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REPORT

Genetic diversity of free-living *Symbiodinium* in the Caribbean: the importance of habitats and seasons

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Abstract Although reef corals are dependent of the dinoflagellate *Symbiodinium*, the large majority of corals spawn gametes that do not contain their vital symbiont. This suggests the existence of a pool of *Symbiodinium* in the environment, of which surprisingly little is known. Reefs around Curaçao (Caribbean) were sampled for free-living *Symbiodinium* at three time periods (summer 2009, summer 2010, and winter 2010) to characterize different habitats (water column, coral rubble, sediment, the macroalgae *Halimeda* spp., *Dictyota* spp., and *Lobophora variegata*, and the seagrass *Thalassia testudinum*) that could serve as environmental sources of symbionts for corals. We detected the common clades of *Symbiodinium* that engage in symbiosis with Caribbean coral hosts A, B, and C using *Symbiodinium*-specific primers of the hypervariable region of the chloroplast 23S ribosomal DNA gene. We also discovered clade G and, for the first time in the Caribbean, the presence of free-living *Symbiodinium* clades F and H. Additionally, this study expands the habitat range of free-living *Symbiodinium* as environmental *Symbiodinium* was detected in *T. testudinum* seagrass beds. The patterns of association between free-living *Symbiodinium* types and habitats were shown to be complex. An interesting, strong association was seen between some clade A sequence types and sediment, suggesting that sediment could be a niche where clade A radiated from a free-living ancestor. Other interesting relationships were seen between sequence types of *Symbiodinium* clade C with *Halimeda* spp. and clades B and F with *T. testudinium*. These relationships highlight the importance of some macroalgae and seagrasses in hosting free-living *Symbiodinium*. Finally, studies spanning beyond a 1-yr cycle are needed to further expand on our results in order to better understand the variation of *Symbiodinium* in the environment through time. All together, results presented here showed that the great diversity of free-living *Symbiodinium* has a dynamic distribution across habitats and time.

Keywords Free-living *Symbiodinium* · cpr23S-HVR · *Thalassia testudinum* · Coral reefs · Macroalgae · Log-linear modeling analysis

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Introduction

Members of the genus *Symbiodinium* (Freudenthal 1962) are among the most studied symbiotic dinoflagellates because of their symbiotic associations with several different phyla (reviewed by Baker 2003; Pochon et al. 2006). The majority of studies have largely focused on understanding the symbiosis between *Symbiodinium* and reef corals (Scleractinia), as this relationship represents an obligate symbiosis for many coral species and is responsible for the success of coral reefs (reviewed by Baker 2003; Coffroth and Santos 2005). The taxonomy of *Symbiodinium* is based

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on different molecular regions including the 28S nuclear and 23S chloroplast ribosomal DNA sequences, which currently recognizes nine large clades designated with the letters A–I (Pochon and Gates 2010). Some of these clades are composed of more than 100 sequence types based on higher resolution markers, such as ITS2 rDNA (Franklin et al. 2012). Differences in geographical distribution, response to environmental stressors, and host specificity have been described for these symbiotic clades (reviewed by Baker 2003; Rodriguez-Lanetty 2003). Constituents of clade C are the most common symbionts of reef corals, except in the Caribbean where clade B has been common since a Pleistocene radiation (LaJeunesse 2005). Representatives of clades A and D are considerably less common, but certain types appear to be adapted to high levels of irradiance (e.g., some members of clade A; Reynolds et al. 2008) and high temperatures (specifically species *S. trenchii* of clade D; e.g., Berkelmans and van Oppen 2006; Jones et al. 2008) when found in symbiosis. Clades F, G, and H are mainly or exclusively found in symbiosis with sponges, foraminiferans, and a few cnidarians (e.g., LaJeunesse 2001; Pochon et al. 2001; Rodriguez-Lanetty et al. 2002; Schonberg and Loh 2005; Granados et al. 2008; Hill et al. 2011). Clades E and I have the least number of genetic types and are the least common symbionts (reviewed by Pochon et al. 2006; Pochon and Gates 2010; Jeong et al. 2014).

Although the symbiosis is obligatory for many corals, *Symbiodinium* is vertically transmitted to the progeny in only 20 % of coral species (reviewed by Harrison 2011). For the other 80 %, symbionts must be acquired de novo each generation. This suggests that an environmental pool of *Symbiodinium* is crucial for the establishment of the new symbiotic relationship (Harrison 2011). Despite reports of *Symbiodinium*-like DNA in environments outside of hosts (hereafter referred to as free-living or environmental *Symbiodinium*) dating back to the late 1970s and early 1980s (Loeblich and Sherley 1979; Chang 1983), free-living *Symbiodinium* did not receive much attention until the late 1990s. The first molecular identification of free-living *Symbiodinium* in 1999 (Carlos et al. 1999) was followed by investigations of their occurrence in the water column, sediments, and on macroalgae (Gou et al. 2003; Coffroth et al. 2006; Thornhill et al. 2006; Koike et al. 2007; Hirose et al. 2008; Littman et al. 2008; Manning and Gates 2008; Porto et al. 2008; Adams et al. 2009; Hansen and Daugbjerg 2009; Pochon et al. 2010; Reimer et al. 2010; Venera-Ponton et al. 2010; Takabayashi et al. 2012; Zhou et al. 2012; Huang et al. 2013; Yamashita and Koike 2013; Jeong et al. 2014). These studies found that free-living *Symbiodinium* occurred in diverse habitats; however, they did not address how *Symbiodinium* assemblages are structured across habitats. Short-term temporal variation has been assessed (Pochon et al. 2010), but has not been examined across periods of more than just a few days. Furthermore, only some of the more recent studies employed pre-filtration procedures to exclude small organisms with symbionts that could be mistaken for free-living *Symbiodinium* (Koike et al. 2007; Pochon et al. 2010; Venera-Ponton et al. 2010; Takabayashi et al. 2012; Huang et al. 2013). In addition, many of these studies based the identification of *Symbiodinium* isolated from the environment on cultures prior to molecular identification (Loeblich and Sherley 1979; Chang 1983; Carlos et al. 1999; Gou et al. 2003; Coffroth et al. 2006; Hirose et al. 2008; Porto et al. 2008; Hansen and Daugbjerg 2009; Reimer et al. 2010; Jeong et al. 2014). Culturing introduces biases due to the tendency of the composition of a culture diverging from the original inoculum (Santos et al. 2001; Coffroth and Santos 2005).

