INTRODUCTION

Long-term ecological analysis is the most effective way to detect temporal changes in community structure, and, while the most basic tool of this trade is natural history, most contemporary efforts are quantitative (Hairston et al. 2005). Studies vary from single-investigator projects to grand initiatives, such as the United States Long-Term Ecological Research network (Gosz et al. 2010), and the temporal scale can extend over decades or centuries (or longer) depending on the length of the study and the potential to exploit legacy data or the fossil record (Jablonski & Sepkoski 1996). Despite advances in understanding of community dynamics that have come from time-series analyses, most studies continue to rely heavily on measurements of organism abundance and size, which are of limited value in elucidating processes that drive patterns of change (Lindenmayer & Likens 2010).
Coral reefs were once best known for their beauty and biological diversity, but over the last 20 yr they have become the poster child for ecosystem degradation. This change in perception reflects the widespread death of corals through disturbances of natural and anthropogenic origin (Hoegh-Guldberg et al. 2007). When the impacts of disturbances on coral reefs were publicized in the 1980s (e.g. Woodley et al. 1981), their effects at the time were contextualized by only a handful of studies documenting the dynamics of coral communities in undisturbed conditions (e.g. Goreau 1959, Loya 1972). Much has changed since these early analyses, including a proliferation of descriptions of the stressors threatening coral reefs (Hoegh-Guldberg et al. 2007), breakthroughs in understanding the functional biology of corals (Baird et al. 2009), and the implementation of large-scale efforts to record the changes underway (e.g. De’ath et al. 2012). It is still rare, however, to find studies that use traditional ecological tools in combination with molecular tools such as those used to identify the genotypes of coral hosts and their symbiotic Symbiodinium (van Oppen & Gates 2006) that collectively inform the processes driving shifts in coral community structure. Where coral reefs have been quantitatively recorded with images, the opportunity remains for retrospective analyses motivated by emerging discoveries, but, in the case of genetic analyses, it is impossible to propose retrospective studies unless fortuitous pre-emptive sampling led to the preservation of historic tissue samples. The likelihood of such sampling on ecologically relevant time scales is low, and in cases where historic tissue samples in usable conditions are available, the opportunity to secure additional information from them must be fully exploited even if the original sampling was less comprehensive than modern-day science would suggest. Such was the case in the present study where single biopsies of 12 corals from 1994 were available.

The present study combines a 16 yr analysis of coral reefs in St. John, US Virgin Islands (Edmunds 2013), with an initiative that began in 1994 to sample corals for genetic identity (Edmunds & Elahi 2007). Our study focuses on the Tektite reef at 14 m depth in Great Lameshur Bay (18°18.585’N, 64°43.373’W), where the benthic community has been monitored since 1987 (Edmunds 2013). The reef consists of a buttress of Orbicella annularis (formerly Montastraea annularis [Budd et al. 2012]) growing in a columnar-lobate morphology (Weil & Knowlton 1994), with lobes capped by live O. annularis tissue and sides encrusted by algae and invertebrates. Adjacent lobes typically share a common skeleton but function autonomously (Hughes & Jackson 1980), and hereafter are defined as colonies (after Connell 1973). Colonies undergo fission to produce multiple copies (ramets) of each host genotype (genets), but ramets and genets cannot be distinguished visually.

*O. annularis* and its relatives (i.e. *O. faveolata* and *O. franksi*) provide an interesting system to study variation in ecological performance and genetic diversity (of host and *Symbiodinium*), for this group of corals is the most important, widespread, and extensively studied hermatypic scleractinian in the tropical western Atlantic (Knowlton et al. 1992). Moreover, *O. annularis* has played a pivotal role in the paradigm shift that has changed the comprehension of the coral–zooxanthella symbiosis from a union containing a single type of algal cells to one hosting a diverse assemblage of *Symbiodinium* (Rowan & Powers 1991, LaJeunesse 2001, Stat et al. 2008, Putnam et al. 2012). It was the *O. annularis* complex that provided the model system for some of the earliest analyses of ecologically meaningful variation of zooxanthella taxa (Rowan & Knowlton 1995, Rowan et al. 1997), and this taxon has remained popular for the analysis of spatio-temporal variation in *Symbiodinium* for more than 2 decades (e.g. Garren et al. 2006, Thornhill et al. 2009, and references therein). Over this period it has become known as one of the most flexible corals with regard to the types of *Symbiodinium* harbored (Garren et al. 2006, Thornhill et al. 2009). Four clades (A, B, C, and D) of *Symbiodinium* (and multiple subtypes) have been found in the *O. annularis* complex (e.g. Rowan & Knowlton 1995, Garren et al. 2006, Thornhill et al. 2006, 2009), together with intra-colony zonations of *Symbiodinium* clades (Rowan & Knowlton 1995) that include variation among polyps (Kemp et al. 2008) and proportional changes in clade abundance over at least 6 yr (Thornhill et al. 2006).

Despite the research attention accorded to *O. annularis*, the opportunity has not been presented to combine concurrent measurements over multiple decades of holobiont performance, *Symbiodinium* diversity, or host identity. Such an opportunity was created by our work in St. John (Edmunds 2013), and in this paper we present the results of an analysis in which we combine ecological and genetic techniques to samples of *O. annularis* collected over 16 yr. While the 2 resulting sets of data each have limitations arising from methodology and demonic intrusion (sensu Hurlbert 1984), together they present a unique perspective to the processes potentially driving patterns of change on coral reefs in the Caribbean. For the
ecological data, the methodological limitations are that the sampled colonies could not all be located in some of the 16 yr of photoquadrats and that slight differences in camera orientation added to the variance in estimating colony area. For the genetic data, the methodological limitations were the small number of replicate colonies, the sampling of each colony with a single biopsy, and the potential for slight differences in biopsy position on the top of colonies at each sampling.

