Coral Reefs (2012) 31:157–167
DOI 10.1007/s00338-011-0832-5

REPORT

Genetic diversity of free-living *Symbiodinium* in surface water and sediment of Hawai'i and Florida

M. Takabayashi · L. M. Adams · X. Pochon · R. D. Gates

Received: 1 April 2011 / Accepted: 6 October 2011 / Published online: 27 October 2011

Abstract Marine dinoflagellates in the genus *Symbiodinium* are primarily known for their symbiotic associations with invertebrates and protists, although they are also found free-living in nanoplankton and microphytobenthic communities. Free-living *Symbiodinium* are necessary for hosts that must acquire their symbionts anew each generation and for the possible reestablishment of endosymbiosis in bleached adults. The diversity and ecology of free-living *Symbiodinium* are not well studied by comparison with their endosymbiotic counterparts, and as a result, our understanding of the linkages between free-living and endosymbiotic *Symbiodinium* is poor. Here, we begin to address this knowledge gap by describing the genetic diversity of *Symbiodinium* in the surface water and reef sediments of Hawai'i and Florida using *Symbiodinium*-specific primers for the hypervariable region of the chloroplast 23S domain V (cp23S-HVR). In total, 29 *Symbiodinium* sequence types were detected, 16 of which were novel. The majority of *Symbiodinium* sequence types in free-living environments belonged to clades A and B, but smaller numbers of sequence types belonging to clades C, D, and G were also detected. The majority of sequences recovered from Hawai'i belonged to clades A and C and those from Florida to clade B. Such distribution patterns are consistent with the endosymbiotic diversity previously reported for these two regions. The ancestral sequence types in each clade were typically recovered from surface water and sediments both in Hawai'i and Florida and have been previously reported as endosymbionts of a range of invertebrates, suggesting that these types have the capacity to exploit a range of very different habitats. More derived sequence types in clades A, B, C, and G were not recovered here, suggesting they are potentially restricted to endosymbiotic environments.

Keywords *Symbiodinium* · Dinoflagellate · Chloroplast ribosomal 23S · Hypervariable region of DomainV (cp23S-HVR)

Communicated by Biology Editor Dr. Mark Warner

Electronic supplementary material The online version of this article (doi:10.1007/s00338-011-0832-5) contains supplementary material, which is available to authorized users.

M. Takabayashi (✉) · L. M. Adams
Marine Science Department, University of Hawai'i at Hilo, 200 West Kawili St., Hilo, HI 96720, USA
e-mail: misakita@hawaii.edu

X. Pochon · R. D. Gates
Hawai'i Institute of Marine Biology, University of Hawai'i at Mānoa, 46-007 Lilipuna Rd., Kāne'ohe, HI 96744, USA

Introduction

The genus *Symbiodinium* is a diverse group of unicellular dinoflagellates best known for their endosymbiotic interactions with scleractinian corals and other marine invertebrates and protists. Genetic studies have revealed that the genus *Symbiodinium* is comprised of nine major groups or clades named clades A–I that each contains multiple subclade types (Pochon et al. 2006; Pochon and Gates 2010). This taxonomic diversity is reflected in differences in the functional attributes of *Symbiodinium*. The nature and composition of endosymbiotic unions, therefore, influence host characteristics such as growth rates, reproductive output, and thermal tolerance (Kinzie and Chee 1979; Fitt 1985; Rowan et al. 1997; Rowan 2004; Little et al. 2004; Stat et al. 2008). Coral–*Symbiodinium* associations are essential to the survival of the host and underpin the productivity and calcification that creates habitat for the

immense biodiversity that coral reefs support (e.g., Muscatine and Porter 1977). The perpetuation of these endosymbioses through time is therefore central to the maintenance of functional integrity in the coral reef ecosystems.

The majority of scleractinian corals produce asymbiotic gametes and larvae that must acquire *Symbiodinium* from the environment anew each generation (Harrison and Wallace 1990). This mode of endosymbiont acquisition, also called horizontal transmission, requires that *Symbiodinium* be present and available for acquisition in the free-living compartments of coral reef environments (sediments and surface water). These free-living *Symbiodinium* have also been proposed to be a source for replenishing endosymbiotic communities in hosts recovering from bleaching (Buddemeier and Fautin 1993; Baker et al. 2008). Although pivotally important to coral reef ecology, our understanding of free-living *Symbiodinium* diversity and ecology is still limited.

Nano-sized dinoflagellates (2–20 μm) are common in both planktonic and benthic communities (Li 2002; Werner et al. 2008). Free-living *Symbiodinium* in these communities have been examined using asymbiotic invertebrate hosts as collectors (Kinzie et al. 2001; Coffroth et al. 2006) and by isolating, culturing, and genotyping *Symbiodinium*-like cells from environmental samples (Carlos et al. 1999; Gou et al. 2003; Coffroth et al. 2006; Hirose et al. 2008b; Porto et al. 2008). Asymbiotic hosts typically acquire one or a few specific *Symbiodinium* genotypes (Baker 2003; LaJeunesse et al. 2004a; Stat et al. 2009) rather than visualizing the full diversity present in the environment. Similarly, diversity estimates using culturing approaches are confounded by the fact that only a subset of *Symbiodinium* types are easily culturable (Santos et al. 2001; Coffroth et al. 2006; Hirose et al. 2008b). Interestingly, the majority of free-living *Symbiodinium* types that have been successfully cultured belong to clades A and B, and some of these have not been identified as endosymbiotic types in these clades. This suggests that *Symbiodinium* types within the same clade may have very different habitat preferences and may be restricted to either free-living or endosymbiotic mode of living (Carlos et al. 1999; Coffroth et al. 2006; Hirose et al. 2008b; Porto et al. 2008). Microscopy, flow cytometry, and genetics are all common methods used to describe the diversity and abundance of specific taxa in the nanoplankton and microphytobenthic communities (Díez et al. 2001; Moreira and López-García 2002; Unrein et al. 2005; Werner et al. 2008). Recent studies applying these approaches to free-living *Symbiodinium* have revealed that they are present in high densities in reef sediments and water column (Littman et al. 2008) and that free-living *Symbiodinium* communities are diverse and contain representatives in clades A, B, and C (Pochon et al. 2010).

The distributions of free-living *Symbiodinium* types are of interest at all spatial scales, especially in relation to the biogeographic patterns that are relatively well characterized for endosymbiotic *Symbiodinium* (LaJeunesse 2002; Baker 2003; LaJeunesse et al. 2004b; Pochon et al. 2004; Goulet et al. 2008). Based on the latter, habitat partitioning and large-scale biogeographic distribution patterns are expected among free-living *Symbiodinium* communities. Habitat partitioning among free-living *Symbiodinium* types has been suggested by previous studies (Coffroth et al. 2006; Adams et al. 2009; Pochon et al. 2010) and likely reflects variability in environmental ranges and habitat preferences of *Symbiodinium*. Large-scale biogeographic patterns of free-living *Symbiodinium* are also expected and may correlate with those of endosymbiotic *Symbiodinium* depending on the degree to which symbiotic hosts rely on horizontal acquisition. For example, locations across the Hawaiian Archipelago may show less overlap between endosymbiotic and free-living *Symbiodinium* communities due to the predominance of symbiotic hosts that perpetuate endosymbiosis via vertical transmission (Krupp 1983; Richmond and Hunter 1990). In contrast, an increased overlap may be more common in the Caribbean, where most hosts acquire symbionts from the environment (Baker 2003; Coffroth et al. 2006). Here, we investigate this hypothesis by exploring the diversity and distribution of *Symbiodinium* in the surface water and sediments of coral reefs from sites on two islands in Hawai‘i and the Florida Keys. The presentation of our data in the context of previous research also provides a summary of all free-living *Symbiodinium* chloroplast 23S domain V hypervariable region sequences (cp23S-HVR) available to date.