The intent of this study was to characterize how the distribution of clades of free-living *Symbiodinium* varies across habitats and to examine changes over time. This will allow us a better understanding of the actual reservoirs of free-living *Symbiodinium* and the temporal dynamics of these populations in the reef ecosystem. The habitats sampled were the water column, sediments, coral rubble, three different genera of macroalgae (*Dictyota* spp., *Halimeda* spp., and *Lobophora variegata*), and for the first time, seagrass beds of *Thalassia testudinum*. The seagrass bed habitat was included in this study because they are known to harbor diverse communities that include dinoflagellates and other epiphytic algae (e.g., Rodriguez et al. 2010; Selina and Levchenko 2011; Hitchcock et al. 2012), and could be acting as a potential reservoir of free-living *Symbiodinium*.

Materials and methods

Field collections

All sampling was carried out on the Caribbean island of Curaçao, Netherlands Antilles. The initial steps of sample processing were carried out at the Caribbean Research and Management of Biodiversity (CARMABI) research station. A total of seven reef sites were surveyed during the summer of 2009, and the summer and winter of 2010 (Fig. 1; Electronic Supplement Material, ESM, 1). At each site, a 50-m line was laid to define a transect of constant depth (10–12 m). Water column, sediment, and coral rubble habitats were sampled every 10 m along the transect. For water column samples, 1-l plastic bottles were emptied using air from the diving tank prior to collection and a sample was taken near a coral colony (1 m above the colony head), usually of *Montastraea cavernsosa* or

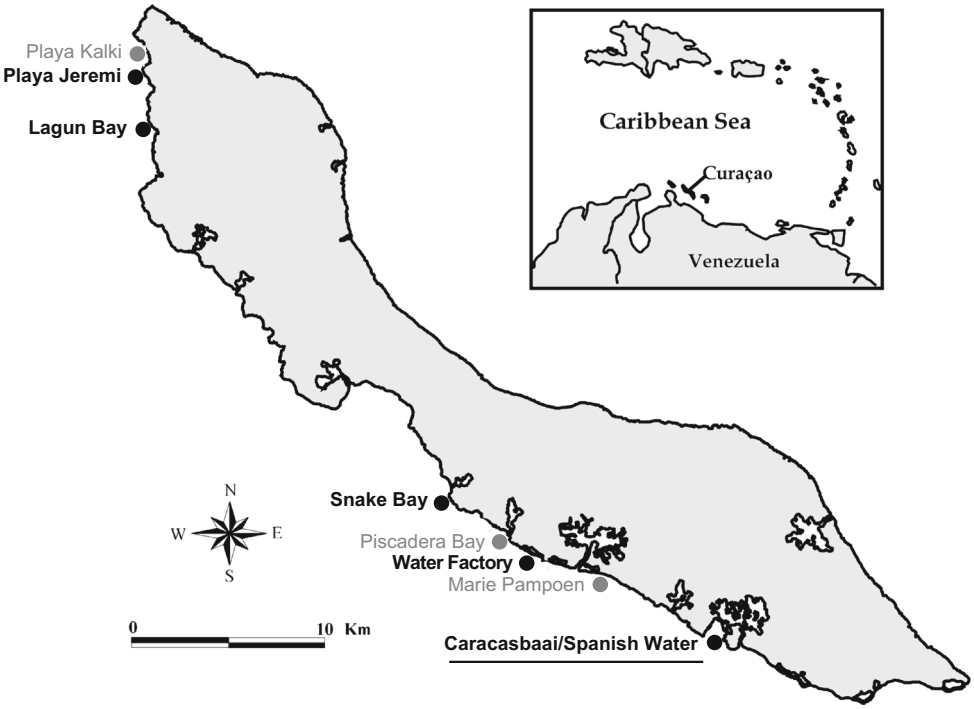


Fig. 1 Map of the Caribbean island of Curaçao, Netherlands Antilles, showing the sampling sites. *Black circles* mark sites that were sampled throughout the three sampling periods (summer of 2009, and summer and winter of 2010). *Gray circles* mark those sites that were sampled on some of the sampling periods. *Underlined* is the seagrass bay where *Thalassia testudinum* was collected. *Inset* shows the geographical location of Curaçao in the Southern Caribbean

Orbicella spp. Although this could be biased toward detecting *Symbiodinium* released by the host, this was done in order to standardize the substratum below. Samples of the upper 2-cm layer of sediment were collected in 250-ml jars. Coral rubble was sampled by aspirating in close proximity to the surface of the rubble using 60-ml syringes. Clumps of macroalgae identified as *Dictyota* spp., *Halimeda* spp., and *L. variegata* or three blades from each plant of *T. testudinum* were placed individually in sterile sampling bags with flat closure (Fisherbrand) with approximately 1 l of water to avoid dehydration during transportation. Epiphytes were collected after vigorous shaking of the macroalgae samples or were scraped from both sides of the *T. testudinum* leafs. All samples were prefiltered successively through 1000-, 500-, 300-, 210-, 100-, and 53-µm nets (WildCo) to remove small multicellular organisms that could contain symbiotic *Symbiodinium*. Prefiltered samples were then drawn through 2.5-µm filter paper by vacuum to collect and concentrate *Symbiodinium*. The paper filters were immediately preserved with 20 % DMSO and stored at −20 °C until further processing.

DNA extraction and analyses

Paper filters were rinsed twice with buffer (50 mM EDTA pH 8.0, 0.4 M NaCl) prior to DNA extraction. Total genomic DNA extraction was carried out according to Vidal et al. (2002), except that centrifugation steps were carried out at 4 °C. DNA was resuspended in 50 µl of TE (10 mM Tris–HCL pH 8.0, 1 mM EDTA pH 8.0). Attempts at PCR amplification of the internal transcribed spacer region 2 of the nuclear ribosomal repeat unit (nrITS2) and the large subunit nuclear ribosomal gene (nr28S) were successful for only a few samples. Additionally, amplifications were unspecific for *Symbiodinium*, as sequences from other organisms were obtained. Therefore, *Symbiodinium* detection was based on amplification and sequencing of the hypervariable region of the domain V of the chloroplast 23S ribosomal DNA gene (cpr23S-HVR), which is a putative *Symbiodinium*-specific marker (see discussions by Santos et al. 2003; Manning and Gates 2008; Pochon et al. 2010). This region was amplified using the forward primer 23SHYPERUP (5′-TCAGTACAAATAATATGCTG-3′, Invitrogen; Santos et al. 2003) and reverse primer 23SHYPERDN (5′-TTATCGCCCCAATTAAACAGT-3′, Invitrogen; Manning and Gates 2008). The PCR profile was as follows: initial denaturation for 3 min at 94 °C, 42 cycles at 94 °C for 20 s, 52 °C for 30 s, 72 °C for 30 s followed by a final extension of 10 min at 72 °C. PCRs contained 1X GreenMaster Mix (Promega), 0.5 µM of each primer, and nuclease-free water for a final volume of 20 µl. For initial PCR attempts with each sample, between 1 and 10 ng μl^{-1} of template DNA was used. A second attempt was made with three times the amount of template DNA for samples that did not yield products in the initial attempt. All samples that resulted in negative amplifications in this second attempt were precipitated with 0.55 mg ml^{-1} GlycoBlue (Invitrogen), 0.10 M sodium acetate, and 93 %

