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COMPARISON OF ENDOSYMBIOTIC AND FREE-LIVING *SYMBIODINIUM* (DINOPHYCEAE) DIVERSITY IN A HAWAIIAN REEF ENVIRONMENT[1]

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Many scleractinian corals must acquire their endosymbiotic dinoflagellates (genus *Symbiodinium*) anew each generation from environmental pools, and exchange between endosymbiotic and environmental pools of *Symbiodinium* (reef waters and sediments) has been proposed as a mechanism for optimizing coral physiology in the face of environmental change. Our understanding of the diversity of *Symbiodinium* spp. in environmental pools is poor by comparison to that engaged in endosymbiosis, which reflects the challenges of visualizing the genus against the backdrop of the complex and diverse micro-eukaryotic communities found free-living in the environment. Here, the molecular diversity of *Symbiodinium* living in the waters and sediments of a reef near Coconut Island, O'ahu, Hawai'i, sampled at four hourly intervals over a period of 5 d was characterized using a *Symbiodinium*-specific hypervariable region of the chloroplast 23S. A comparison of *Symbiodinium* spp. diversity recovered from environmental samples with the endosymbiotic diversity in coral species that dominate the adjacent reef revealed limited overlap between these communities. These data suggest that the potential for infection, exchange, and/or repopulation of corals with *Symbiodinium* derived from the environment is limited at this location, a finding that is perhaps consistent with the high proportion of coral species in this geographic region that transmit endosymbionts from generation to generation.

Key index words: **coral; cp23S; dinoflagellate; ITS-2; *Symbiodinium***

Abbreviations: **cp23S-HVR, chloroplast 23S hypervariable region; ITS, internal transcribed spacer**

Coral reefs are biologically productive and economically important tropical marine ecosystems (van Oppen and Gates 2006). Their ecological success is primarily driven by a mutualistic endosymbiosis between corals and photosynthetic dinoflagellates in the genus *Symbiodinium* (Muscatine et al. 1981). This endosymbiosis is essential to the survival of the host and underpins the productivity and calcification that creates habitat for the immense biodiversity that coral reefs support. Although best known for their relationship with cnidarians, *Symbiodinium* dinoflagellates are also found in association with a myriad of other marine invertebrate and protist hosts, including mollusks, sponges, foraminiferans, and ciliates, and play important functional roles in their hosts (Trench 1993, Glynn 1996, Rowan 1998, Lobban et al. 2002).

The genus *Symbiodinium* contains tremendous taxonomic diversity (Rowan and Powers 1991, LaJeunesse 2001, Pochon et al. 2001), the distribution of which is nonrandom (Iglesias-Prieto et al. 2004, LaJeunesse 2005). Extensive phylogenetic investigations, based primarily on the analyses of nuclear and chloroplast ribosomal (rDNA) genes, have resolved eight subgeneric lineages referred to as clades A through H (Pochon et al. 2004). The use

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of more variable nuclear markers, such as the internal transcribed spacer 1 and 2 (ITS-1 and ITS-2) regions, has further revealed that each *Symbiodinium* clade is composed of a diverse group of subclade types that exhibit distinctive host taxonomic, geographic, and/or environmental distribution patterns (LaJeunesse et al. 2004a, van Oppen et al. 2005, Pochon et al. 2007).

Compared to their counterparts in symbioses, the genetic diversity of free-living *Symbiodinium* spp. in the coral reef environment is virtually unexplored. The existence of a diverse free-living *Symbiodinium* spp. community is inferred by the fact that viable *Symbiodinium* are routinely released into the environment by a range of cnidarians (Steele 1977, Hoegh-Guldberg et al. 1987, Stimson and Kinzie 1991) and by predators feeding on the hosts (Bachman and Muller-Parker 2007), and many cnidarian hosts must acquire *Symbiodinium* anew each generation from the environment (horizontal acquisition) (Baird et al. 2009). The free-living communities are also considered important reservoirs of potential endosymbionts for cnidarian hosts, and more specifically corals, recovering from bleaching.

To date, free-living *Symbiodinium* have been isolated from environmental samples by culturing cells and by using suspending asymbiotic larvae and aposymbiotic adult hosts in the water column until they become infected (Carlos et al. 1999, Coffroth et al. 2001, 2006, Gou et al. 2003, Lewis and Coffroth 2004, Thornhill et al. 2006a, Hirose et al. 2008, Porto et al. 2008). Although the latter studies have confirmed the presence of *Symbiodinium* in the environment, these approaches are not able to fully characterize the free-living *Symbiodinium* spp. diversity because many *Symbiodinium* types cannot be cultured (Santos et al. 2001, Coffroth et al. 2006). As such, environmentally derived cultures do not necessarily represent the diversity of *Symbiodinium* spp. in a particular sample, but rather the types that are most readily cultured (typically *Symbiodinium* in clade A, Hirose et al. 2008). In addition, interactions between cnidarian hosts and *Symbiodinium* are highly specific, and thus asymbiotic and aposymbiotic hosts being used as environmental samplers will select for specific symbiont strains and not fully represent the free-living pool (Coffroth et al. 2001, Rodriguez-Lanetty et al. 2004, Thornhill et al. 2006a).

Developing the capacity to directly genotype free-living communities is essential to assessing the availability of *Symbiodinium* for initial colonization or re-colonization of corals postdisturbance. Molecular phylogenetic techniques make this endeavor tractable and have recently been successfully applied to the analysis of *Symbiodinium* spp. diversity directly from the water column (Manning and Gates 2008). This study demonstrated the utility of this approach in describing *Symbiodinium* spp. diversity in environmental samples; however, the methodology employed did not allow free-living *Symbiodinium* to be distinguished from the endosymbionts of larvae potentially swimming in the sampled water, and of other microscopic symbiont-containing organisms.

The coral reefs in the southern part of the Hawaiian Archipelago are unique in their geographic isolation and are characterized by low coral diversity, a high proportion of species that transmit their endosymbionts from generation to generation (vertical transmission), and high endosymbiont diversity and specificity (LaJeunesse et al. 2004b). In this study, we exploited the very low coral diversity on the reefs in Kāne'ohe Bay, O'ahu, to test the hypothesis that the diversity of free-living *Symbiodinium* occurring in sediments and seawater sampled adjacent to a high cover reef would overlap with the dominant endosymbiotic *Symbiodinium* types associated with the corals on this reef. This step was accomplished using a protocol that allowed free-living *Symbiodinium* spp. diversity in sediment and water samples from a fringing reef in Kāne'ohe Bay to be directly genotyped and compared to the endosymbiotic diversity found in the dominant cnidarian hosts on the same reef. The temporal and spatial sampling in this study was explicitly designed to explore diel migration in the free-living *Symbiodinium* spp. communities by determining how free-living *Symbiodinium* spp. diversity partitions by environment (water depth and sediment) with time (day vs. night).

MATERIALS AND METHODS

Sampling. Water and sediment samples were collected along a vertical transect adjacent to a fringing reef with high coral cover (Fig. 1, a and b) off Coconut Island in Kāne'ohe Bay, O'ahu, Hawai'i, USA (21°25.949′ N, 157°47.358′ W; Fig. 2, a and b). At each of 18 time points during the second week of June 2007 (every 4 h for 48 h, followed by sampling at 0900 and 2100 h for three additional days), 10 L of seawater from each depth (0.5, 2, and 3.8 m depths), and 1 mL of sediment from the benthos at a depth of 4 m were collected using equipment deployed from a 5 m^2 floating platform (Fig. 2c). Water samples were pumped at low flow (2.72 $L \cdot min^{-1}$) from each target depth and passed directly through sterilized 20 μm nylon mesh (Small Parts Inc., Miramar, FL, USA) to remove larger particulates and larvae. Sediment samples were resuspended in 1 L of 0.2 μm filtered seawater and passed through a sterilized 20 μm nylon mesh. Particulates sized between 20 and 5 μm in the filtered water and sediment samples were deposited on nucleopore track etch filters (5 μm; Whatman Inc., Florham Park, NJ, USA) using a vacuum pump at constant −0.34 bar.

Microscopy. Additional samples for microscopic observation were collected from all depths at 0500 and 0900 h on Day 1 as described above ($n = 8$). Filters were mounted on slides and viewed using bright-field and UV illumination on an Olympus BX51 epifluorescent microscope (Olympus, Melville, NY, USA). Cell size, morphology, and chloroplast morphology were used to identify *Symbiodinium*-like cells following LaJeunesse (2001). Images were compared to those obtained from cultured *Symbiodinium* cells and endosymbionts freshly isolated from the sea anemone *Aiptasia pulchella* and used to confirm the presence of *Symbiodinium* in the samples.

