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Diversity in populations of free-living *Symbiodinium* from a Caribbean and Pacific reef

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Abstract

The presence and diversity of free-living dinoflagellates belonging to the endosymbiotic genus *Symbiodinium* were explored in seawater samples collected above coral reefs in Kāne'ohe Bay, O'ahu, Hawai'i, and Puerto Morelos, Quintana Roo, Mexico. Two genetic markers were used to assess *Symbiodinium* diversity in the water column: the internal transcribed spacer 2 region of the nuclear ribosomal array (ITS2), and a hypervariable region in domain V of the large subunit (23S) of the chloroplast ribosomal array (cp23S-HVR). Sequencing of cloned gene fragments reveals that clades B, C, D, and H *Symbiodinium* are detectable in the seawater samples. In addition to the previously described types B1, C3, C15, C21, and D1, novel *Symbiodinium* sequences belonging to clades B and C were also retrieved. The majority of *Symbiodinium* sequences recovered from Kāne'ohe Bay belonged to clade C and those from Puerto Morelos to clade B, a pattern that reflects the dominant types of *Symbiodinium* found in endosymbiosis with scleractinian corals in these two areas. This study represents the first direct assessment of *Symbiodinium* diversity in waters adjacent to coral reefs located in the Caribbean and the Pacific and confirms the presence of *Symbiodinium* in this compartment of the ecosystem. These data provide context for future studies examining spatial and temporal patterns in the availability of *Symbiodinium* in the water column, work that will ultimately promote a greater understanding of the interactions between symbiotic dinoflagellates and their environmentally sensitive benthic hosts.

Marine invertebrates representing at least five phyla form symbiotic relationships with endosymbiotic dinoflagellates belonging to the genus *Symbiodinium* (Stat et al. 2006). As a result of this close coupling of primary producers and consumers, coral reef ecosystems maintain high levels of productivity despite being surrounded by relatively oligotrophic waters (Hoegh-Guldberg 1999). Scleractinian corals form the structural framework of coral reef ecosystems and are the most ecologically important and best-studied examples of these symbioses. The respiratory requirements of the coral host are almost exclusively met by the translocation of photosynthetic materials provided by the endosymbionts and are thus considered to be in obligate symbiosis with *Symbiodinium* (Muscatine and Porter 1977). Therefore, reductions in chlorophyll concentrations per symbiont cell or a loss of resident endosymbionts (or both) results in decreased fitness and possible mortality for the host. This condition, which occurs in response to a variety of environmental stressors, causes the external coloration of the host to appear pale and is commonly described as coral bleaching (Hoegh-Guldberg and Smith 1989). Recovery from bleaching is possible if symbiont populations re-establish in the host in a timely manner.

Until the 1990s, efforts to understand coral–algal associations were hampered by limitations in the methodologies available to assess the identity, diversity, and biogeography of *Symbiodinium* spp. (LaJeunesse 2001). With the introduction of molecular approaches and a focus on a variety of markers on the nuclear and chloroplast ribosomal arrays (18S, 28S, internal transcribed spacer regions [ITS1 and 2], and chloroplast 23S [cp23S]), the genus has now been divided into eight broad clades, A–H, each containing multiple subtypes (Rowan and Powers 1991; Santos et al. 2003; LaJeunesse 2005). At present, ITS2 is arguably the standard for molecular typing of the genus *Symbiodinium* and an extensive database of sequences now exists in GenBank.

Observations of natural bleaching patterns, coral–algal associations under different stress regimes, cultured symbionts under different environmental conditions, and symbiont infectivity studies suggest that different host–symbiont combinations possess different physiological ranges that may reflect, in part, the taxonomic identity of the dinoflagellate symbionts (Kinzie et al. 2001; Rodriguez-Lanetty et al. 2004; Robison and Warner 2006). Building on this framework, the idea that nonlethal bleaching could act as an opportunity for corals to modify their *Symbiodinium* community to adapt to changing environmental conditions has been the subject of much controversy in the field over the past 14 yr (adaptive bleaching hypothesis, Buddemeier and Fautin 1993). This could occur by shifting

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Table 1. Names, dates, times, source descriptions, depth, and total filtered volume of water samples collected in Kāneʻohe Bay, Oʻahu, Hawaiʻi and Puerto Morelos, Mexico for the detection of *Symbiodinium* in the water column.