ethanol. Precipitation was carried out overnight at −20 °C, and the pellet was washed with 70 % ethanol. Between 5–10 and 15–30 ng μl^{-1} of precipitated template DNA was amplified in the third and fourth attempts, respectively. PCR products were treated with ExoSAP (GE Healthcare Life Sciences) or Illustra ExoStar (GE Healthcare Life Sciences). Direct sequencing was carried out at the DNA Analysis Facility at Yale University using the 23SHY-PERUP primer. Sequences were checked for chromatogram quality and edited using CodonCode Aligner (CodonCode Corp.) and BioEdit (Hall 1999). PCR products for six samples were cloned using the Qiagen PCR Cloning^{plus} Kit (Qiagen) to isolate single PCR products, as direct sequencing produced "noisy" traces indicative of multiple DNA templates. Four ampicillin-resistant colonies were selected by blue/white screening, picked using a sterile tip, PCR-amplified, treated with Illustra ExoStar, and sequenced as mentioned above.

Sequence analyses

Sequences obtained by the above methods were used as queries in BLAST v2.2.27+ (Altschul et al. 1990) searches of NCBI's GenBank nonredundant nucleotide database. The results of each BLAST search were used to verify that the highest alignment scores (best match) were from known *Symbiodinium* cpr23S-HVR sequences and also to provisionally assign query sequences to one of the nine recognized *Symbiodinium* clades. To further assign genotypes within *Symbiodinium* clades, a phylogenetic reconstruction using maximum likelihood was carried out, as BLAST is a quick approach to search a database and not phylogenetically supported for assigning genotypes. For this, sequences from each clade were combined along with their matches in GenBank (ESM1, ESM2) and *Heterocaspa triquetra*, a free-living dinoflagellate, as the outgroup sequence. Alignments of sequences were done using MAFFT-FFT-NS-I (Katoh and Toh 2008), edited with GBlocks (Castrana 2000), and then, manually edited in Mesquite v2.75 (Maddison and Maddison 2009). The maximum likelihood tree was built in CIPRES server (www.phylo.org; Miller et al. 2010) using RAxML (Stamatakis 2014) according to the recommended parameters with 1000 bootstrap replicates.

Statistical analyses

Given that two locations were not visited across all sampling periods, statistical analyses were performed on the data collected from the five reef sites that were visited across all sampling periods. Furthermore, in order to determine whether location had an effect on the distribution of *Symbiodinium*, a multivariate ANOVA test based on dissimilarities (Adonis) with 1000 permutations (Oksanen 2011) was carried out fitting habitat, sampling period, and reef site (all seven sites) as environmental variables. Results showed that location was not a significant variable and was not included in further analyses. Adonis analyses were also performed among the other variables (namely *Symbiodinium* clade, habitat, and sampling period) with 1000 permutations fitting habitat and sampling period as environmental variables. Non-metric multidimensional scaling (nMDS) was used to ordinate the data based on the occurrence of free-living *Symbiodinium* by clade, habitat, and sampling period. First, a distance matrix was built using Bray–Curtis dissimilarity with zero values auto-transformed using the package Vegan v3.17 (Oksanen et al. 2010) in the R environment version 2.10.1 (R Development Core Team 2009). Data were then standardized using Wisconsin double standardization and standardized to equal totals, as suggested by the developer. In order to compare the goodness of fit (or stress) from random ordinations, the function metaMDS was used. The solution was rotated so that the largest variance of site scores was on the first axis. Hierarchical clustering of the samples was also carried out and was calculated in R (R Development Core Team 2009) using complete linkage, which finds clusters based on maximum distance between objects (Everitt et al. 2001).

Log-linear modeling analysis was performed to examine associations among *Symbiodinium* clades, sampling periods, and habitats. Models were compared based on the likelihood ratio Chi-squared statistic (Sokal and Rohlf 1995). Models were reduced by removing higher-order interaction terms that did not improve model fit to the data. Sources of significant interactions in the reduced model were determined by obtaining standardized residuals from the null model of no association between variables. Standardized residuals with values <-1.5 or >1.5 indicated combinations of clade, habitat, and time period that were not explained by differences in sampling intensity (which are accounted for in the null model). Log-linear analyses, model comparison, and calculations of standardized residuals were carried out using the MASS package (Ripley 2011) of the R software.

Results

Detection of free-living *Symbiodinium*

DNA was extracted from a total of 303 samples (ESM1), which were collected from three sampling periods; amplification of the cpr23S-HVR was successful for 58 % of these samples (Table 1). This percentage probably represents an underestimation of the "real" presence of free-

Table 1 Percentage of free-living *Symbiodinium* after clade assignment based on a BLAST search and phylogenetic tree (Fig. 2) detected in each habitat

Habitat	Percentage of *Symbiodinium* clades found per habitat across all sampling						Percentage of samples with successful amplification
	A	B	C	F	G	H	
Water column	34.8	56.5	4.3	2.2	2.2	0	62 ($n = 73$)
Sediment	91.7	8.3	0	0	0	0	23 ($n = 43$)
Coral rubble	30.6	52.8	11.1	0	5.6	0	66 ($n = 67$)
Halimeda spp.	11.1	66.7	16.7	0	0	5.6	82 ($n = 22$)
Dictyota spp.	16.7	79.2	0	4.2	0	0	62 ($n = 39$)
L. variegata	0	75	0	0	0	25	80 ($n = 5$)
T. testudinum	7.4	55.6	14.8	18.5	3.7	0	57 ($n = 54$)
Total	58 ($n = 303$)						