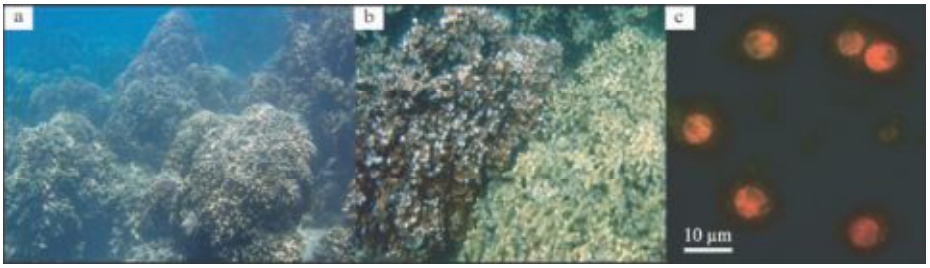

FIG. 1. (a) Reef site where study was conducted, showing (b) high cover of the coral *Montipora capitata* (left) and *Porites compressa* (right) that harbor dominant *Symbiodinium* types C31 and C15, respectively. (c) Representative photomicrograph of *Symbiodinium*-like cells visualized under UV on a <5 µm membrane following filtration of water and sediment samples. Cells appear red due to the autofluorescent properties of chlorophyll.

Reef transects and host collection. The diversity of cnidarian hosts dwelling on the fringing reef directly under and adjacent to the experimental platform (Fig. 2c) was assessed using belt transects 30 m long and 1 m wide ($n = 3$). For each transect, three independent observers identified and counted the cnidarian host species located in the 1 m belt along the length of each transect. These data were averaged to calculate the percentage cover of each species present on the fringing reef. Three randomly selected samples of all endosymbiotic hosts encountered within the belt transects were sampled for endosymbiotic *Symbiodinium* spp. diversity analysis.

DNA extraction. Samples of the endosymbiotic hosts and the 5 µm filters supporting particulates from the sediments and water samples were placed in 1.5 mL microcentrifuge tubes containing 400 µL of guanidinium buffer (50% [w/v] guanidinium isothiocyanate; 50 mM Tris pH 7.6; 10 µM EDTA; 4.2% [w/v] sarkosyl; 2.1% [v/v] β-mercaptoethanol), vortexed for 1 min to saturate the filter, and stored at 4°C for 1 week. The tubes were then placed at −80°C for 10 min, incubated at 72°C for 20 min, vortexed, and then centrifuged at 15,339*g* for 5 min. The supernatant containing the DNA was mixed with an equal volume of isopropanol, and the DNA precipitated at −20°C overnight. The DNA was pelleted by centrifugation at 15,339*g* for 15 min, and the pellet washed in 70% ethanol and resuspended in 50 µL Tris Buffer (0.1 M, pH 8). The concentration and quality of DNA was measured by absorbance at

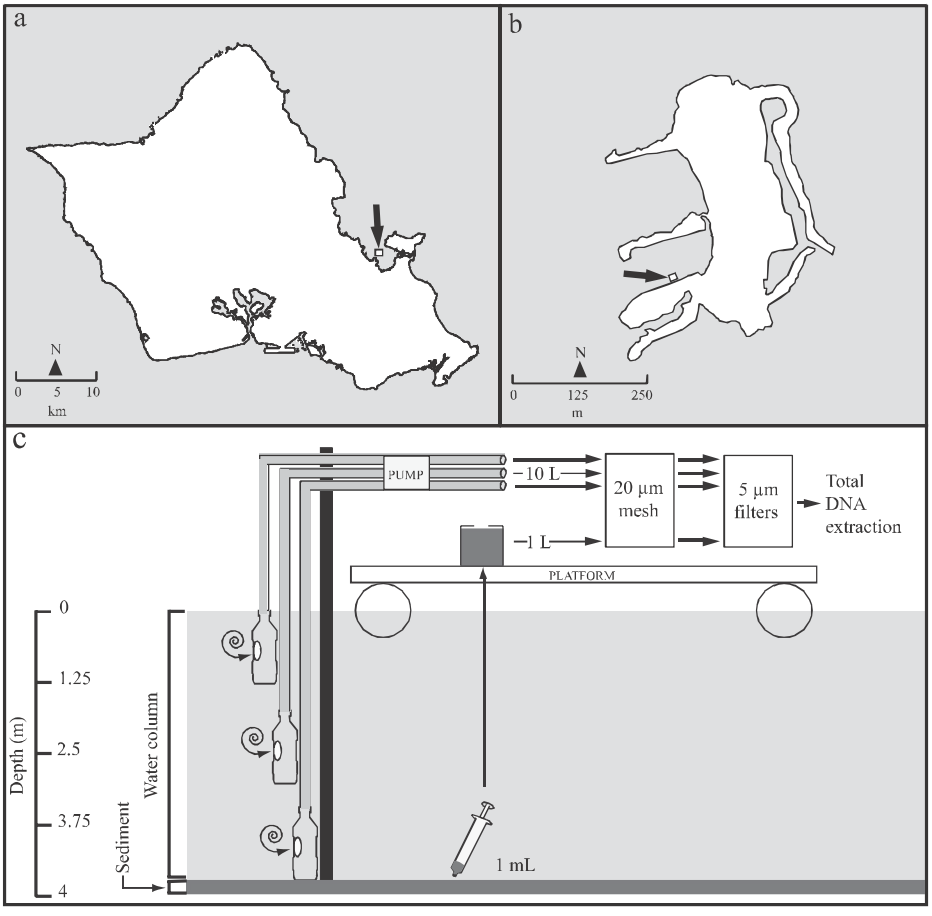

FIG. 2. Geographic location and design of the free-living survey. (a) Map of O'ahu (Hawai'i, USA), showing the location of Coconut Island in Kāne'ohe Bay (black arrow). (b) Detailed map of Coconut Island, showing the location of the floating platform where the survey was conducted (black arrow). (c) Design of the vertical sampling apparatus.

260 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Montchanin, DE, USA), and the DNA was stored at −20°C.

PCR, cloning, and sequencing. The ITS-2 region of the nuclear ribosomal array and Domain V of the LSU of the chloroplast ribosomal array (cp23S-DomainV) were PCR-amplified from extracted DNAs using the *Symbiodinium* ITS-2 primers "itsD" (5′-GTGAATTGCAGAACTCCGTG-3′) and "ITS-2rev2′ (5′-CCTCCGCTTACTTATATGCTT-3′; Pochon et al. 2007), and the cp23S-DomainV primers "23S4F" (5′-GACGGCTGT-AACTATAACGG-3′) and "23S7R" (5′-CCATCGTATTGAACC-CAGC-3′; Pochon et al. 2006) and conditions described in Pochon et al. (2006). The hypervariable region of the cp23S gene (cp23S-HVR) was PCR-amplified using the forward "23SHYPERUP" (5′-TCAGTACAAATAATATGCTG-3′; Santos et al. 2003) and reverse "23SHYPERDN" primers (5′TTATCG-CCCCAATTAAACAGT-3′; Manning and Gates 2008) and the following cycling conditions: 95°C 10 min, followed by 35 cycles (for endosymbiotic samples) or 44 cycles (for environmental samples) of 30 s at 94°C, 30 s at 50°C, and 1 min at 72°C, and a final extension at 72°C for 10 min. The PCR reactions (50 μL) for all primer sets contained 1× NH_4 buffer, 2 mM $MgCl_2$, 200 nM dNTPs, 0.2 μM of each primer, 0.02 μg · μL^{-1} BSA, 1 U Hotstart Immolase *Taq* polymerase (Bioline Inc., London, UK), and 1 μL of a 50× diluted DNA template.

One ITS-2, one cp23S-DomainV, and 16 cp23S-HVR PCR amplifications obtained from environmental samples (water or sediment) and all PCR amplifications from endosymbiotic hosts collected in Hawai'i ($N = 12$) were purified using the QIAquick™ PCR Purification Kit (Qiagen, Valencia, CA, USA) and ligated into the pGEM-T Easy vector™ (Promega, Madison, WI, USA). Positive inserts were verified using plasmid-specific 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, Foster City, CA, USA) at the University of Hawai'i. Approximately 15 clones were sequenced for each of the clone libraries generated for sediment and water samples, and 10 clones sequenced for each of the cnidarian host clone libraries.

For comparative purposes, 16 *Symbiodinium* DNA samples previously isolated from 13 foraminifera and three corals collected from other Pacific localities were also genotyped using either the ITS-2, the cp23S-DomainV, or both. All were sequenced directly (in both directions), and the sequences included in the analysis of ITS-2 and cp23S markers (see Results). Each of these DNA extractions was assigned a unique isolate collection identification number (see Table 4).

Sequence analyses. DNA sequences were inspected and assembled using Sequencher v4.7 (Gene Codes Corporation, Ann Arbor, MI, USA) and identified via the Basic Local Alignment Search Tool (BLAST) in GenBank. *Symbiodinium* sequences belonging to clade C were manually aligned with the BioEdit v5.0.9 sequence alignment software (Hall 1999) and phylogenetically analyzed using statistical parsimony implemented in the program TCS v1.21 (Clement et al. 2000). Two additional 23S-HVR sequence types previously isolated from water samples collected in Kāne'ohe Bay (Manning and Gates 2008), and referred to here as M1 and M2, were also added to this analysis. Networks were delineated with 95% certainty, with gaps being treated as a fifth state. Uncorrected genetic distances within and among analyzed loci were obtained using the program Mega v4.0 (Tamura et al. 2007).