Region	Sample	Date	Time (h)	Source description	Depth (m)	Filtered volume (mL)
Kāneʻohe Bay, Hawaiʻi (Pacific)	Kbay 1	06 Nov 05	10:00	Surface sample, floating bridge, Coconut Island lagoon	0.6	1,800
	Kbay 2	15 Jan 06	10:15	15 cm above mixed *Montipora capitata* and *Porites compressa* reef, Coconut lagoon	8	3,700
	Kbay 3	15 Jan 06	10:30	15 cm above mixed *M. capitata* and *P. compressa* reef, Coconut lagoon	1	2,950
	Kbay 4	15 Jan 06	13:10	Mid-water sample above patch reef in central section of bay	3	2,900
	Kbay 5	21 Jan 06	12:00	15 cm above *M. capitata* colony, patch reef 13	4	3,250
	Kbay 6	09 Jun 06	11:30	30 cm above sediments next to *M. capitata* colony, fringing reef slope in central section of bay	2	2,300
	Kbay 7	09 Jun 06	11:45	15 cm above *M. capitata* colony, fringing reef slope in central section of bay	1	2,500
	Kbay 8	09 Jun 06	14:30	15 cm above *P. compressa* colony, patch reef 27	1.5	4,800
Puerto Morelos, Mexico (Caribbean)	Mex 1	17 May 05	15:30	15 cm above sediments next to *Montastrea faveolata* colony, barrier reef	4	1,800
	Mex 2	17 May 05	15:45	60 cm above *Montastrea annularis* colony, barrier reef	3	1,800
	Mex 3	23 May 05	11:50	15 cm above *Acropora palmata* colony, barrier reef	3	800
	Mex 4	23 May 05	11:50	60 cm above *A. palmata* colony, barrier reef	3	1,800
	Mex 5	23 May 05	12:00	15 cm above *Sideastrea radians* colony, barrier reef	4	1,700
	Mex 6	23 May 05	11:10	15 cm above sediments next to *M. annularis* colony, barrier reef	5	1,550
	Mex 7	23 May 05	11:15	Mid-water sample, 15 cm above barrier reef	3	1,650

the relative abundance of different types of symbionts present within the host coral or exchanging the less environmentally tolerant symbiont community with new symbionts acquired from environmental pools that are more physiologically suited to the prevailing conditions (reviewed in Baker 2003).

Implicit in the adaptive bleaching hypothesis is that free-living pools of *Symbiodinium* are available for corals to select from, and that individuals within these pools are physiologically diverse. The term free-living is defined in this study as exogenous, environmental pools of *Symbiodinium* occurring outside of the host. The importance of these free-living communities is also highlighted by the fact that many corals release asymbiotic (without symbionts) larvae that must acquire their symbiotic complement from environmental pools (Szmant 1986; Rodriguez-Lanetty et al. 2004; van Oppen 2004). This type of horizontal transmission provides new generations of corals with a potential opportunity to select symbionts that might have a greater physiological range with respect to changing environmental conditions (van Oppen 2004).

Surprisingly, our knowledge of free-living *Symbiodinium* pools is relatively limited and only a few studies have documented the existence of these important communities in coral reef ecosystems (Gou et al. 2003; Coffroth et al. 2006; Thornhill et al. 2006*b*). As new discoveries concerning the ecology, physiology, and fitness of the symbiosis are being made, it is increasingly important to fully characterize the availability and diversity of environmental pools of *Symbiodinium*.

To address this knowledge gap, the current study examines the presence and diversity of free-living dinoflagellates belonging to the genus *Symbiodinium* in coastal, tropical reef habitats in the Pacific and Caribbean. Our results confirm that a diversity of dinoflagellates exists in the water column, and that the free-living *Symbiodinium* communities reflect the endosymbiotic diversity found in coral hosts in each of these geographic areas. These data serve as a framework and rationale for future studies that examine the spatial and temporal patterns in the availability of *Symbiodinium* in the water column.

Materials and methods

Sampling—Seawater samples were collected from locations in Kāneʻohe Bay, Oʻahu, Hawaiʻi (21°26′N, 157°47′W) and Puerto Morelos, Quintana Roo, Mexico (20°50′N, 086°52′W) as detailed in Table 1. Samples were collected at the target depth by filling sterile 1.8-liter plastic bottles or gallon Ziploc™ bags.

Sample filtration and deoxyribonucleic acid (DNA) extraction—Each water sample (volume range 0.8 liters to 5 liters) was filtered through a 5-μm nucleopore track etch membrane filter (Whatman) using a vacuum pump at constant −34 kPa. The filters were placed in a 1.5-mL microcentrifuge tube for DNA extraction from the filter using a cetyltrimethylammonium bromide (CTAB) protocol modified from Dempster et al. (1999). An aliquot of 500 μL of CTAB buffer (100 mmol L^{-1} tris-hydrochloride,