Materials and methods

Collection

Seawater and sediment samples were collected from four sites in Hawai‘i and three sites in the Florida Keys during June–July 2008 (Fig. 1, Table 1). The Hawaiian sites and collection times included Lele‘iwi (N19°44′01.11″ W155°01′04.59″; 15:30–17:00 h), Puakō (N19°58′15.67″ W155°50′52.94″; 09:00–10:00 h), and Honokōhau (N19° 40′10.47″ W156°01′37.46″; 11:00–12:00 h) on Hawai‘i Island, and Kāne‘ohe Bay (N 21°26′50.44″ W157°47′ 49.62″; 10:30–11:45 h) on O‘ahu, Hawai‘i. The Florida Keys sites and collection times were at The Cable (N24°28′10.74″ W81°43′25.02″; 09:00–10:00 h), Western Sambo (N24°28′44.88″ W81°42′59.16; 10:30–11:30 h), and Cottrell Key (N24°36′49.14″ W81°55′16.92″; 09:00–10:00 h). At each site, triplicate 10 l samples of surface seawater were collected directly over the reef at a depth of <1 m. Similarly, three to ten replicates of 2 ml surface sediment samples were collected at depths between 5 and

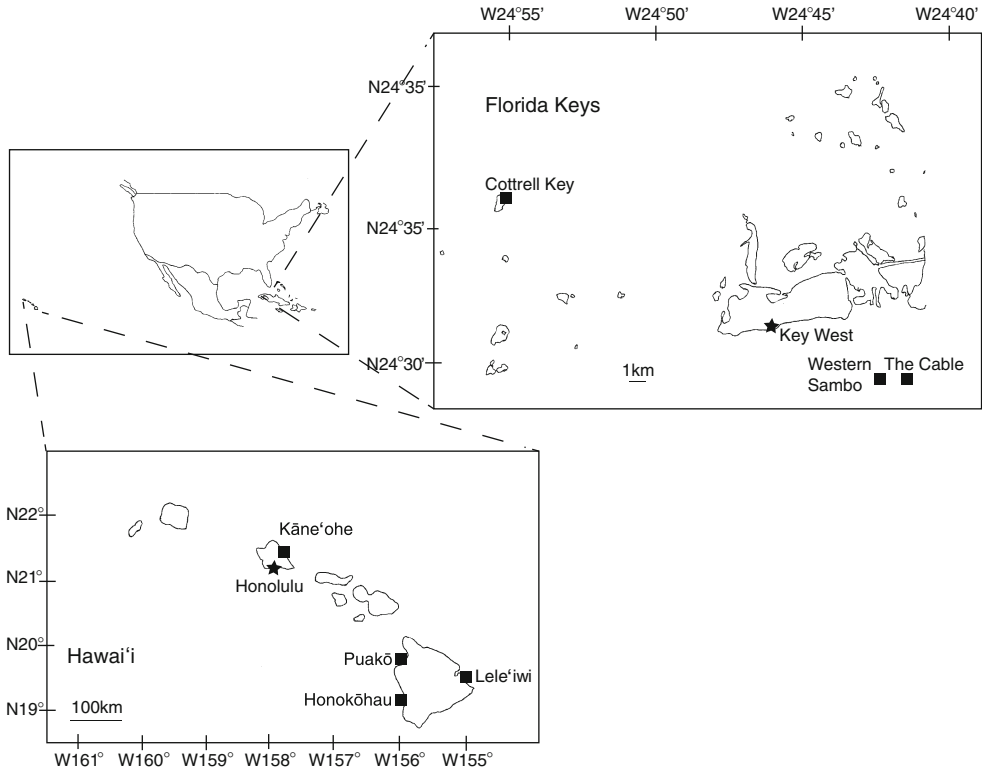

Fig. 1 Map of collection sites in the Hawaiian Islands and the Florida Keys

10 m using a microcentrifuge tube at each site. Only the surface layers of sediments determined as oxic by coloration were collected (i.e., the top 1–5 mm of sediment). Sediment layers of a gray or black coloration indicating anoxic conditions were strictly avoided. Water and sediment samples were immediately stored on ice in the dark and directly transferred to the laboratory for preservation and storage.

Sample preparation

All seawater samples were first filtered by gravity through a 20-µm nylon mesh to exclude endosymbiotic *Symbiodinium* from invertebrate larvae (>100 µm; Edmunds et al. 2005, Nozawa and Harrison 2005, Hirose et al. 2008a), soritid foraminifera (100–15,000 µm in diameter; Pochon et al. 2007), and those trapped in coral mucus. The resulting filtrate was then subjected to further filtration through a 5-µm filter using low constant vacuum filtration at a constant 0.1 bar to capture particles of the size range 5–20 µm, including free-living *Symbiodinium* (5–15 µm; Stat et al. 2006). Each sediment sample was suspended in approximately 200 ml of filtered (0.22 µm) seawater and then sequentially filtered through 20- and 5-µm filters as described above.

DNA analysis

Genomic DNA was extracted from the organisms on the 5-µm filter using 1 ml of a guanidinium protocol described in Adams et al. (2009). DNA was isolated and purified from 200 µl of the total DNA extraction solution. In order to achieve successful PCR amplification, some samples were diluted 1/10 or 1/50. A nested PCR strategy was used to amplify the cp23S-HVR. First, the entire cp23S-DomainV region was amplified from the genomic DNA extraction using the primers "23S1M13" and "23S2M13" (Santos et al. 2002) under the following thermal cycler conditions: 2 min at 94°C, 36 cycles of 94°C for 30 s, 50°C for 1 min, 72°C for 1 min 15 s, followed by 7 min at 72°C. This primer set is not *Symbiodinium*-specific and amplified the cp23S-Domain V region of other dinoflagellates, as well as *Symbiodinium* (Santos et al. 2002). One (1) microliter of the resulting PCR products was used as template in a second PCR to amplify the cp23S-HVR region using the *Symbiodinium*-specific primers "23SHYPERUP" (Santos et al. 2003) and "23SHYPERDN" (same as "23SHYPERDNM13" in Santos et al. 2003 without the M13 sequence, and first applied in Manning and Gates 2008) under the same conditions given above except with an annealing temperature of 55°C. The product of a no-template control from the first PCR was used as template in the second PCR as a

Table 1 Summary of all cp23S-HVR sequence types included in the phylogenetic networks (Fig. 2)