Tissue was sampled in 1994 from *Orbicella annularis* at the long-term study site on Tektite reef, with the objective of describing the clonal population structure using DNA fingerprinting. Some samples were archived, and in 2010 we realized there was a unique opportunity to combine ecological monitoring of a coral population (i.e. with photoquadrats) with a retrospective and comparative analysis of host and *Symbiodinium* genotypes in these specific coral colonies at 2 times separated by 16 yr. We used the detailed records provided by the annual photoquadrats to track over time 12 *Orbicella annularis* colonies that were sampled in 1994 for DNA, so that their tissues could again be biopsied in 2010 for DNA. The tissue samples from 1994 and 2010 were analyzed to explore the relationships among host genotype, *Symbiodinium* assemblages, and colony size (i.e. a measure of ecological success) with the assumption that a single sample of tissue from a standardized location on the colony surface is adequate to characterize host and *Symbiodinium* genotypes. Three hypotheses were tested: (1) *Symbiodinium* communities in *Orbicella annularis* have not changed over time, (2) the relative abundance of *Symbiodinium* genotypes (mostly at the cladal level) is affected equally by the host genotype (the colony in which they are found) and location on the reef (i.e. the photoquadrat in which they are found), and (3) growth of *Orbicella annularis* is independent of the genetic identity of *Symbiodinium* and the coral host.

**MATERIALS AND METHODS**

**Study site and ecology of *Orbicella annularis***

The time-series analysis on the Tektite reef was based on 3 parallel 10 m transects along which 10 contiguous photoquadrats (1 x 1 m) were recorded annually (n = 30 photoquadrats yr⁻¹). Transects crossed the flat upper surface of a single coral butress and did not depart appreciably from 14 m depth across their length. Images were acquired on 35 mm film (Kodachrome 64) from 1987 to 2000, and digitally from 2001 to 2004 (3.34 megapixels) and 2005 to 2010 (6.1 megapixels). Refer to Edmunds (2013) and Edmunds & Elahi (2007) for methodology. Images are archived (http://mcr.lternet.edu/vinp/overview/), and have been analyzed for percentage cover of the benthic community using CPCe software (Kohler & Gill 2006). In addition to photoquadrats, seawater temperature has been recorded in Great Lameshur Bay since 1989, initially at 9 m depth at Yawzi Point (~1 km away from the Tektite reef) from 1989 to 2011, and at the 14 m Tektite site since 2004. The temperature records used here include values from Yawzi Point from 1994 to 2003 and from Tektite from 2004 to 2009. Temperatures were recorded using a Ryan Industries Tempmentor (+0.3°C) from January 1992 to April 1997 and November 1997 to August 1999, an Optic Stowaway logger (+0.2°C accuracy [Onset Industries]) at 9 m depth from May 1997 to October 1997 and from August 1999 to August 2001, and an Aquaprog logger (+0.2°C accuracy [Onset Industries]) at 9 m depth from August 2001 to August 2010. Loggers recorded temperature every 15 to 30 min, and these data were collapsed by day and described using the mean and interquartile ranges by year.

Colonies of *Orbicella annularis* were first sampled on 12 and 16 August 1994 for DNA fingerprinting. In 1994, biopsies of tissue and skeleton were collected in 2 photoquadrats using a 13 mm diameter steel punch. To prevent cross-contamination, a sterile punch was used for each colony and each biopsy was bagged individually underwater. Care was taken to sample biopsies from the horizontal upper surfaces of colonies, and the sampled holes were filled with nontoxic modeling clay. Photoquadrats were then photographed to allow sampled colonies to be located again and to evaluate the effects of sampling on these colonies. Biopsies were transported to the laboratory on ice (~30 min) and crushed in 2 ml of ice-cold guanidine hydrochloride (GHCl) buffer (8 M GHCl, 0.1 M sodium acetate [pH 5.2], 5 mM dithiothreitol, 0.5% N-lauryl sarcosine; Maniatis et al. 1989). After grinding, the slurry was stored at 4°C and transported at room temperature to California State University, Northridge (CSUN). Some samples were processed (Edmunds & Elahi 2007), while others were stored at 4°C until 2010.

Between 5 and 8 August 2010 (i.e. virtually the same time of year as in 1994) the same colonies of *O. annularis* biopsied in 1994 were sampled with the objectives of genotyping the coral host and their *Symbiodinium*. To ensure identical colonies were sampled, the annual photoquadrats were used to
track over time the shape, color, and orientation of the colonies first sampled in 1994. Based on the capacity to relocate colonies for which archived DNA from 1994 was available and the constraints of permits issued by the Virgin Island National Park, 12 colonies were tracked from 1994 to 2009. In 2010, laminated prints of the photoquadrats were used underwater to locate the same colonies for a second sampling and to ensure that the sampling orientations were the same as those employed 16 yr earlier. Sampling in the same position on each colony was important to reduce the likelihood that variation in Symbiodinium genotypes was caused by sampling different microhabitats across the colony surface (Rowan et al. 1997, Garren et al. 2006, Kemp et al. 2008). Our analysis therefore assumes that differences in Symbiodinium genotypes between the 2 samplings reflected the passage of time and not differences in sampling position within each colony.

In 2010, the 12 colonies of O. annularis were sampled using 6 mm diameter punches, and the holes were filled with non-toxic modeling clay. A sterile punch was used for each colony to prevent cross-contamination, and biopsies were bagged individually underwater, stored on ice, and returned to the laboratory for processing. Biopsies were stored in DNA extraction buffer (50% [w/v] guanidinium isothiocyanate, 50 mM Tris [pH 7.6], 10 µM ethylenediaminetetraacetic acid [EDTA], 4.2% [w/v] sarkosyl, and 2.1% [v/v] β-mercaptoethanol) and shipped at room temperature for processing at the University of Hawaii (Symbiodinium) or Florida State University (O. annularis host).