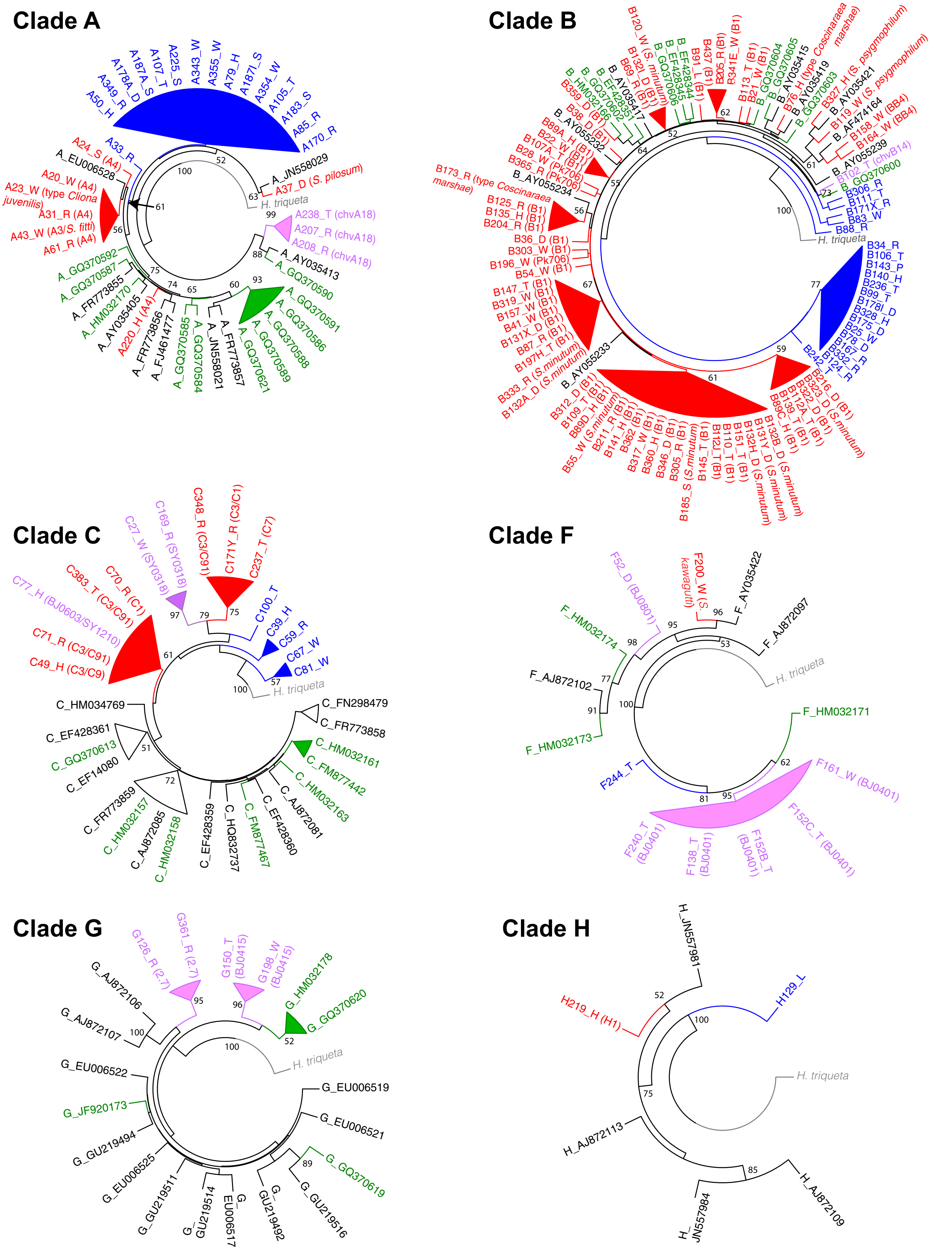
These values were calculated across all sampling periods (summer of 2009, summer of 2010, and winter of 2010) based on the actual number of samples where *Symbiodinium* was detected; *n* indicates the total number of samples collected for each habitat. On average, six replicates of each abiotic habitat were collected per reef site per period, and between 15 and 20 samples of *Thalassia testudinum* were collected per sampling period. The number of macroalgae samples varied depending on the reef site and season

living *Symbiodinium* since factors such as PCR inhibitors present in environmental samples and/or primer bias could have negatively affected PCR amplification success in some of the samples. In every positive case, the highest scoring alignment from a GenBank BLAST search was a known sequence of cpr23S-HVR from *Symbiodinium* (*e*-value $<1 \times 10^{-10}$). Based on their best GenBank matches and the maximum likelihood tree, cpr23S-HVR sequences were assigned to six major clades (A, B, C, F, G, and H) of the nine, genetically diverse clades recognized for *Symbiodinium* (Fig. 2). Of the 143 different sequence types identified, 57 (40 %) represent novel sequences based on the phylogenetic position and the low sequence similarity with reported sequences. Twenty-eight different sequences (17 novel sequences) were recovered of clade A, 87 (33 novel sequences) of clade B, 15 (five novel sequences) of clade C, seven (one novel sequence) of clade F, four sequences of clade G, and two (one novel sequence) of clade H (Fig. 2). Since our cpr23S-HVR sequences were, in general, shorter than those reported by Santos et al. (2003), there was not certainty in the assignment of our sequences to Santos' chloroplast 23S genotype nomenclature, which is based not only on sequence nucleotide information but also on sequence size identified from acrylamide gel separation.

Structural and temporal changes in the assemblages of free-living *Symbiodinium*

The water column contained free-living *Symbiodinium* assigned to clades A, B, C, F, and G (Table 1). Nine of the 31 different sequences found in the water column represented novel sequences (29 %; ESM1). These results are unlikely to be biased by collecting the water sample over *Orbicella* spp. or *M. cavernosa* coral heads, as members of clades F and G have not been reported for these coral species (Yost and Mitchelmore 2010; Green et al. 2014). Additionally, other clades (especially clade D) have been found in symbiosis with these coral species (Santos and LaJeunesse 2006; Correa et al. 2009) and were not found in the water column in the present study. In the case of sediment, initial technical difficulties restricted sample collection of this habitat during the summer of 2009. The single sample that was collected, however, contained *Symbiodinium* clade A. During the other two sampling periods, these technical difficulties were addressed and free-living *Symbiodinium* from clades A and B were detected from the sediment (Fig. 3). From the six different sequences detected in the sediment, four novel sequences were identified, all belonging to clade A (ESM1). The other abiotic habitat sampled was coral rubble, and it contained free-living *Symbiodinium* belonging to clades A, B, C, and G (Table 1). A total of 38 different sequences were detected in the coral rubble of which seven represent novel sequences (18 %; ESM1).

