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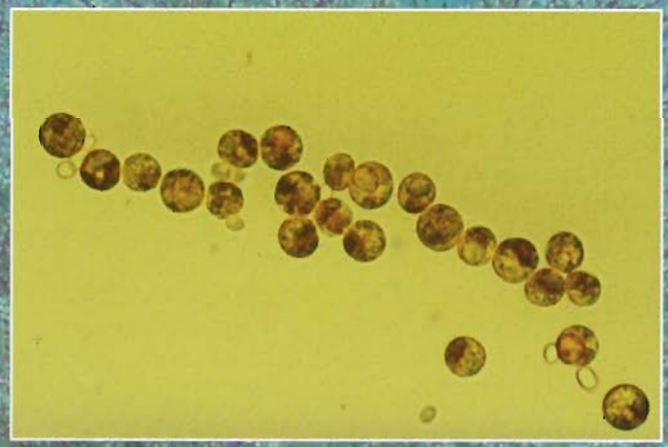

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On the Cover: Many marine invertebrates, such as the Caribbean gorgonian *Pseudopterogorgia elisabethae* (photo by S. Voegeli), harbor symbiotic dinoflagellates belonging to the genus *Symbiodinium* (insert photo by A. Siegel, Microscopy Facility, Dept. biological Sciences, SUNY at Buffalo). Within the chloroplast genome of *Symbiodinium*, the length of a hypervariable region of domain V of the large-subunit (cp23S) ribosomal DNA (rDNA) area is unique among different strains of *Symbiodinium* and can be used as a tool to accurately and rapidly identify members of this dinoflagellate genus. See article by Santos et al. (this issue, pp. 130–140) for details.

SHORT COMMUNICATIONS

ORIGINAL CONTRIBUTIONS

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MARINE BIOTECHNOLOGY

Original Contributions

Phylogenetic Identification of Symbiotic Dinoflagellates via Length Heteroplasmy in Domain V of Chloroplast Large Subunit (cp23S)–Ribosomal DNA Sequences

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Abstract: A protocol that takes advantage of length heteroplasmy in domain V of chloroplast large subunit (cp23S)–ribosomal DNA to identify members of the symbiotic dinoflagellate genus *Symbiodinium* is presented. This protocol is highly specific for *Symbiodinium*, can provide intercladal and intracladal identification of a particular *Symbiodinium* isolate, and can detect multiple *Symbiodinium* chloroplast genotypes simultaneously in the same isolate, making his technique attractive for a variety of research questions. We used this technique to characterize variation among *Symbiodinium* populations associated with a range of phylogenetically diverse and geographically discrete hosts. We also examined symbiont variation within a single host, the Caribbean gorgonian *Pseudopterogorgia elisabethae*, from 9 sites in the Bahamas, and we report a previously undocumented level of symbiont specificity for particular members of *Symbiodinium* clade B in this gorgonian.

Key words: chloroplast, large subunit ribosomal genes, dinoflagellate, length heteroplasmy, *Symbiodinium*, zooxanthellae.

INTRODUCTION

Dinoflagellates are a diverse group of unicellular eukaryotes. In aquatic environments, many dinoflagellates are major photosynthetic and heterotrophic members of the planktonic community. Some dinoflagellates are capable of forming mutualistic symbioses with various invertebrates (reviewed in Taylor, 1974), while others have been implicated in fish kill events or can produce toxins that have adverse effects on fish or humans (reviewed in Bowers et al., 2000). Because of the ecologic and economic importance of dinoflagellates, efforts have been made to develop specific and rapid methodologies to identify members of these protists. Many of these methodologies are based on nuclear ribosomal DNA (nrDNA) sequences. For example, a polymerase chain reaction (PCR) assay for detection of the harmful algal bloom (HAB) species *Pfiesteria piscicida* Steidinger and Burkholder (Steidinger et al., 1996) and related dinoflagellates has been presented based on nuclear small subunit (n18S)–rDNA (Bowers et al., 2000). For the symbiotic dinoflagellates, restriction fragment length polymorphism (RFLP) analysis of n18S–rDNA has been used to differentiate members of the genus *Symbiodinium* Freudenthal (Taylor, 1974) into distinct clades

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A, B, and C (Rowan and Powers, 1991), with each clade probably being composed of many species (Rowan, 1998). RFLP analysis of nuclear large subunit (n28S)–rDNA has also been used to distinguish *Symbiodinium* isolates (Baker, 1999). Techniques such as denaturing-gradient gel electrophoresis (DGGE) of the hypervariable V1 + V2 region of *Symbiodinium* n18S–rDNA have been employed to identify intercladal and intracladal variation in *Symbiodinium* (Carlos et al., 2000; Belda-Baillie et al., 2002), while protocols have been reported that distinguish *Symbiodinium* isolates based on DGGE (Baillie et al., 2000; LaJeunesse, 2001) or single-stranded conformational polymorphism (SSCP) (van Oppen et al., 2001) of nuclear ribosomal internal transcribed spacer (nITS–rDNA) regions.

