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Molecular phylogeny of symbiotic dinoflagellates inferred from partial chloroplast large subunit (23S)-rDNA sequences

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Abstract

Symbiotic associations between invertebrates and dinoflagellates of the genus *Symbiodinium* are a common occurrence in marine environments. However, despite our extensive knowledge concerning the physiological contributions of these algae to their symbiotic partners, our understanding of zooxanthella phylogenetics is still in its early stages. In the past 10 years, studies of *Symbiodinium* phylogenetics have relied solely on nuclear ribosomal (rDNA) genes. To date, organellar DNA sequences have not been employed to infer phylogenies for this genus of symbiotic dinoflagellates. We address this by presenting the first *Symbiodinium* phylogeny based on chloroplast (cp) large subunit (23S)-rDNA sequences. Cp23S-rDNA Domain V sequences were determined for 35 dinoflagellate cultures isolated from a range of invertebrate host species and geographical locations. *Symbiodinium* phylogenies inferred from cp23S-rDNA produced topologies that were not statistically different from those generated from nuclear rDNA, providing the first independent evidence supporting the published major clades of *Symbiodinium*. In addition, comparisons of sequence dissimilarity indicated that cp23S-rDNA Domain V evolves 9–30 times faster than the V1–V4 regions of nuclear small subunit (n18S)-rDNA, 1–7 times as fast as the D1–D3 regions of nuclear large subunit (n28S)-rDNA, and 0.27–2.25 times that of the internal transcribed spacer (ITS)-rDNA region. Our data suggested that cp23S-rDNA Domain V will prove to be a useful molecule for exploring *Symbiodinium* phylogenetics.

Keywords: Chloroplast; Large subunit ribosomal genes; Dinoflagellate; *Symbiodinium*; Symbiosis; Symbiotic dinoflagellates; Zooxanthellae

1. Introduction

Symbiotic associations between invertebrates and dinoflagellates are a common occurrence in marine environments. These dinoflagellates, collectively known as zooxanthellae, predominately belong to the genus *Symbiodinium* (Freudenthal, 1962). The physiological contribution of these dinoflagellates to the symbiosis, including translocation of photosynthetically fixed carbon and assimilation and conservation of nitrogen, have been extensively studied over the past 60 years and has been shown to play a vital role in their host's nutrition (reviewed in Davies, 1993). However, despite our extensive knowledge concerning the physiological contributions of these algae to their symbiotic partners, our understanding of zooxanthella phylogenetics is still in its early stages. This is unfortunate since phylogenetic information is essential in answering important questions concerning these algae and their symbiotic relationships. For example, exploring questions of host–symbiont coevolution and specificity requires explicit knowledge of zooxanthella phylogenetics. Furthermore, an understanding of phylogenetics allows ecological patterns and exceptions to such patterns to be explained within an evolutionary context.

Early researchers assumed that all zooxanthellae belonged to a single pandemic species, *Symbiodinium microadriaticum* (Freudenthal) (Taylor, 1974). However, biochemical (Schoenberg and Trench, 1980a), morpho-

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logical (Schoenberg and Trench, 1980b; Trench and Blank, 1987), infectivity (Schoenberg and Trench, 1980c), motility pattern (Fitt et al., 1981), karyotyping (Blank and Trench, 1985), DNA/DNA hybridization (Blank and Huss, 1989), and DNA base composition (Blank et al., 1988) studies on cultured materials in the 1980s demonstrated that zooxanthellae comprise a heterogeneous group of many species and strains (Trench, 1993; Rowan, 1998). Currently, there are seven genera of dinoflagellates, belonging to four orders, that are recognized as having symbiotic members (Banaszak et al., 1993). Within the genus *Symbiodinium*, four species have been formally described (Trench and Blank, 1987) while six additional taxa have been named but have not been formally described (reviewed in Trench, 1997).

Although these studies established the existence of symbiotic dinoflagellate diversity, many of the techniques applied possessed shortcomings when addressing questions of zooxanthella phylogenetics. For example, application of these techniques requires zooxanthella cultures, which are difficult to establish (Schoenberg and Trench, 1980a) and can result in exclusion of some unculturable forms (Rowan et al., 1996; Carlos et al., 2000; Santos et al., 2001). This would result in some zooxanthellae being inadvertently excluded from phylogenetic studies and restricts studies to small sample sizes. In addition, diagnostic morphological characters of vegetative and motile stages of zooxanthellae may be reduced or not readily apparent depending on life history, stage in the cell cycle, or specifics of the preparation of cells for analysis (Trench and Blank, 1987), making reliance on these characters difficult. Adding to this, morphology does not necessarily delineate evolutionarily cohesive groups within symbiotic dinoflagellates (Wilcox, 1998). For these reasons, these techniques have had limited use in zooxanthella phylogenetics (Schoenberg and Trench, 1980a–c; Blank et al., 1988; Blank and Huss, 1989).

The recent widespread application of DNA-based molecular techniques to phylogenetic studies has lead to more comprehensive phylogenetic studies of *Symbiodinium* from reef-building invertebrates. Rowan and Powers (1991a) were the first to analyze *Symbiodinium* nuclear small subunit (n18S) ribosomal DNA (rDNA) genes using restriction fragment length polymorphisms (RFLPs) and DNA sequencing. Rowan and Powers (1991b) found several distinct n18S-rDNA RFLP patterns distributed within a number of host species. In addition, sequence analysis of n18S-rDNA revealed that diversity within the genus *Symbiodinium* is comparable to that observed among orders of free-living dinoflagellates (Rowan and Powers, 1992). Many authors have also employed n18S-rDNA to explore questions of *Symbiodinium* phylogenetics (McNally et al., 1994; Carlos et al., 1999; Goulet, 1999; Darius et al., 2000). Data from other nuclear rDNAs have also been applied to *Symbiodinium* phylogenetics, including nuclear large subunit (n28S)-rDNA (Wilcox, 1998; Baker, 1999; Pawlowski et al., 2001; Pochon et al., 2001) and the internal transcribed spacer (ITS)-rDNA (Hunter et al., 1997; Baillie et al., 2000; LaJeunesse, 2001), which includes n5.8S-rDNA. These studies separate *Symbiodinium* into several large clades, also known as phylotypes, types, or lineages (e.g., Clades A, B, C (Rowan and Powers, 1991a,b), D (Carlos et al., 1999), and E and F (LaJeunesse, 2001)). A *Symbiodinium* phylogeny generated from n5.8S-rDNA (LaJeunesse, 2001) groups Clade A into one assemblage while Clades B/C/D/E/F form a second, closely related group (Fig. 1). Within B/C/D/E/F, Clades D and E are basal to Clades B/C/F while Clades C and F are sister groups (Fig. 1). Adding to this, Pawlowski et al. (2001) and Pochon et al. (2001) have presented phylogenies that suggest that the symbiotic dinoflagellates from foraminifera form several additional and distinct groups within the *Symbiodinium* species complex, particularly within Clades B/C/D/E/F.

To date, *Symbiodinium* phylogenies have relied solely on members of the nuclear rDNA gene family. Independent genetic evidence, such as sequences from an organellar genome, has not been employed to infer phylogenies for the genus *Symbiodinium*. To address this, we have constructed a *Symbiodinium* phylogeny based on chloroplast large subunit (23S)-rDNA sequences. Such an analysis may shed new light on relationships and evolution within this genus of symbiotic dinoflagellates. For example, chloroplast genes may uncover hidden diversity in *Symbiodinium* since dinoflagellate chloroplast genes exhibit an accelerated evolutionary rate (Zhang et al., 2000; Tengs et al., 2000). In