To evaluate growth, each of the 12 colonies was measured in the annual photoquadrats. Most colonies were found every year, although occasionally it was not possible to measure their size if they were obscured by the camera framer or gorgonians. Colonies were measured using ImageJ 1.42q software (Abramoff et al. 2004) to outline coral tissue and calculate planar area (cm²). Each colony was tracked over 16 yr, and the trajectory of changing size was used as a measure of success. Success of the 12 colonies was placed in a broader context by evaluating overall coral cover using the 30 photoquadrats sampled annually. Coral cover was measured using CPCe software and a grid of 200 randomly located points on each image.

**DNA extraction**

Genomic DNA (coral host and Symbiodinium combined) was extracted following Pochon et al. (2001). The 1994 samples were vortexed, and 200 µl of supernatant was placed in tubes with 400 µl of guanidinium buffer. These tubes, as well as the crushed coral biopsies from 2010, were incubated at 72°C for 20 min, centrifuged at 16,000 × g for 5 min, and 300 µl of the supernatant mixed with an equal volume of isopropanol was incubated at −20°C overnight. DNA was precipitated by centrifugation at 16,000 × g for 15 min, and the DNA pellet was washed in 70% ethanol, resuspended, and stored in Tris buffer (0.1 M pH 8).

**Symbiodinium analysis**

Two genes from different cellular compartments (nuclear and chloroplastic), and 2 analytical approaches (cloning and pyrosequencing) were used to genotype the Symbiodinium in the 24 coral biopsies (12 each from 1994 and 2010). These approaches added the support of multiple markers and methodologies to tests of our hypotheses. Moreover, pyrosequencing provided more resolution to the analysis of temporal variation in Symbiodinium assemblages and its effects on holobiont performance in comparison to studies employing fingerprinting gel-based analysis. Previous studies have focused on banding patterns generated by using gel electrophoresis to separate DNA from Symbiodinium based on denaturing gradients (i.e. DGGE fingerprints) (LaJeunesse 2002, Pochon et al. 2007, Sampayo et al. 2009), and, while such approaches have good resolution for identifying dominant patterns, they have limited capacity to more finely resolve genetic variation or to detect rare genotypes. While interpreting the biological significance of rare Symbiodinium genotypes remains controversial in light of the presence of intragenomic variation associated with ITS2 (LaJeunesse & Thornhill 2011, Stat et al. 2011), the increasing number of examples of Symbiodinium–host symbioses in which rare Symbiodinium genotypes have been found (Franklin et al. 2012, Green et al. 2014) argue strongly for using techniques with the resolution to detect such forms. This argument was compelling to us in the decision to expand our analytical approach to tag pyrosequencing of nrITS2 (nuclear ribosomal internal transcriber spacer 2) amplicons, as was the success of this approach in advancing other fields requiring fine-grained genetic resolution of single-celled organisms (Sogin et al. 2006). An important limitation of this early adoption of new techniques is we assume that the debate over the meaning of rare Symbiodinium genotypes will be resolved in favor of their biological significance.
We PCR amplified the nuclear Internal Transcribed Spacer 2 (nrITS2) region and the chloroplastic 23S domain V (cp23S) region of the ribosomal arrays using the nrITS2 primers ‘its-dino’ and ‘its2rev2’ and the cp23S primers ‘23S4F’ and ‘23STR’ (after Stat et al. 2009, Pochon & Gates 2010). Each 50 µl PCR reaction contained 1 µl of DNA template, 5 µl of 10× ImmoBuffer (Bioline), 0.2 µl IMMOLASE™ Hot-Start DNA Polymerase (Bioline), 2 µl of 50 mM MgCl₂, 1 µl of 10 mM total dNTPs (10 mM each), 1 µl of each primer (10 pmol each), and 38.8 µl of deionized sterile water. A touchdown PCR protocol was performed on a BioRad iCycler™ using the following conditions: 7 min at 95°C, 20 cycles of 94°C for 30 s, 62°C for 30 s (decreased by 0.5°C at each cycle), 72°C for 1 min, and followed by 12 additional cycles with an annealing temperature set at 52°C, and a final extension of 72°C for 7 min. PCR products were purified using the QIAquick™ PCR Purification Kit (Qiagen), and ligated into the pGEM-T Easy vector™ (Promega). A minimum of 10 positive inserts per clone library were amplified using plasmid-specific (M13) primers, and sequenced in both directions using the ABI Prism Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit and an ABI 3100 Genetic Analyzer (Perkin-Elmer Applied Biosystems). Bi-directional sequences were inspected and assembled using Sequencher V4.7 (Gene Codes Corporation) and aligned using BioEdit V7.0.5.3 (Hall 1999), and Symbiodinium were identified to clade and subclade level using the Basic Local Alignment Search Tool (BLAST) in nrITS2 and cp23S databases generated from sequences archived in GenBank (23 April 2013). This database is in Supplement 1 as a fasta file (www.int-res.com/articles/suppl/m506p129_supp/).

The patterns of dominant-cloned Symbiodinium sequence types (i.e. the most abundant sequences in each sample) obtained using nrITS2 and cp23S were similar, but there were a few differences between the abundances in the cloned sequence numbers. This suggested that too few clones had been sequenced to fully capture the diversity of rare Symbiodinium sequence types. To address this issue, all DNA samples were re-sequenced using multiplexed tag pyrosequencing of the nrITS2 amplicons (described as pyrosequencing hereafter). This method, pioneered in the field of microbial ecology, is replacing cloning and DGGE as the technique of choice for addressing diversity (Huse et al. 2008) and has been successfully employed for characterizing cryptic Symbiodinium diversity within *O. faveolata* and *O. franksi* in the Gulf of Mexico (Green et al. 2014). We used pyrosequencing to increase the mean number of Symbiodinium sequences acquired per coral sample from ~10 to >1300. For pyrosequencing, 24 nrITS2 Symbiodinium amplicon libraries were submitted to the Research and Testing Laboratory, LLC (Texas, USA) for analysis. Pyrosequencing involved ligating sample-specific tags for each of the 24 samples during the ITS2 amplification (cycling conditions described above, amplified separately from clone libraries), followed by amplicon purification, emulsion PCR, and high-throughput sequencing on a Roche GS FLX pyrosequencing system.

