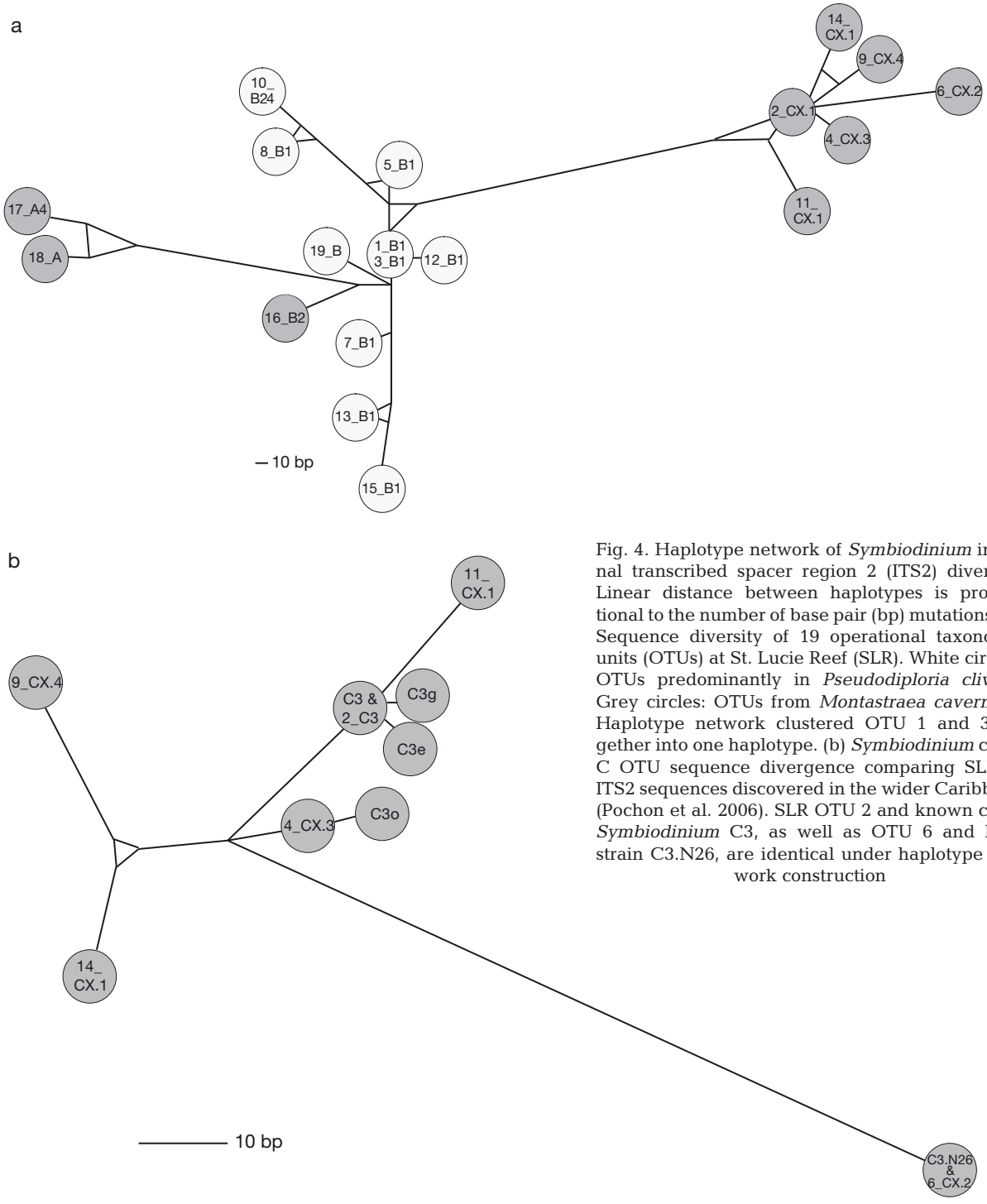

Fig. 4. Haplotype network of *Symbiodinium* internal transcribed spacer region 2 (ITS2) diversity. Linear distance between haplotypes is proportional to the number of base pair (bp) mutations. (a) Sequence diversity of 19 operational taxonomic units (OTUs) at St. Lucie Reef (SLR). White circles: OTUs predominantly in *Pseudodiploria clivosa*. Grey circles: OTUs from *Montastraea cavernosa*. Haplotype network clustered OTU 1 and 3 together into one haplotype. (b) *Symbiodinium* clade C OTU sequence divergence comparing SLR to ITS2 sequences discovered in the wider Caribbean (Pochon et al. 2006). SLR OTU 2 and known clade *Symbiodinium* C3, as well as OTU 6 and ITS2 strain C3.N26, are identical under haplotype network construction

liers from all other OTUs, corroborating a novel *Symbiodinium* ITS2 sequence strain.