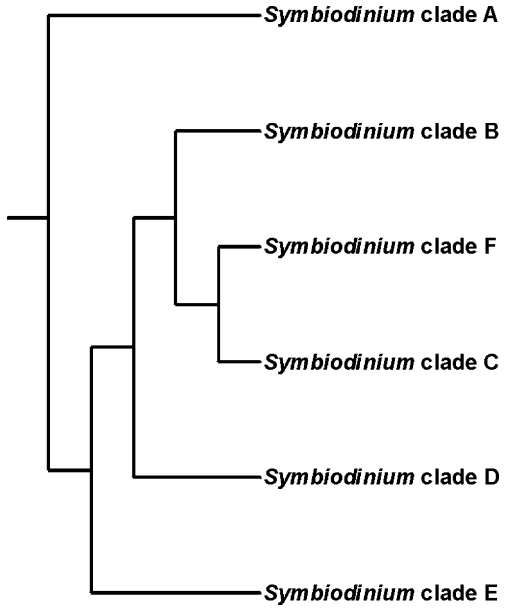

Fig. 1. Phylogenetic relationships between *Symbiodinium* clades inferred from nuclear 5.8S-rDNA (LaJeunesse, 2001).

addition, analyses of chloroplast DNA sequences from bryophytes (Shaw and Goffinet, 2000) and woody perennials (Hargid et al., 2000) have resulted in phylogenies significantly different from those inferred from nuclear DNAs due to events such as reticulate evolution and introgression, respectively. Furthermore, there is a need to acquire sequence data from more symbiotic dinoflagellates, especially those from relatively unstudied geographical regions, to elucidate the molecular systematics of these organisms. For instance, the symbiotic dinoflagellates of only two invertebrate hosts from Okinawa, Japan have been examined at the molecular level (Lee et al., 1995; Baillie et al., 2000). In this study, we present the first molecular phylogeny of *Symbiodinium* based on a region of cp23S-rDNA, expand the molecular characterizations of symbiotic dinoflagellates from a range of invertebrate hosts and geographic locations, and further refine the phylogenetics of this enigmatic group of dinoflagellates.

2. Materials and methods

2.1. Restriction fragment length polymorphism analysis of dinoflagellate nuclear small subunit (n18s)-rDNA

Algal cultures, isolated from a range of invertebrate hosts and geographic locations or acquired from the Provasoli–Guillard National Center for Culture of Marine Phytoplankton (CCMP), were maintained as described in Santos et al. (2001) and employed as a source of DNA (Table 1). These isolates have been in culture for 1–20 years and were initiated either from a single cell (Table 1) or from bulk inoculum from a single host. Approximately 10^6 cells were harvested from 4-week-old cultures and collected by centrifugation at 700*g* for 5 min. Total nucleic acids were extracted and quantified according to the methods of Coffroth et al. (1992). In addition, Dr. Tadashi Maruyama (Marine Biotechnology Institute, Kamaishi Laboratories, Japan) kindly provided us with DNA from the dinoflagellate culture PSP1-05, isolated from the host *Haliclona koremella* (Carlos et al., 1999). Dinoflagellate nuclear small subunit (n18S)-rDNA was amplified by PCR from these isolates using the primers ss5 and ss3z according to Rowan and Powers (1991b) and digested with *Taq*I restriction enzyme. Digestion products were separated by electrophoresis in 2% 0.5× Tris–borate (TBE) agarose gels to generate RFLP patterns. RFLP analysis of n18S-rDNA PCR products separates *Symbiodinium* into several large clades (e.g., *Symbiodinium* Clades A, B, C (Rowan and Powers, 1991a,b), D (Carlos et al., 1999), and E (*S. californium*; LaJeunesse and Trench, 2000; LaJeunesse, 2001)) with each clade probably being composed of many species (Rowan, 1998). RFLP patterns were compared with cloned standards or to the literature to assign each culture to one of these established *Symbiodinium* n18S-rDNA RFLP clades.

2.2. Amplification and sequencing of dinoflagellate cp23S-rDNA domain V

An approximately 0.7-kb region of dinoflagellate cp23S-rDNA, corresponding to Domain V of the cp23S-rDNA molecule (Harris et al., 1994), was PCR amplified from the same isolates as those above using the primer pair 23S1M13 (5′-CACGACGTTGTAAAA CGACGG CTGTAACTATAACGGTCC-3′) and 23S2M13 (5′-GGATAACAATTTCACACAGGCCATCGTATTGA ACCCAGC-3′). These primers were modified from Zhang et al. (2000) (see below). PCRs were performed in 50-μL volumes containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2.0 mM $MgCl_2$, 0.001% gelatin, 200 μM dNTPs, 100 pmol of each primer, 2 U *Taq* polymerase, and 50–70 ng of template DNA. Reactions were carried out in a MJ Research PTC-100 thermocycler (MJ Research Inc., Watertown, MA) under the following conditions: initial denaturing period of 1 min at 95 °C, 35 cycles consisting of 95 °C for 45 s, 55 °C for 45 s, and 72 °C for 1 min, and a final extension period of 7 min. PCR products were purified by electrophoresis in 2% 1× modified Tris–acetate (0.04 M Tris–acetate, 0.0001 M EDTA final concentration) agarose gels and visualized by ethidium bromide staining and long-wavelength ultraviolet light. Purified PCR products were recovered from the excised agarose gel blocks by centrifugation using Spin-X 0.22-μm CA centrifuge tube filters (Costar Corp., Corning, NY) according to the manufacturer's directions.

The first 19 and 20 5′-nucleotides of 23S1M13 and 23S2M13, respectively, allow direct nucleotide sequencing of PCR products using 5′-IRD700 and 5′-IRD800 fluorescent-labeled M13 primers (LI-COR Biotechnology Division, Lincoln, NE). For most samples, nucleotide sequences were determined in this manner. In some cases, cp23S-rDNA PCR products were gel purified as above and cloned using the TOPO TA Cloning kit (Invitrogen, Carlsbad, CA) according to the manufacturer's directions prior to sequencing (Table 1). PCR products employed in cloning were generated with the primer pair 23S1 and 23S2 (Zhang et al., 2000), which lacked the 5′-nucleotides required for direct sequencing, under the same PCR conditions as above. Prior to sequencing, 10 clones were screened by PCR amplification with primers 23S1/23S2 and 2% 0.5× TBE agarose gel electrophoresis for appropriately sized inserts. Bacterial clones chosen for sequencing (4–8 clones per sample) were grown overnight in 1.5 mL of Luria–Bertani broth and plasmids isolated by alkaline lysis extraction (Birnboim, 1983). Bidirectional sequencing of PCR products and plasmids was performed with the SequiTherm EXCEL II DNA Sequencing Kit-LC for

25- to 41-cm gels (Epicentre Technologies, Madison, WI) according to the manufacturer's directions and read with LI-COR's NEN Global IR2 DNA Sequencer System (LI-COR Biotechnology Division). Forward and reverse sequences for each sample were aligned using Sequencher 3.0.1 (Gene Codes Corp., Ann Arbor, MI) and ambiguities corrected by comparison to the reverse sequence or coded using the IUPAC ambiguity codes. Sequences were deposited in GenBank under accession numbers listed in Table 1.

2.3. Phylogenetic analyses of dinoflagellate cp23S-rDNA domain V

For the phylogenetic analyses, cp23S-rDNA sequences from the free-living dinoflagellates *Heterocapsa*

Table 1
Sample information for dinoflagellate cultures included in the chloroplast-23S-rDNA Domain V phylogenetic study