Two biotic habitats were sampled for free-living *Symbiodinium*: macroalgae (*Halimeda* spp., *Dictyota* spp., and *L. variegata*) and seagrass beds of *Thalassia testudinum*. In the case of the *Halimeda* spp., samples contained *Symbiodinium* clades A, B, C, and H (Table 1). From the 16 different sequences identified in *Halimeda* spp., ten represent known sequences (62 %; ESM1). In *Dictyota* spp., clades A, B, and F were identified in the samples collected (Table 1), and 14 of the 22 (64 %) were sequences previously reported (ESM1). Only clades B and H were detected from the few number of *L. variegata* samples (Table 1), of which only one of the three different sequences represents a novel sequence. As for the seagrass beds of *T.*


Clade A
Clade B
Clade C
Clade F
Clade G
Clade H
H. triqueta
A_JN558029
A37_D (S. pilosum)
A238_T (chvA18)
A207_R (chvA18)
A208_R (chvA18)
A_AY035413
A_GQ370590
A_GQ370591
A_GQ370586
A_GQ370588
A_GQ370589
A_GQ370621
A_FR773857
A_JN558021
A_GQ370584
A_GQ370585
A_FJ461477
A_FR773856
A220_H (A4)
A_AY035405
A_HM032170
A_FR773855
A_GQ370587
A_GQ370592
A61_R (A4)
A43_W (A3/S. fitti)
A31_R (A4)
A23_W (type Clione juvenilis)
A20_W (A4)
A_EU006528
A24_S (A4)
A33_R
A50_H
A349_R
A178A_D
A187A_S
A107_T
A225_S
A343_W
A355_W
A79_H
A187I_S
A354_W
A105_T
A183_S
A85_R
A170_R
B173_R (type Coscinaraea marshae)
B_AY055234
B125_R (B1)
B135_H (B1)
B204_R (B1)
B36_D (B1)
B303_W (B1)
B196_W (Pk706)
B54_W (B1)
B_AY055233
B34_R
B106_T
B143_P
B140_H
B236_T
B99_T
B83_W
B88_R
B_GQ370600
B_AY055239
B158_W (BB4)
B164_W (BB4)
B327_H (S. psygmophilum)
B119_W (S. psygmophilum)
B_AF474164
B_AY035421
B_GQ370603
B_AY035419
B_AY035415
B_GQ370605
B_GQ370604
B21_W (B1)
B113_T (B1)
B341E_W (B1)
B205_R (B1)
B437 (B1)
B91_L (B1)
B120_W (S. minutum)
C348_R (C3/C91)
C171Y_R (C3/C1)
C237_T (C7)
C169_R (SY0318)
C27_W (SY0318)
C77_H (BJ0603/SY1210)
C383_T (C3/C91)
C70_R (C1)
C71_R (C3/C91)
C49_H (C3/C9)
C100_T
C39_H
C59_R
C67_W
C81_W
C_FN298479
C_FR773858
C_HM032161
C_FM877442
C_HM032163
C_AJ872081
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C_FM877467
C_HQ832737
C_EF428359
C_HM032158
C_AJ872085
C_HM032157
C_FR773859
C_EF14080
C_GQ370613
C_EF428361
C_HM034769
F52_D (BJ0801)
F200_W (S. kawagutii)
F_AY035422
F_AJ872097
F_HM032174
F_AJ872102
F_HM032173
F_HM032171
F244_T
F161_W (BJ0401)
F152C_T (BJ0401)
F152B_T (BJ0401)
F138_T (BJ0401)
F240_T (BJ0401)
G126_R (2.7)
G361_R (2.7)
G150_T (BJ0415)
G198_W (BJ0415)
G_HM032178
G_GQ370620
G_AJ872106
G_AJ872107
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G_EU006525
G_GU219511
G_GU219514
G_EU006517
G_GU219492
G_GU219516
G_GQ370619
G_EU006521
G_EU006519
H_JN557981
H219_H (H1)
H129_L
H_AJ872113
H_JN557984
H_AJ872109

◀**Fig. 2** Maximum likelihood reconstructions from environmental and symbiotic *Symbiodinium* using cpr23S-HVR from clades A, B, C, F, G, and H. *Numbers* indicate bootstrap support branches above 50 %. For the complete set of data, see ESM 1 and ESM 2. Samples listed in *black* are previously reported *Symbiodinium* sequences from culture and/or hosts, in *green* are previously reported free-living *Symbiodinium* sequences, in *blue* are novel sequences reported in this study, in *red* are sequences from the present study similar to known sequences from hosts and/or cultures, in *light purple* are sequences from the present study similar to known free-living sequences, in *gray* are the outgroup sequences of the free-living dinoflagellate *Heterocaspa triqueta*. For sequences reported in this study, *first letter* indicates clade, *number* indicates sample ID (as seen in ESM 1), and *last letter* corresponds to habitat as follows: *W* water column, *S* sediment, *C* coral rubble, *H Halimeda* spp., *D Dictyota* spp., *L Lobophora variegata*, *T Thalassia testudinum*. For those sequences similar to previously reported sequences, the closest BLAST hit(s) is in *parenthesis*

testudinum, *Symbiodinium* belonging to clades A, B, C, F, and G was detected on this habitat (Table 1). Of the 31 different sequences detected in seagrass, ten represent novel sequences (32 %).

Multivariate ANOVA based on dissimilarities (Adonis) was used to test for associations between *Symbiodinium* clade and habitat or clade and sampling period. Results revealed no significant association between sampling period and *Symbiodinium* clade ($p > 0.05$). However, a significant association was detected between habitat and *Symbiodinium* clade ($p < 0.001$). In order to visualize these patterns of association, an ordination by nMDS was performed (goodness of fit calculated using Kruskal's stress = 13.12; Fig. 4). The clearest distribution is the group of sediment samples near clade A (Fig. 4). The nMDS analysis also suggests an association of symbiont sequence types within clades C and H with *Halimeda* spp., although further statistical testing (below) only supported a significant association with symbionts within clade C. Hierarchical clustering was superimposed onto the nMDS ordination, revealing three clear clusters (Fig. 4). Two clusters were composed of either abiotic or biotic habitats, while the third one was composed of a mixture of habitats (Fig. 4).

