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REPORT

Widespread prevalence of cryptic *Symbiodinium* D in the key Caribbean reef builder, *Orbicella annularis*

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Abstract *Symbiodinium* D, a relatively rare clade of algal endosymbiont with a global distribution, has attracted interest as some of its sub-cladal types induce increased thermal tolerance and associated trade-offs, including reduced growth rate in its coral hosts. Members of *Symbiodinium* D are increasingly reported to comprise low-abundance 'cryptic' (<10 %) proportions of mixed coral endosymbiont communities, with unknown ecological implications. Real-time PCR (RT-PCR) targeted to specific types is sufficiently sensitive to detect these background symbiont levels. In this study, RT-PCR was employed to screen 552 colonies of the key Caribbean reef builder *Orbicella annularis* sampled across a 5.4 million km^2 range for the presence of cryptic *Symbiodinium* 'D1' (i.e., the principal Caribbean ITS2 variants, D1 and D1–4). All but one out of 33 populations analysed were shown to host low abundances of *Symbiodinium* D1, with an average of >30 % of corals per site found to harbour the symbiont. When the same samples were analysed using the conventional screening technique, denaturing gradient gel electrophoresis, *Symbiodinium* D1 was only detected in 12 populations and appeared to be hosted by <12 % of colonies where present (in agreement with other reported low prevalence/absences in *O. annularis*). Cryptic *Symbiodinium* D1 showed a mainly uniform distribution across the wider Caribbean region, although significantly more Mesoamerican Barrier Reef corals hosted cryptic *Symbiodinium* D1 than might be expected by chance, possibly as a consequence of intense warming in the region in 1998. Widespread prevalence of thermally tolerant *Symbiodinium* in *O. annularis* may potentially reflect a capacity for the coral to temporarily respond to warming events through symbiont shuffling. However, association with reduced coral calcification means that the ubiquitous nature of *Symbiodinium* D1 in *O. annularis* populations is unlikely to prevent long-term declines in reef health, at a time when maintaining reef growth is vital to sustain reef ecosystem function.

Keywords Zooxanthellae · Coral bleaching · Clade D · RT-PCR · Climate change · *Montastraea*

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Introduction

In the Caribbean, the massive coral *Orbicella annularis* (formerly *Montastraea*, Budd et al. 2012) is ecologically important in terms of its community dominance and wide geographic range, in addition to playing a key role in

creating habitats associated with biomass and diversity of reef organisms (Knowlton et al. 1992; Mumby et al. 2008). Therefore, understanding the resilience of the *O. annularis* holobiont in a rapidly changing climate is vital when evaluating the future of Caribbean reefs (Ortiz et al. 2013). When *O. annularis* colonies are healthy, photosynthetic dinoflagellates residing in their gastrodermal cells occur at densities of $\sim 2 \times 10^6$ cells cm^{-2} (Fagoonee et al. 1999), contributing to host metabolism and skeletogenesis. The symbiotic relationship between corals and their dinoflagellate endosymbionts, *Symbiodinium*, ultimately sustains the coral reef ecosystem, but is sensitive to environmental change and can be disrupted, causing corals to bleach. During bleaching, endosymbionts may lose pigmentation, become degraded in situ or be expelled from the host (Brown 1997). If not reinstated within a specified time period (dependent on host species, severity of bleaching and other environmental factors), corals will experience partial and occasionally full colony mortality. As global climate change drives sea surface temperatures (SSTs) further above regional norms, the frequency of Caribbean thermal stress events (>2 °C) is predicted to increase by 0.4–0.7 yr^{-1} (Frieler et al. 2013). These events can trigger mass coral bleaching, such as in 2005, where 80 % of Caribbean corals bleached and 40 % experienced mortality (Eakin et al. 2010). However, experimental and observational work has shown that a variety of physiological properties can be attributed to different endosymbiont haplotypes (Rowan et al. 1997; Stat et al. 2008). In particular, some *Symbiodinium* display differential susceptibilities to thermal stress, which can affect the bleaching response of their coral hosts (Warner et al. 2006).

Molecular techniques focused on endosymbiont haplotypes have revealed a suite of internal transcribed spacer 2 (ITS2) gene region variants, classified into nine clades named A–I (Pochon and Gates 2010; Pochon et al. 2014). Clades A–D generally associate with scleractinian corals, with B and C being dominant in the Caribbean. Members of *Symbiodinium* clade D are often found on reefs that experience unusually high SSTs (Baker et al. 2004; Fabricius et al. 2004; Oliver and Palumbi 2011), and in colonies recently impacted by bleaching events (Jones et al. 2008; LaJeunesse et al. 2009), suggesting that temperature stress can, at least temporarily, favour this symbiont (Little et al. 2004; Stat and Gates 2011). Some *Symbiodinium* D types may also be associated with other 'stressful' environmental conditions, including high sedimentation levels (Garren et al. 2006), turbidity (LaJeunesse et al. 2010a), and cool water bleaching events (McGinley et al. 2012). A shift in dominance towards *Symbiodinium* D in stressed *Acropora millepora* has been associated with an acquired tolerance of 1–1.5 °C (Berkelmans and van Oppen 2006). This has led to clade D being described by some as a 'safety parachute' in the face of rapid climate change (Berkelmans and van Oppen 2006; Stat and Gates 2011). However, the benefits of harbouring D are unlikely to outweigh the costs of associated reduced skeletal growth that are also associated with hosting D types (Ortiz et al. 2013).

Symbiodinium D1 (*nomina nuda Symbiodinium glynni*, LaJeunesse et al. 2010b) and D1–4 (previously known as D1a and also designated *Symbiodinium trenchii*, LaJeunesse et al. 2014) are two relatively rare members of clade D with a global distribution and are the only members of clade D that occur in Caribbean corals (Correa et al. 2009a). These symbionts (henceforth referred to as *Symbiodinium* D1, sensu Pochon et al. 2014) have also shown evidence of thermal tolerance (LaJeunesse et al. 2009; Wang et al. 2012). *O. annularis* is known to host a variety of symbiont taxa, commonly clades B and C, but also A and D (Rowan and Knowlton 1995; Toller et al. 2001a; Garren et al. 2006). This association with multiple symbiont taxa is partly due to the fact that *O. annularis* acquires symbionts from its environment (i.e., by horizontal transmission; Szmant 1991). While *Symbiodinium* D1 has been detected in a few *O. annularis* populations, many more studies report no *Symbiodinium* D1 types at all: of 291 colonies screened in previous studies, just 32 hosted clade D (ten studies: see Table 1 for details).