Clade	cp23S-HVR name	Origins	Regions	Accession #
Clade A	*chvA1	C, G, J, S	F, H, J, O, P	AY035405
	*chvA2	C, J, M, S, W	H, O	AY035410
	*chvA3	Z, S	F, H, J, O	AY035413
	chvA4	A	F	AY035404
	chvA5	SP	C	EU006528
	chvA6	C	JA	FJ461476
	chvA7	C, S, W	H, JA, O	FJ461477
	chvA8	C	M	FR773855
	chvA9	C	M	FR773856
	chvA10	C	M	FR773857
	chvA11	S	H	GQ370584
	chvA12	S, W	H, O	GQ370621
	**chvA13	S	H	GQ370585
	**chvA14	S, W	H	GQ370586
	**chvA15	S	F, H	GQ370587
	chvA16	W	H	GQ370588
	chvA17	W	H	GQ370589
	**chvA18	S	H	GQ370590
	chvA19	W	H	GQ370591
	**chvA20	S	F	GQ370592
	chvA21	W	H	GQ370593
	**chvA22	W	H	GQ370594
Clade B	*chvB1	A, C, S, W	F, H, O	AY035416
	*chvB2	A, C, G, S, W	F, H, MX, P	AY055231
	*chvB3	A, G, S	F, H	AY035420
	chvB4	SC	F	AY035415
	chvB5	G	B	AY035417
	chvB6	G	P	AY035419
	chvB7	SC	F	AY055239
	chvB8	SC	F	AF474164
	*chvB9	W	F, MX	EF428345
	*chvB10	G, S	H, P	AY055233
	chvB11	W	MX	EF428344
	chvB12	W	MX	EF428347
	chvB13	W	MX	EF428351
	**chvB14	S	F	GQ370600
	**chvB15	W	F	GQ370601
	chvB16	S, W	H	GQ370602
	**chvB17	S, W	F, H	GQ370603
	**chvB18	S	F, H	GQ370604
	**chvB19	S	F	GQ370605
	**chvB20	S	F	GQ370606
	**chvB21	S	H	GQ370607
	*chvB22	S, W	C, F	EU139605
Clade C	*chvC1	C, F, J, SC, S, W	F, G, GBR, H, JA, M, O, P, PU	AY035424
	chvC2	F, W	GBR, O	AJ872085
	*chvC3	C, S, W	F, H, M	EF428361
	chvC4	W	H	EF428359
	chvC5	F	P	AJ872081
	chvC6	W	H	EF428360
	chvC7	W	H	FM877461
	chvC8	F	R	FM877442
	chvC9	F	H, JA	FN298479
	chvC10	C	JA	FJ461486
	chvC11	C	M	FR773858
	chvC12	C	M	FR773859
	**chvC13	S	H	GQ370613
Clade D	*chvD1	A, C, W	G, GBR, H, JA, M, O	AY035426
Clade G	*chvG1	SP, S, W	C, F, H	EU006517
	chvG2	F	G	AJ872106
	chvG3	F	G	AJ872107
	chvG4	SP	G	EU006519
	chvG5	SP	G	EU006521
	chvG6	SP	G	EU006522
	chvG7	SP	G	EU006524
	**chvG8	S	H	GQ370619
	**chvG9	S	H	GQ370620
	chvG10	SP	F	GU219511
	chvG11	SP	GBR	GU219516
	chvG12	SP	F	GU219492
	chvG13	SP	F	GU219494
	chvG14	SP	GBR	GU219514

One asterisk before the name indicates that the sequence was found in this study as well as previous studies; two asterisks indicate a novel sequence only found in this study. Origins refers to the environmental or symbiotic host sources

One representative accession number is listed for each sequence type. A more detailed version of this table that includes previous names for these sequence types is provided in online supporting information (Table S1)

A sea anemone, *C* scleractinian coral, *F* foraminiferans, *G* gorgonian, *J* jellyfish, *M* mollusk, *O* octocoral, *S* sediment, *SC* soft coral, *SP* sponge, *W* water, *Z* zoanthid. Regions refers to the geographic source, *B* Bahamas, *C* Colombia, *F* Florida, *G* Guam, *GBR* Great Barrier Reef, *H* Hawai'i, *J* Jamaica, *JA* Johnston Atoll, *M* Moorea, *MX* Mexico, *O* Okinawa, *P* Panama, *PU* Palau, *R* Reunion Islands

negative control for the nested PCR protocol employed. In cases where the second round of amplification yielded no products after several attempts, successful amplification of the

first round of PCR with the less specific primer set served as a positive control. This positive control determined that the lack of PCR products with *Symbiodinium*-specific primers was because of the absence of *Symbiodinium* in the samples rather than poor-quality DNA templates. Multiple (8–16) extraction dilutions were amplified, and final amplified products were isolated by gel extraction and purified using the QIAquick Gel Extraction Kit (Qiagen), according to the manufacturer's protocol. Purified PCR products were pooled for each sample and cloned into the pGEM®-T Easy Vector System II (Promega). A total of 18–25 clones per sample were sequenced using an ABI 3730XL capillary-based DNA sequencer (Applied Biosciences) at the Advanced Studies in Genomics, Proteomics and Bioinformatics Sequencing Facility at University of Hawai'i at Mānoa.

Sequence analyses

Sequences were manually edited and aligned, using Sequencher 4.5 (Gene Codes Corporation). Only sequences that were amplified from two or more independent samples or those identical to sequences deposited in GenBank were included in the analysis (Apprill and Gates 2007; Manning and Gates 2008; Pochon et al. 2010; but see Thornhill et al. 2007). Sequence types that were represented by only one clone in any given library were verified by sequencing in the reverse direction. The clade of each sequence type was identified using nucleotide BLAST (Altschul et al. 1990). Sequences belonging to each clade were aligned with closely related published sequences from GenBank, using a Clustal W alignment in BioEdit v5.0.9 (Hall 1999), and the alignment refined manually. A statistical parsimony network was generated for each cladal alignment using TCS v1.21 (Clement et al. 2000) with a maximum of 50-bp connection steps and treating gaps as a fifth state. Each sequence was assigned a unique name reflecting the locus, clade, and sequence number. For example, "chvA1" corresponds to the chloroplast hypervariable, clade A, sequence number 1 (Table 1, Electronic Supplemental Material, ESM Table S1).