One potential problem associated with PCR-based techniques using ribosomal genes is the overestimation of sequence diversity associated with the characterization of unique sequence types that reflect either PCR error or intragenomic variation within these multicopy genes (Apprill and Gates 2006, Thornhill et al. 2007). To address this, and to provide as conservative an estimate of biodiversity as possible, only identical sequences obtained from two or more clone libraries as well as singletons matching existing GenBank entries were used in downstream analysis.

Statistical analyses. Bray–Curtis coefficient of similarity (S) implemented in the software package Primer v6.0 (Clarke and Warwick 2001) was used to determine if the free-living *Symbiodinium* 23S-HVR types recovered from the 16 environmental samples exhibited significant patterns in temporal and/or spatial distribution. The abundance of *Symbiodinium* 23S-HVR sequences were grouped by environment (near-surface water, mid–water column, near-bottom water, sediment) and time (0900 and 2100 h) and standardized to relative frequencies within each grouping. The data were transformed by square-rooting to give more weight to low frequency sequences. *Symbiodinium* 23S-HVR sequences within each grouping were compared using the Bray–Curtis coefficient of similarity (S), which ranges between 0 and 100, where a value of 100 indicates an identical assemblage (Bray and Curtis 1957). The similarity matrix was analyzed using Primer v6.0 in a two-way crossed analysis of similarity (ANOSIM) with factors environment and time. The test statistic (R) in ANOSIM ranges between +1 and −1, where a value of 0 indicates no differences between groups, a value approaching +1 indicates partitioning of variation by group, and a value approaching −1 indicates partitioning of variation within group is greater than between groups.

TABLE 1. PCR amplifications obtained from the 1-week survey at the four sampling habitats and specific sampling times. The majority (1) showed positive amplifications using all primer sets, while a subgroup (2) provided positive amplifications using the primer sets for ITS-2 and 23S-DomainV but negative amplifications using the *Symbiodinium*-specific primer set for 23S-HVR. (S) near-surface: 0.5 m; (M) mid–water column: 2 m; (N) near-bottom: 3.8 m; sediment: 4 m.

Habitat	Sampling dates and times																	
	June 11, 2007						June 12, 2007						June 13, 2007		June 14, 2007		June 15, 2007	
	0500	0900	1300	1700	2100	0100	0500	0900	1300	1700	2100	0100	0900	2100	0900	2100	0900	2100
Water (S)	1	1	1	1	1	1	1	1[a]	1	1	1[a]	1	1[a]	1[a]	1	1	1	1
Water (M)	2	2	1	1	2	1	1	1[a,b]	2	1	1[a]	1	1[a]	1[a]	1	1	1	1
Water (N)	1	1	1	1	1	1	1	1[a]	1	1	1[a]	1	1[a]	1[a]	1	1	1	1
Sediment	2	2	2	2	2	2	2	1[a]	2	2	1[a]	2	2[a]	1[a]	2	2	2	2

[a]Sample selected for cloning and sequencing of the 23S-HVR from *Symbiodinium*.
[b]Sample selected for cloning and sequencing of the ITS-2 and 23S-DomainV.

RESULTS

Microscopic observation of Symbiodinium*-like cells.* Bright-field and UV microscopy confirmed the presence of *Symbiodinium*-like cells in the water and sediment samples. In total, eight >5 μm filters were examined, and *Symbiodinium*-like cells ranging from 6 to 10 μm in diameter were present on all (Fig. 1c).

Reef transects. The transect analysis showed that the coral reefs directly under and adjacent to the sites where water and sediments were sampled were composed of just three coral species. *Porites compressa* accounted for 60% (SD ± 6%) of the cover; *Montipora capitata,* 37.5% (SD ± 6.5%); and *Pocillopora damicornis,* 2.5% (SD ± 2.5%). The anemone *Aiptasia pulchella* was also seen at this location, although it was not recorded in the transects. Three individuals of each of these four species were sampled for the endosymbiont diversity analysis described below.

Molecular survey of free-living Symbiodinium. All DNAs extracted from environmental samples ($N = 72$) yielded positive amplifications of ~350 and 600 base-pair (bp) fragments using the ITS-2 and the cp23S-DomainV primers, respectively. Analysis of cloned fragments (20 clones per marker) from one sample (see Table 1) resolved members of the dinoflagellate genera *Gymnodinium, Heterocapsa, Pentapharsodinium, Prorocentrum,* and *Scrippsiella,* and the diatom genus *Skeletonema* (Fig. 3).

To address the lack of specificity in the ITS-2 and cp23S-DomainV primer sets for *Symbiodinium,* all environmental samples were reanalyzed using the cp23S-HVR primer set, which amplifies a 180 bp hypervariable fragment of the cp23S Domain V gene, and which is *Symbiodinium* specific when applied on environmental samples (Manning and Gates 2008). The cp23S-HVR primers gave positive PCR amplifications in 53 of the 72 environmental samples, a majority of which were water samples (Table 1).

Sixteen cp23S-HVR PCR products obtained from the samples collected at 0900 and 2100 h on the second and third days of the survey (Tables 1 and 2) were selected for cloning and sequencing. The selected PCR products allowed for spatiotemporal comparisons in the four vertical microhabitats. These comparisons included one sediment sample that did not produce a visible PCR amplification, which was processed to evaluate whether a cp23S-HVR fragment was amplified, but at such low concentration that it could not be visualized on the gel. No clones containing inserts were recovered from this sample (Table 2), confirming that no amplified product was present. For the remaining 15 positive amplifications, between 11 and 21 clones were sequenced per sample, resulting in a total of 231 cp23S-HVR sequences. BLASTn searches in GenBank showed that the top hit for each of these sequences was a previously published *Symbiodinium* sequence, confirming the specificity of the cp23S-HVR primer set for this genus.

Five cp23S-HVR sequence types in three *Symbiodinium* clades (A, B, and C) were recovered from the environment (Table 2). A single endosymbiont sequence in clade A (called A-e1) was found in one sediment and one water sample (Table 2). Although this sequence was most closely related to a cultured *Symbiodinium* clade A isolate from the giant clam *Tridacna gigas* in the Indo-Pacific (GenBank AY035431; BLASTn identity 73/80; e-value: 10^{-22}), a 79 bp portion of the sequence did not produce a significant BLASTn match. A single cp23S-HVR belonging to clade B, referred to here as type B-e1, was an exact match to three previously published cultured *Symbiodinium* originally isolated from the corals *Porites evermanni* (GenBank AY035418) and *Pocillopora damicornis* (AY055236) in Hawai'i, and from the anemone *A. pulchella* (AY035416) in Okinawa (Santos et al. 2002). This type was only recovered in the water samples. Lastly, three distinct *Symbiodinium* clade C sequences were retrieved, called C-e1, C-e2, and C-e3. Type C-e1 was the most commonly recovered sequence, and this type and C-e3 occurred in both sediment and the water samples (Table 2). Type C-e2 was only detected in the water column here and matched a previously documented free-living type (Manning and Gates 2008, GenBank EF428361). All GenBank accession numbers for environmental samples are shown in Table 4.

Bray–Curtis similarity coefficient and ANOSIM were employed to statistically test whether the five *Symbiodinium* 23S-HVR sequence types recovered

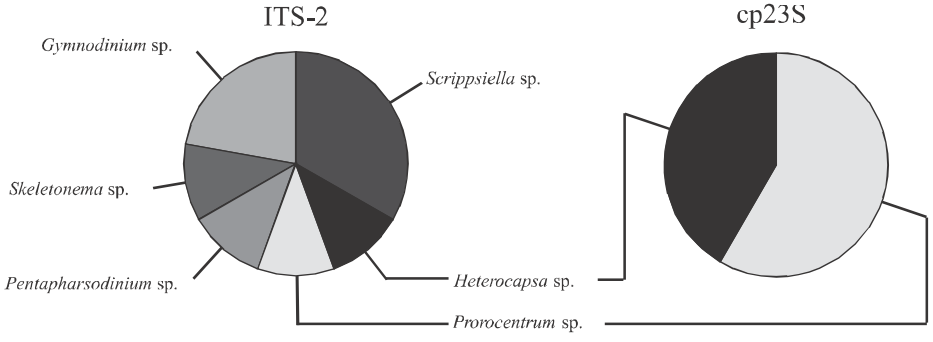

FIG. 3. Pie charts showing the relative abundance of clones representing the diatom and dinoflagellate genera recovered from a water sample using the ITS-2 and the cp23S-DomainV primer sets (a total of 40 clones sequenced).

varied among the four sampling microhabitats (spatial) and/or between the two sampling times (temporal). No significant difference in *Symbiodinium* 23S-HVR groupings was detected between 0900 and 2100 h (ANOSIM global test; $R = 0.125$, $P = 0.346$) or between environments (ANOSIM global test; $R = -0.177$, $P = 0.856$).