n18S-RFLP designation	Host organism	Culture name	Collection location	GenBank accession no.
Symbiodinium Clade A	*Aiptasia pallida*	FLAp#4	Florida Keys	AY035404
	Cassiopea sp.	CassEL1	Kaneohe Bay, HI	AY035410
	Cassiopea sp.	CassKB8	Kaneohe Bay, HI	AY035405
	Cassiopea sp.	CassMJ300	Kaneohe Bay, HI	AY035411
	C. xamachana	Cx	Jamaica	AY035406
	C. xamachana	FLCass	Florida Keys	AY035407
	Plexaura kuna (polyp)[b]	Pk708[a]	San Blas Islands, Panama	AY035408
	Pseudoplexaura porosa (polyp)[b]	Pp719[a]	San Blas Islands, Panama	AY035409
	Tridacna gigas	T[c]	Indo-Pacific	AY035412
	Unknown host	Y109	Okinawa, Japan	AY035413
	Zoanthus sociatus	Zs	Jamaica	AY035414
Symbiodinium Clade B	*A. pallida*	FLAp#3[c]	Florida Keys	AY055235[d]
	A. pulchella	HIAp	Kaneohe Bay, HI	AY035421
	A. pulchella	OkAp#10	Okinawa, Japan	AY035416
	Briareum asbestinum (polyp)[b]	#571[a]	Florida Keys	AY035415
	Briareum asbestinum (polyp)[b]	#579[a]	Florida Keys	AY055239
	Plexaura flexuosa	PurPflex	Florida Keys	AY035420
	P. kuna	Pk13[c]	Florida Keys	AY055231[d]
	P. kuna (polyp)[b]	Pk702[a]	San Blas Islands, Panama	AY035419
	P. kuna (polyp)[b]	Pk706[a,c]	San Blas Islands, Panama	AY055232[d]
	Pocillopora damicornis	Pd[c]	Hawaii	AY055236[d]
	Porites evermanni	Pe	Hawaii	AY035418
	Pseudopterogorgia elisabethae	SSPe	Bahamas	AY035417
Symbiodinium Clade C	*Mastigias* sp.	Mp	Palau	AY035424
	Montipora verrucosa	Mv	Kaneohe Bay, HI	AY035422
	Sinularia sp.	Sin	Guam	AY035423
	Unknown anemone[e]	Ua#31	Okinawa, Japan	AY035425
Symbiodinium Clade D	*Acropora* sp.	A001	Okinawa, Japan	AY035427
	Acropora sp.	A002	Okinawa, Japan	AY035428
	Acropora bruegemanni	A024	Okinawa, Japan	AY035429
	Haliclona koremella	PSP1-05	Palau	AY055241
	Unknown anemone[e]	Ua#2	Okinawa, Japan	AY035426
Symbiodinium Clade E	Free-living	CCMP421[a]	Wellington Harbor, New Zealand	AY055240
Free-living dinoflagellate	*P. kuna* (polyp)[b]	JN120.1[a]	San Blas Islands, Panama	AY035430
	P. damicornis (planula)	Pdam3N	Kaneohe Bay, HI	AY055242

[a] Cultures which were started from a single dinoflagellate cell.
[b] A polyp is defined as a newly settled and metamorphosed planula.
[c] Cultures which possess cp23S-rDNA Domain V molecules with deletion mutations.
[d] GenBank accession number of sequence generated from bacterial clone.
[e] Tentatively identified as *Entacmaea quadricolor* (D. Fautin, University of Kansas, personal communication). This anemone harbors *Symbiodinium* Clade C and a unique genotype of *Symbiodinium* Clade D, which possesses copies of n18S-rDNA that contain deletion mutations. (S.R. Santos, unpublished).

triquetra (AF130039) and *Protoceratium reticulatum* (AF206702) were acquired from GenBank and included in the analyses. *P. reticulatum* was employed as the outgroup. Sequences were initially aligned using ClustalX (Thompson et al., 1997) and manually adjusted using Se-Al v2.0a7b (http://evolve.zoo.ox.ac.uk/software/Se-Al/Se-Al.html) to account for the derived secondary structure of Domain V in *Symbiodinium* cp23S-rDNA (Santos et al., 2002). Area (b) of *Symbiodinium* cp23S-rDNA Domain V (Santos et al., 2002) was excluded from all phylogenetic analyses due to length and sequence variation between the *Symbiodinium* clades that made alignment unreliable. This resulted in 184 characters (positions 207–390) being excluded from all analyses. This alignment is available from TreeBase (http://www.treebase.org) as a Nexus file (study Accession No. S672 and matrix Accession No. M1055) and was employed to construct maximum-parsimony (MP) phylogenetic trees using PAUP*4.0b8 (Swofford, 2000). For the MP analysis, 822 characters (64 parsimony-uninformative characters, 185 parsimony-informative characters) were used under a heuristic search option and gaps treated as missing data. The MP tree was constructed by optimizing the characters with accelerated transformation, 10 repetitions of random sequence additions, starting trees obtained by stepwise addition, and branches swapped by tree bisection–reconnection (TBR). Support for branches in the MP tree was tested by bootstrap analysis of 1000 replicates.

For the maximum-likelihood (ML) analysis, a second, more conservative, cp23S-rDNA alignment was generated from matrix Accession No. M1055 by excluding all gapped positions. This resulted in the exclusion of an additional 309 characters. The final alignment employed in the ML analyses possessed 513 unambiguously aligned characters and is available from TreeBase under matrix Accession No. 1054. To determine the best-fit model of DNA evolution, the alignment was subjected to hierarchical likelihood ratio tests in Modeltest v3.06 (Posada and Crandall, 1998). The ML tree was constructed under the model of evolution and parameters estimated from Modeltest v3.06 ($F81 + \Gamma$, log likelihood $= -2229.9695$; nucleotide frequencies estimated from the data by Modeltest v3.06, four rate categories, $\alpha = 0.3553$) and employed the heuristic search option, sequence additions AS-IS, starting trees obtained by stepwise addition, and branches swapped by TBR. Support for branches in the ML tree was tested by bootstrap analysis of 100 replicates. Tests of topological congruency were conducted with Shimodaira–Hasegawa tests (SH-tests; Shimodaira and Hasegawa, 1999), using a RELL bootstrap of 1000 replicates, as implemented in PAUP*4.0b8. For the SH-tests, ML trees, with (L_1) and without (L_2) constraints, were constructed using the heuristic search option under the same parameters as above.

2.4. Estimation of sequence dissimilarity in Symbiodinium cp23S-rDNA and nuclear rDNAs

Rates of evolution of *Symbiodinium* cp23S-rDNA and nuclear rDNAs were computed for *Symbiodinium* n18S-rDNA RFLP Clades A, B, C, and D according to the methods of Kuzoff et al. (1998). Briefly, comparisons between cp23S-rDNA, n18S-rDNA, n28S-rDNA, and ITS-rDNA were done in the following manner. Nuclear rDNA sequences, generated from the same samples from which cp23S-rDNAs were sequenced, were acquired from two to four representatives of Clades A, B, C, and D (Table 2; *Symbiodinium* n18S-rDNA RFLP Clade E was excluded since only a single sample was available). The V1–V4 regions of n18S-rDNA, the D1–D3 regions of n28S-rDNA, the entire nuclear ITS regions (ITS1, 5.8S, and ITS2), and the cp23S-rDNA Domain V sequences were aligned for each clade using ClustalX (Thompson et al., 1997). The best-fit model of DNA evolution for each molecule was determined using Modeltest v3.06 (Posada and Crandall, 1998). Because the complexity of the best-fit model of DNA evolution differed for the various rDNA molecules and *Symbiodinium* clades (data not shown), the Hasegawa et al. (1985) model of sequence evolution was chosen for the analysis. Sequence dissimilarity was calculated using PAUP*4.0b8. The average sequence dissimilarity values for each molecule were than calculated for *Symbiodinium* n18S-rDNA RFLP Clades A, B, C, and D and employed to compare inferred rates of sequence evolution.

3. Results

RFLP analysis of n18S-rDNA revealed that 33 of the 35 algal cultures included in our study belonged to *Symbiodinium* n18S-rDNA RFLP Clades A, B, C, D, or E (Table 1 and Fig. 2). One of the *Symbiodinium* Clade D isolates, Ua#2, produced copies of n18S-rDNA which contain deletion mutations. These aberrant molecules, which are apparent following PCR amplification and contribute to a unique *Taq*I-generated RFLP pattern (Fig. 2, lane 5), will be characterized elsewhere (S. R. Santos, unpublished). Two cultures, JN120.1 and Pdam3N, isolated from a newly settled polyp of the Caribbean gorgonian *Plexaura kuna* and a planula of the Pacific scleractinian *Pocillopora damicornis*, respectively, produced an RFLP pattern that was not consistent with those of the recognized *Symbiodinium* clades (Fig. 2, lane 8).