Only 2 studies have employed organellar (mitochondrial or plastid, e.g., chloroplast) DNA as a method to rapidly identify and distinguish members of the dinoflagellates. A highly specific PCR protocol to distinguish *P. piscicida* from other closely related dinoflagellates based on mitochondrial cytochrome *b (cob)* has been presented (Zhang and Lin, 2002), while chloroplast small subunit (cp16S)–rDNA has been employed in the specific detection of the toxic dinoflagellate *Gymnodinium galatheanum* Braarud in field samples (Tengs et al., 2001). In both of these studies, organellar DNA was chosen because these molecules *(cob* and cp16S–rDNA) or genomes (the dinoflagellate chloroplast) tend to have an elevated evolutionary rate, leading to sequence variation and differentiation between closely related taxa that may not be apparent from nuclear genes (Zhang et al., 2000; Tengs et al., 2000, 2001; Santos et al., 2002a; Zhang and Lin, 2002). Thus far, a technique to rapidly and specifically identify symbiotic dinoflagellates belonging to the genus *Symbiodinium* using organellar DNA is unavailable. Such a technique would be useful in identifying members of these dinoflagellates as well as determining their population structure and dynamics in their invertebrate hosts. Futhermore, a technique based on the chloroplast genome would circumvent the possibility of contamination from host DNA.

In this study we introduce a novel technique that identifies *Symbiodinium* isolates based on length heteroplasmy in chloroplast large subunit (cp23S)–rDNA domain V. This technique allows rapid intercladal and intracladal identification of *Symbiodinium* isolates as well as their placement in a phylogeny based on cp23S–rDNA domain V sequences. Multiple *Symbiodinium* chloroplast genotypes can be detected simultaneously in the same sample, making this technique attractive for a variety of research questions. We present a broad survey of symbiont populations isolated from hosts collected from around the world to demonstrate the utility of this technique.

Lastly, we present data generated with this technique for the *Symbiodinium* populations of the gorgonian *Pseudopterogorgia elisabethae. P. elisabethae,* widely distributed on coral reefs of the Caribbean Sea, is the sole source of pseudopterosin, a natural product that is currently being used as a topical agent in cosmetic products because of its anti-inflammatory properties (Fenical, 1987). However, there is little knowledge about the biology, ecology, and genetics of *P. elisabethae* or its algal symbionts. It is known that *P. elisabethae* broods asymbiotic planulae that become infected with *Symbiodinium* from the immediate environment following settlement and metamorphosis (Kinzie, 1974) (horizontal transmission of symbionts). Thus *P. elisabethae* colonies could potentially uptake and harbor heterogeneous *Symbiodinium* populations, either within the same colony or between different colonies.

Materials and Methods

Phylogenetic Analysis of *Symbiodinium* cp23S–rDNA Domain V

A *Symbiodinium* phylogeny based on full-length cp23S–rDNA domain V sequences (Santos et al., 2002a) served as a key for the phylogenetic identification of *Symbiodinium* isolates using the technique described here. An additional *Symbiodinium* cp23S–rDNA sequence, obtained from the algal symbionts of a *Briareum asbestinum* colony (a gorgonian) collected in the Florida Keys (designated as BB5), was added to the phylogeny using the procedures outlined in Santos et al. (2002a). This *Symbiodinium* cp23S–rDNA domain V sequence has been deposited into GenBank under accession number AF474164. The free-living dinoflagellate *Protoceratium reticulatum* (AF206702) was employed as the outgroup. Areas a and b of *Symbiodinium* cp23S–rDNA domain V, which have been shown to exhibit length heteroplasmy within, as well as between, the *Symbiodinium* clades (Santos et al., 2002b), were excluded from the phylogenetic analysis as outlined previously (Santos et al., 2002a). This alignment (available from TreeBase at http://www.treebase.org/under study accession number S672 and matrix accession number M1055) was employed to construct a maximum parsimony (MP) phylogenetic tree using PAUP*4.0b8 (Swofford, 2000). For the

MP analysis, 822 characters (118 parsimony-uninformative characters, 83 parsimony-informative characters) were employed under a heuristic search option and gaps were treated as missing data. The MP tree was constructed by optimizing the characters with accelerated transformation (ACCTRAN), 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 were tested by bootstrap analysis of 1000 replicates. Further analyses of *Symbiodinium* phylogenies based on cp23S–rDNA domain V can be found elsewhere (Santos et al., 2002a).