Results

Symbiodinium sequence diversity

The first-round of amplification with the less specific primer set 23S1M13/23S2M13 in the nested PCR was successful in 80 samples. This verified that the quality of extracted DNA was suitable as a PCR template in these samples. Of these, the cp23S-HVR region of *Symbiodinium* was then successfully amplified and sequenced from 62 samples (i.e., 77.5%; Table 1). *Symbiodinium* was detected in 20 out of 21 surface water samples (95.2%) and 42 out of 59 of the sediment samples (71.2%). A total of 1,113 *Symbiodinium* sequences were recovered in our analyses, representing 29 different sequence types (Fig. 2), 13 of which are known and 16 are novel. Each *Symbiodinium* sequence type was found in 1-19 independent samples in this study, with an average of 3.6 samples per sequence type, though many of the sequence types ($N = 18$) were found in only 1 or 2 samples. The number of sequences recovered for each clade varied; 9 belonged to clade A (GQ370580–GQ370582, GQ370585–GQ370587, GQ370590, GQ370592, and GQ370594), 13 to clade B (GQ370595–GQ3705601, GQ370603–GQ370607, and JF327758), 3 to clade C (GQ370609, GQ370611, and GQ370613), 1 to clade D (GQ370614), and 3 to clade G (GQ370618–GQ370620) (Table 1, ESM Table S1). No *Symbiodinium* sequence types belonging to clades E, F, H, or I were found. The majority of novel sequences belonged to clades A and B, while only one novel sequence was recovered in clade C and 2 novel sequences in clade G. Each individual sample yielded 1–5 sequence types, averaging 1.5 *Symbiodinium* sequence types per sample.

The sequence networks for clades A, B, C, and G show multiple sequences radiating out from core sequences, defined here as sequences having 3 or more sequence types radiating from them (Fig. 2). These core sequences are assumed to be ancestral (Correa and Baker 2009; Stat et al. 2009). Only 1 sequence was recovered from clade D and was distantly related (58-bp substitutions and insertions/deletions) to the only other clade D sequence known for this locus. Core sequences included chvA1, chvA2, chvA15, chvB1, chvB2, chvB17, chvC1, chvG1, and chvG10 (Fig. 2).

Sequence diversity among regions

All 5 clades, A, B, C, D, and G, and 21 out of 29 sequence types, recovered in this study were identified in Hawaiian samples, of which 14 sequence types were unique to Hawai'i (Figs. 2a, 3). In comparison, a total of 15 sequence types were recovered from Florida samples. Of these, 8 types were found only in Florida, 7 of which belonging to clade B. Seven sequence types were common to Hawai'i and Florida (chvA3, chvA15, chvB1, chvB2, chvB17, chvB18, and chvG1) (Figs. 2a, 3).

Sequence diversity among habitats

Thirteen sequence types representing clades A, B, C, D, and G were identified in surface water samples. Five sequence types were only found in surface water samples (Fig. 2b), and each water sample contained 1–4 sequence types (average = 2 types/sample). In Hawai'i, 8 sequence

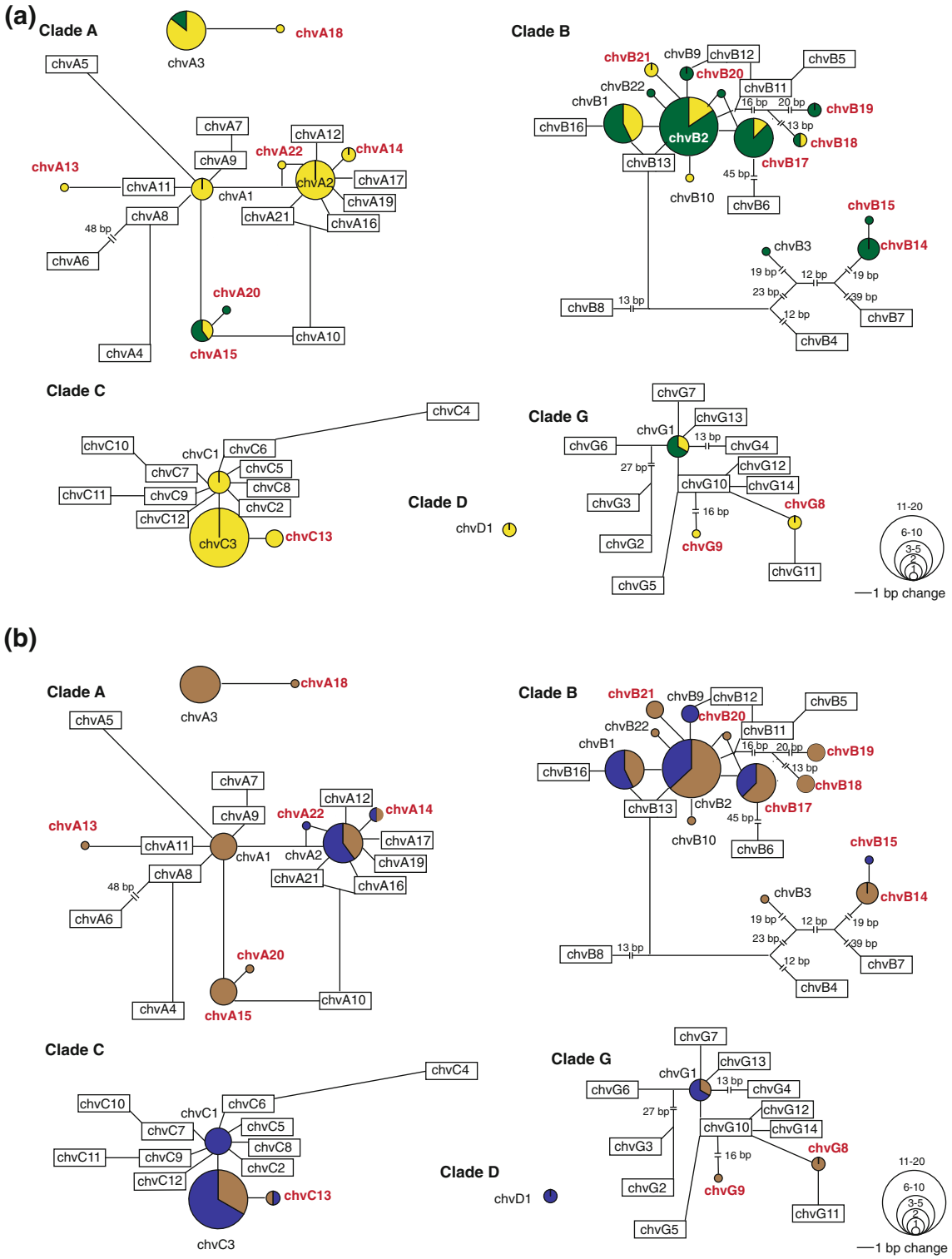

Fig. 2 Statistical parsimony networks of clades *A*, *B*, *C*, *D*, and *G* of *Symbiodinium* comprised of sequence types identified in this study and that of closely related published *Symbiodinium* types, showing **a** diversity of *Symbiodinium* in Hawai'i (*yellow*) versus Florida (*green*) and **b** diversity of *Symbiodinium* in the sediment (*brown*) versus surface water (*blue*). The number of independent samples that contained each sequence type is indicated by the size of the *circle*; indices are given in each figure. Sequence types identified in this study are indicated by *circles* and published sequence types by *squares*. Names of novel sequence types are in *red*