TABLE 2. *Symbiodinium* types recovered from water and sediment samples collected at 0900 and 2100 h during two consecutive days (Table 1). The analysis of 23S-HVR sequences revealed the presence of one, one, and three distinct sequence types in *Symbiodinium* clades A, B, and C, respectively. Sequence type names include an uppercase letter corresponding to the *Symbiodinium* clade followed by the lowercase letter "e" for "environmental sample" and a specific number. Numbers in parentheses represent the number of sequences obtained for each type. (S) near-surface: 0.5 m; (M) mid–water column: 2 m; (N) near-bottom: 3.8 m; sediment: 4 m.

Habitat	Sampling dates and times			
	June 12, 2007		June 13, 2007	
	0900	2100	0900	2100
Water (S)	C-e2 (18)	B-e1 (17)	C-e1 (12)	C-e1 (1) C-e2 (8) C-e3 (2)
Water (M)	C-e1 (16) C-e2 (1)	C-e1 (15) C-e3 (2)	C-e1 (11) C-e2 (1)	C-e2 (3) C-e3 (2) B-e1 (7)
Water (N)	C-e1 (9) B-e1 (9) A-e1 (2)	C-e1 (13) C-e2 (5) C-e3 (1)	C-e2 (1) C-e3 (2) B-e1 (9)	C-e1 (1) C-e2 (1) C-e3 (10)
Sediment	C-e1 (17) A-e1 (2)	C-e1 (21)	N/A	C-e3 (12)

Comparison of endosymbiotic and free-living Symbiodinium *and the diversity resolved using the ITS-2 and cp23S markers.* *P. compressa*, *M. capitata*, *P. damicornis*, and *A. pulchella* were selected for cloning and sequencing of the *Symbiodinium* ITS-2 and cp23S-DomainV markers because these were the dominant cnidarian hosts dwelling on the reef under and adjacent to the site of experiment. Between 5 and 12 sequences per marker were recovered from three individuals of each species, for a total of 236 sequences. A number of *Symbiodinium* ITS-2 types were recovered from each host (Table 3). The numerically dominant ITS-2 types recovered from *P. compressa*, *M. capitata*, *P. damicornis*, and *A. pulchella* were C15, C31, C1d, and B1, respectively, which is consistent with previous symbiont typing for these hosts using ITS-denaturing gradient gel electrophoresis (DGGE) fingerprinting (LaJeunesse et al. 2004b). The minor ITS-2 types (Table 3) include two newly described types, referred to here as C15.2 and C17.2, and six previously published sequence types, KB3, C1, C1c, C3, C21, and C42. Type C15.2 differs from C15 by 2 bp and was detected in all three *P. compressa* colonies. Type C17.2 differs from C17 by 2 bp and was found in two of the three *M. capitata* colonies. The

TABLE 3. Detailed list of *Symbiodinium* sequence types derived from 24 clone libraries of nuclear ITS-2 and chloroplast 23S-DomainV ribosomal genes in three coral species (*Porites compressa*, *Montipora capitata*, and *Pocillopora damicornis*) and one anemone species (*Aiptasia pulchella*) collected at the site of free-living survey. Isolate number refers to the DNA collection identification number shown in Table 4. Numbers in superscript following the sequence type names correspond to the number of obtained sequences per type. The *Symbiodinium* sequence types shown in bold were numerically dominant in each library. GenBank accession numbers are provided within the square brackets for each ITS-2 and cp23S-DomainV sequence type, respectively.

Coral species	Isolate no.	Date	nrITS-2	cp23S-DomainV
P. compressa	6338X	January 25, 2009	**C15**^{8} [FN298448] C15.2^{2} [FN298449]	**C15cp**^{7} [FN298473] C-e2^{3} [FN298474]
P. compressa	6339X	January 25, 2009	**C15**^{6} [FN298450] C15.2^{4} [FN298451]	**C15cp**^{6} [FN298475] C-e2^{4} [FN298476]
P. compressa	6340X	January 25, 2009	**C15**^{6} [FN298452] C15.2^{2} [FN298453] KB3^{2} [FN298454]	**C15cp**^{8} [FN298477] C-e2^{2} [FN298478]
M. capitata	M459	June 15, 2007	**C31**^{4} [FN298455] C17.2^{1} [FN298456]	**C31cp**^{7} [FN298479]
M. capitata	M483	June 15, 2007	**C31**^{4} [FN298457] C17.2^{1} [FN298458]	**C31cp**^{7} [FN298480]
M. capitata	J174	June 15, 2007	**C31**^{8} [FN298459] C21^{1} [FN298460]	**C31cp**^{10} [FN298481]
P. damicornis	6341X	January 25, 2009	**C1d**^{6} [FN298461] C1^{2} [FN298462] C3^{1} [FN298463]	**C1cp**^{11} [FN298482]
P. damicornis	6342X	January 25, 2009	**C1**^{4} [FN298464] C1d^{3} [FN298465] C1c^{2} [FN298466] C3^{1} [FN298467]	**C1cp**^{12} [FN298483]
P. damicornis	6343X	January 25, 2009	**C1d**^{9} [FN298468] C42^{2} [FN298469]	**C1cp**^{11} [FN298484]
A. pulchella	6344X	January 25, 2009	**B1**^{12} [FN298470]	**B184**^{12} [FN298485]
A. pulchella	6345X	January 25, 2009	**B1**^{11} [FN298471]	**B184**^{11} [FN298486]
A. pulchella	6346X	January 25, 2009	**B1**^{11} [FN298470]	**B184**^{12} [FN298487]

detection of minor types reflects the sensitivity of the cloning methodology employed here and contrasts with the ITS-DGGE approach, which cannot detect types representing <10% of the endosymbiont population (Thornhill et al. 2006b). Alternatively, these minor ITS-2 types might represent intragenomic variants within the rDNA operon (see Thornhill et al. 2007).

The *Symbiodinium* cp23S-DomainV primers recovered a single dominant sequence type in all samples except for *P. compressa,* where a minor type was also detected (Table 3). The hypervariable region of this minor sequence type was an exact match to type C-e2 from our environmental survey (see Table 2). The dominant cp23S-DomainV sequence types were named C15cp (*P. compressa*), C31cp (*M. capitata*), and C1cp (*P. damicornis*). The cp23S-DomainV type occurring in *A. pulchella* was an exact match to a previously published cp23S sequence type (GenBank AY035416) originally extracted from *A. pulchella* in Okinawa (Santos et al. 2002) and is commonly referred to as type B184 (Santos et al. 2003).

We hypothesized that the diversity of free-living *Symbiodinium* in sediments and seawater sampled adjacent to *P. compressa, M. capitata,* and *P. damicornis* would overlap the dominant endosymbiont types found in these corals. To test this, 25 *Symbiodinium* samples isolated from 13 foraminiferal and 12 coral samples, and representing nine distinct ITS-2 types in *Symbiodinium* clade C (Table 4), were phylogenetically analyzed and compared using the ITS-2, the cp23S-DomainV, and the cp23S-HVR (Fig. 4). DNA alignments for the three data sets shown in Figure 4, including gaps, were 287, 618, and 180 bp long, respectively (available from the corresponding author upon request). The relative phylogenetic positioning of the nine ITS-2 types shown in Figure 4a is congruent with a previously published network of 85 Indo-Pacific *Symbiodinium* clade C types (Pochon et al. 2007). The analysis of the cp23S-DomainV resulted in similar relationships among types but provides a lower level of phylogenetic resolution (Fig. 4b). For example, ITS-2 types C1, C1d, C3, and C91 all corresponded to the cp23S-DomainV sequence type C1cp. The lower resolution of the cp23S-DomainV is reflected in an average uncorrected genetic distance half (0.006) that of the ITS-2 data set (0.012), with the former containing a total of only 14 bp changes between sequence types. The decrease in phylogenetic resolution is even more pronounced when comparing the ITS-2 types with those obtained using the cp23S-HVR data set (Fig. 4c). Only five out of the nine original ITS-2 types are resolved using this locus. ITS-2 types C1, C1d, C3, C15, and C91 collapse into one core sequence (referred to here as the core 23S-HVR endosymbiotic type), and each of the remaining four types (C19, C31, C64, and C90) diverge from this core sequence by only 1 bp. All free-living sequence types resolved here (C-e1, C-e2, and C-e3) also differed from the core 23S-HVR endosymbiotic sequence type by 1 bp (Fig. 4c). Similarly, a sequence type, named here M2, isolated from two water samples collected in Kāne'ohe Bay (Manning and Gates 2008) was identical to type C-e2 (Fig. 4c, Table 4). Another sequence type reported by Manning and Gates (GenBank EF428359) was most closely related to C-e1 but contained a 10 bp deletion. This sequence type was not included in our analysis because it was only detected in a single water sample (Manning and Gates 2008) and thus does not meet the criteria for inclusion in our analysis. The most common sequence type reported by Manning and Gates (2008) from four water samples, and referred to here as M1, was identical to the core 23S-HVR sequence type (Fig. 4c).