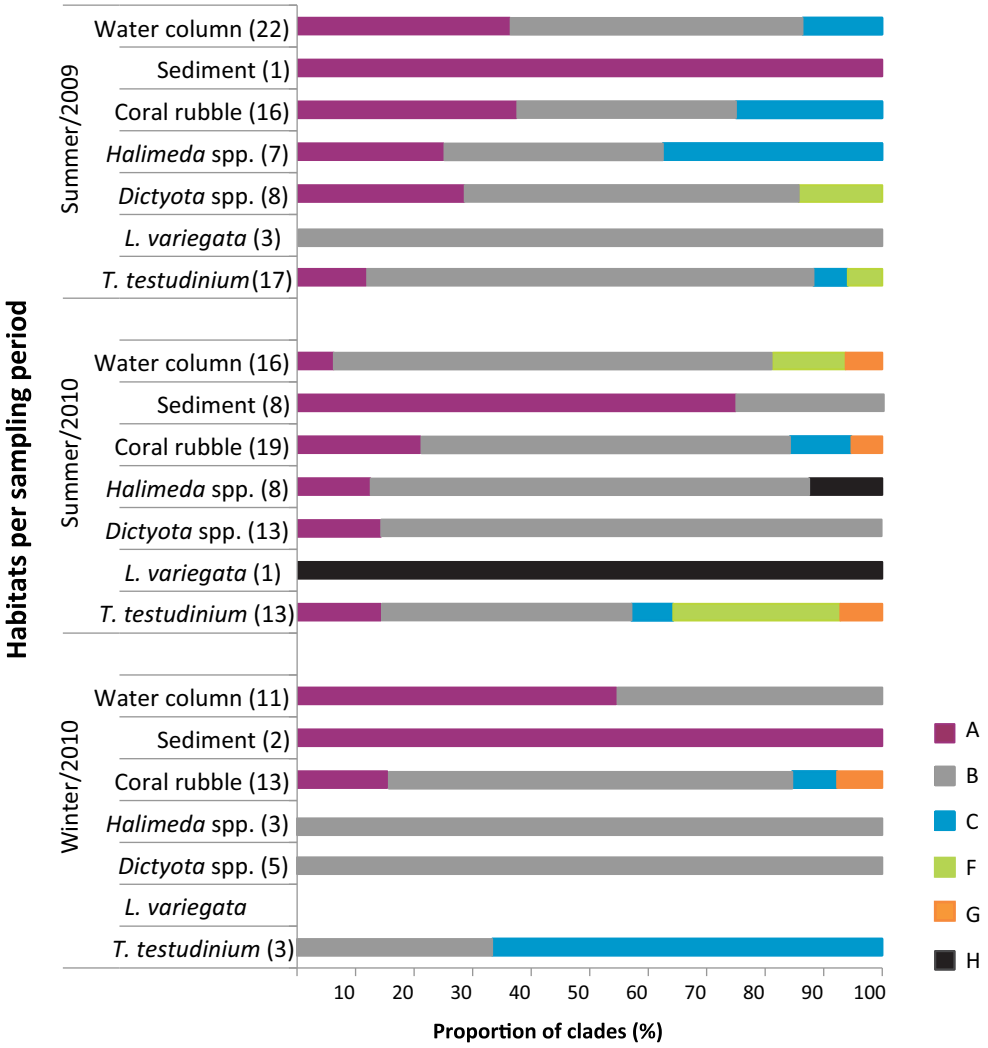

Fig. 3 Relative proportions of free-living *Symbiodinium* found in each habitat during the different sampling periods. These proportions were determined in each sampling period by dividing the number of *Symbiodinium* sequence counts per clade by the total number of *Symbiodinium* sequence counts in each habitat. *Numbers in parenthesis* represent the sample sizes of each habitat that were successfully amplified. *Symbiodinium* clade colored as shown on the lower right. *Lobophora variegata* was not collected during winter of 2010

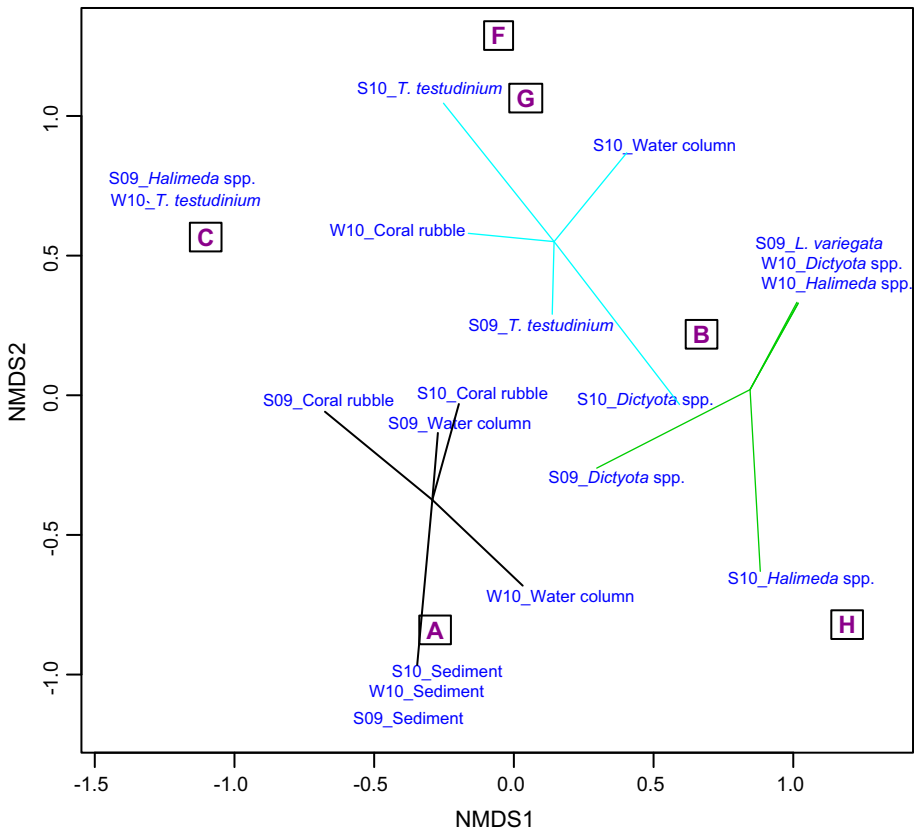

Fig. 4 Non-metric multidimensional scaling (nMDS) of free-living *Symbiodinium*. Six different habitats (water column, sediment, coral rubble, *Halimeda* spp., *Dictyota* spp., *Lobophora* spp., and *Thalassia testudinum*) across three sampling periods (*S09* summer 2009, *S10* summer 2010, and *W10* winter 2010) were sampled. Hierarchical clustering resulted in three clusters (*light blue*, *green*, and *black lines*). Clades A, B, C, F, G, and H are placed based on average weight across sites and are shown in *boxes*. Stress = 13.12 (see text for details)

Log-linear modeling analyses were used to further examine the relationships between *Symbiodinium* clade, habitat, and sampling period that best accounted for the data observed. This analysis excluded samples from *L. variegata* because this habitat was not consistently sampled across all time periods, and clades G and H were not included due to the low number of samples containing these clades. Likelihood ratio tests from the log-linear model analysis indicated that the model that best explained interactions in the data was *habitat* × *clade* ($p < 0.001$) + *sampling date* × *clade* ($p < 0.04$). Unlike the multivariate analysis, this log-linear model analysis showed an effect of sampling period and clade. A plot of standardized residuals was used to determine significant departures from the assumptions of the model stating that clades are distributed independently of sampling date or habitat (Fig. 5). By analyzing the residuals, we were able to appreciate not only the positive associations between clades and habitats, as revealed by the nMDS analysis, but it also allowed detecting significant negative associations. In the water column, there was a strong negative association with clade A during the summer of 2010 and a positive association during the winter of 2010. In the case of sediment, there was a positive association between clade A and sediment throughout the different sampling periods (as seen in the nMDS ordination; Fig. 4), but only significantly during the summer of 2010 as seen in the residuals analysis. Interestingly, all other clades exhibited negative associations with the sediment, particularly clade B, where the standard residuals were lower than −1.5. *Halimeda* spp. had a strong positive association with clade C during the summer of 2009 (Figs. 4, 5). In the case of *Dictyota* spp., only clade A showed a clear negative association during winter of 2010. Finally, *T. testudinum* showed the greatest variation of positive and negative associations of clades and sampling periods. Remarkably, there was a particularly strong positive association between clades B and F during the summers of 2009 and 2010, respectively, and a strong negative association with clades A and B during the winter of 2010.