The apparent low prevalence of *Symbiodinium* D1 in *O. annularis* may be confounded by snapshot sampling. In corals such as *O. annularis* that are associated with multiple symbiont haplotypes, symbiont shuffling—a change in the relative abundance of resident algal populations within a colony—provides a mechanism by which a holobiont may adjust its capacity to respond to environmental change (Baker et al. 2004; LaJeunesse et al. 2009). A study that monitored *Symbiodinium* communities in Barbadian *O. annularis* pre-, during, and post-bleaching demonstrated that corals that harboured 'cryptic' (i.e., low level, <10 % of the total symbiont population) sub-clade D1 endosymbionts later became dominated by them and that these colonies remained unbleached (LaJeunesse et al. 2009). However, most investigations suggest that symbiont communities hosted by *O. annularis* show a high degree of temporal stability (Toller et al. 2001b; Thornhill et al. 2009), with limited evidence for symbiont 'switching' (i.e., changes brought about by uptake of new symbionts from the environment, Baker 2003). Typically, only severe bleaching events are capable of disrupting the symbiont community, and post-disruption, most *O. annularis* colonies regain their original community balance within a matter of months to years (Toller et al. 2001b; Thornhill et al. 2006b; LaJeunesse et al. 2009).

Table 1 Occurrence of *Symbiodinium* clade D in *O. annularis*, as documented by other studies that examine symbiont communities using traditional molecular electrophoresis-based techniques (e.g., RFLP and DGGE)

Sampling Location	Percentage *O. annularis* colonies found to harbour *Symbiodinium* D	Sample size (number of colonies)	Depth (m)	Reef type	Genetic marker employed	Screening technique used	Study
Bahamas (Lee Stocking Island)	13	8	4–14	Bank barrier reef	ITS2	DGGE	LaJeunesse (2002)
Belize	0	103	0–10	Barrier	Nuclear SSU rDNA	RFLP	Garren et al. (2006)
Panama (Bocas del Toro)	11	9	1–8	Lagoonal			
Panama (Bocas del Toro)	0	12	1–15	Lagoonal	Nuclear SSU rDNA	RFLP and sequencing	Toller et al. (2001b)
Panama (San Blas)	100	4	1–12	Coastal fringing			
Panama (San Blas)	0	25	1–7	Outer fringing	Nuclear SSU rDNA	RFLP	Rowan et al. (1997)
Panama (San Blas)	0	11	0–14	Outer fringing	Nuclear SSU rDNA	RFLP	Rowan and Knowlton (1995)
US Virgin Islands (St John—diseased vs healthy)	100	2	4; 8	Healthy coral	ITS2	DGGE	Correa et al. (2009b)
Barbados (prior to bleaching)	36	12	5–15	Bank and fringing reefs	ITS2	DGGE and RT-PCR	LaJeunesse et al. (2009)
Barbados (during bleaching)	85	14	5–15				
Barbados (post-bleaching)	0	9	<5	Fringing reef			
Barbados (post-bleaching)	31	16	>15	Bank reef			
Bahamas (North Normans, Exhumas)	0	12*	4	Patch reef	ITS2	DGGE	Thornhill et al. (2009)
Bahamas (South Perry, Exhumas)	0	6*	12				
Florida Keys (Little Grecian)	35	6**	3	Patch reef			
Florida Keys (Admiral Patch)	4	6$^{\Delta}$	1–2				
Florida Keys (Conch Reef)	0	6$^{\uparrow}$	12				
Barbados (shallow)	0	3	6–10	n/a	ITS2	DGGE (and microsats)	Finney et al. (2010)
Barbados (deep)	0	2	>10				
Belize (Carrie Bow Caye)	100	1	1–5				
Belize	0	18	8	Fore reef	ITS2	DGGE	Warner et al. (2006)
Belize	0	6	25	Reef slope			

In total, 291 *O. annularis* colonies have been sampled across these studies. Of these, 32 (excluding repeated temporal sampling of the same colony)—i.e., roughly 10 % of colonies sampled—were found to contain clade D

* 90 (six colonies sampled 15 times over 4 yrs); ** 108 (six colonies sampled 18 times over 7 yrs); $^{\Delta}$ 96 (six colonies sampled 16 times over 4 yrs); $^{\uparrow}$ 54 (six colonies sampled 9 times over 2 yrs)

Another explanation for the discrepancy in detecting clade D among populations may be the resolution of the screening techniques used. Commonly used molecular techniques for detecting and cataloguing coral endosymbionts (namely PCR-DGGE, SSCP, and RFLP analysis, reviewed by Sampayo et al. 2009) have been successfully employed to detect clades A to D in *O. annularis*, but are not always capable of consistently detecting symbionts at abundances below 5–10 % of the total population (Thornhill et al. 2006b; Mieog et al. 2007; LaJeunesse et al. 2008). Furthermore, in mixed communities, the relatively low copy number of D has meant it may be harder to detect

than other taxa, e.g., clade C (Smith 2008). These limitations mean that symbiont diversity may be underestimated in many species (Loram et al. 2007; Mieog et al. 2007; McGinley et al. 2012; Silverstein et al. 2012). Studies employing real-time PCR (RT-PCR) are now beginning to identify low-abundance 'cryptic' endosymbionts in a number of host species, demonstrating additional complexity of symbiont communities (Silverstein et al. 2012). RT-PCR is 1,000-fold more sensitive than denaturing gradient gel electrophoresis (DGGE) and single strand conformation polymorphism (SSCP; Mieog et al. 2007), and subsequently has the potential to improve estimates of the diversity of coral-associated *Symbiodinium* (Correa et al. 2009a). For example, D has been shown to be prevalent in a low-abundance, background capacity on the Great Barrier Reef (GBR), where it was detected in 71 % of colonies of four coral species tested (Mieog et al. 2007). Meanwhile, in the eastern Pacific, clade D was present at almost imperceptible levels in 40 % of the *Pocillopora* screened (McGinley et al. 2012), and in the Caribbean, cryptic levels of clade D were found in five of six coral genera tested, many of which had never been observed previously to host D (Correa et al. 2009a). Other benefits of RT-PCR include its ability to quantify abundance of types and the ease and speed of the technique compared to traditional methods (Granados-Cifuentes and Rodriguez-Lanetty 2011).