As described above, we acknowledge that ITS2 does not provide the correct characteristics for use as an alpha diversity marker. Prior work (e.g. Stat et al. 2011) has recognized the inability to link an individual biological entity to individual sequences due to duplication in the ribosomal array resulting in the production of paralogs that preclude species assignment. ITS2 can, however, be utilized in a comparative approach to determine patterns in the assemblage of sequences within a given sample. Our approach is primarily focused on identifying genetic variation over time and not on assigning taxonomy to the sequences identified. Reconciling both ecological patterns and taxonomic identity for *Symbiodinium* must be a high priority if the full biological significance of *Symbiodinium* diversity is to be appreciated for coral holobionts.

**Bioinformatic analysis of pyrosequencing results**

The 454 sequencing reads were trimmed to a T threshold of 25, clustered using USEARCH (Edgar 2010), chimera-checked using UCHIME (Edgar et al. 2011), and de-noised based on a quality score of 30 using a standard data analysis pipeline at the Research and Testing Laboratory, LLC. The full fasta file was demultiplexed and adapter-trimmed, and subsequently filtered to remove any terminal regions with a PHRED quality score <20. Sequences were filtered for lengths >180 bp and the maximum number of errors of 1 in the forward primer, and were trimmed of barcodes, adapters, and forward and reverse primers with Integromer (http://course.ics.hawaii.edu/integromer/). A *Symbiodinium*-specific bioinformatic pipeline (symTyper; M. Belcaid unpubl. method, https://github.com/bingo11/symTyper) was then employed to assign each *Symbiodinium* sequence an identity. Briefly, using HMMER V3 (http://hmmer.org/), the resulting sequences were compared to a database of *Symbiodinium* clade hidden Markov model profiles, which were generated from the ITS2
database (Supplement 1). The sequencing reads were subsequently compared to each clade-specific profile, and a read was assigned to clade based on 2 rules: (1) the alignment was significant (e-value ≤ $e^{-20}$ with a sequence alignment over 95% of the read) and (2) the sequences were unlikely to have originated in another clade (e-value for the first hit was at least 5 orders of magnitude smaller than that of the second hit). Sequences failing the first rule were classified as unknown, whereas the sequences failing the second rule were considered ambiguous.

Reads that were successfully assigned to a clade were subsequently compared using BLAST in an nrITS2 database generated from sequences archived in GenBank (Supplement 1) and assigned the subtype of the reference with which they were most similar (97% similarity over 97% of their length). Similar to the clade assignment stage, the non-ambiguity requirement was enforced by requiring that the first hit have a higher raw bit score than that of the second hit. Sequencing reads lacking pairwise similarity with database entries were classified as putatively new and were manually investigated for known systematic biases. Short sequences were dropped from the analysis, while ambiguous sequences aligning with similar quality to 2 or more subtypes were assigned to the lowest common ancestor node in the phylogenetic tree of its clade and reported to clade level only.

Each of the 3 steps described above: (1) clade assignment, (2) sequence subtyping, and (3) tree placement of ambiguous reads were implemented in a python program, available for download from the following github repository: https://github.com/bingo11/ symTyper.

Host genetics

Six microsatellite loci developed by Severance et al. (2004) were used to genotype the O. annularis host tissue. The PCR cocktail consisted of 2.4 μl 5x PCR buffer (Promega), 1.2 μl 1 mM dNTPs, 0.15 μl GoTaq, 1.0 μl 10 M bovine serum albumin, 1.25 to 3.5 μl 1.5 mM MgCl$_2$ (depending on primer), 0.5 μl of fluorescently labeled forward primer, 0.5 μl reverse primer, 2.0 μl DNA (5 ng μl$^{-1}$), and double-distilled water to reach a total volume of 12 μl. PCR amplification was run as follows: 95°C for 3 min, then 30 cycles of 95°C for 1 min, 50°C (primers maMS11, maMS2-4) or 55°C (primers maMS8, maMS12, maMS2-5, and maMS2-8) for 1 min, 72°C for 2 min, then a final extension time of 30 min at 72°C. PCR product from 3 loci with different fluorescent labels was multiplexed (multiplexI: maMS8, maMS2-4, maMS2-5; multiplexII: maMS11, maMS12, maMS2-8) using HiDI formamide (1:12), and 0.5 μl was analyzed with Applied Biosystems 3130xl Genetic Analyzer with Capillary Electrophoresis. Genemapper software (Applied Biosystems, Vers. 4) was used to check for misidentified peaks and stutter bands. All alleles were binned into di- or tri-nucleotide sizes dependent on locus. Colonies with ambiguous genotypes were re-run to confirm binning. Loci were analyzed with MICRO-CHECKER software (van Oosterhout et al. 2004) to calculate the observed and expected heterozygosity and the likelihood of large allele drop-out and null alleles. The probability of colonies having identical genotypes via sexual reproduction (as opposed to being clone-mates through asexual reproduction) was estimated using Gimlet (Valiere 2002).