1.4 mol L^{-1} sodium chloride, 20 mmol L^{-1} ethylendiaminetetra-acetic acid, 2% [wt : vol] CTAB, 1% [wt : vol] polyvinylpyrrolidone, and 0.4% [vol : vol] 2-mercaptoethanol [added just before use]) was added to the filter and vortexed for up to 1 min to saturate the filter with buffer. The sample was placed in a 65°C water bath for 0.5–1 h. After the incubation, the sample was vortexed for 30 s and the supernatant removed (leaving the filter behind) and placed in a new 1.5-mL microcentrifuge tube. An equal volume of chloroform : isoamyl alcohol (24 : 1) was added to the supernatant and the mixture was vortexed for 30 s and placed on a rocker at room temperature for 0.5–1 h. After the incubation, the mixture was vortexed for 30 s and centrifuged at 16,000 × g for 15–30 min. A 400-μL aliquot of the aqueous phase was removed and placed in a new 1.5-mL microcentrifuge tube with an equal volume of chloroform : isoamyl alcohol (24 : 1). The sample was vortexed for 30 s and centrifuged for 15–30 min at 16,000 × g. The aqueous phase was transferred to a new 1.5-mL microcentrifuge tube, mixed with an equal volume of cold isopropanol and half a volume of 5 mol L^{-1} NaCl, vortexed briefly, and DNA precipitated at −80°C for 1–8 h. The precipitated DNA was pelleted by centrifugation at 16,000 × g for 30 min, the DNA pellet washed twice with 200 μL of 70% ethanol, dried for 5–10 min on the bench top, and resuspended in 30 μL of sterile water. The concentration and quality of DNA was measured by absorbance at 260 nm using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies) and the DNA stored at −20°C until analyzed.

Polymerase chain reaction (PCR), cloning, and sequencing—The diversity of *Symbiodinium* in genomic DNAs isolated from the seawater samples was evaluated using the ITS2 and the hypervariable region of domain V in the large subunit of the cp ribosomal array (cp23S-HVR). An approximately 300-base pair (bp) region of the ITS2 rDNA was amplified using the forward primer "ITSintfor2" (5′-GAATTGCAGAACTCCG TG-3′) (LaJeunesse and Trench 2000), and the reverse primer "primer ITS-reverse" (5′-GGGATCCATATGCTTAAGTTCAGCGGGT-3′) (Coleman et al. 1994). Reactions were carried out under the following conditions: an initial denaturation for 2 min at 95°C followed by 35 cycles consisting of 94°C for 45 s, 54°C for 45 s, and 72°C for 30 s, and a final extension at 72°C for 10 min. A 140-bp region of the cp23S-HVR was amplified using the forward primer "23SHYPERUP" (5′-TCAGTACAAA TAATATGCTG-3′; Santos et al. 2003) and the reverse primer "23HYPERDN" (5′-TTATCG CCCCAATTAAAC AGT-3′). The latter primer was modified from "23SHYPERDNM13" (Santos et al. 2003) by removing the first 20 bp representing the -M13 portion of the primer. Reactions were carried out using the following conditions: an initial denaturation for 2 min at 94°C followed by 35 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 5 min.

The PCR reactions (25 μL) for both primer sets contained 1× NH_4 buffer (Bioline), 2 mmol L^{-1} $MgCl_2$, 200 nmol L^{-1} deoxynucleotide triphosphates, 0.2 μmol L^{-1} of each primer, 0.02 μg μL^{-1} bovine serum albumin, 1 unit *Taq* polymerase (Bioline), and 1 μL of template DNA.

Clone libraries were constructed for successfully amplified PCR products using the TOPO TA Cloning kit (Invitrogen) according to the manufacturer's protocol. One clone library was generated per water sample and primer set for a total of 12 libraries for Kāne'ohe Bay water samples (ITS2 = 8; cp23S-HVR = 4) and 10 libraries for the Mexico water samples (ITS2 = 3; cp23S-HVR = 7) as detailed in Table 2. Positive inserts were verified by PCR using M13 primers and plasmids sequenced using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer–Applied Biosystems) and an ABI 3100 genetic analyzer. Each sequence was assigned two letters and a clone number. The first letter representing the gene: I for ITS2 or H for cp23S-HVR, and the second letter representing the location: K for Kāne'ohe Bay or M for Mexico, followed by the clone number. For example, HM-38 represents clone number 38, a cp23S-HVR sequence retrieved from a seawater sample from Puerto Morelos, Mexico.

Sequence analyses—Chromatograms of sequenced clones were checked, edited, and aligned using MacVector® 8.1.1 software (Accelrys division of Symantec). All sequences were analyzed using the BLAST tools in GenBank and those with high match scores to *Symbiodinium* were assembled into alignments for further analysis.

One potential problem associated with PCR-based techniques is the overestimation of sequence diversity resulting from the characterization of unique sequence types that reflect PCR error. To address this, and provide as conservative an estimate of biodiversity as possible, we examined the alignments of all *Symbiodinium* sequences and identified those that were recovered from multiple clone libraries (representing independent water samples) or that differed from a published sequence by three or more bp substitutions. These were included in our analysis and considered true variants on the basis of the extremely low probability of encountering the same PCR error in two independent samples, and the low error rate documented for three or more bp substitutions (Kobayashi et al. 1999). New sequence types found only once in the analysis and differing from published sequences by only one or two substitutions were treated as a match to their top BLAST hit.