MCMC.OTU generalized linear mixed modeling analysis fits the more abundant *Symbiodinium* OTUs and conservatively de-emphasizes OTU absences. OTUs from *M. cavernosa* were fit to the model, and pairwise comparisons between all pairs of factor combinations (species × site, species × season, site × season, species × site × season) revealed that OTU 1_B1, 2_CX.1, 4_CX.3, 6_CX.2, 9_CX.4, and 11_CX.1 were present in >99% of all samples (Fig. 5). After adjusting p-values for multiple comparisons, OTU 1_B1, 4_CX.3, 9_CX.4, and 11_CX.1 in *M. cavernosa* differed by season. The proportion of OTU 1_B1 increased during

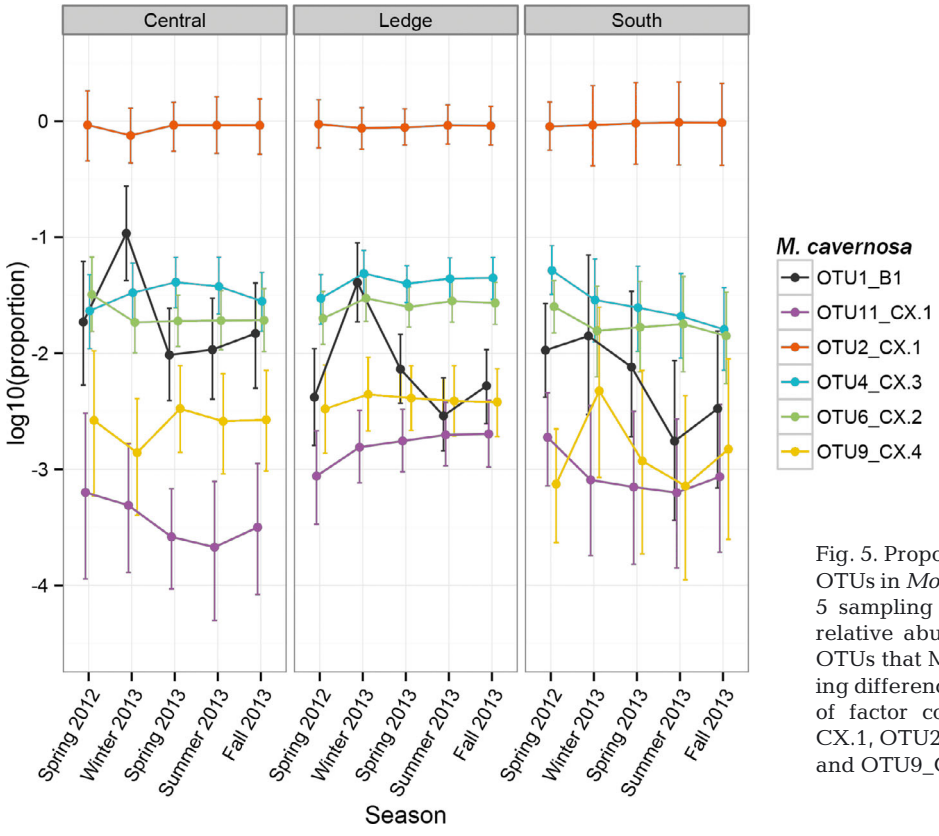

Fig. 5. Proportion of significant *Symbiodinium* OTUs in *Montastraea cavernosa* by site across 5 sampling events, based on distribution of relative abundance (log10 transformed) of 6 OTUs that MCMC.OTU modeled by calculating differences and p-values between all pairs of factor combinations. OTU1_B1, OTU11_CX.1, OTU2_CX.1, OTU4_CX.3, OTU6_CX.2, and OTU9_CX.4 autocorrelate among sample parameter values