RESULTS

Study site and ecology of Orbicella annularis

The Tektite reef had 40 ± 3% coral cover in 1994, 49 ± 3% in 2002 (the highest cover during the study), and 28 ± 2% in 2010 (mean ± SE, n = 30); Orbicella annularis was the dominant coral, accounting for an average of 67% of the coral cover between 1994 and 2010 (Fig. 1). Twelve colonies of O. annularis were followed over time in 2 photoquadrats (Fig. 2), and, at the time of sampling in both years, all colonies were visually indistinguishable in terms of color, overall morphology, and corallite size (Fig. 2, and Fig. S1 in Supplement 2 at www.int-res.com/articles/suppl/m506p129_supp/). There were no apparent differences in reef morphology or physical conditions on this spatial scale, and, while the mean size (± SE) of O. annularis colonies varied between Photoquadrats 1 (76 ± 11 cm$^2$, n = 8) and 2 (132 ± 52 cm$^2$, n = 4) in 1994, the difference was not significant ($t = 1.448$, df = 10, $p = 0.179$). The mean sizes of the 12 colonies were associated positively with coral cover (Fig. 1C; $r = 0.817$, df = 10, $p = 0.001$), and none died over the 16 yr. Eight colonies decreased in size by 41 to 82% between 1994 and 2009 (Fig. 1, and Table S1 in Supplement 2), with declines intensifying after 2002 in both photoquadrats. Three colonies grew by 4 to 68% between 1994 and 2009 (2 in Photoquadrat 1 and 1 in Photoquadrat 2) and represented 3 of the 4 smallest colonies in 1994. One colony was not measured in 2009 (though it was in 2008). Linear regressions of colony size on time were significant for 9 colonies ($F ≥ 8.839$, df = 1, $p ≤ 0.01$).
and ≤14, p ≤ 0.014), but not for colonies 20 and 6 (Photoquadrat 1) or colony 5 (Photoquadrat 2) (p < 0.050); colony 5 did not start to decline in size until 2006–07. Overall, the trajectories of change in size (regardless of statistical significance) ranged from −12.6 cm² yr⁻¹ (Colony 3, Photoquadrat 2) to 1.2 cm² yr⁻¹ (Colony 1, Photoquadrat 2), with a mean rate of change of −2.9 ± 1.1 cm² yr⁻¹ (±SE, n = 12).

**Symbiodinium genetics**

Between 10 and 12 cloned *Symbiodinium* nrITS2 and cp23S sequences were obtained from each colony of *O. annularis* in 1994 and 2010, for a total of 264 nrITS2 and 269 cp23S sequences. The nrITS2 and cp23S cloned sequences predominantly belonged to *Symbiodinium* clades B and C (Fig. 3A,B). A single clade D sequence was found in 1 colony of *O. annularis* in 2010 (too low in abundance to register on Fig. 3B). The *Symbiodinium* clades detected in each coral using both markers were similar, but there were minor differences between markers for colonies 8, 17, and 20 in Photoquadrat 1 in 2010 (Table S1). In these 3 cases, additional clades were detected in low-abundance sequences, where a clade was identified with one marker but not the other. For example in colony 20 in 2010, all 12 cp23S sequences belonged to clade C *Symbiodinium*, but with nrITS2 10 sequences belonged to clade C and 1 to clade D. As described above, such inconsistencies suggested that the number of clones sequenced was insufficient to fully visualize the low-abundance templates, and therefore rationalized the use of pyrosequencing.
Overall, clade-level analysis revealed that the proportional abundance of clade B and clade C *Symbiodinium* was dependent on sampling year for both cp23S ($\chi^2 = 18.058, \text{df} = 1, p < 0.001$) and nrITS2 ($\chi^2 = 16.679, \text{df} = 1, p < 0.001$). Analysis of cp23S revealed that clades B and C accounted for 10 and 90% of the sequences in 1994, respectively, whereas clade B increased to 30% and clade C decreased to 70% of the sequences in 2010 (Fig. 3A). The nrITS2 exhibited a very similar trend with clades B and C representing...
12 and 88% of the sequences in 1994, respectively, and clade B increasing to 33% and clade C decreasing to 67% of the sequences in 2010 (Fig. 3B) (clade D accounted for 0.8% of the sequences). Across the 2 sampling points, subclade level sequence identification included nrITS2 types C7, C12, B1, B101, and D1a (Franklin et al. 2012) and cp23S types cpC12 and cpB1 (cp nomenclature sensu Takabayashi et al. 2012). The fewer cp23S types recovered is consistent with the relatively low taxonomic resolution of this marker as compared to nrITS2.

Pyrosequencing of the nrITS2 region resulted in an average of 1324 ± 142 sequences per sample (±SE), for a total of 31 851 sequences; 99.7% were assigned to clade and ~70% of these to subclade (Table 1). The shift in relative abundance of clade B and C *Symbiodinium* in corals sampled in 1994 and 2010 identified in the cloned cp23S and nrITS2 datasets were confirmed in the pyrosequenced nrITS2 dataset, but the relative abundance of clade B in 1994 and 2010 was higher than that detected using cloning (Fig. 3C). In addition to detecting clades C and B, low numbers of clades A, G, and F (<1%) were also detected using this approach (Table 1, and Table S3 in Supplement 2).

Clades B and C accounted for 99.8% of the pyrosequencing identified to clade level (n = 31 767; Table 1). The abundance of clade B and clade C *Symbiodinium* was again dependent on sampling year (χ² = 471.089, df = 1, p < 0.001), with an increase in clade B in 2010. Of the 12 *O. annularis* colonies sampled in 1994, 3 harbored only clade C and 9 had a mixture of clades B and C (Fig. 4, Table S1). Of these 9, 2 colonies (nos. 7 and 20) had more clade B than clade C sequences, and both of these colonies were found in Photoquadrat 1. In 2010, 2 colonies harbored only clade C, and 10 had a mixture of clades B and C. In contrast to 1994, however, 5 of the 10 mixed-clade colonies in 2010 had a greater abundance of clade B than clade C *Symbiodinium*; again these only occurred in Photoquadrat 1. In Photoquadrat 2, all 4 colonies remaining were dominated by clade C *Symbiodinium*, although a small number of clade B sequences (2.4%) were found in colony 3 and 1 sequence was found in colony 7 (0.2%) (Table S1). With regards to the rarer clades, no clade A *Symbiodinium* were detected in 1994, but clade A sequences were detected in 4 colonies in 2010 (≤0.11% of all sequences; Table S1). Clade G and F sequences were also detected at low frequencies in 4 colonies (<0.09% of all sequences) — 2 in 1994 and 2 in 2010 (Table S1).