PCR amplifications from the various *Symbiodinium* cultures with primer pair 23S1M13 and 23S2M13 produced either one or two PCR products, depending on *Symbiodinium* clade and isolate. The larger PCR product, common to all samples, ranged in size (approxi-

Table 2
Sources of *Symbiodinium* sequences used to compare rates of evolution of chloroplast (cp23S-rDNA) and nuclear (n18S-rDNA, n28S-rDNA, and ITS-rDNA) rDNAs

n18S-RFLP Designation	cp23S-rDNA Taxa	n18S-rDNA[a] Taxa	n28S-rDNA[b] Taxa	ITS-rDNA[c] Taxa
Symbiodinium Clade A	*Aiptasia pallida* (FLAp#4) [AY035404] *Cassiopea xamachana* (Cx) [AY035406] *Tridacna gigas* (T) [AY035412] *Zoanthus sociatus* (Zs) [AY035414]	*Aiptasia pallida* (FLAp#4) [AY427441] *Cassiopea xamachana* (Cx) [AF427442] *Tridacna gigas* (T) [AF427443] *Zoanthus sociatus* (Zs) [AF427444]	*Aiptasia pallida* (FLAp#4) [AF427453] *Cassiopea xamachana* (Cx) [AF427454] *Tridacna gigas* (T) [AF427455] *Zoanthus sociatus* (Zs) [AF427456]	*Aiptasia pallida* (FLAp#4) [AF427465] *Cassiopea xamachana* (Cx) [AF427466] *Tridacna gigas* (T) [AF427467] *Zoanthus sociatus* (Zs) [AF427468]
Symbiodinium Clade B	*Aiptasia pulchella* (HIAp) [AY035421] *Plexaura flexuosa* (PurPflex) [AY035422] *Plexaura kuna* (Pk13) [AY055231] *P. kuna* (Pk702) [AY035419]	*Aiptasia pulchella* (HIAp) [AF427445] *Plexaura flexuosa* (PurPflex) [AF427448] *Plexaura kuna* (Pk13) [AF427446] *P. kuna* (Pk702) [AF427447]	*Aiptasia pulchella* (HIAp) [AF427457] *Plexaura flexuosa* (PurPflex) [AF427460] *Plexaura kuna* (Pk13) [AF427458] *P. kuna* (Pk702) [AF427459]	*Aiptasia pulchella* (HIAp) [AF360564] *Plexaura flexuosa* (PurPflex) [AF360574] *Plexaura kuna* (Pk13) [AF360559] *P. kuna* (Pk702) [AF360575]
Symbiodinium Calde C	*Montipora verrucosa* (Mv) [AY035422] *Sinularia* sp. (Sin) [AY035423] Unknown anemone (Ua#31) [AY035425]	*Mastigias* sp. (Mp) [AF427449] *Montipora verrucosa* (Mv) [AF427450] *Sinularia* sp. (Sin) [AF427451] Unknown anemone (Ua#31) [AF427452]	*Mastigias* sp. (Mp) [AF427461] *Montipora verrucosa* (Mv) [AF427462] Unknown anemone (Ua#31) [AF427463]	*Mastigias* sp. (Mp) [AF427469] *Montipora verrucosa* (Mv) [AF360577] *Sinularia* sp. (Sin) [AF360576] Unknown anemone (Ua#31) [AF427470]
Symbiodinium Clade D	*Acropora bruegemanni* (A024) [AY035429] *Haliclona koremella* (PSP1-05) [AY055241]	*Acropora bruegemanni* (A024) [AF396624] *Haliclona koremella* (PSP1-05) [AB016578]	*Acropora bruegemanni* (A024) [AF396627] *Haliclona koremella* (PSP1-05 [AF427464]	n.d.[d]

Note. Culture name is in parentheses following host name. GenBank accession number is in square brackets.

[a] Amplified with primers ss5M13 (5′-CACGACGTTGTAAAACGACGGTTGATCCTGCCAGTAGTCATATGCTTG-3′) and ssE21.6M13 (5′-GGATAACAATTTCACACAGGCTAGAAACCAACAAAATAGAACTGAGGTC-3′) under standard conditions (Rowan and Powers, 1991a) and sequenced directly from PCR products as described under Materials and methods.

[b] PCR primers (ls1.5 and ls1.3; Wilcox (1998) modified with 5′-nucleotides as described under Materials and methods. Amplification conditions presented in Wilcox (1998). Sequenced directly from PCR products as described Materials and methods.

[c] PCR primers, amplification, and sequencing conditions presented in Santos et al. (2001).

[d] Sequences not determined.

mately 620–800 bp on 2% agarose gels) depending on *Symbiodinium* clade. The smaller PCR product, which appeared in 1 of 11 and 4 of 12 *Symbiodinium* Clades A and B cultures (Table 1), respectively, ranged in size from approximately 300 to 500 bp on 2% agarose gels. These smaller PCR products were consistently amplified from these cultures under various PCR conditions, such as adjusting $MgCl_2$ concentrations and primer annealing temperatures (data not shown), and were sequenced to determine their identity. Sequence analyses of these smaller PCR products suggested that they are cp23S-rDNA gene copies that have suffered internal deletions (Santos et al., 2002). Cp23S-rDNA sequences obtained from internally deleted molecules were excluded from

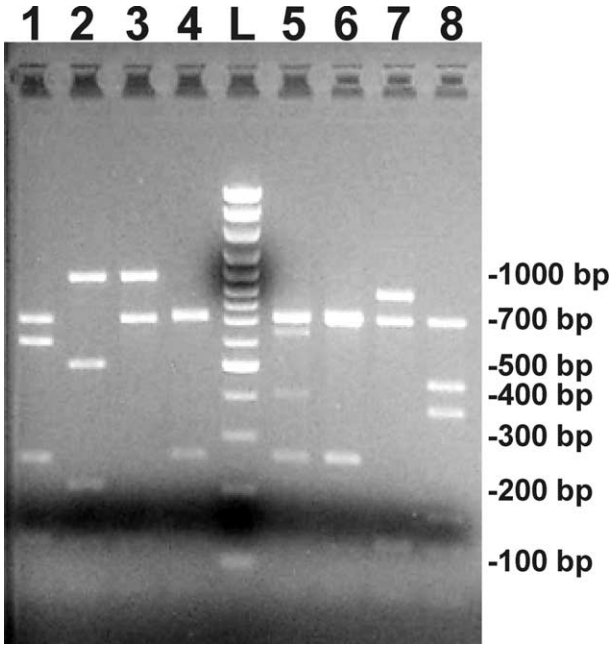

Fig. 2. n18S-rDNA *Taq*I-generated RFLPs from representative dinoflagellate cultures employed in this study. 1, *Cassiopea xamachana* [Cx] (A); 2, *Plexaura flexuosa* [PurPflex] (B); 3, *Montipora verrucosa* [Mv] (C); 4, *Acropora bruegemanni* [A024] (D); L, 100-bp ladder; 5, Unknown anemone [Ua#31] (D); 6, *Haliclona koremella* [PSP1-05] (D); 7, free-living *Symbiodinium* sp. [CCMP421] (E); 8, *Pocillopora damicornis* [Pdam3N]. Square brackets enclose culture names. Letter in parentheses represents *Symbiodinium* n18S-rDNA RFLP clade.

the phylogenetic analyses and are characterized and discussed elsewhere (Santos et al., 2002). Cultures JN120.1 and Pdam3N produced single PCR products of approximately 700 bp.

For the dinoflagellate cp23S-rDNA Domain V sequences, phylogenetic trees constructed under MP and ML criteria gave similar relationships. For both methods of phylogenetic analyses, cultures JN120.1 and Pdam3N were found to group with the free-living dinoflagellate *Heterocapsa triquetra* with strong (100% bootstrap) support (Figs. 3 and 4). These results were consistent with those of the n18S-rDNA RFLP analysis (see above), which suggested that these isolates were not members of the genus *Symbiodinium*. For the dinoflagellate cultures whose n18S-rDNA RFLPs belonged to the recognized *Symbiodinium* clades, these isolates form a distinct assemblage with strong (100% bootstrap) support. Two principle groups, one comprising *Symbiodinium* Clade A while the second encompasses *Symbiodinium* Clades B/C/D/E/F, were identified within the *Symbiodinium* assemblage with moderate to high (54–97% bootstrap) support in the MP and ML analyses (Figs. 3 and 4). For both methods of phylogenetic analyses, the branching orders within *Symbiodinium* Clades B/C/D/E/F were nearly identical. However, subtle topological differences within *Symbiodinium* Clades B/C/D/E/F were apparent in the MP and ML analyses.