Identification of *Symbiodinium* by Length Heteroplasmy of cp23S–rDNA Domain V

To rapidly identify a *Symbiodinium* isolate, as well as determine its placement in the phylogeny based on cp23S–rDNA domain V (see above), an approximately 0.2-kb region of cp23S–rDNA domain V was PCR amplified from each *Symbiodinium* sample used to generate the *Symbiodinium* cp23S–rDNA domain V phylogeny (32 cultures and 1 *in hospite* population, Santos et al., 2002a) using the primer pair 23SHYPERUP (5′-TCAGTACAAATAATATGCTG-3′) and 23SHYPERDNM13 (5′-GGATAACAATTTCACACAGGTTATCGCCCCAATTAAACAGT-3′). These primers were designed to PCR amplify areas a and b of *Symbiodinium* cp23S–rDNA domain V (Santos et al., 2002b; see above). PCR reactions were performed in 10-µl volumes containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.001% gelatin, 200 µM dNTPs, 0.2 pmol 23SHYPERUP, 0.18 pmol 23SHYPERDNM13, 0.02 pmol 5′-IRD800 M13 reverse primer (see below), 1 U *Taq* polymerase, and approximately 10 ng of template DNA. Reactions were carried out in a MJ Research PTC-100 thermocycler (MJ Research Inc., Watertown, Mass.) under the following conditions: initial denaturing period of 2 minutes at 94°C, 35 cycles consisting of 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 30 seconds, and a final extension period of 5 minutes at 72°C. PCR reactions were diluted with 5 µl of loading buffer (95% [vol/vol] formamide, 10 mM EDTA, 0.01% bromophenol blue [final pH, 9.0], denatured at 95°C for 3 minutes, and snap-cooled on ice immediately prior to polyacrylamide gel electrophoresis (see below).

The first twenty 5′-nucleotides of 23SHYPERDNM13 allow incorporation of the 5′-IRD800 fluorescent-labeled M13 reverse primer (LI-COR Biotechnology Division, Lincoln, Neb.) into the PCR product. PCR products (0.3 µl of sample) were separated in 25-cm-long, 0.25-mm-thick 6.5% Long Ranger (FMC Bioproducts, Rockland, Me.) polyacrylamide gels under denaturing (7 M urea) conditions and visualized on LI-COR's NEN Global IR2 DNA Sequencer System. Electrophoresis conditions were as follows: 1500 V, 40 W, 40 mA, 50°C, and a scan speed of 3. A DNA ladder, spanning from 172 to 272 bp at 25-bp increments, was run in every 5th lane across the gel for size reference. The digitized image obtained with LI-COR's NEN Global IR2 DNA Sequencer System is analogous to autoradiographic imaging. cp23S–rDNA amplicons were sized with the fragment analysis program Gene ImaglR Version 3.55 (Scanalytics Inc, Fairfax, Va.) using the DNA ladders as size references. cp23S–rDNA amplicons are designated according to base pair number (i.e., minus the first twenty 5′-nucleotides of 23SHYPERDNM13; see above). A single gel was reloaded up to 3 times over a 5-hour period.

Tests of Specificity for the *Symbiodinium* cp23S–rDNA Primer Set

To test if primers 23SHYPERUP/23SHYPERDNM13 were capable of amplifying the cp23S–rDNA of other dinoflagellates, all available dinoflagellate cp23S–rDNA sequences were downloaded from GenBank. These sequences came from the free-living dinoflagellates *Amphidinium carterae* CS21 (AJ311633), *A. carterae* CCMP 1341 (AF206701), *Heterocapsa* sp. JN120.1 (AY035430), *Heterocapsa* sp. Pdam3N (AY055242), *Heterocapsa niei* CCMP 447 (AF206703), *H. pygmaea* CCMP 1490 (AF130040), *H. rotundata* NEPCC D680 (AF130041), *H. triquetra* CCMP 449 (AF130039), *Protoceratium reticulatum* NEPCC D535 (AF206702), *Scrippsiella trochoidea* NEPCC D602 (AF206705), and *Thoracosphaera heimii* NEPCC D670 (AF206704). The plastid-like 23S–rDNA sequences from the sporozoans *Plasmodium falciparum* (X61660) and *Toxoplasma gondii* (U87145) were also included in the analysis because their highly reduced nonphotosynthetic plastids share a common origin with the chloroplasts of dinoflagellates (Fast et al., 2001), and sporozoa have been found in association with invertebrates that harbor *Symbiodinium* (Upton and Peters, 1986; Nakayama et al., 1998; Goulet, 1999; Toller et al., 2002; A.R. Hannes personal communication). Simulated PCR amplifications were conducted on each of these sequences using the primer sequences for 23SHYPERUP/23SHYPERDNM13 in the computer program AMPLIFY Version 1.2 (Engels, 1993). In addition, DNA from *Heterocapsa* sp. cultures JN120.1 and Pdam3N (Santos et al.,

2002a), as well as *Gymnodinium sanguineum* CCMP 1321 and *Prorocentrum micans* CCMP 1589, were used as templates in PCR reactions under the conditions outlined above.

Simultaneous Detection of Multiple *Symbiodinium* Chloroplast Genotypes

To explore the number of *Symbiodinium* cp23S–rDNA alleles (i.e., number of *Symbiodinium* chloroplast genotypes) that could be simultaneously detected under the PCR reaction and amplification conditions listed above, a series of synthetic mixtures of *Symbiodinium* DNA were constructed and used as PCR templates. These synthetic mixtures were designed to represent 2, 3, 4, or 5 *Symbiodinium* chloroplast genotypes occurring simultaneously at approximately equal concentrations. *Symbiodinium* chloroplast genotypes and mixtures of chloroplast genotypes were chosen to span the entire range of cp23S–rDNA alleles observed from cultures and populations *in hospite* (see below). *Symbiodinium* DNA, isolated from cultures, was diluted to approximately 10 ng/µl prior to being mixed. PCR reactions and polyacrylamide gel electrophoresis conditions of *Symbiodinium* cp23S–rDNA alleles were carried out as described above.

