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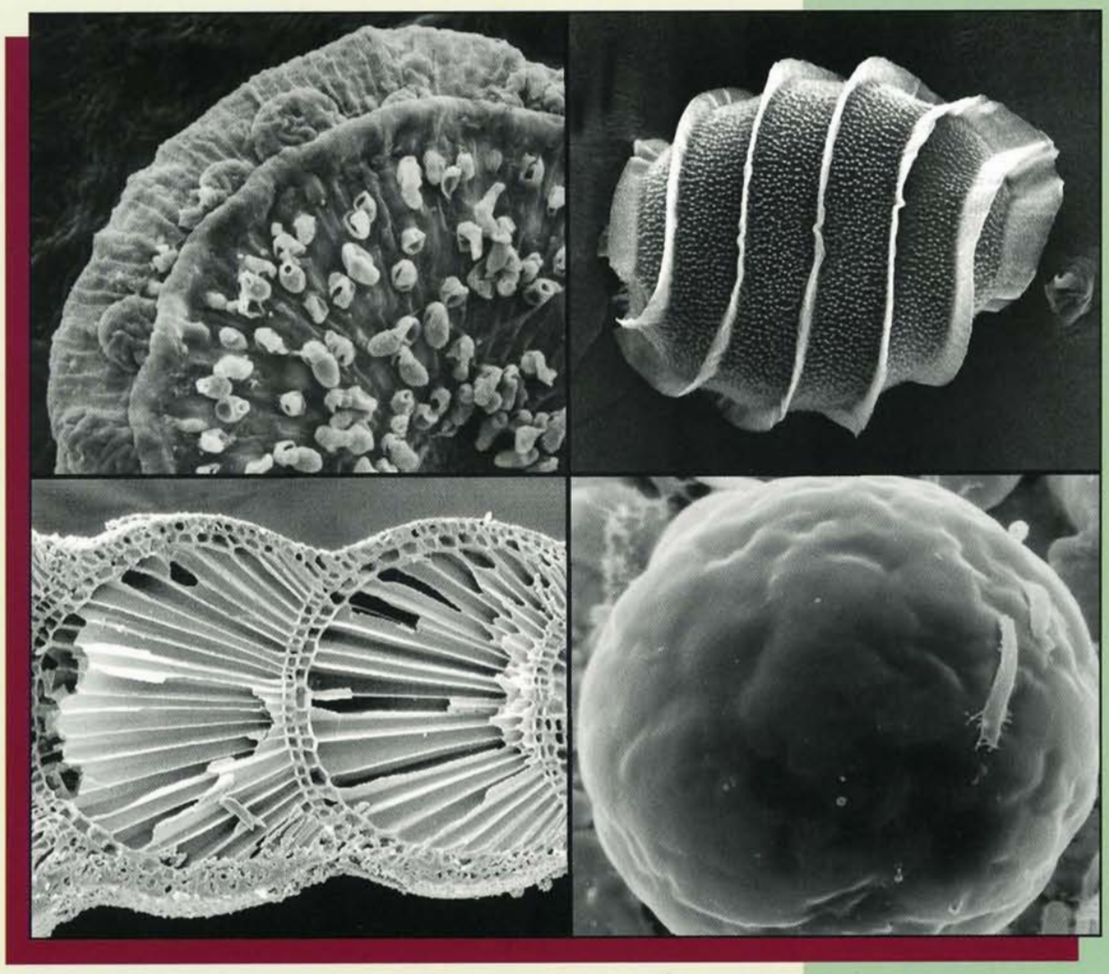

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Front cover: clockwise, from top left: *Phycopeltis arundinaceae* thallus (see Rindi & Guiry, p. 421); *Nitella spiciformis* oospore (see Sakayama *et al.*, p. 397); *Symbiodinium* (symbiotic dinoflagellate) (see Santos *et al.*, p. 311); and *Paulsillvella huveorum* protuberant branch (see Woelkerling *et al.*, p. 358).