For both the MP and the ML analyses, *Symbiodinium* Clades C and F, which possessed identical n18S-rDNA RFLP patterns, were sister groups with moderate to high (67–86% bootstrap) support while *Symbiodinium* Clade B is a sister group to *Symbiodinium* Clades C/F with moderate (65–70% bootstrap) support (Figs. 3 and 4). Branching basal to *Symbiodinium* Clades B/C/F is *Symbiodinium* Clade E, with moderate (52–62% bootstrap) support. Finally, members of *Symbiodinium* Clade D occured basal to *Symbiodinium* Clades B/C/E/F. However, the position of the isolate from *Haliclona koremella*, a member of *Symbiodinium* n18S-rDNA RFLP Clade D, varied depending on the method of phylogenetic reconstruction. In the MP analysis, the *H. koremella* isolate remained as an unresolved lineage, distinct from other members of *Symbiodinium* Clade D (Fig. 3) while the ML analysis placed the same isolate with other members of *Symbiodinium* Clade D at moderate (71% bootstrap) support (Fig. 4). In both phylogenetic trees, the branch leading to the *H. koremella* isolate was extremely long relative to those of the other *Symbiodinium* isolates. For example, in the ML tree (Fig. 4), the branch length of the *H. koremella* isolate (0.18472 substitutions/site) was 6.2× longer than the second longest branch (0.03003 substitutions/site) within the *Symbiodinium* (Clade C isolates). Furthermore, the *H. koremella* branch was approximately 2× longer than the second most divergent *Symbiodinium* isolate (0.09641 substitutions/site) included in the analysis (Clade B isolate *Briareum asbestinum* #579). Excluding the *H. koremella* isolate from the MP and ML analyses resulted in the same phylogenetic relationships within *Symbiodinium* Clades B/C/D/E/F and increased the bootstrap supports within this group (Figs. 3 and 4).

The phylogenetic relationships among the *Symbiodinium* clades inferred from cp23S-rDNA Domain V by MP and ML criteria were nearly identical to those inferred from n5.8-rDNA (Fig. 1). However, the position of *Symbiodinium* Clade E varied between the chloroplast and the nuclear rDNA phylogenies. For cp23S-rDNA Domain V, *Symbiodinium* Clade E was basal to *Symbiodinium* Clades B/C/F while the position of *Symbiodinium* Clade E was basal to *Symbiodinium* Clades B/C/D/F in the n5.8S-rDNA phylogeny (Fig. 1; LaJeunesse, 2001). Shimodaira–Hasegawa tests revealed no significant difference ($\delta = L_1 - L_2 = -4.03634; P = 0.167$) between a topology constrained to reflect the phylogenetic relationships inferred by n5.8S-rDNA (L_1; log likelihood = −2220.88747) and that of cp23S-rDNA Domain V (L_2; log likelihood = −2216.85113).

The rate of evolution of *Symbiodinium* cp23S-rDNA Domain V varied by clade (Table 3). *Symbiodinium* Clades A and B possess similar rates while the rates of

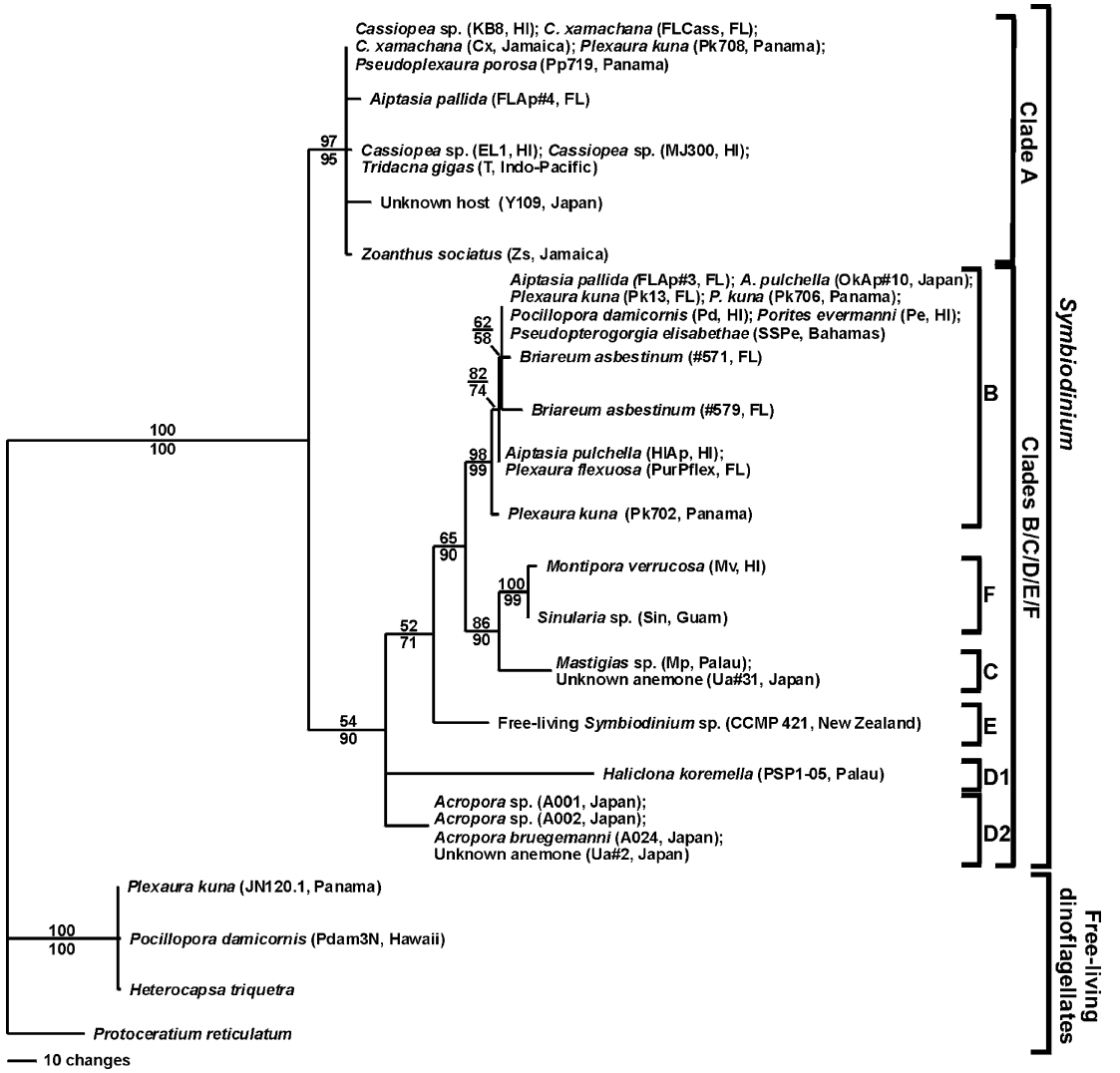

Fig. 3. Phylogenetic tree constructed by maximum-parsimony (MP) of *Symbiodinium* and free-living dinoflagellate cp23S-rDNA Domain V sequences. Values above and below line represent bootstrap support values of nodes when the *Haliclona koremella* isolate (PSP1-05) is included and excluded, respectively, from the MP analysis. Bootstrap support values are percentages of 1000 resamplings. Culture name and collection location (in parentheses) follow the name of the organism from which the dinoflagellate culture was isolated. The length of the most parsimonious tree was 436 steps with a consistency index (CI) of 0.77.

Clades C and D were higher than those of Clades A and B (Table 3). These higher values for *Symbiodinium* Clades C and D, across all of the molecules that were examined, represent the divergence of members within these clades. Examples of this divergence included the ability to spilt *Symbiodinium* n18S-rDNA RFLP Clade C into Clades C and F with cp23S-rDNA Domain V (see above) and n5.8S-rDNA (LaJeunesse, 2001) sequences and the extreme divergence of the *H. koremella* isolate from other *Symbiodinium*, including members of *Symbiodinium* Clade D (see above). For all clades, cp23S-rDNA Domain V exhibited a level of base substitution that was approximately 9–30 times higher than that of the V1–V4 regions of n18S-rDNA. For *Symbiodinium* Clades A, C, and D, the level of base substitution in cp23S-rDNA Domain V is approximately 1–2 times that in the D1–D3 regions of n28S-rDNA, but much lower than that in the nuclear ITS region (e.g., *Symbiodinium* Clades A and C; Table 3). In the case of *Symbiodinium* Clade B, cp23S-rDNA Domain V exhibited levels of base substitution approximately 7 and 2.25 times higher than that of n28S-rDNA and ITS-rDNA, respectively. Finally, *Symbiodinium* cp23S-rDNA Domain V sequences possessed AT compositions ranging between 58 and 67% (Table 4). These values are consistent with those of free-living dinoflagellates and are among the highest recorded for chloroplast and eubacterial 23S-rDNA (Zhang et al., 2000). This high AT composition may play an important role in the creation of aberrant (internally deleted) *Symbiodinium* cp23S-rDNA molecules by replication slippage (Santos et al., 2002).