Symbiodinium cp23S–rDNA Screening of Populations *in hospite*

We employed the *Symbiodinium* cp23S–rDNA screening technique to characterize the symbiotic dinoflagellate populations isolated from a range of phylogenetically diverse hosts including scleractinians (12 species), anemones (4 species), octocorals (13 species), hydrocorals (1 species), and zoanthids (4 species) (Table 1). These specimens were collected from a wide geographic area including samples from the Pacific Ocean, Caribbean Sea, and the Red Sea (see Table 1). Tissue samples were either preserved in 95% ethanol, salt-saturated DMSO (Seutin et al., 1991), or frozen in a liquid nitrogen vapor shipper immediately following collection. Total nucleic acids were extracted and quantified according to the methods of Coffroth et al. (1992) or with a modified extraction protocol (T.L. Shearer and M.A. Coffroth, manuscript in preparation) that uses the Prep-A-Gene DNA Extraction Kit (Bio-Rad Laboratories, Hercules, Calif.). PCR reactions and polyacrylamide gel electrophoresis conditions for *Symbiodinium* cp23S–rDNA screening were carried out as described above.

To look for within-host variation in the number of *Symbiodinium* cp23S–rDNA alleles at one location, a more extensive collection was made. *P. elisabethae* samples were collected from 9 sites in the Bahamas: Sweetings Cay (26°34′N, 77°48′W); Gorda Rock (26°07′N, 77°33′W); South Hampton Reef (25°25′N, 76°48′W); Little San Salvador (24°35′N, 75°58′W); East End Point of Eleuthera (24°37′N, 76°10′W); Cat Island (24°09′N, 75°32′W); San Salvador (24°04′N, 74°32.5′W); Rum Cay (23°37′N, 740°52′W); and Hog Cay (23°37′N, 75°21′W) (n = 20 per site). *P. elisabethae* colonies were sampled in 1995 (Sweetings Cay) or between 1998 and 2000 (all other locations) by SCUBA between depths of 8 and 25 m.

Results and Discussion

Phylogenies based on cp23S–rDNA domain V sequences suggest that the genus *Symbiodinium* is monophyletic and currently resolves the genus into 6 clades (Santos et al., 2002a). The clades, referred to as A, B, C, D, E, and F, were supported by moderate to strong bootstrap supports (68%–94%) in all cases (Figure 1). These *Symbiodinium* clades have also been recognized by phylogenetic studies of nuclear rDNAs, such as nuclear 5.8S–rDNA (LaJeunesse, 2001), while the relationships between the clades inferred by cp23S–rDNA domain V sequences are consistent with those inferred from nuclear rDNAs (Santos et al., 2002a).

PCR amplifications of our *Symbiodinium* isolates using the primers 23SHYPERUP/23SHYPERDNM13, followed by electrophoresis in a polyacrylamide gel, revealed a number of uniquely sized amplicons (hereinafter referred to as cp23S–rDNA domain V alleles) (Figure 2). In most cases uniquely sized cp23S–rDNA domain V alleles are found within, as well as between, *Symbiodinium* clades (Figures 1 and 2). For example, 5 and 6 uniquely sized cp23S–rDNA domain V alleles are found within *Symbiodinium* clades A and B, respectively. Overall, 15 uniquely sized cp23S–rDNA domain V alleles were identified from *Symbiodinium* cultures, with the size of each allele confirmed by simulated PCR amplifications using the full-length *Symbiodinium* cp23S–rDNA domain V sequence and the primer sequences for 23SHYPERUP/23SHYPERDNM13 in the computer program AMPLIFY Version 1.2 (Engels, 1993). For simplicity in referring to a particular cp23S–rDNA domain V allele, the following nomenclature has been adopted: *Symbiodinium,* a capital letter, referring to the major clade that a *Symbiodinium* isolate belongs to in the cp23S–rDNA domain V phylogeny, fol-

Table 1. *Symbiodinium* Isolates Screened by the Chloroplast Large Subunit (cp23S)–rDNA Genotyping Technique[a]