This study aims to reveal hidden cryptic *Symbiodinium* D1 diversity in the Caribbean's most important reef building coral, *O. annularis*, from across the wider Caribbean (Fig. 1). Firstly, the high-resolution technique RT-PCR was used to specifically screen for presence of *Symbiodinium* D1 (i.e., sub-clades D1 and/or D1–4, sensu Pochon et al. 2014) in >500 *O. annularis* colonies. Secondly, conventional DGGE techniques were used to screen the same samples, with a combination of sequencing and gel comparisons used to confirm presence of *Symbiodinium* D1. Detection of *Symbiodinium* D1 in any given sample by RT-PCR, but not DGGE, indicates background or 'cryptic' levels of D1. Finally, a statistical approach called SADIE (Spatial Analysis of Distance Indices) was used to explore patterns in the distribution of cryptic *Symbiodinium* D1.

Quantifying the prevalence of background *Symbiodinium* D1 in coral communities may facilitate refinement of models that predict ecological responses of Caribbean reefs to climate change (Ortiz et al. 2014). Evidence of naturally occurring symbiont shuffling in *O. annularis* has previously been documented at just one Caribbean site (LaJeunesse et al. 2009); by screening samples from across the whole region, our understanding of the ability of *O. annularis* to survive bleaching events can be improved.

Materials and methods

Sample collection and DNA extraction

Fragments of *O. annularis* tissue were collected from 33 sites spanning a 5.4 million km^2 area across the wider Caribbean between 2003 and 2007 (Foster 2007; Fig. 1; Table 2). Approximately 30 small (1 cm^3) fragments were chiselled from the edge of spatially independent ramets at each site and stored in 90 % ethanol at 4 °C, sensu Foster et al. (2012). As bathymetry and irradiance are known to influence the *O. annularis* symbiont community, collections were limited to 2–6 m and only the tops of colonies were sampled. A mix of coral and symbiont DNA was extracted using the DNeasy tissue kit (Qiagen) and then stored at −20 °C.

Screening for *Symbiodinium* D1 using RT-PCR

A 312-base pair target region specific to *Symbiodinium* clade D (including both Caribbean types D1 and D1–4, referred to as '*Symbiodinium* D1' throughout this document sensu Pochon et al. 2014), located in domain 2 of the large subunit (LSU) ribosomal RNA gene, was amplified using published RT-PCR primers (Table 3; Correa et al. 2009a). A 10 µl reaction mix containing 1 mM of both forward and reverse primers, 1 µl DNA template, and 2X Absolute qPCR SYBR Green Fluorescein Mix (Thermo Scientific) was amplified in RT-PCR (CFX96 RT-PCR detection system, Bio-Rad Laboratories, Inc.) using the FAM filter. Reaction conditions were an initial denaturing step of 95 °C for 10 min, followed by 50 PCR cycles of 95, 61, and 72 °C for 30 s each (Correa et al. 2009a). A final high-resolution melting (HRM) step entailed a 55–95 °C temperature ramp of 0.2 °C every 2 s. Fluorescence data were collected during each PCR annealing step and each temperature step of the HRM melt cycle. Each DNA sample was run in duplicate for the *Symbiodinium* D1 primer set, and positive (standard) and negative controls were included on every plate.

Selection of a fixed fluorescent threshold (see Fig. 2a) in the exponential phase of the reaction allowed comparable C_T values to be calculated. Attempts to quantify copy number of *Symbiodinium* D1 in each sample were unsuccessful; however, C_T values can also provide a useful quality control: duplicate values that differed by >1 were discarded. To further assess DNA quality, every sample that was screened for *Symbiodinium* D1 was run through RT-PCR for a second time (again in duplicate) using the ITS2 primer set 'ITSintfor2' and 'ITSrev', Table 3 (cycling conditions: 98 °C for 2 min, followed by 45 cycles of 98 °C (5 s), 55 °C (5 s) and 72 °C (5 s); Granados-Cifuentes and Rodriguez-Lanetty 2011). Any samples that

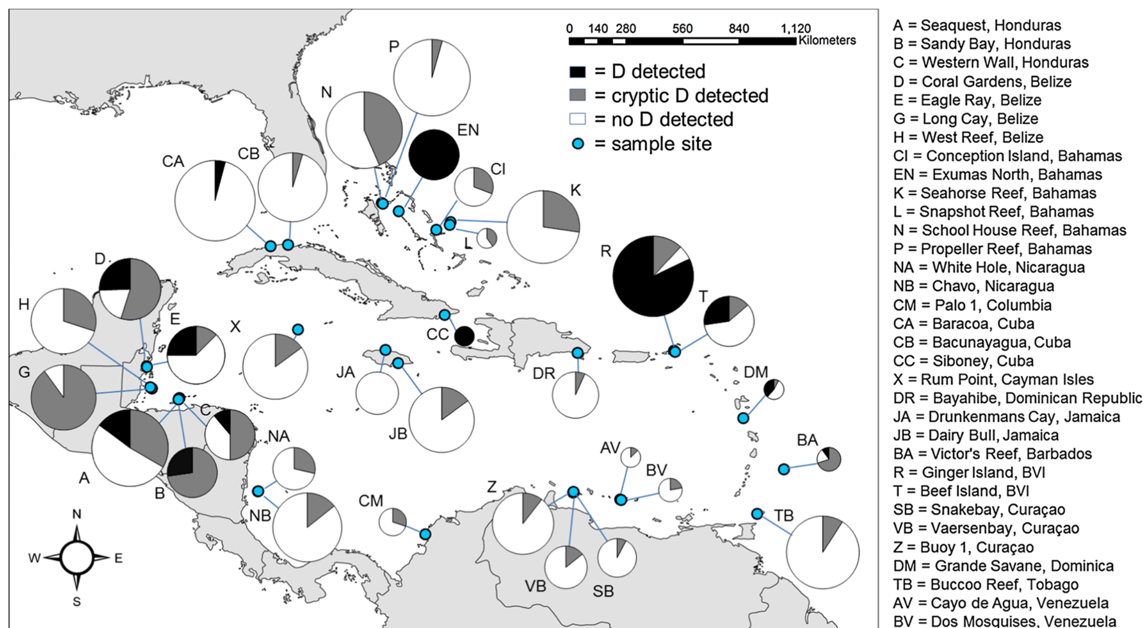

Fig. 1 Proportion of *Orbicella annularis* population hosting *Symbiodinium* D1. Thirty-three sites (each identified by a *letter*) tested. Pie chart size reflects sample size (min = 5 colonies, max = 23), *dark shading* reflects proportion of samples hosting *Symbiodinium* D1 detectable by DGGE, *pale shading* corresponds to low-abundance 'cryptic' *Symbiodinium* D1, detected only by high-resolution technique RT-PCR

failed to generate positive ITS2 amplifications or produced poor quality melt curves were removed from the dataset to avoid false negative D1.