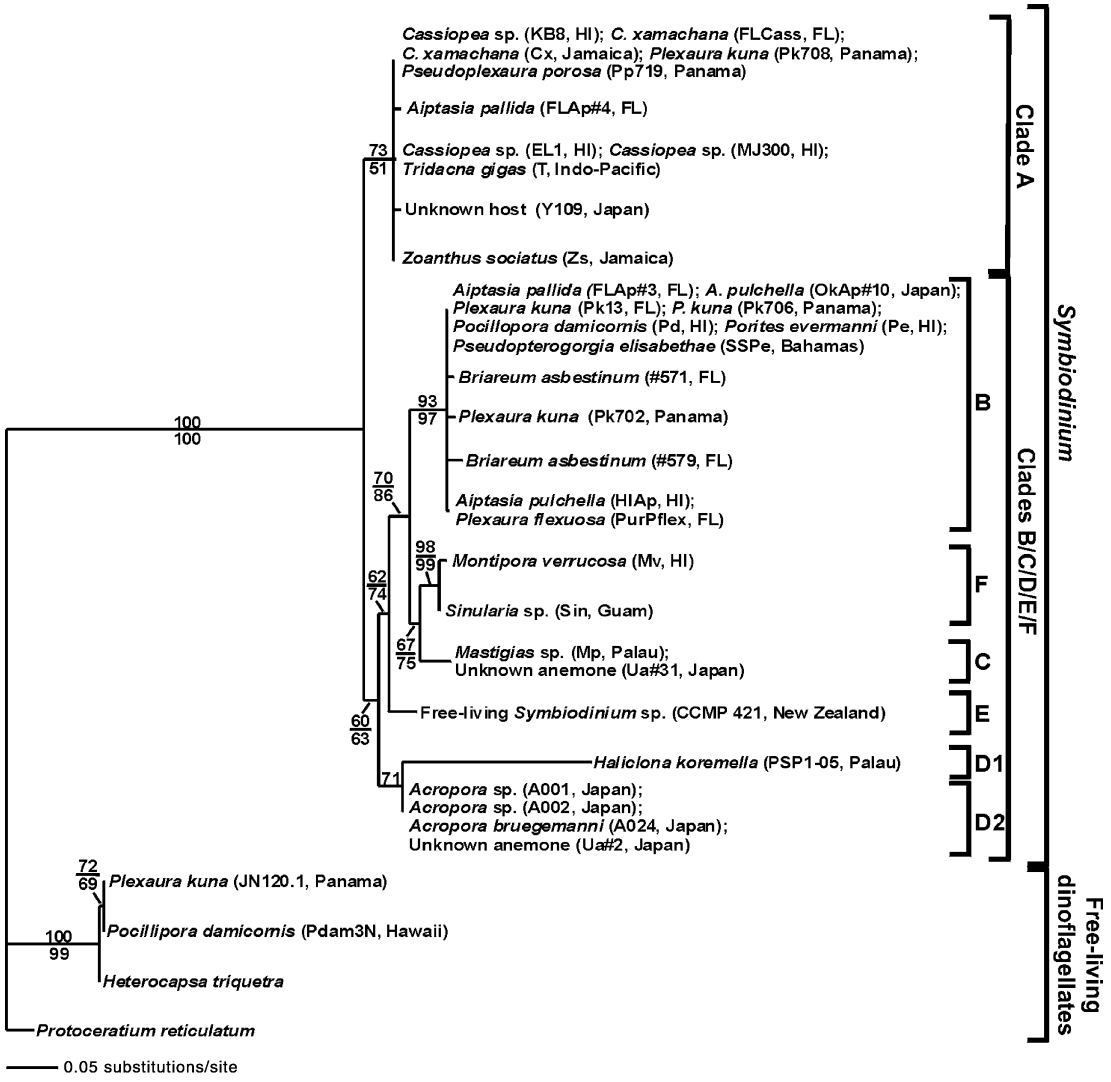

Fig. 4. Maximum-likelihood (ML) phylogenetic tree constructed from *Symbiodinium* and free-living dinoflagellate cp23S-rDNA Domain V sequences (ln likelihood = −2235.13286). Values above and below line represent bootstrap support values of nodes when the *Haliclona koremella* isolate (PSP1-05) is included and excluded, respectively, from the ML analysis. Bootstrap support values are percentages of 100 resamplings. Culture name and collection location (in parentheses) follow the name of the organism from which the dinoflagellate culture was isolated.

Table 3
Average sequence dissimilarities of *Symbiodinium* chloroplast (cp23S-rDNA) and nuclear (n18S-rDNA, n28S-rDNA, and ITS-rDNA) rDNA sequences calculated under the Hasegawa et al. (1985) (HKY85) model of sequence evolution

n18S-RFLP Designation	cp23S-rDNA [Domain V]	n18S-rDNA [V1–V4]	n28S-rDNA [D1–D3]	ITS-rDNA [ITS1,5.8S, ITS2]
Symbiodinium Clade A	0.02488 (629)	0.0027 (747)	0.02588 (758)	0.0911 (709)
Symbiodinium Clade B	0.02848 (697)	0.00094 (747)	0.0041 (748)	0.01265 (664)
Symbiodinium Clade C	0.05763 (629)	0.00643 (752)	0.07264 (793)	0.16813 (738)
Symbiodinium Clade D	0.21165 (752)	0.01769 (750)	0.11434 (800)	n.d.[a]

Note. Areas of each molecule included in the analysis are in square brackets. Values in parentheses are number of characters included in the analysis.

[a] Value not determined.

Table 4
Base composition of chloroplast (cp) 23S-rDNA Domain V from representative *Symbiodinium* and free-living dinoflagellate sequences employed in this study

n18S-RFLP designation	Host organism	AT%	Sites (bp)
Symbiodinium Clade A	*Cassiopea xamachana* (Cx)	58.35	581
	Aiptasia pallida (FLAp#4)	58.24	613
	Tridacna gigas (T)	58.01	624
	Unknown host (Y109)	59.28	609
	Zoanthus sociatus (Zs)	58.33	612
Symbiodinium Clade B	*Briareum asbestinum* (#571)	61.39	619
	Briareum asbestinum (#579)	61.91	659
	A. pulchella (OkAp#10)	62.01	616
	Plexaura kuna (Pk702)	60.64	630
	Plexaura flexuosa (PurPflex)	61.69	663
Symbiodinium Clade C	*Montipora verrucosa* (Mv)	60.93	604
	Sinularia sp. (Sin)	60.43	604
	Unknown anemone (Ua#31)	62.65	597
Symbiodinium Clade D	*Haliclona koremella* (PSP1-05)	66.89	604
	Acropora bruegemanni (A024)	62.43	700
Symbiodinium Clade E	Free-living *Symbiodinium* sp. (CCMP421)	59.62	634
Free-living dinoflagellates	*Heterocapsa* sp. (JN120.1)	63.35	693
	Heterocapsa sp. (Pdam3N)	64.24	674
	Heterocapsa triquetra	64.44	689
	Protoceratium reticulatum	66.14	815

Note. Values presented are for the entire region that was sequenced. Culture name is in parentheses following host name.