The BLAST analysis conducted to subtype for the pyrosequencing dataset revealed that clade B was dominated by B1 (79.7%), B1.1122 (12%), B10 (4.1%), and B101 (1.5%), 4 types that collectively represented 97.3% of the data for this clade (Table S3). The relative abundance of these types was dependent on sampling year (1994 versus 2010) (χ² = 28.030, df = 3, p < 0.001). All B types showed a pattern of increase in 2010, ranging from 1.9-fold in B1 to 2.7-fold increases in B1.1122 (Table S3). Clade C was dominated by subclades C7 (56.1%), C12 (33.4%), and C3.15 (9.2%), which together accounted for 98.7% of the clade C sequences recovered. There was an overall shift in relative abundance between years when all 3 types were compared (χ² = 8.870, df = 2, p = 0.012), driven by an increase in the relative abundance of C3.15 in 2010. The relative proportion of subclades C7 and C12 (89.5% of clade C) was consistent between years (χ² = 0.117, df = 1, p = 0.732), with C7 1.7-fold more abundant than C12 in both years.

Table 1. Breakdown of nuclear ITS2 pyrosequencing results for clade and subclade level identification. Sequences were identified to clade by profiling against each clade within a comprehensive internal nuclear ITS2 database (M. Belcaid unpubl. method). Those filtered out at the clade level did not meet the cutoff values of e−20. Sequences were identified to subclade using BLAST and the internal ITS2 database, where the ‘identified to type’ category includes those hit with >97% similarity and >90% of the target length. The category ‘unidentified to type’ represents new sequences. Sequences filtered out at the subclade level represent those too short to identify (<90% of target length) or those occurring in abundance in <3 independent samples, as a conservative approach to minimize potential PCR or sequencing artifacts. ID: identified; UnID: unidentified.

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<td>6062</td>
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<td>B</td>
<td>46.4</td>
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<td>43.0</td>
<td>69.6</td>
<td>69.5</td>
<td>69.5:30.5</td>
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<tr>
<td>C</td>
<td>53.4</td>
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<td></td>
<td>56.8</td>
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<td>F</td>
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<td>9.1:90.9</td>
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Host genetics

In the 12 colonies of *O. annularis* sampled in 2010, 1 locus was monomorphic (maMS8) and 1 failed to amplify consistently (maMS2-4) and was non-informative. The other 4 loci revealed 6 to 7 alleles. MicroChecker (van Oosterhout et al. 2004) indicated no evidence of deviations from null expectations or null alleles. The 4 variable loci provided support that individuals with the same genotype were not identical by descent (sexual reproduction), but via asexual cloning (Gimlet estimate of probability of sharing genotype by descent was \(7.22 \times 10^{-6}\)). The host microsatellite loci indicated that the 12 colonies represented 5 genetic individuals, to which the Greek letters \(\alpha\) to \(\varepsilon\) were assigned (Fig. 2, Table S1). Photoquadrat 1 contained 8 colonies with 2 genotypes (\(\beta\) and \(\varepsilon\)), and Photoquadrat 2 contained 4 colonies with 3 genotypes (\(\alpha\), \(\gamma\), and \(\delta\)).

Synthesis

*Symbiodinium* communities in coral host genotype \(\beta\) (Photoquadrat 1) were dominated by clade C in 1994, and 2 of these 4 colonies shifted to dominance by clade B in 2010 (Fig. 4). *Symbiodinium* communities in host genotype \(\varepsilon\) (Photoquadrat 1) were diverse, with 2 of the 4 colonies hosting more clade B than clade C *Symbiodinium* in 1994; by 2010, 3 of these colonies were dominated by clade B. The *Symbiodinium* communities in host genotypes \(\alpha\), \(\gamma\), and \(\delta\) (4 colonies, Photoquadrat 2) were dominated by clade C in both years. In Photoquadrat 1, clade B increased in abundance from 1994 to 2010, and independently in both host genotypes \(\beta\) (\(\chi^2 = 111.002, df = 1, p < 0.001\)) and \(\varepsilon\) (\(\chi^2 = 481.412, df = 1, p < 0.001\)), but stayed at low background levels for all genotypes in Photoquadrat 2 (maximum 22 sequences in colony 3).

The change in size (cm\(^2\) yr\(^{-1}\)) of the 12 colonies of *O. annularis* differed qualitatively among host genotypes (Fig. 5A), and differed significantly between genotypes \(\beta\) and \(\varepsilon\), for which 4 replicate colonies were recorded (\(t = 2.679, df = 6, p = 0.037\)); colonies of genotype \(\varepsilon\) barely declined in size, in contrast to colonies of genotype \(\beta\). Colonies belonging to a single host genotype sometimes displayed contrasting growth trajectories with, for example, colonies 6 and 8 of genotype \(\varepsilon\), increasing 4 to 16% between 1994 and 2009, whereas colonies 7 and 20 of the same genotype declined 38 to 41% (Fig. 1). Overall, colonies (pooled among host genotypes) changed in size at similar rates in Photoquadrats 1 and 2 (\(t = 1.214, df = 10, p = 0.253\)). Change in colony size was unaffected by the nrITS2 genetic composition of
Symbiodinium in 1994 (Mann-Whitney U-test, $U = 7.000$, $n_1 = 10$, $n_2 = 2$, $p = 0.519$) and 2010 (Mann-Whitney U-test, $U = 12.000$, $n_1 = 7$, $n_2 = 5$, $p = 0.798$) (Fig. 5B), although of the 3 colonies with positive rates of change in size, 66% were dominated by clade B in 2010.

**DISCUSSION**

The growing number of anthropogenic assaults on the marine environment has impacts over a wide range of spatio-temporal scales, and will continue to affect biological systems for the foreseeable future. These assaults have focused attention on the value of high-resolution time-series analyses of biological systems to record the changes underway and guide effective resource management (Keitt 2008). With this focus, research once described pejoratively as surveillance monitoring (Nichols & Williams 2006) has emerged as a mainstream approach (Nichols & Williams 2006, Lindenmayer & Likens 2010) that benefits from the largesse of governmental funding. This revision of research priorities has been striking for tropical coral reefs where decades of disturbances have reduced coral cover over large areas (Baker et al. 2008, De’ath et al. 2012), and the future threatens to bring more serious losses due to rising temperature and ocean acidification (van Hooidonk et al. 2014). Accordingly, time-series analyses of coral reef community structure have proliferated since the 1990s; yet it is still rare for the functional attributes of corals to be characterized in tandem with ecological monitoring to address processes underlying the patterns of change in community structure (Thornhill et al. 2006, Baker et al. 2013, Tonk et al. 2013).