DISCUSSION

This study was the first to use a PCR-based assessment of genetic diversity in *Symbiodinium* spp. communities sampled from the water column, sediments, and corals on a single reef. The study differs from previous work (Manning and Gates 2008) in incorporating a <20 μm prefiltration step to exclude endosymbionts of larvae present in the water column and reef sediments (Richmond 1987, Morse et al. 1996, Edmunds et al. 2005). This investigation was also the first to formally test the phylogenetic resolution and ecological utility of the ITS-2 and cp23S markers for the analysis of free-living *Symbiodinium* spp. communities.

Molecular survey of free-living Symbiodinium. The use of nuclear ITS-2 and plastid 23S markers are commonly employed in the field for deciphering the diversity, specificity, and ecology of endosymbiotic *Symbiodinium* (reviewed in Baker 2003, Coffroth and Santos 2005). Here, we tested the utility of both markers on a selected water sample (Table 1) and showed that no *Symbiodinium* sequences were recovered using either primer set (Fig. 3), confirming that both lack the specificity to detect *Symbiodinium* in the complex and diverse microeukaryotic communities occurring in this environment. This result is consistent with Manning and Gates (2008) who reported that extensive cloning and sequencing was required to detect *Symbiodinium* in the water column using *Symbiodinium* ITS-2 primers. A similar problem was encountered by Littman et al. (2008), who used *Symbiodinium* 18S rDNA primers in PCR on water and sediment samples and found that only 0.01% of the sequences recovered represented *Symbiodinium* spp. This lack of specificity will likely preclude these markers being used to quantify specific free-living *Symbiodinium* types using real-time PCR approaches (Ulstrup and Van Oppen 2003, Koike et al. 2007) and perhaps helps explain why most studies have relied on culturing *Symbiodinium* cells from environmental samples and/or used asymbiotic larvae or aposymbiotic adult hosts to isolate these

TABLE 4. List of symbiont types shown in Figure 4, their origin, sampling locality, sampling date(s), and corresponding GenBank accession numbers for the ITS-2 and cp23S markers, respectively. Isolate number refers to the DNA collection identification number (see Materials and Methods).

Type[a]	Origin[b]	Isolate no.	Locality[c]	Date	nrITS2-GenBank	cp23S-GenBank[d]
C1	*Ctenactis echinata*	458X	Guam, USA	December 12, 1999	FM877426 (this study)	AJ872078 (Pochon et al. 2006)
C1	*Amphisorus hemprichii*	1932X	GBR, Australia	December 7, 2001	AJ621538 (Pochon et al. 2004)	FM877433 (this study)
C1	*Amphisorus hemprichii*	1933X	GBR, Australia	December 7, 2001	AJ621539 (Pochon et al. 2004)	FM877434 (this study)
C1	*Pocillopora damicornis*	6342X	Hawaii, USA	January 25, 2009	FN298464 (this study)	FN298483 (this study)
C1d	*Pocillopora damicornis*	6341X	Hawaii, USA	January 25, 2009	FN298461 (this study)	FN298482 (this study)
C1d	*Pocillopora damicornis*	6343X	Hawaii, USA	January 25, 2009	FN298468 (this study)	FN298484 (this study)
C3	*Marginopora vertebralis*	591X	GBR, Australia	December 27, 1999	AJ621534 (Pochon et al. 2004)	FM877435 (this study)
C3	*Heliopora cerulea*	50X	Guam, USA	November 24, 1999	FM877427 (this study)	AJ872080 (Pochon et al. 2006)
C3	*Marginopora vertebralis*	602X	GBR, Australia	December 27, 1999	AJ621533 (Pochon et al. 2004)	FM877436 (this study)
C3	*Marginopora vertebralis*	1833X	GBR, Australia	December 3, 2001	AJ621535 (Pochon et al. 2004)	FM877437 (this study)
C15	*Porites compressa*	6338X	Hawaii, USA	January 25, 2009	FN298448 (this study)	FN298473 (this study)
C15	*Porites compressa*	6339X	Hawaii, USA	January 25, 2009	FN298450 (this study)	FN298475 (this study)
C15	*Porites compressa*	6340X	Hawaii, USA	January 25, 2009	FN298452 (this study)	FN298477 (this study)
C15	*Marginopora vertebralis*	610X	GBR, Australia	December 27, 1999	AJ621540 (Pochon et al. 2004)	FM877438 (this study)
C15	*Sorites* sp.	489J	GBR, Australia	December 15, 1999	AJ291516 (Pochon et al. 2006)	AJ872084 (Pochon et al. 2006)
C15	*Porites cylindrica*	8X	Guam, USA	November 22, 1999	FM877428 (this study)	AJ872083 (Pochon et al. 2006)
C19	*Marginopora vertebralis*	490J	GBR, Australia	December 15, 1999	AJ291515 (Pochon et al. 2006)	AJ872085 (Pochon et al. 2006)
C31	*Montipora capitata*	M459	Hawaii, USA	June 15, 2007	FN298455 (this study)	FN298479 (this study)
C31	*Montipora capitata*	M483	Hawaii, USA	June 15, 2007	FN298457 (this study)	FN298480 (this study)
C31	*Montipora capitata*	J174	Hawaii, USA	June 15, 2007	FN298459 (this study)	FN298481 (this study)
C90	*Sorites* sp.	1479X	Panama	September 4, 2001	AJ621128 (Pochon et al. 2006)	AJ872081 (Pochon et al. 2006)
C90	*Sorites* sp.	1466X	Panama	September 4, 1999	AJ620945 (Pochon et al. 2006)	AJ872082 (Pochon et al. 2006)
C91	*Amphisorus hemprichii*	84X	Guam, USA	November 27, 1999	FM877429 (this study)	AJ872086 (Pochon et al. 2006)
C91	*Amphisorus hemprichii*	85X	Guam, USA	November 27, 1999	AJ621543 (Pochon et al. 2004)	FM877439 (this study)
C64	*Marginopora vertebralis*	989X	Reunion, France	December 16, 2000	FM877432 (this study)	FM877442 (this study)
C-e1	Water and sediment	5470-5524X	Hawaii, USA	June 11–15, 2007	–	FM877443 (HVR) (this study)
C-e2	Water	5470-5524X	Hawaii, USA	June 11–15, 2007	–	FM877453 (HVR) (this study)
C-e3	Water and sediment	5470-5524X	Hawaii, USA	June 11–15, 2007	–	FM877461 (HVR) (this study)
M1	Water	HK-18	Hawaii, USA	November 6, 2005–June 9, 2006	–	FM877475 (HVR) (Manning and Gates 2008)
M2	Water	HK-31	Hawaii, USA	November 6, 2005–June 9, 2006	–	EF428361 (HVR) (Manning and Gates 2008)

[a]Symbiont types originally extracted from a host were named based on ITS-2 molecular taxonomy. Environmental samples received new names. GenBank accession numbers for types A-e1 and B-e1 are FM877468 and FN377572, respectively.

[b]Symbiont origin indicates "host species name" if extracted from a host, and "water and/or sediment" if extracted from the environment.

[c]All Hawaiian coral samples (*Porites compressa*, *Montipora capitata*, and *Pocillopora damicornis*) were collected from the locality where the "free-living" survey was conducted. *Symbiodinium* spp. in these samples were cloned prior to sequencing, and the numerically dominant sequence type was retained here as the dominant symbiont in these samples (see Table 3).

[d]All sequences consist of the cp23S-DomainV, except for the environmental sequences that are shorter (cp23S-HVR).

communities prior to genotyping (e.g., see Coffroth et al. 2006).

To address the lack of specificity in the ITS-2 and cp23S-DomainV primer sets for *Symbiodinium*, all environmental samples were reanalyzed using the cp23S-HVR primer set, following Manning and Gates (2008). Only three out of the 18 sediment samples gave positive PCR amplifications, which is surprising given recent literature that suggests that free-living *Symbiodinium* are mainly benthic (Coffroth et al. 2006, Littman et al. 2008), and that asymbiotic coral larvae in the species *Acropora monticulosa* from Okinawa, Japan, primarily acquired *Symbiodinium* when exposed to sediments as compared to seawater alone (Adams et al. 2009). One potential explanation is that free-living *Symbiodinium* display considerable variability in their spatial distribution and show preference for sediments of specific compositions that vary within reefs and between geographic regions. Indeed, Littman et al. (2008) revealed high spatial variability in *Symbiodinium* spp. abundance within and between reef sites, suggesting that the distribution of free-living *Symbiodinium* is influenced by complex interactions between physical, chemical, and biological factors. The sediments sampled in the present study were very fine in grain size composition (between 63 and 250 μm in range), conditions that may not favor the aggregation of *Symbiodinium*. It is also plausible that *Symbiodinium* aggregates were present, but that we missed them by collecting such small sediment samples. Also, in addition to removing larvae, the prefiltration step in our sampling protocol may have removed clumps of *Symbiodinium*, dividing cells, and those entrapped in mucus and other aggregates. Future studies will need to address spatial heterogeneity of *Symbiodinium* by sampling larger volumes of sediment or representatives of different sediment compositions.