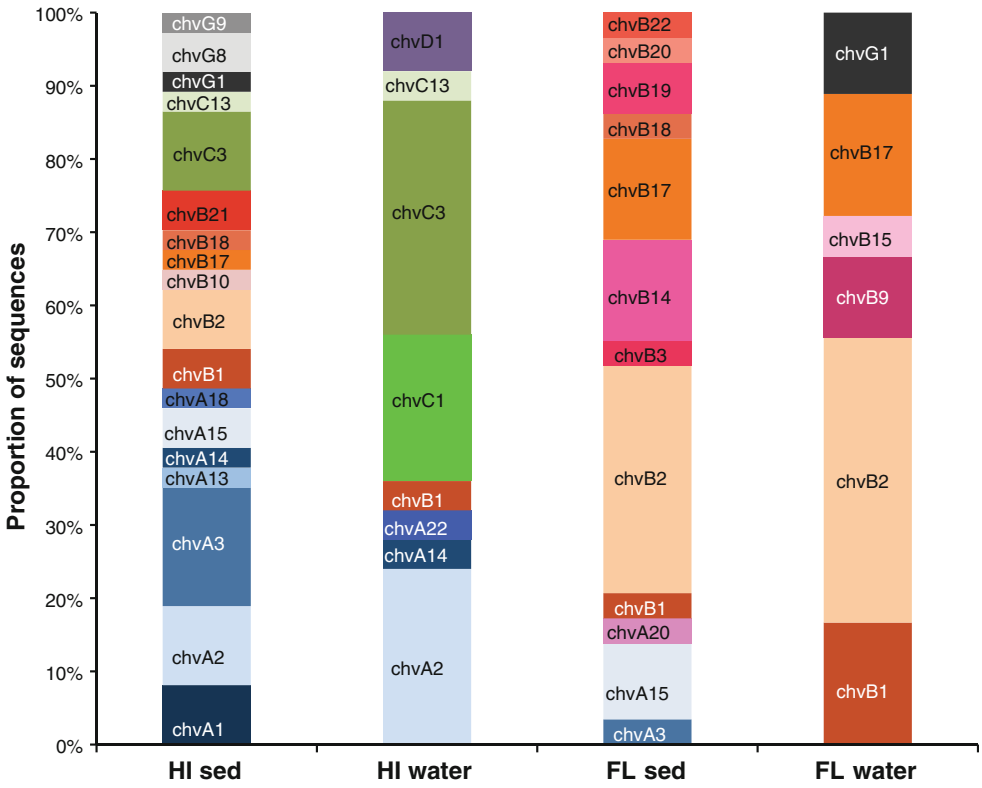

Fig. 3 Proportions of free-living *Symbiodinium* sequence types recovered from sediment ("sed") and surface water ("water") samples in Hawai'i (HI) and Florida (FL)

types from clades A–D were identified in surface water (Fig. 3), though clade B (chvB1) was only identified in one water sample from Kāne'ohe Bay and clade D was only found at Puakō. Sequence types chvA2, chvC1, and chvC3 were the most common in Hawai'i, and 2 of these 3 sequences were always recovered at a site. Surface water samples in Florida were dominated by clade B, which was found in every sample at every site. In Florida, 5 clade B types were identified, and type chvB2 was the most commonly encountered, being present in every sample but one. Clade G, specifically type chvG1, was found in only 2 water samples at 2 different sites, Cottrell Key and The Cable, in Florida.

In sediment samples, 21 out of the 24 sequence types identified were from clades A–C and 16 of these were found solely in sediment samples (Figs. 2b, 3). Each sediment sample yielded 1–5 sequence types, averaging 1.8 types per sample. In Hawai'i, 18 sequence types from clades A, B, C, and G were found, though clade C was identified in only 4 (out of 21) sediment samples and clade G was only identified in samples from Puakō. In addition, clade A was the most ubiquitous, being present at every site and in most samples (13); however, no one type was particularly common, as was found for the surface water samples. In Florida, sediment samples contained 14 sequence types from clades A, B, and C; however, clade A was only present in 4 samples (out of 21) and clade C in 1 sample (at The Cable). Clade B was the most frequently found clade in Florida; however, as with Hawai'i, no one sequence type was more prevalent than another. Of the 29 sequence types identified in this study, 8 (chvA2, chvA14, chvB1, chvB2, chvB17, chvC3, chvC13, and chvG1) were found in both surface water and sediment samples (Figs. 2b, 3); however, no sequence type was found in all habitats and regions (Fig. 3).

Discussion

Free-living *Symbiodinium* are necessary to establish or re-establish endosymbiosis with many marine organisms and are also likely to have important ecological roles in benthic and pelagic ecosystems (GuoFu et al. 2008; Werner et al. 2008). A number of previous studies have identified free-living *Symbiodinium* from coral reefs globally, but detailed characterization of their diversity is only now beginning to emerge (Carlos et al. 1999; Gou et al. 2003; Coffroth et al. 2006; Koike et al. 2007, GuoFu et al. 2008; Hirose et al. 2008b; Littman et al. 2008; Manning and Gates 2008; Porto et al. 2008; Pochon et al. 2010; Venera-Ponton et al. 2010). The overall irregularity in the detection of *Symbiodinium* in our environmental samples and the fact that new studies continue to find previously uncharacterized sequence types suggest that the distribution patterns of free-living *Symbiodinium* are spatially heterogeneous. This is not surprising given that the distribution patterns of many dinoflagellates

are similarly spatially heterogeneous (Mouritsen and Richardson 2003). Part of spatiotemporal heterogeneity in free-living *Symbiodinium* diversity at any given location may also depend on diel vertical migration observed in cultured *Symbiodinium* (Yacobovitch et al. 2004) and commonly seen in other dinoflagellates in the field (e.g., Blasco 1978). Sample collection in our study generally occurred in late morning to minimize temporal variability. Expanded sampling in the future is needed to gain a more comprehensive understanding of the ecology of free-living *Symbiodinium* over dynamic spatiotemporal scales.

The hypervariable region of the chloroplast 23S domain V (cp23S-HVR) was selected as the molecular marker in this study because it is the only marker specific enough to distinguish *Symbiodinium* from other closely related dinoflagellates in environmental samples (Manning and Gates 2008; Pochon et al. 2010). Although cp23S-HVR provides less taxonomic resolution than either the nuclear ITS, or cp23S-Domain V within which it resides, the phylogenetic patterns resolved by all of these markers are largely consistent with one another (Stat et al. 2009; Pochon et al. 2010). An additional advantage of this locus is that it is coding and may be directly linked to physiological performance. The cp23S gene transcribes for the chloroplastic ribosomal RNA (rRNA), which is strongly conserved through evolution (Harris et al. 1994). Chloroplastic 23S-rRNAs are most closely related to eubacterial 23S-rRNAs (Gray 1988), which forms the peptidyl transferase center that helps protein folding and could ultimately affect physiology (Samanata et al. 2008). Interestingly, *Symbiodinium* types that were distinguished by cp23S-DomainV sequences have different growth rates under different temperatures in culture (Kinzie et al. 2001, Santos et al. 2002), suggesting that this marker links to phenotypic traits that are important.