Host organism	Sample size	Collection location	*Symbiodinium* cp23S–rDNA designation
Scleractinaria			
Acropora palita	1	Japan	D206
Dichocoenia sp.	2	Florida Keys	B184
Favia fragum	2	Florida Keys	C180
Galaxia sp.	1	Japan	C180
Gonopora sp.	1	Japan	C180
Madracis decactis	1	Florida Keys	C180
Meandrina meandrites	1	Florida Keys	B184
Mycetophyllia sp.	1	Florida Keys	B184
Porites lutea	1	Japan	C180
Psammocora sp.	1	Japan	C180
Solenastrea sp.	1	Florida Keys	C180
Turbinaria sp.	1	Japan	C180
Actiniaria			
Aiptasia pulchella	50	Japan; Hawaii; Israel	B184
Aiptasia pallida	12	Bermuda	B184
A. pallida	18	Florida Keys	A193
A. pallida	1	Long Key, Florida	A193/C180
A. pallida	1	Long Key, Florida	A193/B184
A. pallida	1	Long Key, Florida	B224
Unknown anemone	1	Florida Keys	C180
Unknown anemone	3	Hawaii	C180
Octocorallia			
Briareum asbestinum (adult colony)	12	Florida Keys	B178
B. asbestinum (adult colony)	5	Florida Keys	B184
B. asbestinum (adult colony)	1	Florida Keys	B224
B. asbestinum (polyp)	58	Florida Keys	A194/B184
B. asbestinum (polyp)	113	Florida Keys	B184
B. asbestinum (polyp)	5	Florida Keys	A194
B. asbestinum (polyp)	2	Florida Keys	A194/B184/B224
B. asbestinum (polyp)	1	Florida Keys	A194/B224
B. asbestinum (polyp)	5	Florida Keys	B184/B223
B. asbestinum (polyp)	1	Florida Keys	B184/B211/B223
B. asbestinum (polyp)	2	Florida Keys	A194/B184/B223
B. asbestinum (polyp)	2	Florida Keys	B223
Eunicea sp.	11	Florida Keys	B184
Gorgonia sp.	8	Florida Keys	B184
Muricea sp.	4	Florida Keys	B184
Plexaura flexuosa	1	Florida Keys	B184
Plexaura kuna	70	San Blas Islands, Panama	B184
Pseudoplexaura sp.	1	Florida Keys	B184
Pseudopterogorgia acerosa	2	Florida Keys	B184
Pseudopterogorgia americana	9	Florida Keys	B184
Pseudopterogorgia elisabethae	178	Bahamas	B184
Sinularia sp.	1	Guam	C180
Sinularia sp.	1	Japan	C180
Unknown soft coral	1	Japan	C180

Table 1. Continued

Host organism	Sample size	Collection location	*Symbiodinium* cp23S–rDNA designation
Others			
Millipora sp.	1	Japan	C180
Palythoa sp.	1	Japan	C180
Palythoa sp.	1	Florida Keys	C180/D206
Palythoa sp.	2	Florida Keys	D206
Unknown sponge	2	Florida Keys	B184
Unknown zoanthid	1	Florida Keys	A194

[a]All samples are *Symbiodinium* populations *in hospite.*

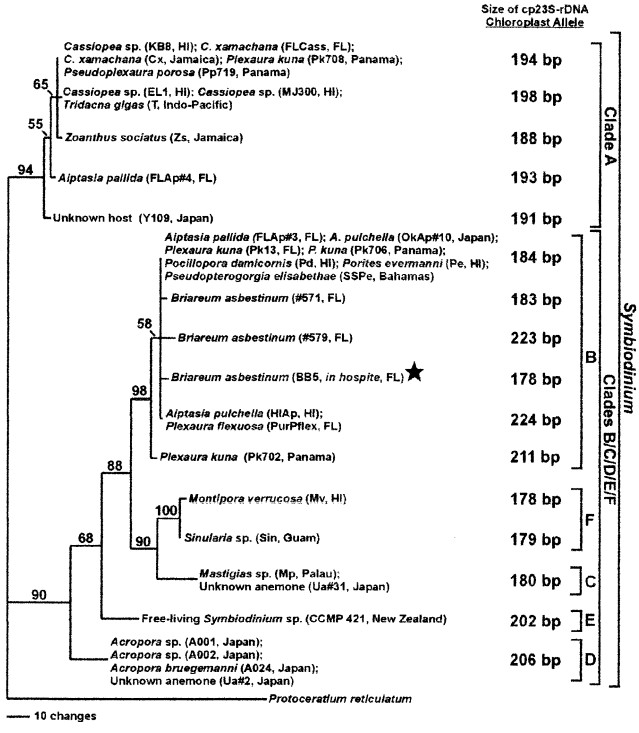

Figure 1. Fragment sizes (bp) of *Symbiodinium* cp23S–rDNA domain V alleles and their relationship to the *Symbiodinium* cp23S–rDNA phylogeny. The phylogenetic tree was constructed by maximum parsimony (MP). Values above line represent bootstrap support values of nodes as percentages of 1000 resamplings. Culture name and collection location (in parentheses) follow the name of the organism from which the dinoflagellate culture was isolated. Sample name followed by a black star represents sequence of *Symbiodinium* population *in hospite*; all others are from cultures. The length of the most parsimonious tree was 310 steps with a consistency index (CI) of 0.8194.

lowed by a number representing the base pair size of the particular cp23S–rDNA domain V allele that the *Symbiodinium* isolate possesses.