Results

Success of DNA extractions and PCR—The quality and quantity of DNA obtained from our extraction protocol varied among samples (Table 2). Extractions from Kāne'ohe Bay water samples provided good yields of high-quality DNA, whereas those for Mexico varied in both quality and quantity, perhaps reflecting the storage of filters at −20°C for 10 months before processing.

The success of PCR also varied between samples and the primers sets used (Table 2). The ITS2 region was successfully amplified from all Kāne'ohe Bay samples. These primers are reported to be *Symbiodinium* specific with respect to host DNA (LaJeunesse and Trench 2000). Here,

Table 2. Total DNA recovered from each water sample taken in Kāne'ohe Bay, Hawai'i (Kbay) and Puerto Morelos, Mexico (Mex), the total number of clones sequenced, and the resulting number of *Symbiodinium* sequences found per clone library (one library per sample), grouped by ITS2 and cp23S-HVR genes. Dashes (-) indicate failure of a primer set to amplify the targeted gene from the total DNA recovered, resulting in no clone library generated.

		ITS2		cp23S-HVR	
Sample/ clone library	Total DNA recovered (ng)	No. of clones sequenced	No. of *Symbiodinium* sequences	No. of clones sequenced	No. of *Symbiodinium* sequences
Kbay 1	3,500	23	7	17	17
Kbay 2	1,125	15	0	–	–
Kbay 3	3,600	13	1	15	14
Kbay 4	500	7	2	8	8
Kbay 5	925	12	2	–	–
Kbay 6	5,400	14	0	–	–
Kbay 7	3,400	17	0	–	–
Kbay 8	1,825	20	2	7	7
Totals:		121	14	47	46
Mex 1	23.25	–	–	6	6
Mex 2	34.25	11	10	9	9
Mex 3	50.5	–	–	4	4
Mex 4	26	–	–	12	12
Mex 5	40.5	16	16	11	11
Mex 6	78.75	–	–	6	6
Mex 7	103	8	8	7	7
Totals:		35	34	55	55

in addition to *Symbiodinium*, the ITS2 primers amplified a variety of dinoflagellate and diatom genera from the water sampled in Kāne'ohe Bay (data not shown). This accounts for the discrepancy between the total number of sequences analyzed and the number of *Symbiodinium* sequences retrieved from Kāne'ohe Bay (Table 2). *Symbiodinium* was detected in five of the eight Kāne'ohe Bay samples (Kbay 1, 3, 4, 5, and 8; Table 2). For the Mexico samples, the ITS2 was successfully amplified from three of the seven water samples (Mex 2, 5, and 7) and all but one of the ITS2 clones sequenced for these samples were *Symbiodinium*. The one exception was *Prorocentrum triestinum* (GenBank: AF208246) amplified from water sample Mex 2. Attempts to optimize the PCR and amplify the ITS2 from the other four Mexico water samples failed.

The cp23S-HVR was amplified from all seven Mexico samples and four of the eight Kāne'ohe Bay water samples (Table 2). Attempts to optimize the PCR and amplify the cp23S-HVR from the other four Kāne'ohe Bay water samples failed. Our data for the cp23S-HVR primer set confirms previous reports that these primers are highly specific to *Symbiodinium* (Santos et al. 2003). Of a total of 103 cp23S-HVR sequences obtained from both locations, all but one hit *Symbiodinium* in BLAST searches, with the one exception representing a partial match to *Heterocapsa triquetra* (GenBank: AF130032; match 72/82 bp; e-value: 10^{-13}; Table 2).

ITS2 sequence data—In Kāne'ohe Bay, a total of 121 ITS2 sequences was generated from clone libraries representing Kāne'ohe Bay water samples. *Symbiodinium* belonging to clade C were the most frequent *Symbiodinium* sequence types found in Kāne'ohe Bay (Fig. 1A) and a total of seven clade C variants were recovered (Fig. 2B). These included the published *Symbiodinium* types C3 (GenBank: AF499789; Kbay 1) and C15 (GenBank: AY239369; Kbay 3 and Kbay 4) and new variants that differed from published sequences by 3–10 bp (Figs. 1A, 2B). *Symbiodinium* type B1 was also detected in Kāne'ohe Bay (GenBank: AF333511; Kbay 5).