4. Discussion

During the past 10 years, our understanding of the evolutionary relationships among the symbiotic dinoflagellates has expanded dramatically, due in large part to the use of molecular sequence data. Currently, 12 clades of *Symbiodinium* are recognized. These include *Symbiodinium* Clades A, B, C, D, E, and F, with additional clades appearing to be specific to the soritid foraminifera (Pawlowski et al., 2001; Pochon et al., 2001). The relationships between these *Symbiodinium* clades have been established using a variety of nuclear rDNAs, such as n18S-rDNA (Carlos et al., 1999; Darius et al., 2000; McNally et al., 1994; Rowan and Powers, 1991a), ITS-rDNA (Baillie et al., 2000; Hunter et al., 1997; LaJeunesse, 2001), and n28S-rDNA (Baker, 1999; Pawlowski et al., 2001; Pochon et al., 2001; Wilcox, 1998). In all cases, *Symbiodinium* Clade A forms one group while *Symbiodinium* Clades B/C/D/E/F and the foraminifera symbionts form a second, closely related group. Our *Symbiodinium* phylogeny, the first based on an organellar genome for these organisms, possesses a nearly identical topology for *Symbiodinium* Clades A/B/C/D/E/F. However, the relationships within the *Symbiodinium* B/C/D/E/F group, based on cp23S-rDNA Domain V sequences, appear to differ from those inferred from nuclear rDNAs.

In our cp23S-rDNA phylogeny, the positions of *Symbiodinium* Clades D and E are reversed compared to those from nuclear rDNA (compare Fig. 1 to Figs. 3 and 4). Tests of topological congruency (SH-tests) reveal no significant difference between our *Symbiodinium* cp23S-rDNA phylogeny and one constrained to reflect the phylogenetic relationships inferred by n5.8S-rDNA. The lack of significant difference between the topologies may be attributed to the low amount of divergence between the clades of the *Symbiodinium* B/C/D/E/F group inferred from cp23S-rDNA Domain V. Cp23S-rDNA Domain V was chosen for this analysis since it is fairly well conserved in sequence (Harris et al., 1994), even in the dinoflagellates (Zhang et al., 2000; see below), and it will be interesting to determine whether additional sequence data from cp23S-rDNA or other chloroplast genes will statistically support this alternative *Symbiodinium* phylogeny. However, until additional chloroplast sequences or phylogenies become available, the *Symbiodinium* cp23S-rDNA Domain V phylogenies presented here are congruent with phylogenies constructed from nuclear rDNAs and provide the first independent genetic evidence that supports the cladal relationships of *Symbiodinium*.

The congruence between *Symbiodinium* chloroplast and nuclear phylogenies found in our study was unexpected. In the free-living dinoflagellates, phylogenies based on chloroplast genes do not always result in the same topology as nuclear genes. For example, phylogenies based on the chloroplast-encoded protein *psbA* ordered dinoflagellate taxa according to their branch lengths and not to their known affinities based on n18S-rDNA (Zhang et al., 2000). The reasons for these discrepancies are thought to stem from marked differences in evolutionary rates in taxa due to the systematically ultrarapid evolutionary rate of the dinoflagellate chloroplast genome (Tengs et al., 2000; Zhang et al., 2000) and resulting long-branch artifacts (Zhang et al., 2000) during phylogenetic reconstruction. This has led to the idea that chloroplast genes are less suitable than nuclear genes for accurately reconstructing phylogenies for the dinoflagellates (Zhang et al., 2000). However, this is not the case for the genus *Symbiodinium*. Our results are similar to what has been observed for plastid and nuclear phylogenies inferred for members of the Apicomplexa (Lang-Unnasch et al., 1998), whose nonphoto- synthetic plastids share a common origin with dinoflagellate chloroplasts (Fast et al., 2001). Lang-Unnasch et al. (1998) found that plastid rDNA phylogenies supported the cladal divisions and relationships among the five major groupings of Apicomplexa established by nuclear rDNA. Thus, our study suggests that *Symbiodinium* chloroplast and nuclear rDNAs share a

common evolutionary history. Taken together, these observations suggest that chloroplast genes may not be suitable for inferring phylogenetic relationships among widely divergent dinoflagellate taxa. However, it appears that for closely related groups of dinoflagellates, such as *Symbiodinium*, chloroplast genes may be useful molecules with which to address phylogenetic questions. Research into this area deserves further attention.

Comparisons of sequence dissimilarity for *Symbiodinium* nuclear rDNAs suggest that levels of base substitution vary substantially between the molecules. For example, the V1–V4 regions of n18S-rDNA exhibit much lower values of sequence dissimilarity than those of the D1–D3 regions of n28S-rDNA. These data are consistent with the idea that the variable domains of n28S-rDNA are more appropriate than those of n18S-rDNA for phylogenetic studies of symbiotic dinoflagellates (Wilcox, 1998). Cp23S-rDNA Domain V also evolves at a much faster rate than the V1–V4 regions of n18S-rDNA but appears to evolve at approximately the same rate as the D1–D3 regions of n28S-rDNA. These results suggest that cp23S-rDNA Domain V provides approximately the same amount of phylogenetically informative characters as the D1–D3 region of n28S-rDNA. However, this is only true for intracladal comparisons since one region of *Symbiodinium* cp23S-rDNA Domain V (area (b) of Santos et al., 2002) cannot be reliably aligned between the clades (see Materials and methods). ITS-rDNA exhibits a much higher level of base substitution than cp23S-rDNA Domain V. This is not unexpected since ITS-rDNA, being a noncoding spacer region, is predicted to have much fewer selective constraints than coding regions (Schlotterer et al., 1994). As with area (b) of cp23S-rDNA Domain V, however, *Symbiodinium* ITS-rDNA sequences are highly divergent and cannot be aligned between *Symbiodinium* clades (LaJeunesse, 2001). Thus, *Symbiodinium* cp23S-rDNA Domain V appears to be as useful a molecule as the D1–D3 regions of n28S-rDNA for exploring intracladal *Symbiodinium* phylogenetics while being less informative than ITS-rDNA at the same level.

For *Symbiodinium* Clade B, comparisons of sequence dissimilarity reveal a trend not seen in the other clades. *Symbiodinium* B exhibits higher levels of base substitution in cp23S-rDNA Domain V relative to any of the nuclear rDNAs that were examined, including ITS-rDNA. However, *Symbiodinium* Clade B cp23S-rDNA Domain V possesses sequence dissimilarity values similar to those of *Symbiodinium* Clade A, suggesting that cp23S-rDNA Domain V is evolving at approximately the same rate in these clades. For the nuclear rDNA molecules, *Symbiodinium* Clade B exhibits a level of base substitution approximately three to seven times lower than that of *Symbiodinium* Clades A and C. Taken together, these results suggest that the rate of evolution has slowed in the nuclear genome of *Symbiodinium* Clade B. The reasons behind this reduced evolutionary rate for the nuclear rDNAs of *Symbiodinium* Clade B are unknown but research into this phenomenon may provide important information into the molecular evolution of symbiotic dinoflagellates.

Several of the dinoflagellate isolates used in our study were found to be members of the free-living dinoflagellates or unusual members of the genus *Symbiodinium*. Cultures JN120.1 and Pdam3N, which were isolated from a newly settled polyp of the Caribbean gorgonian *Plexaura kuna* and a planula of the Pacific scleractinian *Pocillopora damicornis*, respectively, produced an RFLP pattern that was not consistent with those of the recognized *Symbiodinium* clades. Phylogenetic analyses of cp23S-rDNA Domain V also excluded these isolates from the genus *Symbiodinium* and strongly supported their membership in the free-living dinoflagellate genus *Heterocapsa*. The most likely explanation for these cultures is that JN120.1 and Pdam3N were inadvertently isolated from the surface or within the digestive system of these cnidarians while attempting to isolate and culture members of *Symbiodinium*. These data expand the idea that nonrepresentative isolates can result from attempts to culture *Symbiodinium* (Santos et al., 2001). These results may also extend to culture PSP1-05, isolated from the Palauan sponge *H. koremella*. Carlos et al. (1999) questioned whether this isolate is a symbiont of *H. koremella* or a free-living dinoflagellate because of the manner in which it was isolated. Although our cp23S-rDNA Domain V phylogeny, along with the nuclear rDNA phylogenies of other authors (Carlos et al., 1999; Pochon et al., 2001), places culture PSP1-05 within the genus *Symbiodinium*, the reasons behind its high level of divergence relative to other members of the *Symbiodinium* warrants exploration. One possibility is that PSP1-05 represents a free-living dinoflagellate lineage that is derived from a member of *Symbiodinium* Clade D. The derivation of free-living forms from symbionts may be common in groups where symbionts have some capacity for living independently and hosts have multiple potential symbionts (Hibbett et al., 2000). Hibbett et al. (2000) suggest that invertebrate–dinoflagellate symbioses fit the description of such associations. Unfortunately, while evolutionary rate changes have been observed to occur in transitions to a symbiotic lifestyle (Moran, 1996; Lutzoni and Pagel, 1997), data to determine whether changes in evolutionary rate occur when symbionts make a transition to the free-living state are currently unavailable. This possibility and the determination of whether culture PSP1-05 is part of the *H. koremella* symbiont population *in hospite* or whether PSP1-05 is capable of forming stable symbiotic associations with invertebrate hosts should be explored.