Screening for *Symbiodinium* D1 using DGGE

DGGE was used to identify *Symbiodinium* ITS2 types within each individual *O. annularis* fragment following LaJeunesse (2002). DNA was amplified in a PCR (95 °C for 5 min; followed by 30 cycles of 94 °C (45 s), 57 °C (45 s), and 72 °C (60 s); with a final annealing step of 59 °C for 20 min) using *Symbiodinium*-specific rDNA primers 'ITS2 Clamp' and 'ITSintfor2' (Table 3). PCR products, mixed with 5 µl bromophenol blue loading buffer (15 % Ficoll, 0.25 % xylene cyanol FF, 0.25 % bromophenol blue), were electrophoresed at 114 V on a polyacrylamide denaturing gradient gel (40 to 60 % denaturant) at 60 °C (Ingeny System). An ITS2 standard (with B1, C1, and C3, provided by the Coral Reef Ecosystems lab, University of Queensland, Australia) was run in the first lane of each gel. After 14 h, the gel was stained with SybrGreen I (Invitrogen) nucleic acid gel stain at room temperature for 20 min, before imaging in a UV transilluminator. Imaged gels were examined by eye and scored for types, in comparison to a database of other gels used to help identify haplotypes. Dominant bands from each DGGE gel were excised, cleaned, and sent for sequencing (Macrogen) to resolve ITS2 type.

Data analysis

Examination of HRM melt curves from the final step of the RT-PCR process allowed purity of the reaction products to be assessed. Amplified product generated a melt peak at the correct temperature range (e.g., 84.4 °C ± 0.5 (mean ± SD) for D1 fragments targeted by RT-PCR primers), whereas melting at lower temperatures was indicative of primer dimer. Where both RT-PCR duplicates produced a melt peak around 84.4 °C, the sample was scored as containing *Symbiodinium* D1 (Fig. 2a). Where bands were observed on the DGGE gel in either the D1 or D1a position (or both), the sample was also scored as hosting *Symbiodinium* D1 (Fig. 2b). Samples that scored positively for D1 in RT-PCR, but negatively in DGGE, were said to contain 'cryptic' or background levels of *Symbiodinium* D1.

SADIE, a statistical approach designed for assessing the patterning of count data from spatially referenced locations, was used to quantify spatial patterns in the data (Perry 1995). SADIE measures the spatial pattern at each sampled unit using an index of clustering based on geographic distance—assigning each site either a positive patch cluster (v_i) or a negative gap cluster (v_j) value. These can then be mapped with filled and empty circles, representing local quantification of spatial patterning. Interpolation between the data points (using a universal kriging method) produces a red-blue contour plot (e.g., Fig. 3), indicating clustering of spatial data (Perry et al. 1999).

Table 2 Summary of RT-PCR and DGGE outputs by site, grouped by marine eco-region

Caribbean eco-region	Site identifier	Reef site	Sample date	Sample size	% Colonies hosting D (RT-PCR)	% Colonies hosting D (DGGE)	Dominant symbiont
Mesoamerican Barrier Reef	A	Seaquest, Honduras	Oct 2004	23	39	17	B1
	B	Sandy Bay, Honduras	Oct 2004	16	100	38	B1/C1
	C	Western Wall, Honduras	Oct 2004	16	56	13	B1
	D	Coral Gardens, Belize	Jan 2006	19	74	26	B17
	E	Eagle Ray, Belize	Jan 2006	18	17	28	B1/B17
	G	Long Cay, Belize	Jan 2006	20	90	0	B1/B17
	H	West Reef, Belize	Jan 2006	20	30	0	B1/B17
The Bahamas	CI	Conception Island, Bahamas	May 2007	13	31	0	B1
	EN	Exumas North, Bahamas	Apr 2007	16	56	100	D1
	K	Seahorse Reef, Bahamas	Jun 2006	22	27	0	B1
	L	Snapshot Reef, Bahamas	Jun 2006	5	40	0	B1
	N	School House Reef, Bahamas	Jun 2006	23	43	0	B1
	P	Propeller Reef, Bahamas	Jun 2006	23	4	0	B1
South-western	NA	White Hole, Nicaragua	Sep 2006	14	29	0	B1
	NB	Chavo, Nicaragua	Sep 2007	21	14	0	B1/C12
	CM	Palo 1, Colombia	Oct 2005	10	30	0	C
Greater Antilles	CA	Baracoa, Cuba	Sep 2007	24	4	4	B10
	CB	Bacunayagua, Cuba	Sep 2007	21	5	0	B10
	CC	Siboney, Cuba	Sep 2007	22	5	100	D1a
	X	Rum Point, Cayman	Jul 2007	20	15	0	B1/C7
	DR	Bayahibe, Dominican Republic	Oct 2007	15	7	0	C12
	JA	Drunkenmans Cay, Jamaica	Sep 2007	14	0	0	B1
	JB	Dairy Bull, Jamaica	Sep 2007	20	15	0	B1/B8
Lesser Antilles	BA	Victor's Reef, Barbados	Jul 2007	9	78	11	B1j/B1
	R	Ginger Island, BVI	Nov 2007	24	67	96	D1a
	T	Beef Island, BVI	Nov 2006	16	19	38	B1/D1a
	SB	Snakebay, Curaçao	Aug 2007	13	8	0	C7
	VB	Vaersenbay, Curaçao	Oct 2005	14	14	0	C7
	Z	Buoy 1, Curaçao	Oct 2005	19	11	0	B1/C7
	DM	Grande Savane, Dominica	Oct 2005	8	13	63	C12
	TB	Buccoo Reef, Tobago	Sep 2007	22	9	0	C1
	AV	Cayo de Agua, Venezuela	Aug 2007	8	13	0	B1j/C12
	BV	Dos Mosquises, Venezula	Aug 2007	9	22	0	B1j/C12

Dominant symbiont represents the main symbiont type (or types) that occurred at a site. Little within-site variability was detected

Results

Detection of *Symbiodinium* D1 in *O. annularis*

Five hundred and fifty-two coral colonies were successfully screened for *Symbiodinium* D1 using both techniques. RT-PCR produced positive amplifications (indicating presence of *Symbiodinium* D1) in 170 colonies (31 % of all corals), and *Symbiodinium* D1 was scored as being present at 97 % (32 of 33) of sampling sites (Fig. 1). Melt curves revealed almost all (>98 %) samples with positive amplifications had a characteristic dissociation signature with melting point at 84.4 °C: the melting temperature of the target fragment (Fig. 2a). Those that did not were removed from the dataset.