Symbiodinium belonging to clade B were the most frequent sequence types found in the Mexico samples (Fig. 1A), with *Symbiodinium* type B1 being detected in all three water samples that were successfully amplified with the ITS2 primers. One new sequence variant was also retrieved that differed from *Symbiodinium* type B1 by eight bp (GenBank: EF428335; Mex 2). *Symbiodinium* types D1 (GenBank: AF334660) and C21 (GenBank: AY239372) were also recovered from this region (Mex 7; Fig. 1A).

cp23S-HVR sequence data—Among the cp23S-HVR *Symbiodinium* sequences recovered from Kāne'ohe Bay, the majority were clade C, although clades B and H were also detected (Fig. 1B). One cp23S-HVR *Symbiodinium* sequence recovered from all four water samples was an exact match to a published clade C chloroplast 23S sequence (GenBank: AY035424). Two new clade C variants were also obtained, one differing from the above published sequence by 11 bp (GenBank: EF428359; Kbay 4) and the other differing by only one bp (GenBank: EF428361; Kbay 3 and Kbay 4). An additional sequence recovered from three of the four Kāne'ohe Bay water samples was an exact match to a published *Symbiodinium* clade B chloroplast 23S sequence (GenBank: AY055236). The *Symbiodinium* clade

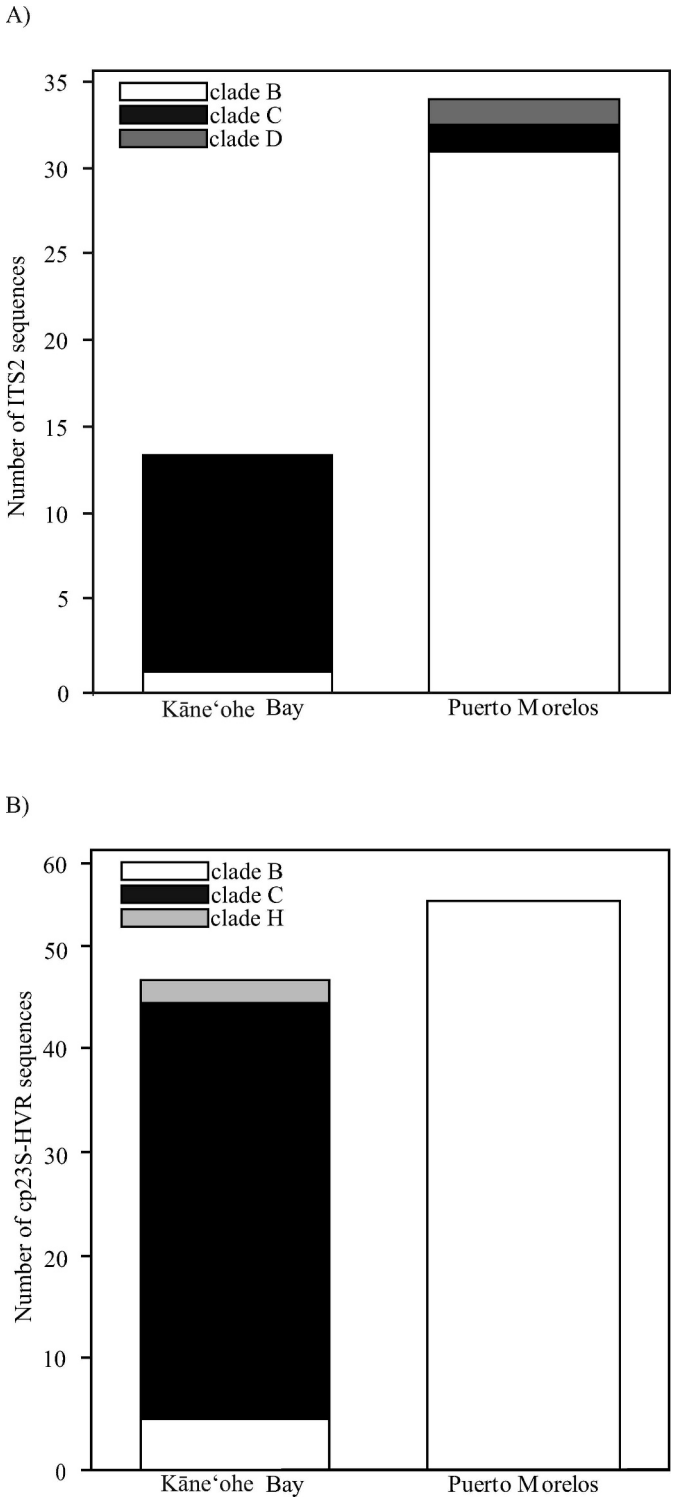

Fig. 1. The total number of *Symbiodinium* sequences retrieved from water samples taken in Kāne'ohe Bay, Hawai'i and Puerto Morelos, Mexico. (A) ITS2 *Symbiodinium* sequences grouped by clade B (white bars), clade C (black bars), and clade D (dark gray bar). (B) cp23S-HVR *Symbiodinium* sequences grouped by clade B (white bars), clade C (black bars), and clade H (light gray bar).

H sequence retrieved matched a sequence isolated from a foraminiferan host sampled in Florida (GenBank: AJ872109; Kbay 3).