The identity of culture CCMP421, which was obtained from the CCMP, also needs to be clarified. Historically, this culture has been considered to be an

isolate of *Gymnodinium varians*, cultured from a water sample taken in Wellington Harbor, New Zealand by Dr. F. Hoe Chang. However, over the past several years, phylogenetic studies based on n18S-rDNA (Rowan and Powers, 1992) and n28S-rDNA (Wilcox, 1998) have placed this isolate within the genus *Symbiodinium*. In addition, recent work further supports the idea that culture CCMP421 has been misidentified as a member of the genus *Gymnodinium* and is, in fact, a member of the genus *Symbiodinium* (Saldarriaga et al., 2001). Our cp23S-rDNA Domain V phylogenies also support the idea that CCMP421 has been misidentified. With this overwhelming evidence, CCMP421 should be referred to as a member of the *Symbiodinium*. Future work should be done to characterize the relationship between CCMP421 and *S. californium* (Banaszak et al., 1993), the only other symbiotic dinoflagellate (recognized to date) that possesses a *Symbiodinium* Clade E n18S-rDNA RFLP pattern (LaJeunesse and Trench, 2000).

The results of our cp23S-rDNA Domain V study also demonstrate the ability to discriminate between different strains of *Symbiodinium*. For example, 16 unique cp23S-rDNA Domain V sequences were recovered from the 33 *Symbiodinium* isolates included in the study. Given the fact that *Symbiodinium* culturing is highly selective and that many members of *Symbiodinium* have, so far, proved to be unculturable (reviewed in Santos et al., 2001), the diversity uncovered in this study is conservative. Thus, it is probable that additional cp23S-rDNA diversity will be discovered from *Symbiodinium* populations *in hospite*. On the other hand, within some clades (e.g., *Symbiodinium* Clades A and B), many *Symbiodinium* cultures analyzed in this study shared identical cp23S-rDNA sequences. For example, identical cp23S-rDNA Domain V sequences were obtained from *Symbiodinium* Clade A cultures isolated from *Cassiopea* sp. (jellyfish) and newly settled polyps of *P. kuna* and *Pseudoplexaura porosa* (both gorgonians). For *Symbiodinium* Clade B, isolates from *Aiptasia pulchella* (anemone) and *Plexaura flexuosa* (gorgonian) possessed the same cp23S-rDNA Domain V sequences. In many cases, cultures that possessed identical cp23S-rDNA Domain V sequences were obtained from the Pacific and the Caribbean (Figs. 3 and 4). For example, we have found that the *Symbiodinium* Clade D cultures isolated from Okinawa possess cp23S-rDNA Domain V sequences identical to those of the *in hospite* symbionts from *Palythoa* sp. in the Florida Keys (data not shown).

Table 5
Studies that have employed the *Symbiodinium* cultures whose chloroplast (cp) 23S-rDNA domain V sequences are presented in this analysis

n18S-RFLP designation	Culture name in this study	Culture name in previous studies	Previous studies
Symbiodinium Clade A	CassKB8	*Cassiopea medusa* (Oahu)	Kinzie and Chee (1982)
		Cassiopea KB8 (Hawaii)	Kinzie et al. (2001), Taguchi and Kinzie (2001)
	Cx	*Cassiopea xamachama* (Caribbean)	Kinzie and Chee (1979), Kinzie et al. (2001)
		Cassiopea sp. (Jamaica)	Kinzie and Chee (1982)
	FLAp#4	FLAp#4	Santos et al. (2001)
	Pk708	*Plexaura kuna* 93.3.1, 93.3.2	Coffroth et al. (2001)
	Pp719	*Pseudoplexaura porosa* 42.9.1, 42.9.2, 42.9.3	Coffroth et al. (2001)
	T	*Tridacna gigas* (Central Pacific)	Kinzie et al. (2001), Santos et al. (2001)
	Zs	*Zoanthus sociatus* (Caribbean)	Kinzie et al. (2001)
Symbiodinium Clade B	FLAp#3	FLAp#3	Santos et al. (2001)
	HIAp	*Aiptasia pulchella* (Hawaii)	Kinzie and Chee (1979), Kinzie and Chee (1982), Kinzie et al. (2001), Santos (1995), Santos et al. (2001)
	Pd	*Pocillopora damicornis* (Hawaii)	Kinzie et al. (1984), Santos et al. (2001)
	PurPflex	PurPflex	Santos et al. (2001)
	Pk13	Pk13	Goulet and Coffroth (1997), Santos et al. (2001)
	Pk702	Pk702	Goulet and Coffroth (1997), Santos et al. (2001)
		Plexaura kuna 21.4.1, 21.4.2	Coffroth et al. (2001)
	Pk706	Pk706	Goulet and Coffroth (1997), Santos et al. (2001)
		Plexaura kuna 72.2.1, 72.2.2	Coffroth et al. (2001)
	SSPe	SSPe	Santos et al. (2001)
Symbiodinium Clade C	Mp	*Mastigias* sp. (Palau)	Santos et al. (2001)
	Mv	*Montipora verrucosa* (Hawaii)	Kinzie et al. (1984), Kinzie et al. (2001), Santos et al. (2001)
	Sin	Sin	Santos et al. (2001)
Symbiodinium Clade D	PSP1-05	PSP1-05	Carlos et al. (1999)
Symbiodinium Clade E	CCMP421	CCMP421	Rowan and Powers (1992), Wilcox (1998), Saldarriaga et al. (2001)

Note. See references for additional details.

Many authors (Baillie et al., 2000; Baker, 1999; Carlos et al., 1999; Darius et al., 2000; LaJeunesse, 2001; Rowan and Powers, 1991a,b; Rowan and Powers, 1992; Santos et al., 2001) have reported similar results from nuclear rDNA data. Thus, our cp23S-rDNA data and other authors' nuclear rDNA data imply that groups of closely related zooxanthellae are cosmopolitan and have the ability to form relationships with phylogenetically diverse hosts. However, it remains unknown whether *Symbiodinium* isolates that possess identical rDNA sequences, either in the chloroplast or in the nuclear genomes, are equivalent at the physiological and/or infectivity level.

The *Symbiodinium* cultures whose cp23S-rDNA Domain V sequences are presented here have been employed in a number of previous studies (Table 5). By comparing the results of these previous studies to the relationships inferred from *Symbiodinium* cp23S-rDNA Domain V, additional insight into both studies can be gathered. For example, Kinzie et al. (2001) demonstrated that growth rates of cultured zooxanthellae from different hosts varied according to temperature. Four *Symbiodinium* Clade A isolates (cultures CassKB8, Cx, T, and Zs) were included in Kinzie et al. (2001) and the cultures responded in the following ways: CassKB8 and Cx responded in a similar fashion while the growth rates of T and Zs decreased and increased with increasing temperature, respectively. Thus, three unique responses are evident, with two of the four cultures exhibiting the same response. Comparison of these results with the cp23S-rDNA Domain V phylogenies presented in this study reveals that cultures CassKB8 and Cx possessed identical sequences while cultures T and Zs possessed unique sequences of their own. These preliminary results suggest a correlation between physiological response and cp23S-rDNA Domain V sequence. Additional research into the correlation between chloroplast rDNA and protein gene sequences and physiological response may provide unique insight into important ecological processes, such as coral bleaching.

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