Only five cp23S-HVR sequence types (A-e1, B-e1, C-e1, C-e2, and C-e3) in three *Symbiodinium* clades (A, B, and C) were recovered from the environment (Table 2). This is surprising given the diversity of *Symbiodinium* clades reported for this region. For example, clade D *Symbiodinium* occurs in endosymbiosis with *M. capitata* in Hawai'i (LaJeunesse et al. 2004b), and clade F is common in cultures originally extracted from clade C–dominated Hawaiian hosts (R. A. Kinzie, unpublished). Also, clade H was previously detected in a water sample from Kāne'ohe Bay (Manning and Gates 2008). Here, no *Symbiodinium* sequences representing clades D, F, or H were detected in the sediments or water column. Free-living *Symbiodinium* in clade C were more diverse and more frequently encountered than those in clades A and B. This observation is consistent with the overall abundance of these clades in the ecosystem and the fact that clade C endosymbionts largely dominate reef invertebrate communities in this region, whereas clade B and particularly clade A are relatively rare (Stat et al. 2008). This finding, however, contrasts with a

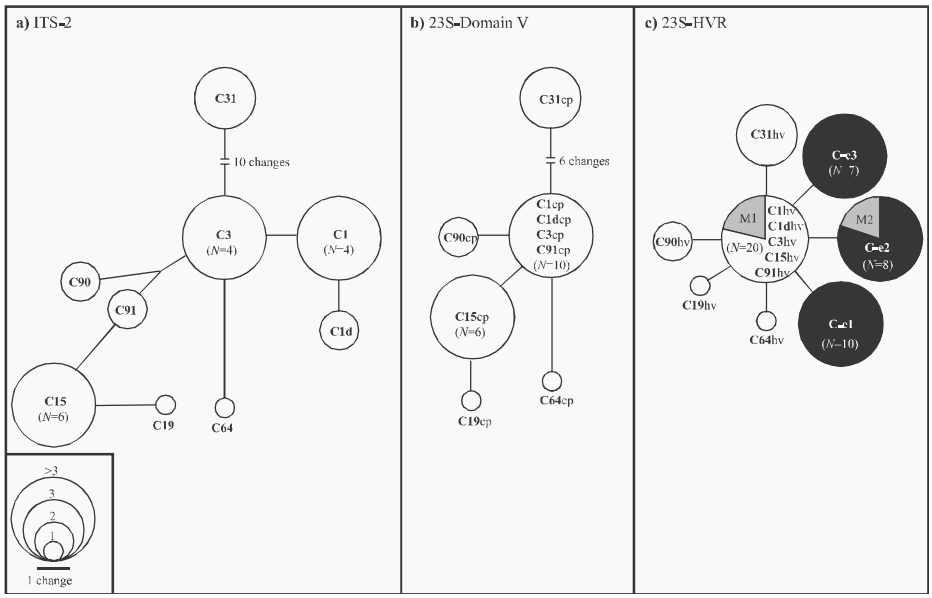

FIG. 4. Statistical parsimony networks of Hawaiian clade C *Symbiodinium*. (a) Nine endosymbiotic types analyzed using the ITS-2 marker, (b) the same nine endosymbiotic types analyzed using the cp23S-DomainV marker (the letters "cp" following type names refer to the DomainV of this "chloroplast" gene), (c) the same nine endosymbiotic types, plus the three free-living types recovered here (black color) and the two free-living types (gray color) reported in Manning and Gates (2008; Table 4) analyzed using the cp23S-HVR marker ("hv" refers to the hypervariable region of the cp23S-DomainV). Numbers of independent samples with identical sequences are indicated by circle size at each node. Where more than three identical sequences were recovered, the total number is given in parentheses following the names of the *Symbiodinium* types.

number of recent studies investigating free-living *Symbiodinium*. For example, Coffroth et al. (2006) primarily identified *Symbiodinium* clades A and B using asymbiotic octocoral recruits (*Briareum* sp.) as "symbiont samplers" and in cultures of >380 "*Symbiodinium*-like" dinoflagellates from reef rubble. Although some of these sequence types were clearly related to *Symbiodinium* clade A, they were different from the previously described endosymbiotic members of the clade. Similarly, Hirose et al. (2008) established 16 *Symbiodinium* cultures from three sampling locations of coral reef sand in Okinawa, Japan, and again identified all isolates as belonging to *Symbiodinium* clade A. These results speak directly to the differential culturability of the clades and reflect the fact that clade A *Symbiodinium* is the most easily cultured group in the genus. Although the sequence type A-e1 detected in our survey is highly divergent from *Symbiodinium* strains currently known in clade A, further comparisons between clade A endosymbionts associated with marine invertebrates in Hawai'i and this strain using the cp23S-HVR marker are needed to confirm whether this type is exclusively free living. Type B-e1 was detected in four water samples (Table 2). This type has been detected in water samples from Kāne'ohe Bay before (Manning and Gates 2008), is identical to the endosymbionts hosted by *Aiptasia pulchella* at our experiment site, and corresponds to the published type B184 occurring in endosymbiosis with this anemone in Okinawa (Santos and Coffroth 2003), also known as type B1 (LaJeunesse 2001). This type is reportedly the most ubiquitous lineage in the Caribbean (LaJeunesse 2002, but see Santos et al. 2004) and has been shown to be quite resilient in a bleaching-induced experiment (Lewis and Coffroth 2004) and common among macroalgal-associated demersal free-living *Symbiodinium* spp. communities in the Caribbean (Porto et al. 2008). As such, the finding of type B-e1 in our environmental survey is consistent with previous reports and adds to the body of findings suggesting that this *Symbiodinium* type has the ability to alternate between a free-living and endosymbiotic lifestyle.

The question of whether distinct *Symbiodinium* clades and/or distinct strains within a clade show differences in their spatiotemporal distributions is relevant to understanding the timing and types of free-living *Symbiodinium* available to asymbiotic larvae and bleached hosts. Although laboratory experiments on *Symbiodinium* cultures have demonstrated that they undergo daily transitions from the nonmotile (coccoid) to a motile form and spend 3–7 h in the "water column" of the flask (Fitt et al. 1981, Santos et al. 2001, Yacobovitch et al. 2004), such observations are still lacking from the field. Two studies now suggest that *Symbiodinium* types in clade A more likely occur in the benthos than types in clade B and C (Coffroth et al. 2006, Adams et al. 2009). Although similar patterns were observed in our spatiotemporal survey with type A-e1 either associated with or directly above the benthos and type B-e1 restricted to the water column (Table 2), these patterns were not statistically supported. Overall, our analysis suggests that there is no partitioning of *Symbiodinium* spp. between morning and evening samples or between water and sediment samples.

Comparison of endosymbiotic and free-living Symbiodinium *and the diversity resolved using the ITS-2 and cp23S markers.* In the Pacific, *Symbiodinium* belonging to clade C are the most ecologically dominant in cnidarians (Baker 2003, LaJeunesse 2005), and members of this group occur in endosymbiosis with all corals from O'ahu, Hawai'i (LaJeunesse et al. 2004b). At the location where our survey was performed, the two dominant coral species, *P. compressa* and *M. capitata* (Fig. 1, a and b), and the less abundant *P. damicornis*, hosted *Symbiodinium* spp. communities dominated by C15, C31, and C1d, respectively, confirming earlier work (LaJeunesse et al. 2004b). Although these corals transmit their symbionts vertically, adult host colonies are known to release thousands of symbionts daily (Titlyanov et al. 1996, Stimson et al. 2002). We therefore hypothesized that the diversity of free-living *Symbiodinium* in sediments and seawater sampled adjacent to these corals would overlap the dominant endosymbiont *Symbiodinium* types in the corals.

None of the 231 sequences obtained from our environmental survey matched the dominant types found in *P. compressa, P. damicornis* (core 23S-HVR sequence type), or *M. capitata* (C31). Given the lower taxonomic resolution afforded by the cp23S-HVR marker, it is significant that all the free-living *Symbiodinium* sequence types resolved in our survey were distinct from the dominant endosymbionts in neighboring corals (Fig. 4c). This finding contradicts that of Manning and Gates (2008) who isolated the core endosymbiotic 23S-HVR sequence type from four water samples (referred to here as M1). A possible explanation for this discrepancy resides in the sampling protocol used in Manning and Gates (2008), which did not exclude endosymbiotic larvae originating from surrounding symbiont-containing organisms. Indeed, these authors also detected other endosymbiotic (ITS-2) types that are common in Hawai'i, such as C3 and C15. In the present study, samples were prefiltered to exclude symbiotic larvae. With this new level of methodological stringency, the core 23S-HVR sequence type was not detected in environmental samples. Thus, these data lead us to reject our working hypothesis and conclude that there is no overlap between the diversity of free-living *Symbiodinium* occurring in sediments and seawater sampled adjacent to a high cover reef and the dominant endosymbiotic *Symbiodinium* types associated with the corals on this reef.