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Evolution of length variation and heteroplasmy in the chloroplast rDNA of symbiotic dinoflagellates (Symbiodinium, Dinophyta) and a novel insertion in the universal core region of the large subunit rDNA

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S.R. SANTOS, D.J. TAYLOR, R.A. KINZIE III, K. SAKAI AND M.A. COFFROTH. 2002. Evolution of length variation and heteroplasmy in the chloroplast rDNA of symbiotic dinoflagellates (Symbiodinium, Dinophyta) and a novel insertion in the universal core region of the large subunit rDNA. *Phycologia* 41: 311–318.

Marine dinoflagellates are a diverse and ecologically important group of unicellular protists responsible for events such as 'red tides' and for forming mutualistic symbioses with various invertebrates. However, little is known about the evolution of organellar DNA in these organisms. We analysed domain V of the chloroplast large subunit ribosomal DNA (cp23S-rDNA) from a variety of symbiotic dinoflagellates and uncovered several unique features. These included length heteroplasmy of cp23S-rDNA that resulted from deletion mutations and the presence of a 1–13 bp nucleotide insertion in the universal core region. The insertion in the large subunit rDNA is novel across all kingdoms and appears to be a unique derived state for the symbiotic genus *Symbiodinium*. In addition, analysis of expansion segments (ES) revealed that the cp23S-rDNA domain V of symbiotic dinoflagellates is the most size variable of any known plastid-harbouring organism. Our results demonstrate that the universal core secondary structure and the chloroplast rDNA ES are less constrained in size than previously thought.

INTRODUCTION

Dinoflagellates are a diverse group of aquatic, unicellular protists. In marine environments, many dinoflagellates are major photosynthetic and heterotrophic members of the plankton; others form mutualistic symbioses with various invertebrates or are responsible for 'red tides' and fish kills. Despite the fact that dinoflagellates constitute a major and important group of protists, little is known about the evolution of organellar DNA in these organisms. For example, at present, only a single mitochondrial gene, cytochrome oxidase subunit 1 (*cox1*), has been sequenced and analysed from dinoflagellates (Inagaki *et al.* 1997; Norman & Gray 1997). Zhang *et al.* (1999) studied the chloroplast genome of the free-living dinoflagellate *Heterocapsa triquetra* (Ehrenberg) F. Stein and found a reduced gene complement comprising seven protein-encoding and two ribosomal genes. Furthermore, investigators found that each dinoflagellate chloroplast gene is located separately on an individual minicircular chromosome (Zhang *et al.* 1999; Barbrook & Howe 2000), which contrasts with the arrangement of many genes on a single circular chromosome, which is the typical architecture of chloroplast genomes (McFadden 1999).

Along with this unusual gene complement and organization, the chloroplast ribosomal DNA (cp-rDNA) of free-living dinoflagellates is the most divergent in sequence and length among organisms with chloroplasts (Zhang *et al.* 1999, 2000; Tengs *et al.* 2000). Tengs *et al.* (2000) excluded the *H. triquetra* chloroplast small subunit (cp16S)-rDNA sequence from their phylogenetic analysis because of its extremely derived nature. Furthermore, Zhang *et al.* (2000) found it very difficult to align some regions of the dinoflagellate chloroplast large subunit (cp23S)-rDNA with cp23S-rDNAs from other organisms. It is not known why the evolutionary rate of the cp-rDNA is accelerated in free-living dinoflagellates (Tengs *et al.* 2000), nor whether the acceleration also applies to symbiotic species. To date, information regarding the chloroplast genome of symbiotic dinoflagellates is unavailable. The purpose of our study was to characterize the cp-rDNA length variation in symbiotic dinoflagellates and to examine the possible mechanisms for this length variation. To explore these questions, we sequenced and analysed domain V of cp23S-rDNA from members of the dinoflagellate genus *Symbiodinium* Freudenthal (Taylor 1974). These dinoflagellates, commonly referred to as zooxanthellae, are intra- or intercellular symbionts of marine invertebrates, including foraminiferans, sponges, scleractinian corals, sea anemones, octocorals, zoanthids, hydrocorals and molluscs (Glynn 1996), and have been shown to play a vital role in their host's nutrition and physiology (reviewed in Davies 1993). We show that size variation in symbiotic dinoflagellates is produced by length heteroplasmy from deletion mutations, a novel universal core insertion and length variable expansion segments (ES).