Any C_T values over 45 were also discarded, as at a level of C_T of >45 rare cells could be contaminants. This improved reproducibility estimates by >10 %. Although signals were often low (again, common in mixed assemblages), template-free controls were consistently negative.

The proportion of coral samples hosting *Symbiodinium* D1 detected by RT-PCR ranged from 0 to 100 %, with an

average of 30 % hosting cryptic *Symbiodinium* D1 per site (SE = 4.67). Only Drunkenman's Cay in Jamaica (site JA) was found to have no cryptic *Symbiodinium* D1 ($n = 14$), despite these samples having a good quantity of starting DNA template (ITS2 = 12.8 ng μl^{-1}).

In comparison, DGGE detected *Symbiodinium* D1 in a total of 12.5 % of corals (69 colonies) at just 12 of the 33 sites. At six of these 12 sites (DM, CC, CA, EN, BVI, T, and R; Table 2), *Symbiodinium* D1 was manifested on DGGE gels as paired bright bands in the D1 (upper band) and D1a position (lower band), see Fig. 2b. This banding profile represents a D1 ITS2 variant known as D1–4 (GenBank Accession AF499802), which was confirmed by sequencing. At the remaining six sites (A–E and BA; Table 2), clade D was not the dominant symbiont type. Here, D1 bands were pale and were rarely accompanied by a second band in the D1a position. It is possible that these symbionts may be a closely related sequence variant D1 (AF334660), which differs from D1–4 by a few bp (Pochon et al. 2014), as RT-PCR primers cannot distinguish these types, and melt curves were unable to discriminate without a pure reference.

In addition to *Symbiodinium* D1, DGGE also distinguished numerous other ITS2 *Symbiodinium* types (Electronic Supplementary Materials, ESM, Table S1), nested within clades A–D. All *Symbiodinium* D1 appeared more commonly in mixed assemblages with B and C types (44 and four colonies, respectively) than alone (21 colonies). ITS2-B1 (AF333511) was the most frequently occurring sub-clade, found in 71 % of colonies (see ESM Table S1 for other types). Thirty-one percent of samples harboured clade C (13 % exclusively), 65 % exclusively harboured clade B, and a further 17 % hosted a mix of B and C, compared with the 4 % of colonies that hosted *Symbiodinium* D1 alone.

Spatial distribution of *Symbiodinium* D1

DGGE-detectable *Symbiodinium* D1 showed a lack of spatial structuring across the Caribbean (SADIE Index of Aggregation, $I_a = 1.57$, $P_a = 0.05$), indicating an apparently random distribution of this symbiont among coral populations. A second spatial analysis of the overall pattern of distribution of low-abundance (RT-PCR-detectable) *Symbiodinium* D1 was also close to random ($I_a = 1.42$, $P_a = 0.09$), again indicating absence of regional-scale structuring in its arrangement. However, a high SADIE patch cluster index (v_i) of 2.22 ($p = 0.01$), indicative of localised clustering at the site level, revealed higher-than-expected occurrences of cryptic *Symbiodinium* D1 in some local areas (i.e., sites A–C, G, H, K, and L). The location of these sites created a patch cluster around the Mesoamerican Barrier Reef System, MBRS (red area, Fig. 3). Several more sites (CB, T, SB, VB, and BV) hosted less *Symbiodinium* D1 than might be expected by chance, producing a significant gap cluster around the southern Caribbean (Fig. 3, blue area), although there were no significant gaps in the overall distribution ($v_i = -1.25$, $p = 0.192$).

Discussion

RT-PCR revealed that at all but one site, ~30 % of *O. annularis* harboured *Symbiodinium* D1 at low-abundance 'cryptic' levels. In comparison, out of the 552 individuals screened, DGGE identified only 69 instances of *Symbiodinium* D1 at just 12/33 sites, indicating that this technique did not detect D1 at low levels in a further 101 coral colonies and missed its presence at 20 sites.

The discrepancy in the resolution of the two techniques is unsurprising: the strength of DGGE is in its ability to detect a breadth of types, rather than its sensitivity. ITS2-DGGE detection levels of just 5–10 % have been reported in the Caribbean (tested on mixed cultures of B1 and C2; Thornhill et al. 2006b), while in Pacific populations, *Symbiodinium* D1 were only identified at 10–30 % of the total symbiont population (LaJeunesse et al. 2008). Limited detection of *Symbiodinium* D1 by DGGE may be due to a swamping effect of other symbiont types during the PCR step or preferential amplification of alternative clades. The genomes of clade D *Symbiodinium* are known to have a low ribosomal copy number (e.g., 3–5 times lower than clade C), making them harder to distinguish in mixed samples (Smith 2008). For example, one ITS-DGGE study detected *Symbiodinium* C3-e at proportions as low as 1 %, but only detected D when it existed in proportions >20 % (LaJeunesse et al. 2009). DGGE banding showed 48/69 colonies hosting *Symbiodinium* D1 contained mixed symbiont assemblages, which would exacerbate this issue. The distribution and abundances of other observed types corroborate well with the findings of numerous smaller-scale studies into *O. annularis* symbiont community composition (e.g., Toller et al. 2001a; LaJeunesse et al. 2009; Thornhill et al. 2009). *Symbiodinium* B1 dominated >70 % of sampled corals, supporting the literature that shows B1 to be the most common Caribbean ITS2 type in a variety of cnidarian genera (LaJeunesse 2002), including *O. annularis* (Thornhill et al. 2009).