The chloroplast cp23S-HVR *Symbiodinium* sequences recovered from all seven Mexico water samples belonged to clade B (Fig. 1B). One sequence found in three water samples was an exact match to the published *Symbiodinium* clade B chloroplast 23S sequence (GenBank: AY055231; Mex 1, 2, and 4) and four variants differing from this published sequence by one to two bp were detected in multiple water samples (GenBank: EF428344, EF428345, EF428347, and EF428351).

Discussion

The availability of a diversity of *Symbiodinium* in the water column has important implications for coral–dinoflagellate symbioses. Unlike environmental pools that exist in the sediments or are deposited on the reef via the feces of coralivorous fish or periodic expulsion events from nearby adult polyps, *Symbiodinium* in the water column have a dispersive capacity that is independent of these local vectors (Muller-Parker 1984; Carlos et al. 1999; Coffroth et al. 2006). It is presumed that in sediments *Symbiodinium* exist as they do in culture, periodically shifting from nonmotile to motile stages for short periods of time. Early observations made on cultured *Symbiodinium* isolated from a variety of hosts have reported a daily transition from the nonmotile to the motile stage, with peak duration of motility lasting from 3 to 7 h depending on host origin (Fitt et al. 1981). Although the dispersal capabilities of *Symbiodinium* living in the benthos are presumably not as great as those present in the water column, this compartment of the environment likely represents an important and understudied reservoir of diversity. Thus, asymbiotic larvae and adult corals clearly have the opportunity to interact with, and select from, a well-mixed source pool of *Symbiodinium* in perpetuating their endosymbioses.

The diversity of *Symbiodinium* found in the water column adjacent to a Pacific and a Caribbean reef reflects established patterns of diversity seen within cnidarian hosts in these two areas. Clade C *Symbiodinium* represented the most frequent types found in Kāne'ohe Bay and clade B in Puerto Morelos, Mexico, a distribution that was confirmed using both the ITS2 and cp23S-HVR. In addition to previously published types, new *Symbiodinium* sequence types were detected in the free-living communities from both regions. Five novel clade C ITS2 sequences and two novel clade C cp23S-HVR sequences were retrieved from seawater sampled in Kāne'ohe Bay. Likewise, one novel clade B ITS2 sequence and four novel clade B cp23S-HVR sequences were recovered from Mexico. None of the clone libraries generated in this study were sequenced to completion and thus the sequence diversity reported here is likely an underestimation of what exists in nature.

Phylogenetic reconstructions of *Symbiodinium* using the ITS2 show that clade C is the most derived clade in the genus, encompassing over 100 ITS2 subtypes (LaJeunesse 2005). LaJeunesse et al. (2004) report that several distinctive clade C types are found in Hawai'i and that