MATERIAL AND METHODS

Restriction fragment length polymorphism analysis of the dinoflagellate nuclear small subunit–rDNA

Algal cultures, isolated from a range of invertebrate hosts and geographical locations and maintained as described by Santos

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Table 1. Dinoflagellate cultures from which the chloroplast (cp) 23S-rDNA was sequenced in this study.[1]

n18S-RFLP designation	Host organism	Culture name	Collection location	GenBank accession number
Symbiodinium				
Clade A	*Aiptasia pallida* Verrill	FLAp#4	Florida Keys	AY035404
Clade A	*Cassiopea* sp.	CassEL1	Kaneohe Bay, Hawaii	AY035410
Clade A	*Cassiopea* sp.	CassKB8	Kaneohe Bay, Hawaii	AY035405
Clade A	*Cassiopea* sp.	CassMJ300	Kaneohe Bay, Hawaii	AY035411
Clade A	*C. xamachana* Bigelow	Cx	Jamaica	AY035406
Clade A	*C. xamachana*	FLCass	Florida Keys	AY035407
Clade A	*Plexaura kuna* (polyp)[2]	Pk708[3]	San Blas Islands, Panama	AY035408
Clade A	*Pseudoplexaura porosa* Houttuyn (polyp)[2]	Pp719[3]	San Blas Islands, Panama	AY035409
Clade A	*Tridacna gigas*	T[4]	Indo-Pacific	AY035412; AY035431[5]
Clade A	unknown host	Y109	Okinawa, Japan	AY035413
Clade A	*Zoanthus sociatus* Ellis & Solander	Zs	Jamaica	AY035414
Symbiodinium				
Clade B	*A. pallida*	FLAp#3[4]	Florida Keys	AY055235;[6] AY035434[5]
Clade B	*A. pulchella* Calgren	HIAp	Kaneohe Bay, Hawaii	AY035421
Clade B	*A. pulchella*	OkAp#10	Okinawa, Japan	AY035416
Clade B	*Briareum asbestinum* Pallas (polyp)[2]	#571[3]	Florida Keys	AY035415
Clade B	*Plexaura flexuosa* Lamouroux	PurPflex	Florida Keys	AY035420
Clade B	*P. kuna*	Pk13[4]	Florida Keys	AY055231;[6] AY035433[5]
Clade B	*P. kuna* (polyp)[2]	Pk702[3]	San Blas Islands, Panama	AY035419
Clade B	*P. kuna* (polyp)[2]	Pk706[3,4]	San Blas Islands, Panama	AY055232;[6] AY055233;[5,6] AY055234[5,6]
Clade B	*Pocillopora damicornis* Linnaeus	Pd[4]	Hawaii	AY055236;[6] AY035432;[5] AY055237;[5,6] AY055238[5,6]
Clade B	*Porites evermanni* Vaughan	Pe	Hawaii	AY035418
Clade B	*Pseudopterogorgia elisabethae* Bayer	SSPe	Bahamas	AY035417
Symbiodinium				
Clade C	*Mastigias* sp.	Mp	Palau	AY035424
Clade C	*Montipora verrucosa* Lamarck	Mv	Kaneohe Bay, Hawaii	AY035422
Clade C	*Sinularia* sp.	Sin	Guam	AY035423
Clade C	unknown anemone[7]	Ua#31	Okinawa, Japan	AY035425
Symbiodinium				
Clade D	*Acropora* sp.	A001	Okinawa, Japan	AY035427
Clade D	*Acropora* sp.	A002	Okinawa, Japan	AY035428
Clade D	*A. brueggemanni* Brook	A024	Okinawa, Japan	AY035429
Clade D	unknown anemone[7]	Ua#2	Okinawa, Japan	AY035426
Free-living dinoflagellate	*Plexaura kuna* (polyp)[2]	JN120.1[3]	San Blas Islands, Panama	AY035430