That said, it is important to note that a great deal of our current knowledge regarding the endosymbiotic *Symbiodinium* spp. diversity in the reef

invertebrate communities of Hawai'i comes from a single study that sampled a few individuals of a broad range of species found at two locations on the island of O'ahu (LaJeunesse et al. 2004b). Similarly, the collection of environmental samples was carried out only in Kāne'ohe Bay over a limited time period in the present study. Given typical free-living dinoflagellates' mobility and diurnal migration, free-living *Symbiodinium* spp. distribution is likely to vary spatially and temporally. As sampling effort increases in spatial scale and frequency, it is likely that additional *Symbiodinium* spp. diversity will be discovered in Hawai'i and possible that the dominant endosymbionts of reef invertebrates will also be identified in free-living compartments of the ecosystem. Our finding of type C-e2 as both a minor endosymbiotic type in *P. compressa* (see Table 3) and a member of the free-living *Symbiodinium* spp. diversity in the water column suggests that this type, like B-e1, has the ability to survive and exploit both endosymbiotic and free-living lifestyles. This is interesting in the light of previous studies suggesting that endosymbiotic type C15 and closely related variants are more resilient in extreme environmental conditions as compared to other clade C types (LaJeunesse et al. 2003, Pochon et al. 2004).

The comparison of markers presented here highlights the need to develop new markers that provide subclade taxonomic resolution for describing diversity in all compartments of coral reef ecosystems. The inability of the cp23S-HVR to distinguish between closely related *Symbiodinium* ITS-2 types (Fig. 4c) indicates that it is a clade-level diagnostic marker but has limited utility for exploring diversity within *Symbiodinium* clades. The cp23S-HVR is, however, the only marker currently available that is specific enough to directly target *Symbiodinium* cells in the environment.

Our findings support the existence of potentially nonsymbiotic *Symbiodinium* spp. diversity (see Coffroth et al. 2001). The absence of the dominant scleractinian endosymbionts C1, C1d, C3, C15, and C31 in the water column and sediments in this study raises questions as to whether these *Symbiodinium* types can survive outside their scleractinian hosts for an extended time. Some coral endosymbionts must persist outside the host, at least transiently, because asymbiotic larvae of many coral species must acquire compatible endosymbionts from the environment or perish (Lewis and Coffroth 2004, Coffroth and Santos 2005). However, it is plausible that the endosymbionts of some corals have evolved such high levels of biological integration with their hosts that they cannot survive independently and are obligate endosymbionts of their hosts. Such a scenario would necessitate vertical transmission of endosymbionts, which is the dominant mode of endosymbiont transmission in the corals of Hawai'i and would certainly explain the absence of coral endosymbionts in the environment found here.

The absence of overlap between free-living and the dominant endosymbiont types in *P. compressa*, *M. capitata*, and *P. damicornis* is provocative in terms of the "Adaptive Bleaching Hypothesis" (Buddemeier and Fautin 1993) and suggests that the potential for exchange between symbiotic and environmental pools of *Symbiodinium* in Kāne'ohe Bay may be more limited than previously thought. These observations point indirectly to endosymbiont shuffling (sensu Baker 2003) as a potentially important mechanism by which Hawaiian corals might modify their endosymbiotic assemblages in response to environmental change.

The present study indicates that spatial and temporal complexity exists in free-living *Symbiodinium* spp. communities, ecological patterns that potentially have dramatic implications for the resilience of coral reef ecosystems worldwide. A thorough characterization of free-living *Symbiodinium* spp. diversity through extensive local and regional sampling of the environment represents an important component of the future research necessary to link the presence and distribution of specific *Symbiodinium* strains to the resilience of reef corals. Current efforts are focused on evaluating temporal patterns of *Symbiodinium* spp. availability in the environment associated with mass spawning events and characterizing these communities on reefs dominated by horizontally transmitting hosts.

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Adams, L. M., Cumbo, V. R. & Takabayashi, M. 2009. Enhanced primary acquisition of *Symbiodinium* by asymbiotic coral larvae exposed to sediments when compared to the water column. *Mar. Ecol. Prog. Ser.* 377:149–56.

Apprill, A. M. & Gates, R. D. 2006. Recognizing diversity in coral symbiotic dinoflagellate communities. *Mol. Ecol.* 16:1127–34.

Bachman, S. & Muller-Parker, G. 2007. Viable algae released by the seastar *Dermasterias imbricata* feeding on the symbiotic sea anemone *Anthopleura elegantissima*. *Mar. Biol.* 150:369–75.

Baird, A. H., Guest, J. R. & Willis, B. L. 2009. Biogeographical and evolutionary patterns in the reproductive biology of scleractinian corals. *Annu. Rev. Ecol. Evol. Syst.* 40:551–71.

Baker, A. C. 2003. Flexibility and specificity of coral-algal symbiosis: diversity, ecology, and biogeography of *Symbiodinium*. *Annu. Rev. Ecol. Syst.* 34:661–89.

Bray, J. R. & Curtis, J. T. 1957. An ordination of the upland forest communities of southern Wisconsin. *Ecol. Monogr.* 27:325–49.

Buddemeier, R. W. & Fautin, D. G. 1993. Coral bleaching as an adaptive mechanism. *Bioscience* 43:320–6.

Carlos, A. A., Baillie, B. K., Kawachi, M. & Maruyama, T. 1999. Phylogenetic position of *Symbiodinium* (Dinophyceae) isolates from tridacnids (Bivalvia), cardiids (Bivalvia), a sponge