winter 2013, and it became less frequent during the subsequent sampling seasons. OTU 4_CX.3 became less abundant at the South site throughout the sampling period, and proportions of OTU 11_CX.1 were lowest in the Central site over time. OTU 9_CX.4 fluctuated across all reef sites. The most abundant OTUs within *P. clivosa*, OTUs 1, 3, 5, 7, 8 (all B1), fit the MCMC.OTU model reliably. After calculating differences between all pairs of factor combinations, these 5 OTUs retained significant abundances (>99% of all samples) within *P. clivosa* by site over time (Fig. 6). Only OTU 8_B1 was significantly different over time and by reef site; its proportion increased during the first 12 mo at the South site and was lower overall at the Central site (Fig. 6). The remaining OTUs from *P. clivosa* remained stable throughout the study at all sites, and this consistency is evident in Figs. 3 & 6.

DISCUSSION

Symbiodinium density and chlorophyll content

Symbiodinium abundance and photosynthetic pigments are important for overall holobiont fitness. The potential effects of changes in local water quality on coral performance are substantiated by direct phototropic responses of *Symbiodinium* (Brown et al. 1999). The present results of 5 sampling events spanning 17 mo show variation in *Symbiodinium* density and photosynthetic pigments over time. Previous long-term (Fagoonee 1999, Fitt et al. 2000) and short-term (Chen et al. 2005, Kemp et al. 2014) observations reported higher *Symbiodinium* densities in the winter and spring under reduced temperature and irradiance, contrary to results from the SLR system (Fig. 2), which showed slight increases following the discharge events of summer and fall 2013. Freshwater discharge is generally characterized by increased surface water temperature, reduced salinity, increased dissolved inorganic nutrients, and lowered light availability (Beal et al. 2012). Light attenuation from low ambient light or suspended materials have been shown to cause an increase in cell numbers (Falkowski & Dubinsky 1981, Costa et al. 2004). Costa et al. (2004) observed an increase in cell density during northeast Brazil's wet season (fall/winter), while chlorophyll increased during the dry season (summer). *Symbiodinium* densities and chlorophyll in Brazilian corals were drastically reduced following heavy summer rains (Costa et al. 2004), contradicting the observations at SLR.