Discussion

With the pioneer work of Brandt (1881), more than a century ago, we learned about the existence of the intracellular symbiosis between *Symbiodinium* and scleractinian corals, which doubtlessly is the driving force of coral reef ecosystems. Surprisingly, it took 100 yr from Brandt's work to discover that the large majority of corals spawn gametes to the water column where external fertilization occurs (Harrison et al. 1984) and that most of the coral

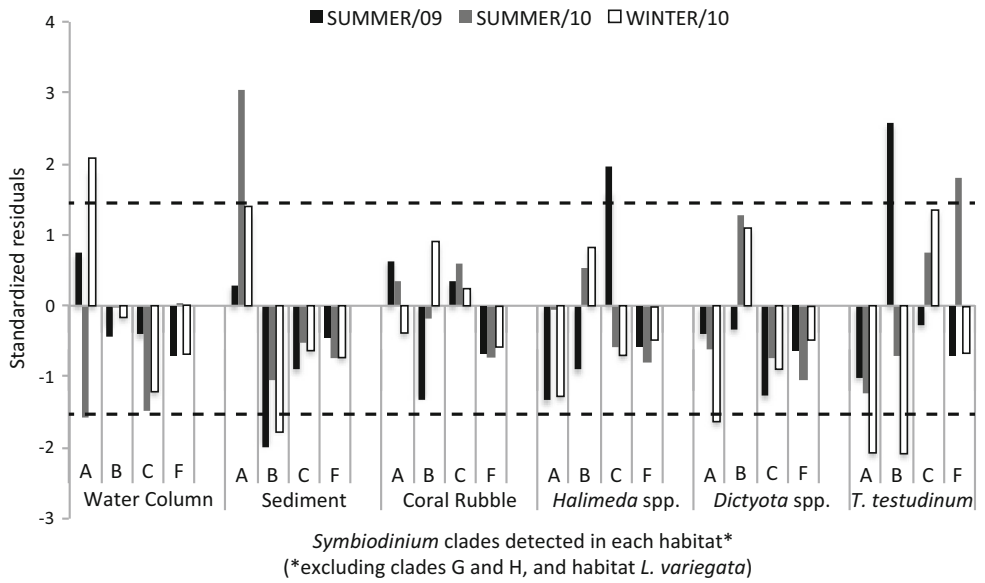

Fig. 5 Standardized residuals from the model *clade* × *habitat* + *clade* × *sampling period*. *Dashed lines* represent the cutoff values <−1.5 or >1.5 indicating combinations of *clade*, *habitat*, and *sampling period* that were not explained by differences in sampling intensity (accounted in the null model). Sampling period colored as shown. *Lobophora variegata*, clade G, and clade H were not included in this residual analysis (see text for details)

offspring lack their precious microalgae symbionts in their early developmental stages (Harrison 2011). This clearly implies the existence of a free-living stage in the life cycle of these symbiotic *Symbiodinium*. However, over the last 30 yr, we have learned so little of the ecological aspects of this important phase of the *Symbiodinium* life cycle. Having said that, various studies have documented, through time snapshot explorations, the composition of *Symbiodinium* in various habitats within the reef (Carlos et al. 1999; Gou et al. 2003; Coffroth et al. 2006; Thornhill et al. 2006; Koike et al. 2007; Hirose et al. 2008; Littman et al. 2008; Manning and Gates 2008; Porto et al. 2008; Adams et al. 2009; Hansen and Daugbjerg 2009; Pochon et al. 2010; Reimer et al. 2010; Venera-Ponton et al. 2010; Takabayashi et al. 2012; Zhou et al. 2012; Huang et al. 2013; Yamashita and Koike 2013; Jeong et al. 2014). Nevertheless, none of these studies, except one (Pochon et al. 2010) over a timeframe of 2 d, used systematic sampling approaches to understand the spatial and temporal distribution of *Symbiodinium* in the free-living environment. With this in mind, this study set out to systematically investigate the composition of *Symbiodinium* in multiple reef habitats (water column, sediment, coral rubble, associated with macroalgae and seagrasses) over various seasons within 2 yr. It is important to note that some of the *Symbiodinium* types detected in this and previous studies may not necessarily be "free-living" but transient since they could be recently released from corals. Adding to the contribution of previous studies, we revealed that not only the common clades of *Symbiodinium* A, B, C, and to a lesser extent G are found in the free-living environment, but also discovered, for the first time for a Caribbean reef environment, the presence of *Symbiodinium* clades F and H. The detection of members of clades F and H as free-living (Zhou et al. 2012; Huang et al. 2013; this study) thus implies a pool of symbionts that might be shared among hosts from different phyla in the Caribbean. The discovery of new *Symbiodinium* clades in the environment in the present study highlights the value of using culture-independent techniques to assess diversity, compared to culturing-based surveys conducted in the past that have underestimated the "real" diversity in the reef environment (Coffroth et al. 2006).

The data showed a great diversity of *Symbiodinium* chloroplast rDNA sequences in the environment, particularly from clade B, which could be expected as this clade is highly diverse among the symbiotic *Symbiodinium* associated with reef invertebrate hosts in the Caribbean (LaJeunesse 2005). Moreover, *Symbiodinium* clade B was identified in all habitats and was the clade most consistently present over all three sampling periods (Fig. 3). This ubiquity of clade B is in agreement with its reported dominance *in symbio* in the Caribbean (Baker and Rowan 1997; LaJeunesse 2002). Conversely, considerably less diversity within clade C was detected in the environment even though this clade is also a highly diverse lineage in host symbioses (for a phylogeographic synthesis see LaJeunesse 2005). It was also unexpectedly found in a small number of environmental samples (Fig. 3), which contrasts with the prevalence of this clade not only in the

Caribbean but also worldwide (LaJeunesse 2005). The disparity in diversity and commonness between these two clades could be attributed to differences in population dynamics among the *Symbiodinium* types when present in the free-living stage. Clade C types could have a peak in the free-living environment on a different season from the sampling periods included in the present study, or it is possible that clade C uses other habitats not sampled here. Alternatively, it is plausible that free-living *Symbiodinium* (non-symbiotic) in clade B could have radiated in the free-living environment. This could explain the discovery of more novel sequences within clade B compared to clade C (Fig. 2). A similar case could occur for *Symbiodinium* within clade A, in which we detected a larger number of new *Symbiodinium* sequence types than clade C (Fig. 3). Whether these novel *Symbiodinium* types discovered in the free-living environment are potentially able to associate in symbiosis with an invertebrate host could not be measured in this study and therefore remains elusive. However, previous studies have shown that some novel *Symbiodinium* types found only in the free-living environment can engage in symbiosis with some corals (Lewis and Coffroth 2004; Coffroth et al. 2006; Thornhill et al. 2006), but it still remains unknown whether these experimental symbioses are stable over time.