Free-living *Symbiodinium* diversity as compared to symbiotic counterpart

The general structure of the statistical parsimony networks of free-living *Symbiodinium* based on cp23S-HVR is similar to networks generated for *Symbiodinium* in other studies for the ITS-2 and cp23S-DomainV in that the derived types radiate out from few ancestral types in each clade (LaJeunesse 2005; Correa and Baker 2009; Stat et al. 2009) (Fig. 2). The diversity of free-living sequence types in clade A recovered in this study is high and much greater than, and in part distinct from, the endosymbiotic *Symbiodinium* diversity known for this clade (Stat et al. 2006; Correa and Baker 2009). Although our understanding of both endosymbiotic and free-living *Symbiodinium* diversity is far from complete, this difference supports the suggestion of Coffroth et al. (2006) and Hirose et al. (2008b) that at least some clade A *Symbiodinium* do not engage in endosymbiosis and are exclusively free-living. Similarly, the high number of free-living clade B sequence types, mostly previously uncharacterized by this marker, is in agreement with a recent study (Manning and Gates 2008) and suggests that some clade B *Symbiodinium* may also be exclusively free-living. The contrastingly low diversity of free-living clade C *Symbiodinium* is surprising. Many corals, particularly those in the Pacific, associate with clade C *Symbiodinium* (e.g., Pochon et al. 2006), and thousands of *Symbiodinium* cells are expelled from hosts on a daily basis (Stimson and Kinzie 1991; Jones and Yellowlees 1997; Baghdasarian and Muscatine 2000). Consequently, the diversity of clade C *Symbiodinium* in the environment is expected to be as high as found in endosymbioses. Our results indicate otherwise and suggest that some clade C *Symbiodinium* may be confined to endosymbiotic habitats. Occurrence of free-living clade G *Symbiodinium* in both Hawai'i and Florida is intriguing. Although clade G *Symbiodinium* has been found in association with a wide range of invertebrates and protists, their distribution range was previously considered to be restricted to the Indo-Pacific (van Oppen et al. 2005; Pochon et al. 2006; Goulet et al. 2008; Granados et al. 2008). Recent phylogenetic analyses of the genus *Symbiodinium* using the nuclear large subunit ribosomal DNA have revealed that clade G contains two genetically divergent sub-clades. One of these has so far only been found in soritid foraminifera from Guam, Micronesia (Pochon et al. 2001, 2007), and the other sub-clade is most commonly associated with excavating sponges of the genus *Cliona* (Schönberg and Loh 2005; Schönberg et al. 2008). Recently, the latter sub-clade has also been found in symbiosis with excavating sponges in the Caribbean (Granados et al. 2008; Hill et al. 2011). Interestingly, clade G had not been recovered from the free-living *Symbiodinium* community in Hawai'i and the Caribbean (Manning and Gates 2008; Porto et al. 2008), but our study retrieved three clade G sequence types, one of which (chvG1) was found in both Hawai'i and Florida, and is identical to numerous cp23S-HVR *Symbiodinium* sequences previously reported from Caribbean excavating sponges (see Table 1, ESM Table S1).

Of the 29 free-living *Symbiodinium* sequence types recovered in this study, 11 types (chvA1, chvA2, chvA3, chvB1, chvB2, chvB3, chvB10, chvC1, chvC3, chvD1, and chvG1) have previously been identified as endosymbiotic. In Hawai'i, 5 sequence types (chvA1, chvA2, chvB1, chvC1, and chvC3) previously known as endosymbiotic (Santos et al. 2001; Pochon et al. 2010) were identified as free-living in this study. The overlap between free-living and symbiotic communities in Hawai'i was greater than was found in Pochon et al. (2010), which is likely due to the additional sampling in Hawai'i in this study. Nonetheless, the overlap

between symbiotic and free-living *Symbiodinium* communities in Hawai'i is relatively small, perhaps suggesting that some *Symbiodinium* are restricted and optimized to the endosymbiotic environment and have a limited ability to survive outside their hosts once expelled (Hill and Ralph 2007). Interestingly, most coral hosts in Hawai'i transmit their dinoflagellate symbionts vertically without relying on the environmental *Symbiodinium* pools (Krupp 1983, Richmond and Hunter 1990); therefore, the limited overlap between symbiotic and free-living communities is expected. A larger overlap would then be predicted in Florida where much more symbiotic hosts acquire *Symbiodinium* from the environment than those in Hawai'i (Baker 2003; Pochon et al. 2010). The endosymbiotic *Symbiodinium* communities in Florida, however, have not been thoroughly characterized using the cp23S-HVR marker (Hill et al. 2011), so it is impossible to test this prediction at this time.

Free-living *Symbiodinium* diversity patterns in different regions

Comparison of free-living *Symbiodinium* sequence diversity from Hawai'i and Florida reveals some interesting biogeographic patterns. The free-living *Symbiodinium* sequence types belonging to clades A, B, C, D, and G were recovered in Hawai'i, and those of clades A, B and G were found in Florida (Figs. 2a, 3). Furthermore, 85.1% of the free-living diversity in Florida was found in clade B. In contrast, 82.3% of the free-living diversity in Hawai'i was found in clades A and C (sequences in circles in Figs. 2, 3). The dominance of clade B in free-living *Symbiodinium* community in Florida interestingly mirrors the endosymbiotic *Symbiodinium* community for the Caribbean (LaJeunesse 2002; Baker 2003; Goulet et al. 2008). Additionally, higher genetic diversity of free-living *Symbiodinium* was detected in Hawai'i (21 sequence types) as compared to Florida (15 sequence types) (Fig. 3). However, it is unclear if this pattern reflects the ecology or is correlated with the uneven sample sizes. Lastly, the sequence types that were found in both Hawai'i and Florida were all ancestral sequence types, except for two, chvA3 and chvB18 (Fig. 2a). These 'generalists' may have been widespread before the Indo-Pacific and Atlantic oceans were separated and remained undifferentiated because of limited sexual reproduction (LaJeunesse 2005). However, because dinoflagellate endemism is generally considered rare (Taylor et al. 2008, but see Thornhill et al. 2009), more derived types may be found elsewhere with further sampling.

Diversity patterns in different habitats

Some interesting patterns also emerge when genetic diversity of free-living *Symbiodinium* in surface water and sediment are compared with one another. The most abundant and ancestral sequence types (except chvA14) were found both habitats, while the less abundant and more derived sequence types were generally found in sediments (Fig. 2b). Further, a relatively smaller number of sequence types dominated surface waters, chvA2, chvC1, and chvC3 in Hawai'i and chvB2 in Florida (Fig. 3). In contrast, sequence types were almost equally represented in sediments of each region (Fig. 3). Given the differences in sample size and collection protocols to accommodate the solid and liquid nature of environmental samples, the *Symbiodinium* diversity in the two habitats cannot be directly compared. However, the observed diversity of *Symbiodinium* sequence patterns characteristic for each environment is noteworthy. It is possible that such diversity patterns are indicative of ecological differences in free-living *Symbiodinium* communities in these habitats. The specificity of certain *Symbiodinium* sequence types to a particular habitat, and their abundances and capacity to move between these habitats are largely unknown at this stage but certainly warrant further investigation.