**Have Symbiodinium in Orbicella annularis changed over 16 years?**

The present study focused on an ecologically dominant hermatypic coral and combined decadal-scale ecological surveys with genetic analyses to evaluate the extent to which the dynamics of individual colonies are associated with the genetic identity of the animal host and their algal symbionts (Symbiodinium). In so doing, we applied targeted monitoring (Nichols & Williams 2006) to test 3 hypotheses relevant to identifying biological events associated with the change in coral community structure in one reef habitat on the south coast of St. John. First, we tested the hypothesis that genetic variants of *Symbiodinium* in *Orbicella annularis* have remained unchanged over 16 yr. This hypothesis was rejected, with a clear demonstration that the relative abundances of *Symbiodinium* clades and subclade types changed over time in the majority of coral colonies studied. The rapidly emerging descriptive landscape of phenotypic variation in *Symbiodinium* genotypes demonstrates the potential for coral success to be affected by the kinds of algal symbionts they harbor. For example, *Symbiodinium* genotypes differ in their photophysiology (Iglesias-Prieto et al. 2004, Warner et al. 2006), carbon fixation and translocation to the coral host (Stat et al. 2008, Cantin et al. 2009), and thermal tolerance (Rowan 2004, Warner et al. 2006). Moreover, variation in *Symbiodinium* genotypes is associated with the phenotypic traits of the holobiont, including susceptibility to disease (Stat et al. 2008, Correa et al. 2009) and colony growth (Little et al. 2004). The biological significance of our finding lies in demonstrating that the composition of the coral holobiont changed between 1994 and 2010. These changes provide a mechanistic rationale to infer that the functional capacity that these corals possessed in 2010 to respond to contemporary (and future) conditions is different from the capacity they possessed in 1994 to respond to previous conditions.

Our findings are, however, inconsistent with the directional and predictable shifts in *Symbiodinium*
composition that might be anticipated by the adaptive bleaching hypothesis (ABH) (Buddemeier & Fautin 1993). This hypothesis posits that bleaching provides a means for corals to change the composition of their *Symbiodinium* communities to maximize performance in the face of new conditions (Buddemeier & Fautin 1993). Although initially met with resistance (Hoegh-Guldberg 1999), research has emerged demonstrating that at least some corals can acquire resistance to thermal stress through changes in the genetic composition of their *Symbiodinium* (Berkelmans & van Oppen 2006, Jones et al. 2008), with members of clade D conferring resistance to high temperature (Berkelmans & van Oppen 2006). There is, however, more to resisting environmental challenges than the capacity to change genetic variants of *Symbiodinium*, since symbiont flexibility (i.e. the capacity to contain a varied sequence assemblage of *Symbiodinium*) appears to be a trait associated with species of corals that are sensitive to environmental conditions (Putnam et al. 2012). Moreover, host tissue alone plays an important role in resisting bleaching stressors (Baird et al. 2009).

Although the sampling for *Symbiodinium* genotypes in our study is temporally sparse, our findings are inconsistent with the ABH, particularly since the 1994 and 2010 samplings spanned a period characterized by warming seawater with several exceptionally warm years (Fig. 1C) that were associated with thermal bleaching (McWilliams et al. 2005) and, on the study reef, outbreaks of bleaching and coral disease affecting *O. annularis* (Miller et al. 2009). Of the 8 corals in Photoquadrat 1, 7 showed changes in *Symbiodinium* community composition between 1994 and 2010, with the trend most conspicuous for the increased prevalence of clade B in 2010. Less than 5 m away in Photoquadrat 2, all 4 sampled corals showed the reverse trend of stability in *Symbiodinium* community composition, hosting clade C almost exclusively in both years. Shifts in *Symbiodinium* community composition to favor clade B are perhaps unsurprising given reports that clades A and B typically dominate the upper surfaces of *O. annularis* in shallow water (Rowan et al. 1997, Toller et al. 2001a, Thornhill et al. 2006). Nevertheless, in the context of the ABH, it is noteworthy that patterns of change in *Symbiodinium* communities differed in colonies separated by only a few meters in a uniform habitat and that there was a low occurrence of at least 1 thermally resistant clade (i.e. D in 2010) (Berkelmans & van Oppen 2006). These observations must, however, be interpreted with caution, as we have no knowledge of how *Symbiodinium* clades changed in abundance in the 16 yr between samplings, and it is impossible to know what selective forces elicited the changes we observed in 2010, or the adaptive consequences with regards to tolerance to thermal regimes.