[1] rDNA, ribosomal deoxyribonucleic acid; RFLP, restriction fragment length polymorphism.
[2] A polyp is defined as a newly settled and metamorphosed planula.
[3] Cultures that were started from a single dinoflagellate cell.
[4] Cultures that possess cp23S-rDNA domain V molecules with deletion mutations.
[5] GenBank accession number for cp23S-rDNA domain V molecules with deletion mutations.
[6] GenBank accession number of the sequence from bacterial clones.
[7] Tentatively identified as *Entacmaea quadricolor* Ruppell & Leuckart (D. Fautin, University of Kansas, personal communication). This anemone harbours *Symbiodinium* clade C, as well as a unique genotype of *Symbiodinium* clade D, characterized by the presence of truncated molecules of the n18S-rDNA gene (S.R. Santos, unpublished observations).

et al. (2001), were employed as a source of DNA (Table 1). Total nucleic acids were extracted and quantified according to the methods of Coffroth *et al.* (1992). The dinoflagellate nuclear small subunit (n18S)-rDNA was amplified by polymerase chain reaction (PCR), using the primers ss5 and ss3z, according to Rowan & Powers (1991), and digested with the *Taq* I restriction enzyme. Digestion products were separated by electrophoresis in 2% 0.5× Tris–borate (TBE) agarose gels to generate restriction fragment length polymorphism (RFLP) patterns. RFLP analysis of n18S-rDNA PCR products separates *Symbiodinium* into several large clades, viz. *Symbiodinium* clades A, B and C (Rowan & Powers 1991), D (Carlos *et al.* 1999), and E ('*S. californium*': LaJeunesse & Trench 2000; LaJeunesse 2001), with each clade probably comprising many species (Rowan 1998).

Amplification and sequencing of dinoflagellate cp23S-rDNA domain V

An approximately 0.7 kb region of the dinoflagellate cp23S-rDNA, corresponding to domain V of the cp23S-rDNA molecule (Harris *et al.* 1994), was PCR amplified from the same cultures as aforementioned using the primer pair 23S1M13 (5′-CACGACGTTGTAAAACGACGGCTGTAACTA-

TAACGGTCC-3′) and 23S2M13 (5′-GGATAACAATTTCACACAGGCCATCGTATTGAACCCAGC-3′). These primers were modified from those in Zhang *et al.* (2000) (see subsequently). PCR reactions 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 deoxynucleotide triphosphates, 100 pmol of each primer, 2 U *Taq* polymerase and 50–70 ng of the template DNA. Reactions were carried out in an MJ Research® PTC-100 thermocycler (MJ Research, Watertown, MA, USA) 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 (TAE) (0.04 M TAE, 0.0001 M ethylenediaminetetraacetic acid 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, Corning, NY, USA), 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′-IRD800 and 5′-IRD700 fluorescent-labelled M13 primers (LI-COR Biotechnology Division, Lincoln, NE, USA). For most samples, nucleotide sequences were determined in this manner. In some cases, the cp23S-rDNA PCR products were gel purified as above and cloned using the TOPO TA Cloning® kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's directions, before 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 aforementioned. Ten clones were screened by PCR amplification with primers 23S1 and 23S2, followed by 2% 0.5× TBE agarose gel electrophoresis, for the presence of inserts of the appropriate size. Bacterial clones chosen for sequencing (4–8 clones per sample) were grown overnight in 1.5 ml of Luria–Bertani broth and plasmids were 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 25–41 cm gels (Epicentre Technologies, Madison, WI, USA), 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, USA) and ambiguities corrected by comparison with the reverse sequence or coded using the International Union of Pure and Applied Chemistry ambiguity codes. Sequences were deposited in GenBank under accession numbers listed in Table 1.