Some cp23S–rDNA alleles (e.g., Figure 2, *Symbiodinium* B184, F179, and F178; lanes 9, 22, and 23) may produce an artifact, probably owing to imperfect replication of the allele during PCR amplification. We have found that these artifacts, which are consistently 2 bp shorter than the cp23S–rDNA allele, do not interfere with the determination of allele sizes since they are typically of weaker intensity. In addition, culture Pk706 (Figure 2, lane 21), a *Symbiodinium* B184 culture which possesses aberrant, internally deleted cp23S–rDNA molecules (see Santos et al., 2002b), produced 3 distinct amplicons. The largest amplicon (184 bp) is the true cp23S–rDNA allele, while the smaller amplicons (181 and 179 bp) result from aberrant cp23S–rDNA molecules serving as PCR amplification template. However, *Symbiodinium* isolates that possess aberrant, internally deleted cp23S–rDNA molecules that will amplify with this technique are uncommon (S.R. Santos, personal observation). To aid in interpretation of experimental results, we suggest the running of previously characterized *Symbiodinium* cp23S–rDNA genotypes in all polyacrylamide gels or sequence analysis of questionable cp23S–rDNA alleles.

No amplicons were generated from any of the free-living dinoflagellate or sporozoan DNA templates included in this study. Although these data suggest that primers 23SHYPERUP/23SHYPERDNM13 are specific for dinoflagellates belonging to the genus *Symbiodinium,* we cannot discount the possibility that dinoflagellates closely allied to *Symbiodinium* may also amplify with these primers. When new cp23S–rDNA alleles are discovered using these primers, investigators should sequence the entire cp23S–rDNA domain V region and subject it to phylogenetic analyses. This process is vital to properly identifying the unknown entity as well as its phylogenetic position relative to members of the genus *Symbiodinium.*

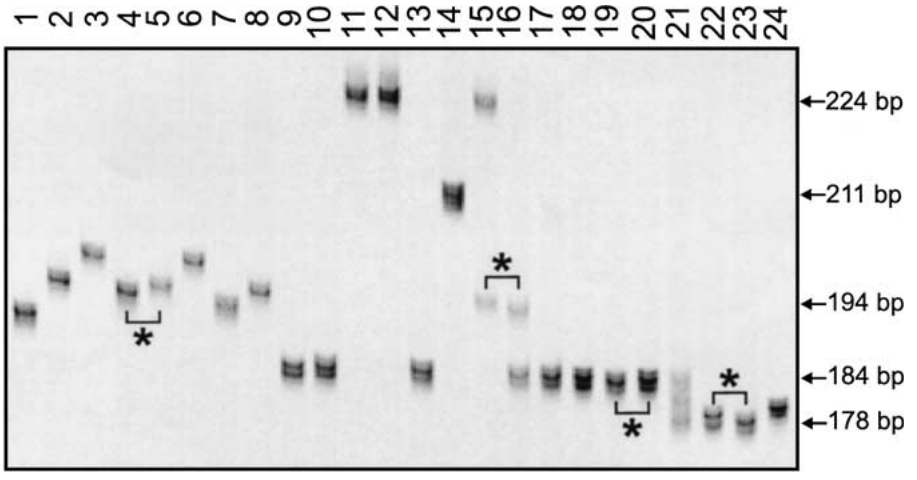

Figure 2. Polyacrylamide gel electrophoresis of cp23S–rDNA domain V alleles from *Symbiodinium* cultures. Lane 1, *Zoanthus sociatus* (Zs) [A188]; lane 2, Jamaican *Cassiopea xamachana* (Cx) [A194]; lane 3, *Tridacna gigas* (T) [A198]; lane 4, FL. *Aiptasia pallida* (FLAp#4) [A193]; lane 5, FL. *C. xamachana* (FLCass) [A 194]; lane 6, *Cassiopea* sp. (CassELl) [A198]; lane 7, unknown host (Y109) [A191]; lane 8, *Plexaura kuna* (Pk708) [A194]; lane 9, *Pseudopterogorgia elisabethae* (SSPe) [B184]; lane 10, Okinawan *Aiptasia pulchella* (OkAp#10) [B184]; lane 11, *Plexaura flexuosa* (PurPflex) [B224]; lane 12, Hawaiian *A. pulchella* (HIAp) [B224]; lane 13, *P. kuna* (Pk13) [B184]; lane 14, *P. kuna* (Pk702) [B211]; lane 15, synthetic mixture of Jamaican *C. xamachana* and *P. flexuosa* (Cx/PurPflex) [A194/B224]; lane 16, natural mixture of *Symbiodinium* clades A and B from *A. pallida* (see Santos et al., 2001) (FLAp#1) [A193/B184]; lane 17, *Porites evermanni* (Pe) [B184]; lane 18, *Pocillopora damicornis* (Pd) [B184]; lane 19, *Briareum asbestinum* (#571) [B183]; lane 20, FL. *A. pallida* (FLAp#3) [B184]; lane 21, *P. kuna* (Pk706) [B184 + 181 + 179]; lane 22, *Sinularia* sp. (Sin) [F179]; lane 23, *Montipora verrucosa* (Mv) [F178]; lane 24, *Mastigias* sp. (Mp) [C180]. Names in parentheses are culture names. Information in brackets represents *Symbiodinium* clade (capital letter) and cp23S–rDNA domain V allele size (in base pairs). Samples marked by an asterisk (*) indicate pairs of cp23S–rDNA alleles that differ in length by a single base pair.