The patterns of association between *Symbiodinium* types discovered in the free-living environment with specific habitats within the reef were complex, as in some cases, the type of association between *Symbiodinium* and habitat was reversed depending on the sampling period. For instance, clade A was positively associated with the water column in winter 2010 but negatively associated in summer 2010. Clade B was positively associated with the seagrass *T. testudinium* in summer 2009 but negatively associated in winter 2010. Longer-term studies that span beyond 2-yr cycles are needed to better understand the temporal effect on the population dynamics of some *Symbiodinium* clades in the free-living environment.

Other significant relationships between clade and habitat were less affected by time. A positive association was revealed between sediment and *Symbiodinium* clade A. While *Symbiodinium* clade A was also found in other habitats, most of the sediment samples were dominated by novel *Symbiodinium* clade A types, which corroborates findings from other studies. In the Caribbean, Hawaii, and off the Japanese coast, clade A has been commonly found in sediments (Carlos et al. 1999; Pochon et al. 2010; Takabayashi et al. 2012; Yamashita and Koike 2013), even though this clade is not the most common symbiont for corals on those reefs (LaJeunesse et al. 2004; Pochon et al. 2010; Yamashita and Koike 2013). The fact that sediment-associated *Symbiodinium* clade A types detected in our studies and others nest under different non-symbiotic groups when inferring phylogenetic relationships (Fig. 2) supports the postulation that some members of clade A found the sediment as a niche to radiate from a possibly exclusively free-living ancestor (also suggested by Yamashita and Koike 2013).

Another interesting positive association was discovered between *Symbiodinium* clade C and the green macroalgae *Halimeda* spp. While clades including A, B, F, and H were also identified in association with this macroalgae, the associations were not significant with the exception of clade H, which was not tested due to low sample size. This finding supports previous discoveries of *Symbiodinium* associations with macroalgae (Porto et al. 2008; Venera-Ponton et al. 2010). In the southern Great Barrier Reef, *Symbiodinium* clade C was associated with samples from the green macroalgae *Chlorodesmis fastigiata*, *Halimeda opuntia,* and *H. discoidea*, the red macroalgae *Hypnea spinella*, *H. pannosa*, *Laurencia intricata,* and *Asparagopsis taxiformis*, the brown macroalgae *Padina* sp. and *Lobophora variegata*, and algal turfs (Venera-Ponton et al. 2010). Interestingly, this previous study did not detect *Symbiodinium* types other than clade C. This could have resulted from a bias in the molecular DNA markers used (D1/D2 28S nrDNA) by Venera-Ponton et al. (2010), which may have preferentially amplified *Symbiodinium* clade C or perhaps due to the predominance of C types in Great Barrier Reef corals. In the Caribbean, Porto et al. (2008) used the same molecular marker used in our study (cpr23S-HVR) and were able to identify the presence of several clades (A, B, and C) associated with the macroalgae *Halimeda* spp., *Lobophora variegata*, *Amphiroa* spp., and *Caulerpa prolifera*. However, the few number of collected samples prevented them from finding additional cryptic *Symbiodinium* clades found in our study, and, for the same reason, they were unable to detect a significant specific association between *Symbiodinium* clade and macroalgae species as in the present study. Taken together, all of these studies provide clear evidence of the association of *Symbiodinium* in the free-living environment and macroalgae. Several factors may explain why macroalgae could be a reservoir for some free-living *Symbiodinium*. It has been shown that macroalgae release organic substrates that are utilized by epiphytes including dinoflagellates (Haas et al. 2010). It is possible that this leakage of nutrients is favorable to *Symbiodinium* in an otherwise nutrient-poor environment (Porto et al. 2008). Additionally, macroalgae may provide a large surface area, making them ideal for colonization attachment (Parson and Preskitt 2007). While more studies are required to further broaden and confirm that particular sequence types associate with certain species of macroalgae, the findings presented here suggest that not all groups of macroalgae have negative effects on coral reefs, since some microalgae, such as

Halimeda spp., seem to act as environmental reservoirs for some *Symbiodinium* clades during an important phase of the life cycle of these organisms.

Although previous studies have identified other dinoflagellates as common epiphytes of seagrasses (e.g., Marasigan et al. 2001; Rhodes 2011; Selina and Levchenko 2011; Hitchcock et al. 2012), this study is the first to document the presence of *Symbiodinium* dinoflagellates (A, B, F, and G) associated with the blades of the seagrass *T. testudinum*. Some of these *Symbiodinium*, including clades B and F, showed significant positive associations with *T. testudinum* in some periods of the year. Future studies are needed in order to gain a better understanding of the ecology and dynamics of *Symbiodinium* within *Thalassia* seagrass beds, and between seagrasses and coral reefs.

Besides the significant association found between some clades and habitats within the reef environment, the analyses also revealed that some free-living *Symbiodinium* clades are differentially distributed through time; however, the temporal patterns are complex and longer-timeframe studies are needed to better understand the temporal dynamics through several annual cycles. Having said that, the time interaction might suggest the possible existence of phenology patterns in free-living *Symbiodinium*. We hypothesize that the phenology of free-living *Symbiodinium* could potentially include a peak near spawning season, which is the time when newly released larvae would be engaging in symbiosis with *Symbiodinium*. Future studies are required to confirm the existence of such phenological phenomenon and to determine its close proximity in time to spawning season.

In conclusion, the present study provides a baseline required for understanding the patterns and distribution of free-living *Symbiodinium* populations. Results presented here highlight the variety of habitats, including seagrass beds, that might be acting as reservoirs of free-living *Symbiodinium*. More studies are required to increase the number of sampling periods as well as to use quantitative tools to determine the abundance of free-living *Symbiodinium*. This will improve our understanding of the phenology of these populations in the environment. Further experiments will be required in order to determine which of these cpr23S sequence types are actually capable of engaging in symbiosis and would therefore display both life-history strategies (symbiosis and free-living). Additionally, it will be important to understand how factors such as temperature, salinity, wind, and precipitation may affect free-living *Symbiodinium* assemblages as seen in other dinoflagellates (e.g., Villanoy et al. 2006; D'Costa et al. 2008; Bouimetarhan et al. 2009). Answering these questions is fundamental for coral reefs, an ecosystem that is changing at an incredibly fast pace (Gardner et al. 2003).

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