Secondary structural analysis of dinoflagellate cp23s-rDNA domain V

For comparisons between *Symbiodinium* and other dinoflagellates, the cp23S-rDNA sequences of the free-living dinoflagellates *H. rotundata* (Lohmann) G. Hansen (AF130041) and *Protoceratium reticulatum* (Claparède & Lachmann) Bütschli (AF206702) were acquired from GenBank. Additionally, the plastid-like 23S-rDNA sequence from the apicomplexan *Plasmodium falciparum* Welsh (X61660) was included in the analysis because its highly reduced nonphotosynthetic plastid shares a common origin with the chloroplasts of dinoflagellates (Fast *et al.* 2001). Sequences were 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 secondary structure (see subsequently). Alignments of representative sequences were deposited in TreeBase (http://www.treebase.org) as Nexus files under the study accession number S663 and the matrix accession numbers M1040 and M1041.

Structural comparisons of domain V of cp23S-rDNA between *Symbiodinium,* free-living dinoflagellates and other eukaryotes were made as follows. Given the relationship between apicomplexan and dinoflagellate plastids, along with the lack of the cp23S-rDNA secondary structure models for dinoflagellates, the plastid-like 23S-rDNA secondary structure for *P. falciparum* was downloaded from the Gutell Lab Comparative RNA web site (http://www.rna.icmb.utexas.edu/) and *Symbiodinium* and free-living dinoflagellate sequences were superimposed onto this 23S-rDNA secondary structure. Areas of high sequence conservation in cp23S-rDNA between *Symbiodinium* and other eukaryotes (see subsequently) served as reference points. Less conserved regions, with compensatory nucleotide changes or small (1–3 bp) length differences, were folded by eye to conserve secondary structural characteristics. In regions of sequence heterogeneity or large (> 3 bp) length differences, or in parts of the molecule that did not form structures consistent with those of other organisms, the limits of the variable regions were identified and the number of nucleotides in them counted to allow comparisons to be made among groups. Organisms that represent the two kingdoms that possess plastids (Protista and Plantae), and for which secondary structure maps are available, served as representatives of the eukaryotic cp23S-rDNA secondary structure. These organisms included *Astasia longa* Pringsheim (a colourless flagellate; X14386), *Chlamydomonas reinhardtii* Dangeard (X15727), *Cyanophora paradoxa* Korshikov (a glaucocystophyte; U30821), *Euglena gracilis* Klebs (X12890), *P. falciparum* and *Zea mays* Linnaeus (Z00028). The cp23S-rDNA secondary structures for these organisms were also downloaded from the Gutell Lab Comparative RNA web site.

RESULTS AND DISCUSSION

RFLP analysis of dinoflagellate n18S-rDNA

RFLP analysis of n18S-rDNA revealed that 30 of the 31 algal cultures included in our study belonged to *Symbiodinium* clades A, B, C or D (Table 1). One culture, JN120.1, isolated from a newly settled polyp of the Caribbean gorgonian *Plexaura kuna* Lasker, Kim & Coffroth, produced an RFLP pattern that was not consistent with those of the recognized *Symbiodinium* clades. Phylogenetic analysis of the cp23S-rDNA sequence from this culture suggests that it is a member of the free-living dinoflagellate genus *Heterocapsa* Stein (S.R. Santos, unpublished observations). The most likely explanation for this result is that JN120.1 was inadvertently isolated from the surface or within the digestive system of the *P. kuna* polyp

during attempts to isolate and culture members of *Symbiodinium.*

Aberrant cp23S-rDNA molecules in *Symbiodinium*

Amplification of the various *Symbiodinium* cultures with the primers 23S1M13 and 23S2M13 produced one or two PCR products, depending on the *Symbiodinium* clade and isolate. The larger PCR product, common to all samples, varied in size (*c.* 620–800 bp on 2% 0.5× TBE agarose gels) depending on the *Symbiodinium* clade. This size range for the larger PCR products is consistent for domain V of the cp23S-rDNA in dinoflagellates (Zhang *et al.* 2000) and is hereinafter referred to as the full-length cp23S-rDNA domain V product. The smaller PCR product, which appeared in one of 11 and four of 11 *Symbiodinium* clade A and B cultures (Table 1), respectively, ranged in size from *c.* 300 to 500 bp on 2% 0.5× TBE agarose gels. These smaller PCR products were consistently amplified from these cultures under various PCR conditions, such as different $MgCl_2$ concentrations and primer annealing temperatures (data not shown). For these *Symbiodinium* isolates, both products were isolated and sequenced to determine their identity. The *Heterocapsa* sp. culture, JN120.1, produced a single PCR product of approximately 700 bp on 2% 0.5× TBE agarose gels.