Analyses of synthetic mixtures of *Symbiodinium* DNA demonstrate that at least 5 uniquely sized *Symbiodinium* cp23S–rDNA domain V alleles can be detected simultaneously when present at approximately equal concentrations (Figure 3). The final DNA concentration for each *Symbiodinium* chloroplast genotype in the 5 genotypic mixtures was approximately 2 ng per reaction. How much of this DNA represents template cp23S–rDNA is unknown, but given that most of the DNA was probably nuclear in origin and that dinoflagellate nuclei contain between 2 and 200 pg DNA (Spector, 1984), these results suggest that between 10 and 1000 *Symbiodinium* cells of a particular cp23S–rDNA chloroplast genotype could be detected in this sample. Since amount of nuclear DNA (e.g., number of chromosomes) and chloroplast number can vary between *Symbiodinium* isolates (reviewed in LaJeunesse, 2001), *Symbiodinium* cell numbers beyond those presented here should be quantified on a case-by-case basis. We suggest PCR amplification and examination of DNA dilution series or mixtures tailored to an investigator's particular experimental question and PCR reagents (e.g., thermostable DNA polymerase). In our work, we have documented numerous cases of field-collected host individuals simultaneously infected with up to 3 distinct *Symbiodinium* cp23S–rDNA chloroplast genotypes, particularly in newly infected gorgonian polyps (Table 1). Coffroth et al. (2001) demonstrated that newly settled octocoral polyps rapidly take up multiple *Symbiodinium* clades. Screening of recently settled polyps with the technique presented here showed a previously undocumented level of within-clade variation during the initial ontogeny of the symbiosis (see *B. asbestinium* polyps in Table 1). Thus this technique not only has the ability to distinguish *Symbiodinium* isolates at the intracladal and intercladal levels, but also can detect the presence of multiple *Symbiodinium* cp23S–rDNA chloroplast genotypes of a single clade in a single sample.

Thus far, approximately 700 symbiotic dinoflagellate samples have been examined using this technique. These samples were collected from the Red Sea, Pacific Ocean, and Caribbean Sea; represent cultured (n = 92) as well as *Symbiodinium* populations *in hospite* (n = 603); and were isolated from a range of phylogenetically diverse hosts (Table 1). Analyses of *Symbiodinium* populations *in hospite*

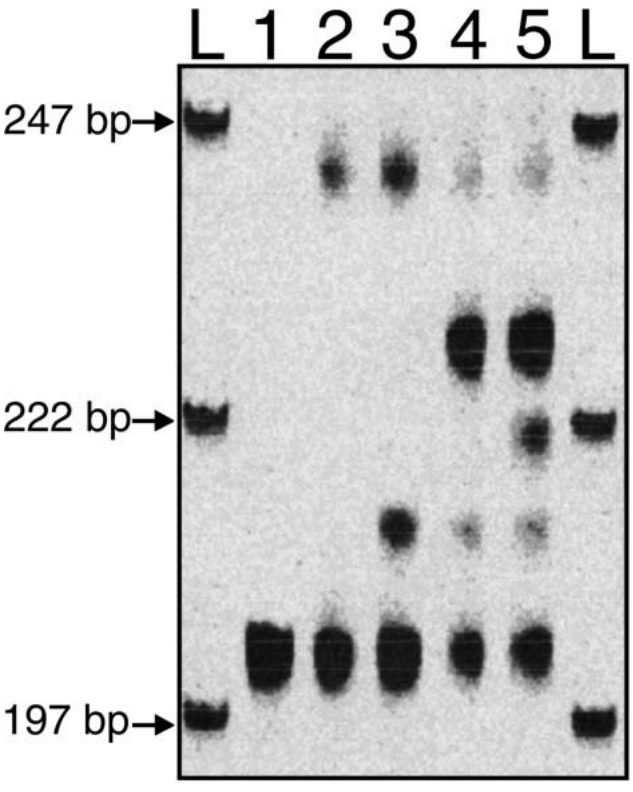

Figure 3. Polyacrylamide gel electrophoresis analysis of synthetic mixtures of *Symbiodinium* cp23S–rDNA domain V alleles. Numbers above lanes represent number of alleles included in each synthetic mixture. *Symbiodinium* samples mixed in each lane are as follows: lane 1, *Symbiodinium* B184 only; lane 2, *Symbiodinium* B184/B224; lane 3, *Symbiodinium* B184/B224/A194; lane 4, *Symbiodinium* B184/B224/A194/B211; lane 5, *Symbiodinium* B184/B224/A194/B211/E202. L represents DNA ladder lane.

using the cp23S–rDNA screening technique led to the discovery of only a single allele that has not been documented from *Symbiodinium* cultures *(B. asbestinum* sample BB5, cp23S-rDNA domain V allele B178, Table 2). Unexpectedly, we have found more *Symbiodinium* diversity from cultured materials than from samples *in hospite* (Table 2). These results are consistent with the finding that some strains of *Symbiodinium* are cryptic and have only been characterized from cultured materials (Santos et al., 2001; LaJeunesse, 2002). Taken together, these results suggest that a large proportion of the *Symbiodinium* cp23S–rDNA domain V diversity present in nature has been described. However, it is highly probable that additional *Symbiodinium* cp23S–rDNA domain V alleles remain to be documented, particularly within *Symbiodium* clades C, D, E, and F, which have not been extensively sampled.