(Porifera), a soft coral (Anthozoa), and a free-living strain. *J. Phycol.* 35:1054–62.
Clarke, K. R. & Warwick, R. M. 2001. *Change in Marine Communities: An Approach to Statistical Analysis and Interpretation.* 2nd ed. PRIMER-E Ltd., Plymouth, UK, 88 pp.
Clement, M., Posada, D. & Crandall, K. A. 2000. TCS: a computer program to estimate gene genealogies. *Mol. Ecol.* 9:1657–9.
Coffroth, M. A., Lewis, C., Santos, S. & Weaver, J. 2006. Environmental populations of symbiotic dinoflagellates in the genus *Symbiodinium* can initiate symbioses with reef cnidarians. *Curr. Biol.* 16:R985–7.
Coffroth, M. A. & Santos, S. R. 2005. Genetic diversity of symbiotic dinoflagellates in the genus *Symbiodinium*. *Protist* 156:19–34.
Coffroth, M. A., Santos, S. R. & Goulet, T. L. 2001. Early ontogenetic expression of specificity in a cnidarian algal symbiosis. *Mar. Ecol. Prog. Ser.* 222:85–96.
Edmunds, P. J., Gates, R. D., Leggat, W., Hoegh-Guldberg, O. & Allen-Requa, L. 2005. The effect of temperature on the size and population density of dinoflagellates in larvae of the reef coral *Porites astreoides*. *Invertebr. Biol.* 124:185–93.
Fitt, W. K., Chang, S. S. & Trench, R. K. 1981. Motility patterns of different strains of the symbiotic dinoflagellate *Symbiodinium* (=*Gymnodinium*) *microadriaticum* Freudenthal in culture. *Bull. Mar. Sci.* 31:436–43.
Glynn, P. W. 1996. Coral reef bleaching: facts, hypotheses and implications. *Glob. Change Biol.* 2:495–509.
Gou, W. L., Sun, J., Li, X. Q., Zhen, Y., Xin, Z., Yu, Z. G. & Li, R. X. 2003. Phylogenetic analysis of a free-living strain of *Symbiodinium* isolated from Jiaozhou Bay, P.R. China. *J. Exp. Mar. Biol. Ecol.* 296:135–44.
Hall, T. A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* 41:95–8.
Hirose, M., Reimer, J. D., Hidaka, M. & Suda, S. 2008. Phylogenetic analyses of potentially free-living *Symbiodinium* spp. isolated from coral reef sand in Okinawa, Japan. *Mar. Biol.* 155:105–12.
Hoegh-Guldberg, O., McCloskey, L. R. & Muscatine, L. 1987. Expulsion of zooxanthellae by symbiotic cnidarians from the Red Sea. *Coral Reefs* 5:201–4.
Iglesias-Prieto, R., Beltran, V. H., LaJeunesse, T. C., Reyes-Bonilla, H. & Thome, P. E. 2004. Different algal symbionts explain the vertical distribution of dominant reef corals of the eastern Pacific. *Proc. R. Soc. Lond.* 271:1757–63.
Koike, K., Yamashita, H., Oh-Uchi, A., Tamaki, M. & Hayashibara, T. 2007. A quantitative real-time PCR method for monitoring *Symbiodinium* in the water column. *Galaxea* 9:1–12.
LaJeunesse, T. C. 2001. Investigating the biodiversity, ecology, and phylogeny of endosymbiotic dinoflagellates in the genus *Symbiodinium* using the ITS region: in search of a "species" level marker. *J. Phycol.* 37:866–80.
LaJeunesse, T. C. 2002. Diversity and community structure of symbiotic dinoflagellates from Caribbean coral reefs. *Mar. Biol.* 141:387–400.
LaJeunesse, T. C. 2005. "Species" radiations of symbiotic dinoflagellates in the Atlantic and IndoPacific since the Miocene-Pliocene transition. *Mol. Biol. Evol.* 22:570–81.
LaJeunesse, T. C., Bhagooli, R., Hidaka, M., deVantier, L., Done, T., Schmidt, G. W., Fitt, W. K. & Hoegh-Guldberg, O. 2004a. Closely related *Symbiodinium* spp. differ in relative dominance in coral reef host communities across environmental, latitudinal and biogeographic gradients. *Mar. Ecol. Prog. Ser.* 284:147–61.
LaJeunesse, T. C., Loh, W. K. W., van Woesik, R., Hoegh-Guldberg, O., Schmidt, G. W. & Fitt, W. K. 2003. Low symbiont diversity in southern Great Barrier Reef corals, relative to those of the Caribbean. *Limnol. Oceanogr.* 48:2046–54.
LaJeunesse, T. C., Thornhill, D. J., Cox, E. F., Stanton, F. G., Fitt, W. K. & Schmidt, G. W. 2004b. High diversity and host specificity observed among symbiotic dinoflagellates in reef coral communities from Hawaii. *Coral Reefs* 23:596–603.
Lewis, C. L. & Coffroth, M. A. 2004. The acquisition of exogenous algal symbionts by an octocoral after bleaching. *Science* 304:1490–2.
Littman, R. A., van Oppen, M. J. H. & Willis, B. L. 2008. Methods for sampling free-living *Symbiodinium* (zooxanthellae) and their distribution and abundance at Lizard Island (Great Barrier Reef). *J. Exp. Mar. Biol. Ecol.* 364:48–53.
Lobban, C. S., Schefter, M., Simpson, A. G. B., Pochon, X., Pawlowski, J. & Foissner, W. 2002. *Maristentor dinoferus* n. gen., n. sp., a giant heterotrich ciliate (Alveolata, Ciliophora) with zooxanthellae, from Pacific coral reefs. *Mar. Biol.* 140:411–23.
Manning, M. & Gates, R. D. 2008. Diversity in populations of free-living *Symbiodinium* from a Caribbean and Pacific reef. *Limnol. Oceanogr.* 53:1853–61.
Morse, A. N. C., Iwao, K., Baba, M., Shimoike, K., Hayashibara, T. & Omori, M. 1996. An ancient chemosensory mechanism brings new life to coral reefs. *Biol. Bull.* 191:149–54.
Muscatine, L., McCloskey, L. R. & Marian, R. E. 1981. Estimating the daily contribution of carbon from zooxanthellae to coral animal respiration. *Limnol. Oceanogr.* 26:601–11.
van Oppen, M. J. H. & Gates, R. D. 2006. Conservation genetics and the resilience of reef-building corals. *Mol. Ecol.* 15:3863–83.
van Oppen, M. J. H., Mahiny, A. J. & Done, T. J. 2005. Geographic distribution of zooxanthella groups in three coral species on the Great Barrier Reef sampled after the 2002 bleaching event. *Coral Reefs* 24:482–7.
Pochon, X., Garcia-Cuetos, L., Baker, A. C., Castella, E. & Pawlowski, J. 2007. One-year survey of a single Micronesian reef reveals extraordinarily rich diversity of *Symbiodinium* types in soritid foraminifera. *Coral Reefs* 26:867–82.
Pochon, X., LaJeunesse, T. C. & Pawlowski, J. 2004. Biogeographic partitioning and host specialization among foraminiferan dinoflagellate symbionts (*Symbiodinium*; Dinophyta). *Mar. Biol.* 146:17–27.
Pochon, X., Montoya-Burgos, J. I., Stadelmann, B. & Pawlowski, J. 2006. Molecular phylogeny, evolutionary rates, and divergence timing of the symbiotic dinoflagellate genus *Symbiodinium*. *Mol. Phylogenet. Evol.* 38:20–30.
Pochon, X., Pawlowski, J., Zaninetti, L. & Rowan, R. 2001. High genetic diversity and relative specificity among *Symbiodinium*-like endosymbiotic dinoflagellates in soritid foraminiferans. *Mar. Biol.* 139:1069–78.
Porto, I., Granados, C., Restrepo, J. C., Sánchez, J. A. & Humphries, S. 2008. Macroalgal-associated dinoflagellates belonging to the genus *Symbiodinium* in Caribbean reefs. *PLoS ONE* 3:e2160.
Richmond, R. H. 1987. Energetics, competency, and long-distance dispersal of planula larvae of the coral *Pocillopora damicornis*. *Mar. Biol.* 93:527–33.
Rodriguez-Lanetty, M., Krupp, D. A. & Weis, V. M. 2004. Distinct ITS types of *Symbiodinium* in Clade C correlate with cnidarian/dinoflagellate specificity during onset of symbiosis. *Mar. Ecol. Prog. Ser.* 275:97–102.
Rowan, R. 1998. Diversity and ecology of zooxanthellae on coral reefs, a review. *J. Phycol.* 34:407–17.
Rowan, R. & Powers, D. A. 1991. Molecular genetic identification of symbiotic dinoflagellates (zooxanthellae). *Mar. Ecol. Prog. Ser.* 71:65–73.
Santos, S. R. & Coffroth, M. A. 2003. Molecular genetic evidence that dinoflagellates belonging to the genus *Symbiodinium* Freudenthal are haploid. *Biol. Bull.* 204:10–20.
Santos, S. R., Gutierrez-Rodriguez, C. & Coffroth, M. A. 2003. Phylogenetic identification of symbiotic dinoflagellates via length heteroplasmy in domain V of chloroplast large subunit (cp23S) ribosomal DNA sequences. *Mar. Biotech.* 5:130–40.
Santos, S. R., Shearer, T. L., Hannes, A. R. & Coffroth, M. A. 2004. Fine-scale diversity and specificity in the most prevalent lineage of symbiotic dinoflagellates (*Symbiodinium*, Dinophyceae) of the Caribbean. *Mol. Ecol.* 13:459–69.
Santos, S. R., Taylor, D. J. & Coffroth, M. A. 2001. Genetic comparisons of freshly isolated versus cultured symbiotic dinoflagellates: implications for extrapolating to the intact symbiosis. *J. Phycol.* 37:900–12.
Santos, S. R., Taylor, D. J., Kinzie, R. A., Hidaka, M., Sakai, K. & Coffroth, M. A. 2002. Molecular phylogeny of symbiotic

dinoflagellates inferred from partial chloroplast large subunit (23S)-rDNA sequences. *Mol. Phylogenet. Evol.* 23:97–111.

Stat, M., Morris, E. & Gates, R. D. 2008. Functional diversity in coral-dinoflagellate symbiosis. *Proc. Natl. Acad. Sci. U. S. A.* 105:9256–61.

Steele, R. D. 1977. The significance of zooxanthella containing pellets extruded by sea-anemones. *Bull. Mar. Sci.* 27:591–4.

Stimson, J. & Kinzie, R. A., III. 1991. The temporal pattern and rate of release of zooxanthellae from the reef coral *Pocillopora damicornis* Linnaeus under nitrogen-enrichment and control conditions. *J. Exp. Mar. Biol. Ecol.* 153:63–74.

Stimson, J., Sakai, K. & Sembali, H. 2002. Interspecific comparison of the symbiotic relationship in corals with high and low rates of beaching-induced mortality. *Coral Reefs* 21:409–21.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* 24:1596–9.

Thornhill, D. J., Daniel, M. W., LaJeunesse, T. C., Schmidt, G. W. & Fitt, W. K. 2006a. Natural infections of aposymbiotic *Cassiopea xamachana* scyphistomae from environmental pools of *Symbiodinium*. *J. Exp. Mar. Biol. Ecol.* 338:50–6.

Thornhill, D. J., LaJeunesse, T. C., Kemp, D. W., Fitt, W. K. & Schmidt, G. W. 2006b. Multi-year, seasonal genotypic surveys of coral-algal symbioses reveal prevalent stability or post-bleaching reversion. *Mar. Biol.* 148:711–22.

Thornhill, D. J., LaJeunesse, T. C. & Santos, S. R. 2007. Measuring rDNA diversity in eukaryotic microbial systems: how intragenomic variation, pseudogenes, and PCR artifacts confound biodiversity estimates. *Mol. Ecol.* 16:5326–40.

Titlyanov, E. A., Titlyanov, T. V., Leletkin, V. A., Tsukahara, J., van Woesik, R. & Yamazato, K. 1996. Degradation of zooxanthellae and regulation of their density in hermatypic corals. *Mar. Ecol. Prog. Ser.* 139:167–78.

Trench, R. K. 1993. Microalgal-invertebrate symbiosis: a review. *Endocytobiol. Cell Res.* 9:135–75.

Ulstrup, K. E. & Van Oppen, M. J. H. 2003. Geographic and habitat partitioning of genetically distinct zooxanthellae (*Symbiodinium*) in *Acropora* corals on the Great Barrier Reef. *Mol. Ecol.* 12:3477–84.

Yacobovitch, T., Benayahu, Y. & Weis, V. M. 2004. Motility of zooxanthellae isolated from the Red Sea soft coral *Heteroxenia fuscescens* (Cnidaria). *J. Exp. Mar. Biol. Ecol.* 298:35–48.