DNA sequences obtained directly from the smaller PCR products suggest that they are cp23S-rDNA molecules that have suffered internal deletion mutations. In the case of the *Symbiodinium* clade A isolate from *Tridacna gigas* Linne, alignment of the sequences revealed that the smaller (hereinafter referred to as aberrant) cp23S-rDNA molecule was identical in sequence to the full-length cp23S-rDNA domain V product, except for a deletion of 164 nucleotides in the middle of the molecule. In the case of the *Symbiodinium* clade B isolates FLAp#3 and Pk13, single long stretches of 250 and 298 bp, respectively, were also absent from the middle of the aberrant cp23S-rDNA molecules. For the third clade B isolate, culture Pd, the aberrant cp23S-rDNA molecule lacked two short (6 and 23 bp) regions, along with a stretch of 208 nucleotides near the 3′ end of the molecule. Attempts to sequence the aberrant cp23S-rDNA molecule of the fourth *Symbiodinium* clade B culture (culture Pk706) directly from the PCR product resulted in sequences that were initially clear but then deteriorated rapidly in both the forward and reverse directions. In addition, attempts to sequence the full-length cp23S-rDNA domain V products of the four *Symbiodinium* clade B cultures discussed earlier gave similar results. Results such as these have been observed when attempting to sequence mixtures of PCR products that differ in length (Williams & Knowlton 2001). To explore this possibility, the PCR products from these samples were cloned and sequenced.

Sequence analysis of clones from the full-length cp23S-rDNA domain V products of the *Symbiodinium* clade B cultures FLAp#3, Pk13 and Pk706 revealed that clones (seven clones each for cultures FLAp#3 and Pk13; four clones for Pk706) from the same sample possessed identical or nearly identical sequences. For some cloned sequences, single transitions were observed at nucleotide positions conserved between the *Symbiodinium* clades, suggesting that these substitutions represent PCR error. However, no length differences were observed between cloned sequences from the same sample. Thus, the rapid deterioration of the sequence observed from the PCR products probably originated from contamination of the full-length cp23S-rDNA domain V products with the aberrant cp23S-rDNA product from the same culture. On the other hand, sequence analysis of clones from the full-length cp23S-rDNA domain V product of the Pd culture revealed three types of distinct sequences. The first type of sequence (three of eight clones) lacked the same 23 bp region as the sequence of the aberrant cp23S-rDNA molecule from the same culture. The second type of sequence (two of eight clones) lacked this 23 bp region, along with the same 6 bp region that was missing in the aberrant cp23S-rDNA molecule. Both of these molecules possessed the 208 bp region that was absent in the aberrant cp23S-rDNA molecule (see earlier). The third type of sequence (three of eight clones) was full-length and identical or nearly identical in sequence to the full-length cp23S-rDNA domain V clones obtained from cultures FLAp#3, Pk13 and Pk706 (isolates that produced the aberrant cp23S-rDNA domain V molecules) and full-length cp23S-rDNA domain V PCR products from cultures SSPe, Ap10 and Pe (isolates that did not produce the aberrant cp23S-rDNA domain V molecules). For the aberrant cp23S-rDNA PCR product of culture Pk706, clones contained one out of two distinct sequences. One type of sequence (one of five clones) lacked a stretch of 105 bp and the second type of sequence (four of five clones) lacked the same stretch of 105 bp, along with a region of 6 bp. However, the 6 bp region absent in the aberrant cp23S-rDNA molecule of the culture Pk706 was distinct from the 6 bp region absent in the various molecules recovered from the culture Pd (see earlier). Again, single transitions were observed in conserved nucleotide positions between the cloned sequences and are thought to represent PCR error.