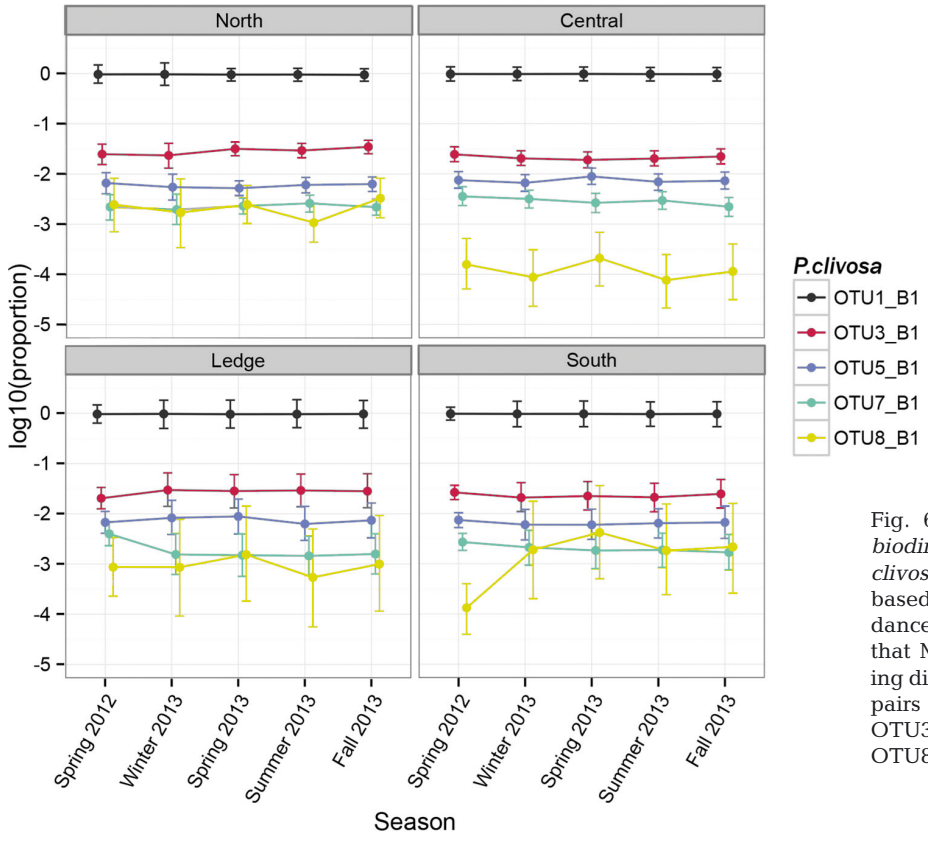

Fig. 6. Proportion of significant *Symbiodinium* OTUs in *Pseudodiploria clivosa* by site across 5 sampling events, based on distribution of relative abundance (log10 transformed) of 6 OTUs that MCMC.OTU modeled by calculating differences and p-values between all pairs of factor combinations. OTU1_B1, OTU3_B1, OTU5_B1, OTU7_B1, and OTU8_B1 autocorrelate among sample parameter values

Turbid freshwater runoff over SLR alters light attenuation, thereby reducing down-welling light to resident coral colonies (Beal et al. 2012). In addition to increasing cell numbers, *Symbiodinium* are known to acclimatize to reduced light availability by either increasing their cellular chlorophyll content or by changing the size of photosynthetic antennae (Falkowski & Dubinsky 1981, Iglesias-Prieto & Trench 1994). Wilkerson et al. (1988) observed larger *Symbiodinium* cells from *Montastraea cavernosa* in comparison to other coral species, in agreement with preliminary investigation in this study and recent chlorophyll per volume results using flow cytometry (Williams & Voss 2015). Larger cells could provide more space for either more or larger photosynthetic units (Iglesias-Prieto & Trench 1994). Moreover, pigment changes in the photosynthetic unit could be a compensatory mechanism in response to changes in water clarity (Falkowski & Dubinsky 1981) following freshwater runoff. Increases in accessory pigments (e.g. peridinin) extend the light capturing capacity of *Symbiodinium* (Hofmann et al. 1996), and may explain why cellular chlorophyll content in the present study did not change over time. Quantification of accessory pigments and the ratio of peridinin:chl *a* in future studies can elucidate pigment modifications under variable light regimes. Self-shading from increased *Symbiodinium* densities can also partly explain why chlorophyll increases were not observed following freshwater discharge. Fluctuations in other abiotic factors (PAR, salinity and nutrients levels) have the potential to influence *Symbiodinium* densities and pigment content, and should thus be incorporated into future studies to provide further insight on correlations between water clarity and *Symbiodinium* responses.

Symbiodinium assemblage sequencing reveals stable symbiont assemblages and novel symbiotic associations

M. cavernosa predominantly hosts *Symbiodinium* types CX.1 at 91%, B1 at 2%, and CX.3 at 4% of all mapped ITS2 sequence reads. Conversely, *P. clivosa* contained type B1 (LaJeunesse et al. 2012) at 97% of mapped reads, followed by a genetic variant of B1 at 3%, while OTUs assigned to clade C types account for only 0.07%. There were no significant changes in the dominant symbiont proportion among the 4 reef

sites despite extensive freshwater runoff, suggesting that the types discovered at SLR are adapted to local regimes at Florida's northernmost reef (Savage et al. 2002, Rodriguez-Lanetty et al. 2003, LaJeunesse et al. 2010a). Type B1 in *M. cavernosa* significantly increased in abundance at the Central and Ledge sites during winter 2013. Proportions of type B1 that generally accounted for 0.5 to 3% of total mapped reads from other sampling events increased to 8%. Type B1 has been shown to be a host-generalist (LaJeunesse 2002, Finney et al. 2010), and may have the potential to become abundant in *M. cavernosa* if the local environment remains unperturbed. However, the abundance of type B1 sequence reads declined thereafter within the dry season (December–May), so other resident *Symbiodinium* may have increased. Quantifying relative abundances of *Symbiodinium* sequences cannot provide conclusions about absolute assemblage composition unless sequence copy number per cell is determined; therefore, shifts in representation are speculative in this study.