Precision of RT-PCR can also be lower in samples hosting multiple clades (Loram et al. 2007). Mixed communities were common in our dataset, with 27 % of colonies hosting >3 sub-clades. However, the primers used work well in mixed assemblages (Correa et al. 2009a), and reproducibility (among replicates) of RT-PCR detection of *Symbiodinium* D1 was 63 %. Another way in which the reported RT-PCR results may have misrepresented

Table 3 Primer pairs used for RT-PCR and PCR-DGGE analyses

Primer name	Direction	Primer sequence (5′–3′)	Primer size (bp)	Primer melting temperature (*Tm*)	Primer GC content (%)	Reference
Clade D	*Forward*	GCCGTGTACGGTGCTCGCTCTCAA	24	63	62.5	Correa et al. (2009a)
Clade D	*Reverse*	GGCCACTCGCAAATGGACAGC	21	58	61.9	
ITSintfor2	*Forward*	GAATTGCAGAACTCCGTG	18	48	50.0	LaJeunesse and Trench (2000)
ITS2 CLAMP	*Reverse*	CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCC GGGATCCATATGCTTAAGTTCAGCGGGT	67	87	77.6	
ITSrev	*Forward*	GGGATCCATATGCTTAAGTTCAGCGGGT	28	61	50.0	Coleman et al. (1994)

The first set (*Clade D*) is nuclear DNA primer sets developed to amplify portions of the multi-copy ribosomal LSU gene family in *Symbiodinium* clade D using RT-PCR. The second set targets the ITS2 region of *Symbiodinium* for DGGE analysis: the internal primer 'ITSintfor2' anneals to a conserved region of the 5.8S rDNA and is paired with the conserved 3′ flanking primer 'ITS-rev' that has been modified for DGGE by addition of a 39-bp GC clamp (underlined) in 'ITS2CLAMP'

Symbiodinium D1 diversity is through the sampling design. Collection of coral fragments was limited to unshaded colony tops at <6 m, in order to avoid sampling intra-colony irradiance-driven zonation. One consequence of this standardised sampling protocol is that *Symbiodinium* D1 diversity on colony sides or shaded parts may have been missed. Since shallow-water *O. annularis* colonies have been shown to bleach more in shaded areas (Rowan et al. 1997), and clade D is often associated with bleaching, the results presented may be an underestimate of the true prevalence of *Symbiodinium* D1 in *O. annularis*.

DGGE detection of *Symbiodinium* D1 in *O. annularis*

DGGE detected *Symbiodinium* D1 in just 12 % of the total *O. annularis* colonies screened (69 colonies, representing 40 % of the 'true' *Symbiodinium* D1 prevalence). Studies employing similar molecular techniques have reported comparable abundances of D in the Caribbean, which generally represent <10 % of the endosymbiotic community in the host population (Thornhill et al. 2006b). At 21 of 33 sites, DGGE did not detect *Symbiodinium* D1 at all (Fig. 1), a result directly comparable to several smaller-scale studies (e.g., Belize: Garren et al. 2006; Warner et al. 2006; Panama: Rowan and Knowlton 1995, Rowan et al. 1997; and in the Bahamas: Thornhill et al. 2009; see Table 1) that reported absences of clade D in *O. annularis* when employing similar molecular techniques (DGGE and RFLPs). In cases where clade D has been detected using DGGE and RFLPs (e.g., at Lee Stocking Island, Bahamas by LaJeunesse 2002; in Panama by Garren et al. 2006; and Barbados by LaJeunesse et al. 2009), it often presents at similar frequencies to the 12 % of colonies in this study (12.5, 11, and 3 % (by DGGE), respectively). Higher reported abundances are often associated with corals stressed by temperature (e.g., >60 % *O. annularis* in Barbados and Florida during bleaching; LaJeunesse et al. 2009; Finney et al. 2010) and/or disease (e.g., 100 % *O. annularis* in Panama; Toller et al. 2001b). In this study, sporadic response to stress events could explain the high dominance of *Symbiodinium* D1 (90–100 %) detected by DGGE at sites EN, R, and CC.

DGGE banding profiles (Fig. 2b) and sequencing suggest that the majority of the clade D detected in this study is ITS2 sequence variant D1-4 (*S. trenchii*; LaJeunesse et al. 2010a), although in some cases, distinguishing D1–4 from the closely related sub-type D1 was problematic. Further structural, biochemical, and physiological investigations would be required to determine the ecological significance of the molecular differences between D1 and D1–4 to the host (Stat et al. 2012, but also LaJeunesse et al. 2014). Examination of these types with fine-scale genetic markers may reveal further diversity, just as higher-resolution markers have demonstrated that *Symbiodinium*

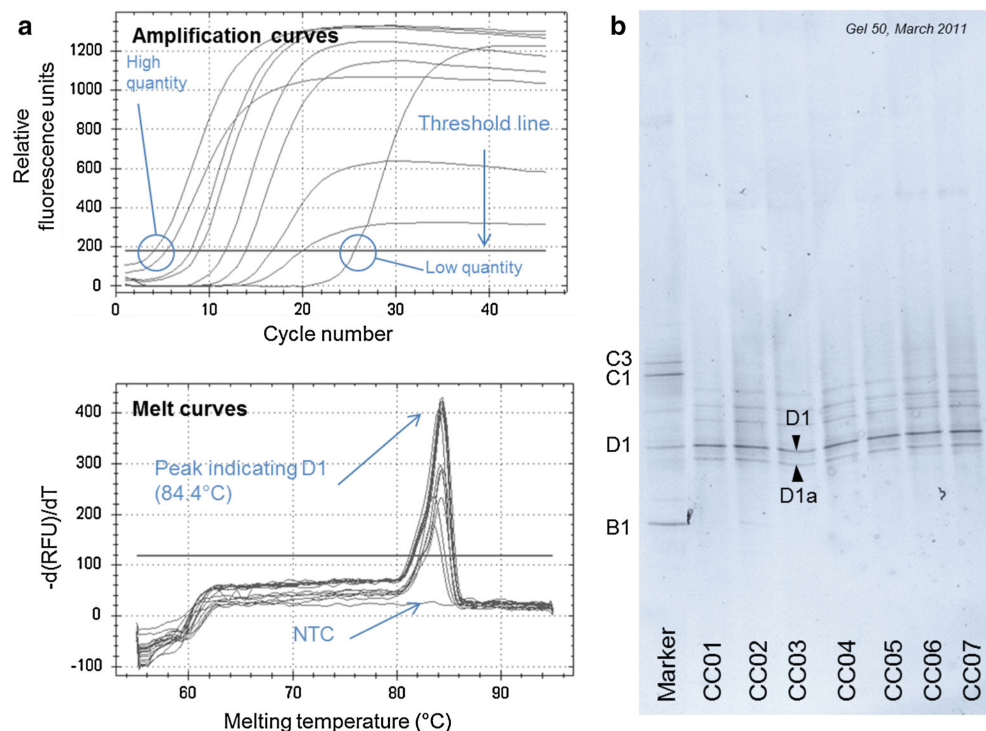

Fig. 2 Comparing outputs of the two *Symbiodinium* clade D screening methods. **a** RT-PCR output. Amplification curves (*top panel* from RT-PCR) and melt curves (*bottom panel* from HRM analysis) were generated for every reaction. Positive amplification (*top panel*) of duplicates and generation of a melting peak around 84.4 °C (*bottom panel*) indicates that *Symbiodinium* D1 is present in the sample, while template-free controls (NTC) and samples without D1 did not amplify. **b** DGGE gel. Each *lane* represents the symbiont community hosted by an individual *O. annularis* colony (from Cuban samples CC, see Fig. 1). Paired bands in the D1 and D1a position on the gel indicate presence of type D1-4

B1 consists of several specialised lineages (Finney et al. 2010). An improved understanding of how *Symbiodinium* genetic variation among sub-types is manifested in terms of quality as a mutualistic partner will be key to understanding the coral-algal symbiosis, and the ecological and evolutionary drivers that maintain such a variety of genetic types (Heath and Stinchcombe 2013).