The internal deletions present in these cp23S-rDNA molecules and the consistency with which we were able to amplify these molecules from the same five *Symbiodinium* cultures provide evidence that the variation results from length heteroplasmy rather than artefacts of the PCR amplification. Additional evidence comes from the fact that the *Symbiodinium* isolates that produced aberrant cp23S-rDNA domain V products also possessed full-length cp23S-rDNA domain V products that were identical or nearly identical to those from cultures that did not produce aberrant molecules. Examples of this include two *Symbiodinium* clade A cultures (isolates CassEL1 and CassMJ300) that possessed full-length cp23S-rDNA domain V sequences identical to the *T. gigas* isolate and the *Symbiodinium* clade B isolates discussed earlier. If these aberrant cp23S-rDNA molecules resulted from errant primer–template interactions or other PCR artefacts, they would be expected to occur in samples that possessed similar templates. However, this is not the case. Furthermore, cp23S-rDNA molecules with internal deletions have also been sequenced from *Symbiodinium* clades B and D populations *in hospite* (S.R. Santos, unpublished observations), demonstrating that these molecules are not limited to *Symbiodinium* cultures. Taken together, these data imply that these molecules are genuine and that some *Symbiodinium* strains harbour 1–3 types of aberrant cp23S-rDNA molecules. Zhang *et al.* (1999) found truncated copies of *psb*C and cp16S-rDNA genes in the free-living dinoflagellate *H. triquetra.* Our data, combined with the observations of Zhang *et al.* (1999), imply that ab-

errant protein and ribosomal genes may be common in the chloroplast genomes of dinoflagellates.

The existence of cp23S-rDNA molecules with internal deletions in *Symbiodinium* raises the question of how these molecules form. Recent work by Zhang *et al.* (2001) found that minicircular chromosomes in *H. triquetra* undergo recombination, resulting in probably nonfunctional, aberrant minicircular chromosomes that represent fragments of different chloroplast genes (Zhang *et al.* 2001). In these aberrant minicircular chromosomes, the fragments remain identical or nearly identical in sequence to the corresponding regions of the normal minicircular chromosomes (i.e. functional genes) by gene conversion (Zhang *et al.* 2001). Gene conversion also appears to occur in the chloroplast genome of *Symbiodinium* because the sequences of aberrant and full-length cp23S-rDNA domain V molecules are identical or nearly so. If these aberrant cp23S-rDNA molecules in *Symbiodinium* were pseudogenes, located in the nucleus, one would expect sequence divergence from the full-length molecule. Alternatively, these aberrant cp23S-rDNA molecules in *Symbiodinium* may have arisen recently, leaving little time for sequence divergence to occur. Although our data cannot rule out the possibility that the aberrant *Symbiodinium* cp23S-rDNA molecules occur as chimeric minicircular chromosomes, this phenomenon does not seem to explain the pattern of length heteroplasmy observed in *Symbiodinium.* The cp23S-rDNA fragments in the aberrant minicircular chromosomes of *H. triquetra* possess duplications as well as deletions (Zhang *et al.* 2001), which is in contrast to the aberrant cp23S-rDNA molecules of *Symbiodinium,* which have only suffered deletions. Furthermore, only two regions of *H. triquetra* cp23S-rDNA are involved in aberrant minicircular chromosomes (Zhang *et al.* 2001), neither of which encompasses domain V. These data suggest that the molecular and evolutionary processes by which aberrant cp23S-rDNA molecules are created and maintained in *Symbiodinium* are probably not identical to the processes that operate on the aberrant minicircular chromosomes of *H. triquetra.*