Acknowledgments This research was supported in part by the National Oceanic and Atmospheric Administration, Project #R/CR-16, which is sponsored by the University of Hawai'i Sea Grant College Program (NA05OAR4171048 to M.T.), a National Science Foundation Award (OCE-0752604 to R.D.G.), the Swiss National Science Foundation (PBGEA-115118 to X.P.), the School of Ocean and Earth Science and Technology at the University of Hawai'i and the Edwin Pauley Foundation. Nathaniel Olson, Renee Shutt, and Monika Frazier provided assistance and were supported by Research Experience for Undergraduate internship program (NSF #0453630; PI D.K. Price) and the University of Hawai'i Experimental Program to Stimulate Competitive Research (NSF EPS0554657). We thank Michiko Ojimi, Vivian Cumbo, Paula Ayotte, Mark Manuel, Nakoa Goo, Nancy Chaney, and Kevin Kaluna for field support. Florida Keys National Marine Sanctuary permitted our collections (permit number FKNMS-2008-049). This is Hawai'i Sea Grant publication JC-08-32, Hawai'i Institute of Marine Biology publication contribution #1437, and School of Ocean and Earth Science and Technology contribution #8116.

References

Adams LM, Cumbo VR, Takabayashi M (2009) Exposure to sediments enhances primary acquisition of *Symbiodinium* by asymbiotic coral larvae. Mar Ecol Prog Ser 377:149–156

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic logical alignment search tool. J Mol Biol 215:403–410

Apprill AM, Gates RD (2007) Recognizing diversity in coral symbiotic dinoflagellate communities. Mol Ecol 16:1127–1134

Baghdasarian G, Muscatine L (2000) Preferential expulsion of dividing algal cells as a mechanism for regulating algal-cnidarian symbiosis. Biol Bull 199:278–286

Baker AC (2003) Flexibility and specificity in coral-algal symbiosis: Diversity, ecology and biogeography of *Symbiodinium*. Annu Rev Ecol Evol Syst 34:661–689

Baker AC, Glynn PW, Riegl B (2008) Climate change and coral reef bleaching: An ecological assessment of long-term impacts,

recovery trends and future outlook. Estuar Coast Shelf Sci 80: 435–471
Blasco D (1978) Observations on the diel migration of marine dinoflagellates off the Baja California coast. Mar Biol 46:41–47
Buddemeier RW, Fautin DG (1993) Coral bleaching as an adaptive mechanism. Bioscience 43:320–325
Carlos AA, Baillie BK, Kawachi M, Maruyama TA (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 35:1054–1062
Clement M, Posada D, Crandall KA (2000) TCS: a computer program to estimate gene genealogies. Mol Ecol 9:1657–1659
Coffroth MA, Lewis CL, Santos SR, Weaver JL (2006) Environmental populations of symbiotic dinoflagellates in the genus *Symbiodinium* can initiate symbiosis with reef cnidarians. Curr Biol 16:R985–R988
Correa AMS, Baker AC (2009) Understanding diversity in coral-algal symbiosis: a cluster-based approach to interpreting fine-scale genetic variation in the genus *Symbiodinium*. Coral Reefs 28: 81–93
Díez B, Pedrós-Alió C, Massana R (2001) Study of genetic diversity of eukaryotic picoplankton in different oceanic regions by small-subunit rRNA gene cloning and sequencing. Appl Environ Microbiol 67:2932–2941
Edmunds PJ, Gates RD, 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 124:185–193
Fitt WK (1985) Effect of different strains of zooxanthellae *Symbiodinium microadriaticum* on growth and survival of their coelenterate and molluscan hosts. Proc 5th Int Coral Reef Symp 6:131–136
Gou W, Sun J, Zhen Y, Xin Z, Yu Z, Li R (2003) Phylogenetic analysis of a free-living strain of *Symbiodinium* isolated from Jiaozhou Bay, P.R. China. J Exp Mar Biol Ecol 296:135–144
Goulet TL, Simmons C, Goulet D (2008) Worldwide biogeography of *Symbiodinium* in tropical octocorals. Mar Ecol Prog Ser 355: 45–58
Granados C, Camargo C, Zea S, Sánchez JA (2008) Phylogenetic relationships among zooxanthellae (*Symbiodinium*) associated to excavating sponges (*Cliona* spp.) reveal an unexpected lineage in the Caribbean. Mol Phylogenet Evol 49:554–560
Gray JS (1988) Organelle origins and ribosomal RNA. Biochem Cell Biol 66:325–348
GuoFu C, GuangCe W, ChunYun Z, BaiCheng Z (2008) Morphological and phylogenetics analysis of a *Gymnodinium*-like species from the Chinese Coast. Chin Sci Bull 53:561–567
Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp 41:95–98
Harris EH, Boynton JE, Gillham NW (1994) Chloroplast ribosomes and protein synthesis. Microbiol Rev 58:700–754
Harrison PL, Wallace CC (1990) Coral reproduction. In: Dubinsky Z (ed) Ecosystems of the world: coral reefs. Elsevier, Amsterdam
Hill R, Ralph PJ (2007) Post-bleaching viability of expelled zooxanthellae from the scleractinian coral *Pocillopora damicornis*. Mar Ecol Prog Ser 352:137–144
Hill M, Allenby A, Ramsby B, Schonberg C, Hill A (2011) *Symbiodinium* diversity among host clionaid sponges from Caribbean and Pacific reefs: evidence of heteroplasmy and putative host-specific symbiont lineages. Mol Phylogenet Evol 59:81–88
Hirose M, Yamamoto H, Nonaka M (2008a) Metamorphosis and acquisition of symbiotic algae in planula larvae and primary polyps of *Acropora* spp. Coral Reefs 27:247–254
Hirose M, Reimer JD, Hidaka M, Suda S (2008b) Phylogenetic analyses of potentially free-living *Symbiodinium* spp. isolated from coral reef sand in Okinawa, Japan. Mar Biol 155:105–112
Jones R, Yellowlees JD (1997) Regulation and control of intracellular algae (zooxanthellae) in hard corals. Philos Trans R Soc Lond Ser B 352:457–468
Kinzie RA III, Chee GS (1979) The effect of different zooxanthellae on the growth of experimentally reinfected hosts. Biol Bull 156: 315–327
Kinzie RA III, Takayama M, Santos SR, Coffroth MA (2001) The adaptive bleaching hypothesis: Experimental tests of critical assumptions. Biol Bull 200:51–58
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
Krupp DA (1983) Sexual reproduction and early development of the solitary coral *Fungia scutaira* (Antozoa: Scleractinia). Coral Reefs 2:159–164
LaJeunesse TC (2002) Diversity and community structure of symbiotic dinoflagellates from Caribbean coral reefs. Mar Biol 141: 387–400
LaJeunesse TC (2005) "Species" radiations of symbiotic dinoflagellates in the Atlantic and Indo-Pacific since the Miocene-Pliocene transition. Mol Biol Evol 22:570–581
LaJeunesse TC, Thornhill DJ, Cox EF, Stanton FG, Fitt WK, Schmidt GW (2004a) High diversity and host specificity observed among symbiotic dinoflagellates in reef coral communities from Hawaii. Coral Reefs 23:596–603
LaJeunesse TC, Bhagooli R, Hidaka M, deVantier L, Done T, Schmidt GW, Fitt WK, Hoegh-Guldberg O (2004b) Closely related *Symbiodinium* spp. differ in relative dominance in coral reef host communities across environmental, latitudinal and biogeographic gradients. Mar Ecol Prog Ser 284:147–161
Li WKW (2002) Macroecological patterns of phytoplankton in the northwestern North Atlantic Ocean. Nature 419:154–157
Little F, van Oppen MJH, Willis BL (2004) Flexibility in algal endosymbioses shapes growth in reef corals. Science 304:1492–1494
Littman RA, van Oppen MJH, Willis BL (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
Manning M, Gates RD (2008) Diversity in populations of free-living *Symbiodinium* from a Caribbean and Pacific reef. Limnol Oceanogr 53:1853–1861
Moreira D, López-García P (2002) The molecular ecology of microbial eukaryotes unveils a hidden world. Trends Microbiol 10:31–38
Mouritsen LT, Richardson K (2003) Vertical microscale patchiness in nano- and microplankton distributions in a stratified estuary. J Plankton Res 25:783–797
Muscatine L, Porter JW (1977) Reef corals: Mutualistic symbioses adapted to nutrient-poor environments. Bioscience 27:454–460
Nozawa Y, Harrison PL (2005) Temporal settlement patterns of larvae of the broadcast spawning reef coral *Favites chinensis* and the broadcast spawning and brooding reef coral *Goniastrea aspera* from Okinawa, Japan. Coral Reefs 24:274–282
Pochon X, Gates RD (2010) A new *Symbiodinium* clade (Dinophyceae) from soritid foraminifera in Hawai'i. Mol Phylogenet Evol 56:492–497
Pochon X, Pawlowski J, Zaninetti L, Rowan R (2001) High genetic diversity and relative specificity among Symbiodiniumlike endosymbiotic dinoflagellates in soritid foraminiferans. Mar Biol 139:1069–1078
Pochon X, LaJeunesse TC, Pawloski J (2004) Biogeographic partitioning and host specialization among foraminiferan