In addition to *Symbiodinium* abundance homogeneity among the 4 reef sites, *Symbiodinium* assemblages within both coral species remained relatively constant across all sites during this study period, despite environmental variability occurring near SLR. Only type B1 and CX.4 within *M. cavernosa* significantly changed during winter 2013 (Fig. 5), but these low frequency strains account for only 7 and 0.3% of mapped reads, and may not be functionally significant for overall holobiont performance. Determining fine-scale genetic responses in symbiont composition following disturbance events is one potential advantage of using highly sensitive molecular techniques such as deep sequencing (Gust et al. 2014). Jones & Yellowlees (1997) report that complete replacement of symbiont assemblages ('shuffling' or 'switching', Baker 2003) following disturbance can take at least 1 mo (see also Dimond et al. 2013). Coral sampling after the 2 major freshwater discharges in summer and fall 2013 occurred 3 to 4 wk after flow rate increased. The time frame for *Symbiodinium* modifications following disturbances at SLR, as well as whether symbiont populations within these 2 host species actually 'switch' or 'shuffle' (Thornhill et al. 2006a, LaJeunesse et al. 2009) were previously unknown. The results of this study indicate that the *Symbiodinium* assemblage in each coral remains remarkably stable. To elucidate whether the stable associations observed are sustained, future samplings should be done closer to the discharge and repetitively during subsequent months.

Next-generation sequencing detected novel *Symbiodinium* types and associations within *M. cavernosa* and *Pseudodiploria clivosa*, suggesting that genetic drift and/or differentiation occurred in Caribbean populations to produce unique *Symbiodinium* assemblages found in corals at SLR. Although *Symbiodinium* phylotype B1 has been reported in *P. clivosa* within the Caribbean (Finney et al. 2010; Fig. 7), this is the first report of its occurrence in *M. cavernosa*. Additionally, the clade C variants detected at SLR represent divergent lineages of clade C3 detected in previous studies, but only within *M. cavernosa* and not within *P. clivosa* (LaJeunesse 2002, Savage et al. 2002, Finney et al. 2010). Haplotype network analysis between these variants assigned type CX.1 to C3 and type CX.2 to C3.N26, and the remaining C types from SLR have differentiated from type C3. Type CX.2 identified at SLR identically matched the recently identified ITS2

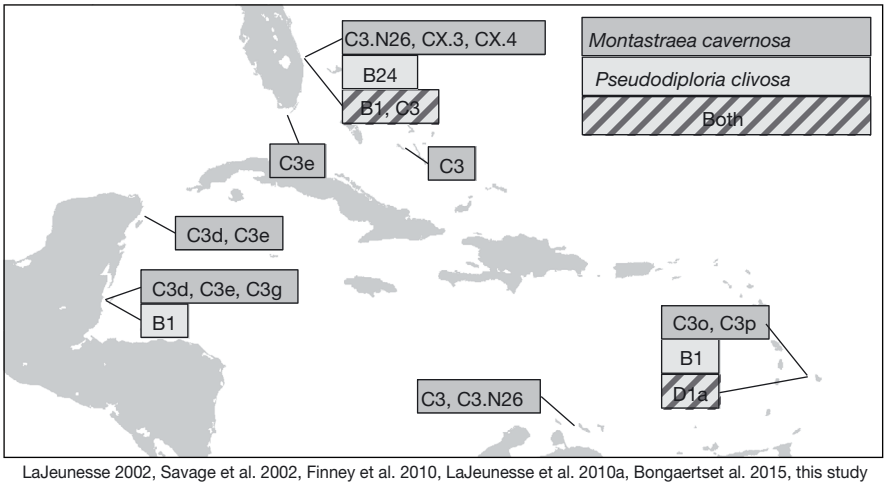

Fig. 7. *Symbiodinium* diversity in the wider Caribbean. Previous studies determining *Symbiodinium* types in corals *Montastraea cavernosa* and *Pseudodiploria clivosa* were used for comparison with this study. Following Network analysis, type CX.1 was updated to type C3. Map of Caribbean generated from www.iho-machc.org