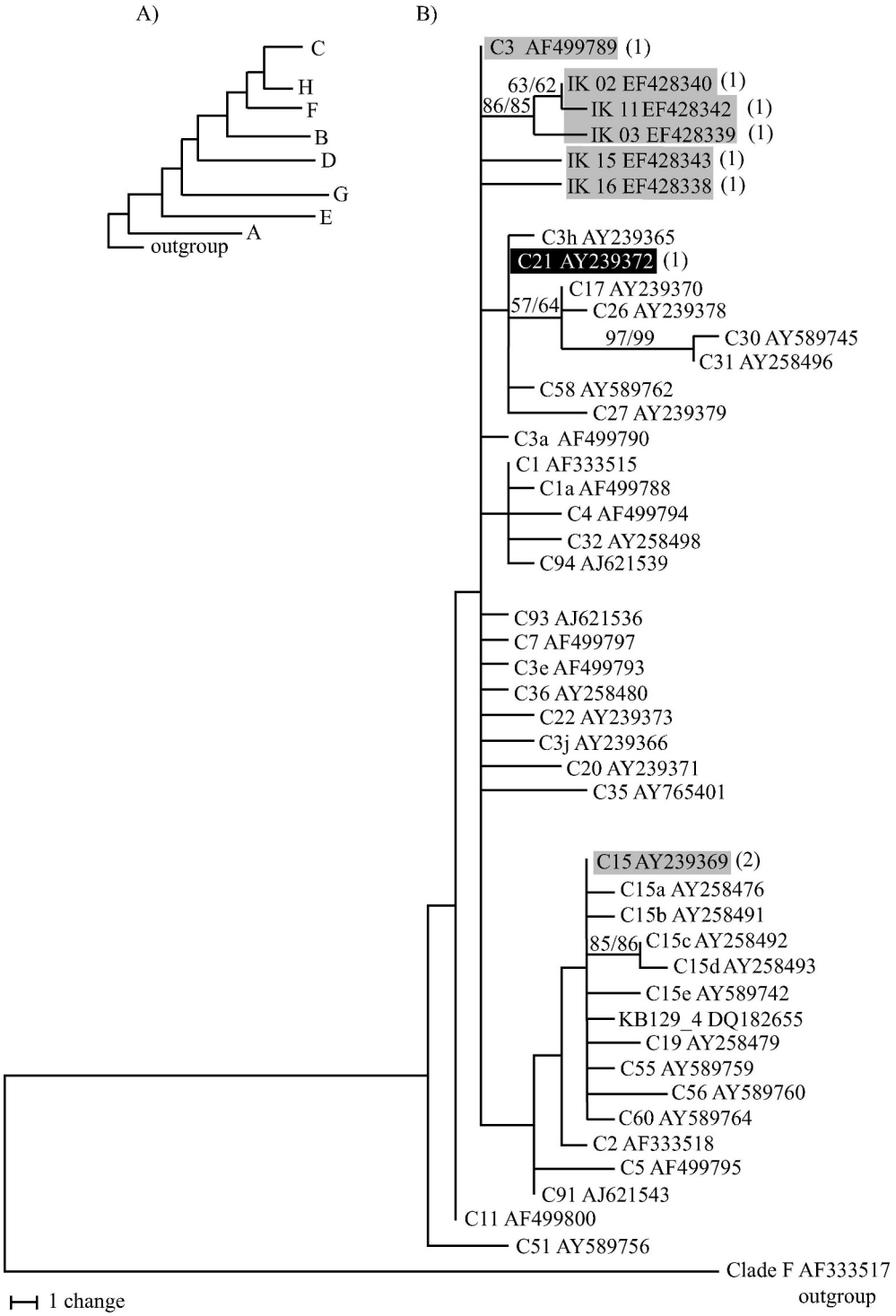

Fig. 2. Relationships in the genus *Symbiodinium* resolved using the ITS2 nuclear marker. (A) Representative phylogeny of all clades in the genus *Symbiodinium* adapted from Pochon et al. (2004). Note the relative position of clade C in the genus. (B) Maximum parsimony (MP) phylogenetic reconstruction of clade C *Symbiodinium* sequence types using the ITS2 nuclear marker. Kāneʻohe Bay (highlighted in gray) and Puerto Morelos (highlighted in black) sequence types found in this study were compared with known sequence types from GenBank. Sequence types found in this study as well as previously published types are followed by their GenBank accession number. The numbers in parentheses indicate the number of times a particular sequence type was detected in different water samples. Neighbor-joining (NJ) methods yielded similar tree topology. The tree is rooted using a representative clade F sequence. MP/NJ bootstrap values are indicated for internal nodes with probabilities above 50%.

C3, a global generalist that forms associations across broad latitudinal scales and with multiple host species, is relatively rare in this area. Interestingly, *Symbiodinium* type C3 was detected in water samples taken from Kāne'ohe Bay, and five of the 14 total *Symbiodinium* ITS2 sequences retrieved were novel clade C sequences that were most closely related to type C3 (Fig. 2B).

The coral genera *Porites* and *Montipora* are extremely abundant in Kāne'ohe Bay and are known to host ITS2 type C15 (and ITS2 sequence variants related to C15; Apprill and Gates 2007) and C31, respectively (LaJeunesse et al. 2004). Given the dominance of these corals in Kāne'ohe Bay, one might expect their endosymbionts (or related variants of C15 and C31) to be prevalent in the free-living community. We encountered C15 in two different water samples and C31 in none. Interestingly, the *Porites* and *Montipora* spp. in Kāne'ohe Bay pass the endosymbionts on to the next generation in the eggs (vertical transmission). Although it is not clear whether these vertical transmitters interact with environmental pools as adults, the specificity of their interactions with their endosymbionts could limit C3 to a mostly free-living lifestyle in Hawaiian waters.

The majority of ITS2 sequences retrieved from the Puerto Morelos water samples were B1, the most prevalent clade B symbiont type found among different host species in the Caribbean (LaJeunesse 2002; Fig. 1A). Given the number of clones sequenced, the low retrieval of novel sequence variants in clade B is consistent with previous observations of relatively low diversification within this clade as compared with clade C (LaJeunesse 2005). *Symbiodinium* C21 was the only clade C sequence found in the Mexico samples and is an established type known to form associations with *Montipora* spp. found in the western Pacific. The fact that this *Symbiodinium* type was detected in Caribbean waters further exemplifies the need to fully characterize free-living populations. It is possible that many *Symbiodinium* types found in hosts in one region are limited to free-living lifestyles in other locations, as found with C3 in Kāne'ohe Bay.

One D1 ITS2 sequence was also found in Puerto Morelos. Corals belonging to the genus *Montastrea* are an ecologically important component of Caribbean reefs and they are known to harbor multiple ITS2 types from clades A, B, C, and D (Rowan et al. 1997; Thornhill et al. 2006*a*). Historically, clade D appears to exhibit a patchy distribution among hosts but members of this clade are considered opportunistic or weedy symbionts that take advantage of marginal environmental conditions that may be unsuitable for other symbiont types (Baker 2003). Given that this symbiont exhibits an indiscriminate distribution and associates with hosts found in compromised habitats, D1 may have the physiological capacity to exist in the water column until conditions favor its union with a health-compromised host.