RT-PCR detection of *Symbiodinium* D1 in *O. annularis*

RT-PCR revealed a widespread prevalence of *Symbiodinium* D1, both between (at 32/33 sites) and within sites (in more than half of all populations, 11–40 % of colonies harboured cryptic *Symbiodinium* D1). Prevalence of cryptic *Symbiodinium* D1 in an average of 29.8 % of colonies per site supports evidence from Barbados that reported 28 % of healthy *O. annularis* colonies contained D1 that was detectable only by RT-PCR (LaJeunesse et al. 2009). In Barbados, D1 prevalence later increased to 60 % of colonies in response to a bleaching event. Colonies harbouring D1 remained unbleached, demonstrating the ability of *O. annularis* to respond to temperature anomalies through symbiont shuffling. Correa et al. (2009a) also used high-resolution techniques to record low levels of *Symbiodinium* D1 in 21 % of sampled Caribbean corals (but not *O. annularis*) across a geographic range comparable to this study. Our findings demonstrate the breadth of the association of *O. annularis* with *Symbiodinium* D1 shown in Barbados, across a Caribbean scale comparable to Correa et al. (2009a).

The spatial arrangement of RT-PCR-detectable *Symbiodinium* D1 across the Caribbean was fairly uniform (Figs. 1, 3), supporting the consensus that clade D exhibits random geographic distribution (Stat and Gates 2011). Its presence might mirror environmental availability of *Symbiodinium* D1 at the time of symbiont uptake during coral settlement. This would suggest an innate region-wide low-level presence of *Symbiodinium* D1, which could imply a natural ability to respond to future warming events (if shuffling allows D1 to increase in abundance and be maintained in response to warming). Site-level spatial analyses revealed a high number of colonies hosting *Symbiodinium* D1 at MBRS sites (Fig. 3, red areas) and, to a lesser extent, a relative scarcity of *Symbiodinium* D1 in corals in the southern Caribbean (Fig. 3, blue areas). Bleaching history has been proposed as an explanation for the occurrence of clade D (Baker et al. 2004), and observed site-level variability may be explained by thermal stress history at different Caribbean locations. Higher frequencies of *Symbiodinium* D1 at MBRS sites may reflect the severity of the 1998 mass bleaching event in the region, where an extended +1.5 °C warming event was experienced in the western Caribbean in September 1998. Here, 76 % of *O. annularis* colonies experienced bleaching (Kramer et al. 2000), which may have facilitated the establishment of *Symbiodinium* D1 in these communities. The 1998 bleaching event was not as severe in the southern Caribbean, possibly explaining fewer *Symbiodinium* D1 at these sites. Timespans ranging from months to years are