In a single case, an identically sized cp23S–rDNA domain V allele was found in 2 different *Symbiodinium* clades. A 178-bp allele was recovered from sample *Briareum asbestinum* BB5 *(Symbiodinium* clade B) as well as *Montipora verrucosa* culture Mv *(Symbiodinium* clade F) (Figure 1). In spite of this case of size homoplasy, the identity of samples that possess a 178-bp allele can rapidly be established by amplification and *Taq*I-RFLP analysis of n18S–rDNA (Rowan and Powers, 1991) from the same sample or by sequencing either the 178-bp allele itself or the entire cp23S–rDNA domain V region (Santos et al., 2002a).

In addition to the example discussed above, we have sequenced *Symbiodinium* cp23S–rDNA domain V alleles belonging to the same size class from cultures and populations *in hospite* and found no sequence variability between alleles of identical size from different *Symbiodinium* isolates. This observation suggests that sequence variability between *Symbiodinium* cp23S–rDNA domain V alleles of the same size is uncommon. The lack of sequence variability prompted us to employ standard fragment analysis protocols that separate alleles only by size (see ''Materials and Methods'') rather than by DGGE or SSCP, which separate alleles predominately by sequence differences. However, cp23S–rDNA alleles, generated with primer pair 23SHYPERUP/23SHYPERDN, could also be separate using DGGE (Muyzer, 1999) or SSCP (Sunnucks et al., 2000) protocols, if desired by an investigator.

Screening of 178 of the 180 *P. elisabethae* colonies from across the Bahamas using the *Symbiodinium* cp23S–rDNA screening technique revealed a single allele in all samples (DNA from 2 colonies collected from South Hampton Reef did not amplify and were removed from further analysis). These results suggest that each *P. elisabethae* colony harbored a single, numerically dominant *Symbiodinium* cp23S–rDNA chloroplast genotype. This allele was identified as *Symbiodinium* B184, which placed the symbiotic dinoflagellates inhabiting *P. elisabethae* as members of *Symbiodinium* clade B (Figure 2), consistent with cladal identification data generated from n18S–rDNA RFLP analyses (Goulet, 1999; Santos et al., 2001) and phylogenies based on ITS-rDNA (Santos et al., 2001). However, given that 6 *Symbiodinium* clade B chloroplast genotypes have currently been identified using this technique, all of which occur in the Caribbean Sea (Figure 1 and Table 1) and are harbored by gorgonian hosts (Figure 1), these data suggest a previously undocumented level of symbiont specificity in adult *P. elisabethae* for members of *Symbiodinium* B184.

Table 2. Summary of *Symbiodinium* Chloroplast Large Subunit (cp23S)–rDNA Genotypes Identified to Date from Cultures and *in hospite*

Symbiodinium cp23S–rDNA genotype	Culture	*In hospite*
A188	XX	
A191	XX	
A193	XX	XX
A194	XX	XX
A198	XX	
B178		XX
B183	XX	
B184	XX	XX
B211	XX	XX
B223	XX	XX
B224	XX	XX
C180	XX	XX
D206	XX	XX
E202	XX	
F178	XX	
F179	XX	

Thus the *Symbiodinium* cp23S–rDNA screening technique not only identified the clade of *Symbiodinium* harbored by *P. elisabethae*, but also provided data regarding the symbionts' intracladal identity.

We have presented a novel technique that employs length heteroplasmy in cp23S–rDNA domain V to identify members of the symbiotic dinoflagellate genus *Symbiodinium* and detect within-clade variation. This technique offers high throughput (approximately 150 samples can be screened in 5 hours on LI-COR's NEN Global IR2 DNA Sequencer System under the conditions described above) and is amendable to other automated or manual DNA sequencing systems. In addition, the technique can be easily modified to incorporate DGGE and SSCP technologies. Because the technique targets a symbiont-specific genome, separation of host and symbiont DNA is not necessary. These characteristics make the *Symbiodinium* cp23S–rDNA screening technique ideal for exploring a range of research questions involving these dinoflagellates, particularly those that require resolution at the intracladal level. For example, we are currently using this technique to track multiple infection and differential retention of *Symbiodinium* chloroplast genotypes in the ontogeny of *B. asbestinum*, a Caribbean gorgonian that produces asymbiotic planulae. Application of techniques such as the one presented in this report will offer new insight into the biology of these enigmatic dinoflagellates.

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