LaJeunesse 2002, Savage et al. 2002, Finney et al. 2010, LaJeunesse et al. 2010a, Bongaertset al. 2015, this study

strain C3.N26 (Bongaerts et al. 2015) isolated from *M. cavernosa* at 5 m of water depth in Curaçao. Sequencing-based approaches to measuring *Symbiodinium* diversity has increased resolution at lower cost as compared to Sanger-based ITS2 diversity assessments, but this technique has the tendency to recover intragenomic variants at low abundance (Thornhill et al. 2007, Sampayo et al. 2009, Arif et al. 2014). Based on BLAST results and Network analysis, it is conceivable that cryptic OTUs 11 and 14 (both designated as type CX.1) are intragenomic variants of abundant OTU 2 (type CX.1), particularly considering the immense diversity and radiation of clade C (Arif et al. 2014). Integration of additional molecular markers, coupled with morphological and physiological data, can facilitate improved characterization of *Symbiodinium* diversity.

Both *M. cavernosa* and *P. clivosa* can associate with multiple types not previously identified in association with each coral host, and these novel associations could represent selection of types at an ecologically unusual location such as SLR. It is also possible that *Symbiodinium* types at SLR persisted at prevailing environmental conditions and became incorporated into the symbiosome to promote coral host survival. Since these 2 coral species acquire their *Symbiodinium* horizontally (Szmant 1986), they have the propensity to build stable assemblages with types that are adapted to the local environmental conditions. Stable patterns in symbiotic associations have been observed in several coral species when exposed to environmental variability (Thornhill et al. 2006b, McGinley et al. 2012, Putnam et al. 2012, Dimond et al. 2013, Kenkel et al. 2013). Acclimatory responses to stressors may be suppressed in holobionts located in stable environments when exposed to infrequent stress events (Thornhill et al. 2006b), and the results of this study and other research (McGinley et al. 2012, Putnam et al. 2012) indicate that historical exposure to environmental stressors could generate adapted associations. Moreover, flexible symbioses are suggested to be maladaptive and may be metabolically costly for coral hosts via reduced host growth or increased interspecific competition among *Symbiodinium* types following stress events (Jones & Berkelmans 2010, Putnam et al. 2012). Therefore, corals with preconditioned symbioses may be able to outperform conspecific corals from homogeneous environments (Kenkel et al. 2013), and the associations observed at SLR may be representative of future coastal, subtropical reef symbiont composition if reefs are increasingly subject to terrestrial influences.

Although SLR corals contain mixed, background populations of *Symbiodinium*, it is uncertain whether these background populations offer any functional advantage to the coral host, contributing to stress tolerance (Barshis et al. 2010, Silverstein et al. 2012). Analyzing *Symbiodinium* assemblages from other coral species at SLR may reveal additional symbiotic associations that include these background symbionts, and could aid in explaining their prevalence at this reef. While fine-scale diversity can be measured via deep sequencing (Quigley et al. 2014), the proportion of symbionts detected cannot yet elucidate functionality (Silverstein et al. 2012, Green et al. 2014). In addition, sequence diversity and abundance does not directly correlate with cellular quantity of ITS2 symbiont types, so care should be taken when assigning overall holobiont fitness to *Symbiodinium* type abundances.

CONCLUSIONS

The results of this study reveal changes in *Symbiodinium* density and photosynthetic pigments within 2 different corals species, *Montastraea cavernosa* and *Pseudodiploria clivosa*, under environmental variability at Florida's northernmost reef. Conversely, *Symbiodinium* assemblage diversity and type relative abundances do not change over time, providing evidence of stable symbioses during episodic and seasonal environmental changes. Next-generation sequencing of *Symbiodinium* ITS2 types revealed that types B1, CX.1, CX.4, and CX.3 differentially associate with the 2 dominant coral species at SLR. *Symbiodinium* dynamics within *M. cavernosa* and *P. clivosa* differ, but changes in density or pigment content are not strong indicators of coral persistence in anthropogenically influenced environments. *Symbiodinium* responses were similar at all sites, suggesting that any potential effects from discharge events could extend to the entire reef tract. This has important implications for influences that contribute to coral–*Symbiodinium* symbioses from unique environments, and effective management of Florida coral reef ecosystems should not focus on a particular coral species but should include the entire reef system for holistic strategies. Local cultivated associations could be reflective of associations in future reefs adjacent to coastal communities, especially if the rate of selection in a more sensitive coral holobiont is outpaced by expanding anthropogenic practices and climate change.

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