**Factors associated with changes in *Symbiodinium* community composition**

Our second objective was to test the hypothesis that *Symbiodinium* community composition was affected equally by the host genotype and the location of each colony on the reef. This analysis was opportunistic in that host genotypes were not selected for analysis *a priori* and the spatial analysis contrasted photoquadrats spatially separated by ~5 m. This hypothesis was, however, also rejected. Our results showed that variation in *Symbiodinium* community composition in *O. annularis* in St. John was more strongly influenced by location on the reef on a scale of meters than by host genotype. Given the apparent spatial homogeneity of the reef where the study was conducted, we foresaw no physical driver of variation in *Symbiodinium* genotypes on a scale of meters, even though variation at this spatial scale has been found among colonies of Montipora capitata in Hawaii (Stat et al. 2011, but see LaJeunesse & Thornhill 2011) and Acropora millepora on a single reef flat (Jones et al. 2008). While discordant environmental conditions over the surface of mounding corals creates opportunities for variation in *Symbiodinium* genotypes (Rowan et al. 1997, Garren et al. 2006), such effects are unlikely to have influenced the *Symbiodinium* communities in the present study because care was taken to sample corals in similar locations on the upper surface of all colonies. This does not, however, exclude the possibility that micro-scale variation (e.g. among polyps) in *Symbiodinium* genotypes confounded our contrast of *Symbiodinium* genotypes in 1994 and 2010, although there is no evidence that such variation occurs on the relatively smooth upper surfaces of *O. annularis* on the Tektite reef of St. John. Variation in *Symbiodinium* genotypes among closely spaced polyps has been detected using syringe-needle sampling on *Orbicella faveolata* from Belize (Kemp et al. 2008), and application of this technique to *O. annularis* in St. John would be valuable in determining how much of the variation in *Symbiodinium* genotypes between 1994 and 2010 was caused by polyp-scale differences in sampling location within individual colonies.
Many of the *Symbiodinium* types encountered in the present study, including C7, C12, C3, B1, B10, and A3, have previously been reported in the *O. annularis* species complex, and show zonation as a function of depth and position on the colony surface, with clade C typically more abundant at greater depths and in shaded microhabitats (Rowan et al. 1997, Garren et al. 2006, Thornhill et al. 2006, 2009, Warner et al. 2006). The apparent temporal stability of clade C type *Symbiodinium* in Photoquadrat 2, yet strong shifts in types and relative abundances just 5 m away in Photoquadrat 1, is inconsistent with these previous studies and suggests factors independent of depth contributed to the *Symbiodinium* community composition. Assuming that the physical conditions defining the optimal niches for *Symbiodinium* success—for example, light intensity, seawater temperature, and nutrients (Cooper et al. 2011, Tonk et al. 2013)—are similar on the upper surfaces of conspecific colonies across a topographically uniform reef at a constant depth and that the host genotype has relatively little effect on the types or abundances of *Symbiodinium* genotypes (this study), then location-specific variation in *Symbiodinium* genotypes could arise from at least 3 sources.

First, stochastic processes (Stat et al. 2011) could influence *Symbiodinium* genetic structure by the random supply of genotypes to coral hosts. Secondly, the hidden effects of history could determine genetic variation in *Symbiodinium* as a result of events occurring prior to sampling in 1994, or between the 2 samplings in 1994 and 2010. And third, biotic processes unique to each photoquadrat and independent of *O. annularis* colonies could indirectly influence *Symbiodinium* community composition in the corals. Such processes could include non-scleractinian sources and sinks of *Symbiodinium* genotypes (Takabayashi et al. 2012), or biological events indirectly affecting the interaction of coral hosts and *Symbiodinium* (e.g. Jeong et al. 2012). Bleaching and coral diseases can also mediate changes in the association between the coral host and its symbionts (Toller et al. 2012), and, interestingly, a severe outbreak of bleaching and subsequent coral disease occurred on the Tektite reef in 2005 (Miller et al. 2009). These events ultimately led to a 53% reduction in coral cover between 2005 and 2006 over the entire Tektite reef (which included an area larger than that sampled here) (Miller et al. 2009). Conceivably, therefore, the differences in the *Symbiodinium* found in *O. annularis* in 2010 versus 1994 could reflect a persistent response to bleaching and disease in 2005, with the patchiness of these phenomena driving the small-scale spatial variation we detected in 2010 for *Symbiodinium* types found in closely spaced colonies of *O. annularis*. While the present study cannot distinguish among the aforementioned possibilities, our results suggest that the compositions of *Symbiodinium* communities in *O. annularis* at this location are strongly influenced by extrinsic processes.

**Do variants of *Symbiodinium* affect the growth of *Orbicella annularis***?

Finally, we tested the hypothesis that the growth of *O. annularis* is independent of the genetic identity of *Symbiodinium* and the animal host. This hypothesis was rejected, and our results demonstrated that the growth of *O. annularis* differed among host genotypes and showed a weak trend to vary depending on the genetic complement of *Symbiodinium*. The effect of host genotype reinforces earlier studies emphasizing the importance of phenotypic variation among clonal genotypes of corals (Bruno & Edmunds 1997), and, in the present case, reveals a mechanism that could potentially drive shifting abundances of host genotypes over time. Based on the growth rates of the 5 host genotypes that were resolved, genotypes $\delta$ and $\epsilon$ would likely be the only 2 to thrive in the future, with the other 3 locally extirpated. This result is similar to that found in other *Orbicella* spp. where corals with higher biomass survived following bleaching (Thornhill et al. 2011). Genotype $\epsilon$ corals had the highest proportion of clade B symbionts in 2010, a trend that encapsulated the greatest within-genotype *Symbiodinium* shift from clade C to clade B. While not statistically significant, it is striking that corals containing a predominantly clade B *Symbiodinium* represented 66% of the corals that displayed positive trajectories of changing size over 16 yr.

**Summary and future work**

This study reveals the benefits that can be realized by combining routine ecological tools of coral reef monitoring with contemporary techniques to characterize the genetic identity of the dominant corals and their *Symbiodinium* symbionts. We have been able to combine what has become a common pattern on tropical coral reefs—declining coral cover and shrinking coral colonies—with a process-oriented analysis to shed light on the association between genetic variation in the coral host, genetic diversity of *Symbiodinium* symbionts, and changes in coral pop-
ulation structure. In light of our results, it may be timely to focus attention on the role of reef micro-environments in affecting the Symbiodinium complement of corals and to evaluate the conditions under which the Symbiodinium complement has the strongest effect on holobiont phenotype. Further, given the advances made here through the analysis of legacy tissue samples fortuitously preserved with DNA analyses in mind, it might be valuable to consider archiving coral tissue samples in concert with ongoing major coral reef monitoring efforts. Finally, the present analysis underscores the importance of resolving the biological significance of rare genotypes in Symbiodinium communities within coral hosts and of reaching a consensus regarding the best analytical tools for this task.

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