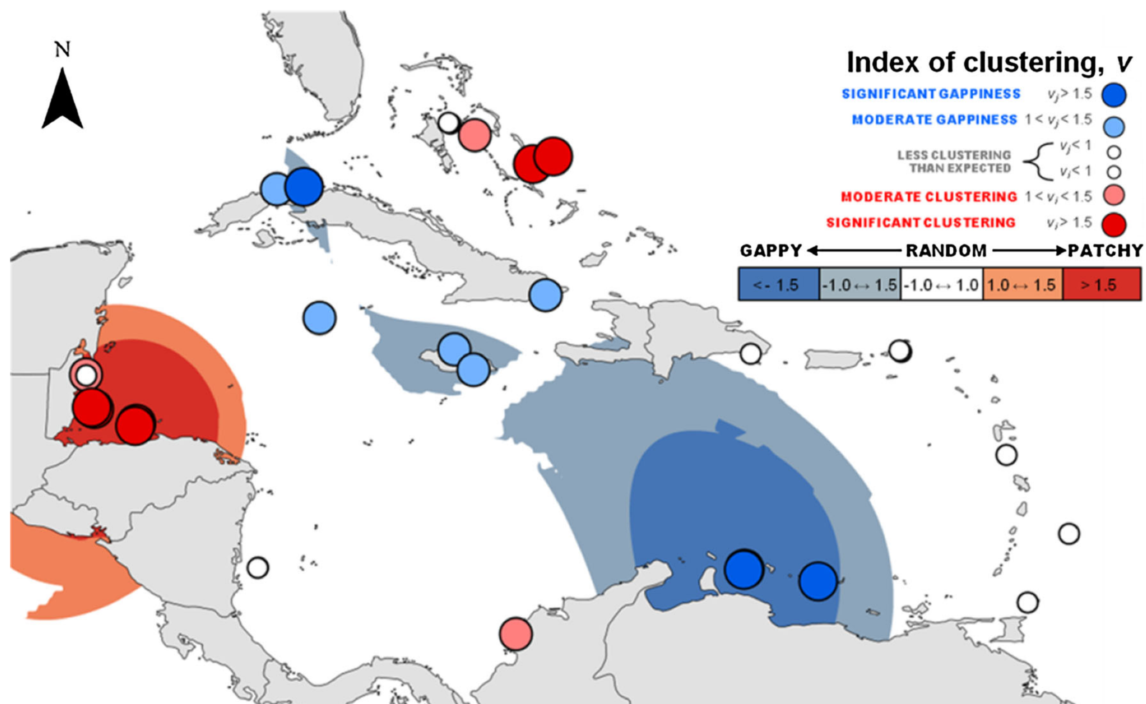

Fig. 3 *Red-blue* plot generated by SADIE analysis, indicating spatial clustering in the *Symbiodinium* D1 distribution among sampled *Orbicalla annularis* populations. Each *circle* represents one site where *O. annularis* was sampled ($n = 33$ sites). *Red circles* indicate sites with a higher-than-expected prevalence of *Symbiodinium* D1, *blue circles* indicate sites with significant scarcity of *Symbiodinium* D1, and *white circles* represent populations hosting the same amount of D1 as might be expected by chance when compared to >5,000 random permutations of the spatially referenced data. The size of each *circle* represents the statistical significance of the clustering index value, i.e., *largest filled circles* represent a unit with clustering index that exceeds the 95th percentile for patches (*red*) or gaps (*blue*), from the mean of the randomised distributions; medium-sized circles denote a unit that exceeds the 90th percentile; *small circles* denote unit with clustering below expectation (<1 or >−1). The 33 data points are superimposed onto a contour plot, with coloured areas denoting clustering beyond expectation, e.g., *red shaded areas* are geographic regions of higher-than-expected prevalence of *Symbiodinium* D1 in *O. annularis* and *blue areas* regions of lower-than-expected (or ‘gappy’ prevalence), while the *darkest areas* indicate highly significant clustering/gappiness. The large amount of *white space* on the map is indicative of a lack of significant spatial patterning in the occurrences of *Symbiodinium* D1 in *O. annularis* across the majority of the Caribbean region (exceptions being the Mesoamerican Barrier Reef and southern Caribbean, particularly around Curaçao and Venezuela)

required for original symbiont communities to re-establish dominance following bleaching-associated increases in clade D in *O. annularis* (Thornhill et al. 2006b; LaJeunesse et al. 2009), making it conceivable that our sampling period for the MBRS (October 2004) could coincide with recovering symbiont communities. Thus, temporal sampling would be needed to resolve the stability of cryptic *Symbiodinium* D1 at each site and to indicate whether shuffling or local uptake best explains the observed spatial distribution.

Wider implications of high *Symbiodinium* D1 prevalence

This study demonstrates that the prevalence of background *Symbiodinium* D1 reported in Barbados in the sentinel study of LaJeunesse et al. (2009) can be extrapolated to *O. annularis* across the entire wider Caribbean region. This suggests that the demonstrated ability of Caribbean colonies to recover from severe bleaching events may be more geographically extensive than first supposed. However, there are several caveats. Firstly, uncertainty exists around the ecological significance of background levels of *Symbiodinium* to the host. Despite 30 % of colonies hosting cryptic *Symbiodinium* D1, the majority of symbiont communities (65 %) in this study were dominated by *Symbiodinium* B1. The relationship between ITS2-B1 and Caribbean corals has existed since the Pleistocene and has been used to demonstrate stable symbioses and a lack of support for symbiont shuffling in the past (Baker et al. 2013). Evidence for the evolution of these long-term stable, mutual cooperations between coral and dominant *Symbiodinium* types presents a paradox in the face of an extremely large amount of variation in potential symbiont partners. An improved understanding of the traits imparted to hosts by different symbiont types might go some way to elucidating the significance of maintaining background ‘cryptic’ abundances of these types and help explain persistence of

variation in *Symbiodinium* (Heath and Stinchcombe 2013). The flexibility of *O. annularis* in hosting varied symbionts certainly does not appear to confer a greater diversity of potential responses to environmental threats in this important reef builder; it remains ranked among the most bleaching susceptible of Caribbean species (Manzello et al. 2007; van Hooidonk et al. 2012).

Secondly, not all species are as flexible in their associations as *Orbicella* spp.; many are 'specifist', exhibiting low flexibility and hosting taxonomically narrow assemblages (Putnam et al. 2012). Brooding corals (including common Caribbean species *Agaricia* sp., *Siderastrea* sp., and *Porites* sp.) inherit symbionts from parents, usually resulting in more specific and more stable symbioses, implying fewer opportunities for shuffling (Thornhill et al. 2006a). Other D1 hosts do not show comparable 'shuffling' in response to stress events (e.g., LaJeunesse et al. 2009; McGinley et al. 2012). Host-related factors, such as abundance of UV-absorbing compounds and the ability of coral to mop up hydrogen peroxide that triggers the response to expel, are also important in determining differential bleaching responses (Baird et al. 2009; Wooldridge 2014); this undermines the importance of the symbiont community in dictating future reef responses to thermal stress.

Thirdly, the benefits incurred by hosting D1 in terms of resilience to thermal stress may be countered by severe costs in terms of trade-offs, including a reduction in energy to juvenile corals, reduced growth rate and reproductive output, and increased disease susceptibility (e.g., Little et al. 2004; Jones and Berkelmans 2010; Littman et al. 2010). Not all clades are equally valuable as symbiotic partners, e.g., clade A is understood to be functionally less beneficial to *Acropora* in Hawaii than clade C (Stat et al. 2008). The ability of scleractinian corals to form calcium carbonate skeletal structures is linked to symbiosis; *A. millepora* colonies harbouring clade D grew 38 % slower than those hosting C (Little et al. 2004; Jones and Berkelmans 2010). Trade-offs such as this may help to explain the stable coexistence of multiple symbiont types when evolution should favour the development of long-term cooperation in coral-algal mutualisms (Heath and Stinchcombe 2013). Symbionts such as D1 that translocate fewer resources to their host may be maintained in low abundances as long as greater numbers of higher-quality mutualistic partners exist that can provide for the fitness of the host. However, *Symbiodinium* D1 may use stressful conditions as an opportunity to exploit the host as a habitat rather than engage in an interactive and mutually beneficial partnership, which may affect fitness of coral hosts and accelerate reef decline (Heath and Stinchcombe 2013; Ortiz et al. 2013). Ecosystem models have been used to explore the likelihood of the evolution of hypothetical 'super symbionts' that possess a combination of traits (and trade-offs) capable of maintaining Caribbean coral cover in the face of rising SSTs (Ortiz et al. 2014). In these models, the time required for establishment of the 'super-symbiont' into coral populations was a critical factor in their success in maintaining future Caribbean coral cover. The empirical findings of this study—that a symbiont with traits comparable to those required in the 'super-symbiont' is already established in at least 30 % of the Caribbean population of the reef builder *O. annularis*—are encouraging in this context and also may help refine the super-symbiont model. Unfortunately, the model concludes unless the new symbiont could provide 33 % more benefit than existing D1–4 in terms of reducing mortality during bleaching, at 20 % of the cost (of reduced growth rates), any increase in dominance is unlikely to ensure the persistence of Caribbean reefs. In the Caribbean, where maintenance of coral growth rates is important in competitive interactions with macroalgae (Roff and Mumby 2012) and is synonymous to healthy ecosystem functioning (Kennedy et al. 2013), increasing the dominance of cheating *Symbiodinium* D1 may cause more harm to reefs than good (Ortiz et al. 2013, 2014).

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