The *Symbiodinium* diversity described using the cp23S-HVR marker supports the clade level patterns of diversity described using the ITS2 in that the majority of cp23S-HVR sequences from Kāne'ohe Bay belonged to clade C and all from Puerto Morelos belonged to clade B. The genotyping on the basis of the cp23S-HVR confirms that this marker is highly specific for *Symbiodinium* when used with environmental samples. This marker therefore represents a powerful and inexpensive tool for screening environmental samples for the presence or absence of *Symbiodinium* using a simple PCR protocol.

As is the case with all studies that use the rapidly evolving portions of the ribosomal arrays to examine diversity, the presence of multiple copies of these genes within an individual makes it possible that some of the diversity recovered here reflects intragenomic variation (multiple sequence types within individual cells). This has previously been discussed as a potential source of sequences that could drive an overestimation of biodiversity (Apprill and Gates 2007); however, it is currently not clear to what degree intragenomic variation influences our understanding of biodiversity in *Symbiodinium* (van Oppen and Gates 2006).

Alternative methods for studying free-living populations—Free-living *Symbiodinium* have previously been examined by culturing single cells from environmental samples or using asymbiotic larvae and aposymbiotic adult hosts to cull symbionts from the seawater as a means to evaluate *Symbiodinium* diversity in the environment (Carlos et al. 1999; Coffroth et al. 2006; Thornhill et al. 2006*b*). These techniques have been successful in confirming that free-living *Symbiodinium* are present in both water and sediment samples. However, both approaches have constraints that prevent a comprehensive survey of symbiont diversity in this compartment of the ecosystem. For example, the majority of symbiotic dinoflagellates, and particularly those found in association with scleractinian corals, have never been successfully cultured. In addition, the interactions between anthozoans and *Symbiodinium* are highly specific. As such, although infections of asymbiotic larvae and aposymbiotic adults confirm the presence of ''suitable'' types, selective factors limit the detection of the broader *Symbiodinium* diversity present in the environment. Thus, the amplification of *Symbiodinium* sequences directly from water samples provides a more comprehensive means of characterizing the diversity of *Symbiodinium* free living in the environment. These recovered sequences also ultimately serve as a basis for designing quantitative protocols to examine the abundance of specific types within these communities across temporal and spatial scales.

The importance of characterizing free-living populations—The ability to thoroughly characterize free-living *Symbiodinium* diversity represents an important component in the global effort to link the presence and distribution of distinct *Symbiodinium* ''types'' with the resilience of reef corals. We have demonstrated for the first time that diverse populations of symbionts exist in the seawater adjacent to reefs, a circumstance that provides coral hosts the opportunity to interact with and potentially select from these populations, thereby perpetuating their endosymbioses. It is likely that free-living *Symbiodinium* populations represent temporally dynamic communities that experience shifts in the relative

abundance of physiologically different types in response to changes in their physical environment. Specific individuals within these free-living assemblages may currently be excluded from engaging in endosymbiotic interactions with anthozoan hosts depending on such factors as geographic partitioning and rarity. However, should environmental conditions select against the current endosymbiotic complement, individuals in the free-living assemblage may be capable of exploiting the newly available "endosymbiotic niche." As future work characterizing free-living *Symbiodinium* continues, it is likely that the diversity revealed will uncover strictly nonsymbiotic lineages. Current phytoplankton research uses a variety of techniques to study the abundance and diversity of phytoplankton communities including morphological identification, cell culturing, PCR, and microsatellites, as well as flow cytometry, pigment concentrations, and pigment composition (Medlin et al. 2000; Estrada et al. 2004; Iglesias-Rodriquez et al. 2006). Incorporating this suite of techniques in future studies may enhance our understanding of free-living *Symbiodinium* and their roles in both symbiotic systems and in the phytoplankton. More thoroughly describing the free-living *Symbiodinium* populations is vitally important to exploring the scope for change in endosymbiotic interactions on the reef.

Multiple long-term studies have monitored populations of *Symbiodinium* in hospite over temporal and spatial scales, and among multiple host species (Fagoonee et al. 1999; Goulet and Coffroth, 2003; Thornhill et al. 2006*b*). The extension of these efforts to include environmental pools of *Symbiodinium* will enhance our understanding of the interactions of cnidarian hosts with symbiotic dinoflagellates. Monitoring the variability in free-living *Symbiodinium* communities and phytoplankton through space and time may also uncover patterns of change that serve as useful indicators of coral reef health. Uncovering the full spectrum of *Symbiodinium* diversity, especially those found free-living in the environment, is crucial in understanding the ecological range in which these dinoflagellates can persist and the potential ability of symbiotic hosts to adapt to a future of environmental change.

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