dinoflagellate symbionts (*Symbiodinium*; Dinophyta). Mar Biol 146:17–27

Pochon X, Montoya-Burgos JI, 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, Garcia-Cuetos L, Baker AC, 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 26:867–882

Pochon X, Stat M, Takabayashi M, Chasqui L, Chauka L, Logan D, Gates R (2010) Comparison of endosymbiotic and free-living *Symbiodinium* (Dinophyceae) diversity in a Hawaiian reef environment. J Phycol 46:53–65

Porto I, Granados C, Restrepo JC, Sanchez JA (2008) Macroalgal-associated dinoflagellates belonging to the genus *Symbiodinium* in Caribbean reefs. PLoS ONE 3:e2160

Richmond RH, Hunter CL (1990) Reproduction and recruitment of corals: comparisons among the Caribbean, the tropical Pacific and the Red Sea. Mar Ecol Prog Ser 60:185–203

Rowan R (2004) Thermal adaptation in reef coral symbionts. Nature 430:742

Rowan R, Knowlton N, Baker A, Jara J (1997) Landscape ecology of algal symbionts creates variation in episodes of coral bleaching. Nature 388:265–269

Samanata D, Mukhopadhyay D, Chowdhury S, Ghosh J, Pal S, Basu A, Bhattacharya A, Das A, Das D, DasGupta C (2008) Protein folding by Domain V of *Escherichia coli* 23S rRNA: Specificity of RNA-protein interactions. J Bacteriol 190:3344–3352

Santos SR, Taylor DJ, Coffroth MA (2001) Genetic comparisons of freshly isolated versus cultured symbiotic dinoflagellates: Implications for extrapolating to the intact symbiosis. J Phycol 37: 900–912

Santos SR, Taylor DJ, Kinzie RA III, Hidaka M, Sakai K, Coffroth MA (2002) Molecular phylogeny of symbiotic dinoflagellates inferred from partial chloroplast large subunit (23S)-rDNA sequences. Mol Phylogenet Evol 23:97–111

Santos SR, Guiterrez-Rodriguez C, Coffroth MA (2003) Phylogenetic identification of symbiotic dinoflagellates via length heteroplasmy in Domain V of chloroplast large subunit (cp23S)-ribosomal DNA sequences. Mar Biotechnol 5:130–140

Schönberg CHL, Loh W (2005) Molecular identity of the unique symbiotic dinoflagellates found in the bioeroding demosponge *Cliona orientalis* Thele, 1900. Mar Ecol Prog Ser 299:157–166

Schönberg CHL, Suwa R, Hidaka M, Loh WKW (2008) Sponge and coral zooxanthellae in heat and light: preliminary results of photochemical efficiency monitored with pulse amplitude modulated fluorometry. Mar Ecol 29:247–258

Stat M, Carter D, Hoegh-Guldberg O (2006) The evolutionary history of *Symbiodinium* and scleractinian hosts-symbiosis, diversity, and the effect of climate change. Perspect Plant Ecol Evol Syst 8:23–43

Stat M, Morris E, Gates RD (2008) Functional diversity in coral-dinoflagellate symbiosis. Proc Nat Acad Sci USA 105:9256–9261

Stat M, Pochon X, Cowie ROM, Gates RD (2009) Specificity in communities of *Symbiodinium* in corals from Johnston Atoll. Mar Ecol Prog Ser 386:83–96

Stimson J, Kinzie R (1991) The diel pattern of release of zooxanthellae by colonies of *Pocillopora damicornis* maintained under control and nitrogen-enriched conditions. J Exp Mar Biol Ecol 153:63–74

Taylor FJR, Hoppenrath M, Saldarriaga JF (2008) Dinoflagellate diversity and distribution. Biodivers Conserv 17:407–418

Thornhill DJ, Lajeunesse TC, Santos SR (2007) Measuring rDNA diversity in eukaryotic microbial systems" how intragenomic variation, pseudogenes, and PCR artifacts confound biodiversity estimates. Mol Ecol 16:5326–5340

Thornhill DJ, Xiang Y, Fitt WK, Santos SR (2009) Reef endemism, host specificity and temporal stability in populations of symbiotic dinoflagellates from two ecologically dominant Caribbean corals. PLoS One 4:e6262

Unrein F, Izaguirre I, Massana R, Balagué V, Gasol JM (2005) Nanoplankton assemblages in maritime Antarctic lakes: characterization and molecular fingerprinting comparison. Aqua Microb Ecol 40:269–282

van Oppen MJH, Mahiny AJ, Done TJ (2005) Geographic distribution of zooxanthella types in three coral species on the Great Barrier Reef sampled after the 2002 bleaching event. Coral Reefs 24:482–487

Venera-Ponton DE, Diaz-Pulido G, Rodriguez-Lanetty M, Hoegh-Guldberg O (2010) Presence of *Symbiodinium* spp. in macroalgal microhabitats from the southern Great Barrier Reef. Coral Reefs 29:1049–1060

Werner U, Blazejak A, Bird P, Eickert G, Schoon R, Abed RMM, Bissett A, de Beer D (2008) Microbial photosynthesis in coral reef sediments (Heron Reef, Australia). Estuar Coast Shelf Sci 76:876–888

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