A possible mechanism that could generate deletion mutations in *Symbiodinium* cp23S-rDNA domain V is replication slippage. In rat mitochondrial DNA, replication slippage and deletion mutations of various lengths have been proposed to occur in regions of strong secondary structure (VanTuyle *et al.* 1996). In addition, these regions are typically flanked by AT-rich sequences (VanTuyle *et al.* 1996). For *Symbiodinium,* the cp23S-rDNA domain V is AT-rich (58–63%; S.R. Santos, unpublished observations) and forms secondary structures (Fig. 1). Furthermore, comparisons between the deletions from *Symbiodinium* clade B (TreeBase matrix accession number M1040) reveal no common length to the deletions or nucleotide motifs (e.g. direct repeats) at the junctions of the deletions. These characteristics make replication slippage a possible mechanism for the formation of the deletion mutations in *Symbiodinium* cp23S-rDNA. However, additional studies are required to test this hypothesis. Questions concerning the exact mechanisms by which these aberrant cp23S-rDNA molecules are created in *Symbiodinium,* the reasons behind the high incidence of these molecules in *Symbiodinium* clade B, and the fates of these molecules deserve further examination.

Secondary structure of *Symbiodinium* cp23S-rDNA domain V

The proposed secondary structure of *Symbiodinium* cp23S-rDNA domain V is consistent with those published for other cp23S-rDNAs (Fig. 1). However, seven areas that were identified by comparisons between *Symbiodinium,* free-living dinoflagellates and other plastid-harbouring organisms deserve special mention. These areas (labelled a–g in Fig. 1) varied mainly in size (Table 2) and are discussed later. For simplicity, we have chosen to employ the *Escherichia coli* (Migula) Castellani & Chalmers coordinate system (nucleotide positions 1915–2592) (Brosius *et al.* 1980), when defining an area, and the ES numbering system employed by Gerbi (1996), when describing variable size regions. An alignment of representative cp23S-rDNA sequences is available from TreeBase under matrix accession number 1041.

AREA A (POSITIONS 2041–2044): This area is located in the universal core secondary structure of 23S-rDNA (Gerbi 1996). In *Symbiodinium* clades A, C and D, 11–13 bp are inserted into this area, whereas *Symbiodinium* clade B possesses a single additional nucleotide (Table 2). These nucleotides (boxed region in Fig. 2) display sequence variability within, as well as between, the *Symbiodinium* clades. ES regions are not known to occur at these positions in other 23S-rDNAs (Gerbi 1996; Schnare *et al.* 1996), suggesting that this is a novel, and previously undocumented, insertion in the universal core secondary structure of 23S-rDNAs. Furthermore, phylogenetic data suggest that the insertion is a unique derived state for the genus *Symbiodinium* that has been reduced in size in *Symbiodinium* clade B. *Symbiodinium* phylogenies inferred from cp23S-rDNA gene sequences suggest that the genus is monophyletic and comprises two principal groups. One group comprises members of *Symbiodinium* clade A; the second group encompasses members of *Symbiodinium* clades B, C and D, with clade D being basal to clades B and C (S.R. Santos, unpublished observations). Whether this novel insertion is confined to *Symbiodinium* cp23S-rDNA or whether it is also found in the cp23S-rDNAs of dinoflagellates closely related to *Symbiodinium* remains to be determined.

AREA B (POSITIONS 2081–2239): This area contains three ES regions (ES 29–31) and also contributes 10 and 11 bp to the universal core secondary structure of 23S-rDNAs (Gerbi 1996; Schnare *et al.* 1996), which are localized towards the 5′ and 3′ ends of the area, respectively. Little size variation is observed in the cp23S-rDNA of other plastid-harbouring eukaryotes (Table 2). However, the dinoflagellates display a wide range of sizes (Table 2). Members of *Symbiodinium* possess the smallest number of nucleotides described to date for this area, ranging from 86 to 142 bp. On the other hand, the *Heterocapsa* sp. culture JN120.1 possesses the largest number of nucleotides (195 bp) described to date for this area of cp23S-rDNA. Additional nucleotide sequences and detailed comparative studies are required to deduce the secondary structure of this area because of the extreme variability in primary sequence and length within *Symbiodinium.*

AREA C (POSITIONS 2296–2322): This entire area is considered as an ES region (ES 33) in the 23S-rDNA (Gerbi 1996). In other plastid-harbouring eukaryotes, two size classes have been observed (Table 2). Length variation in *Symbiodinium* is

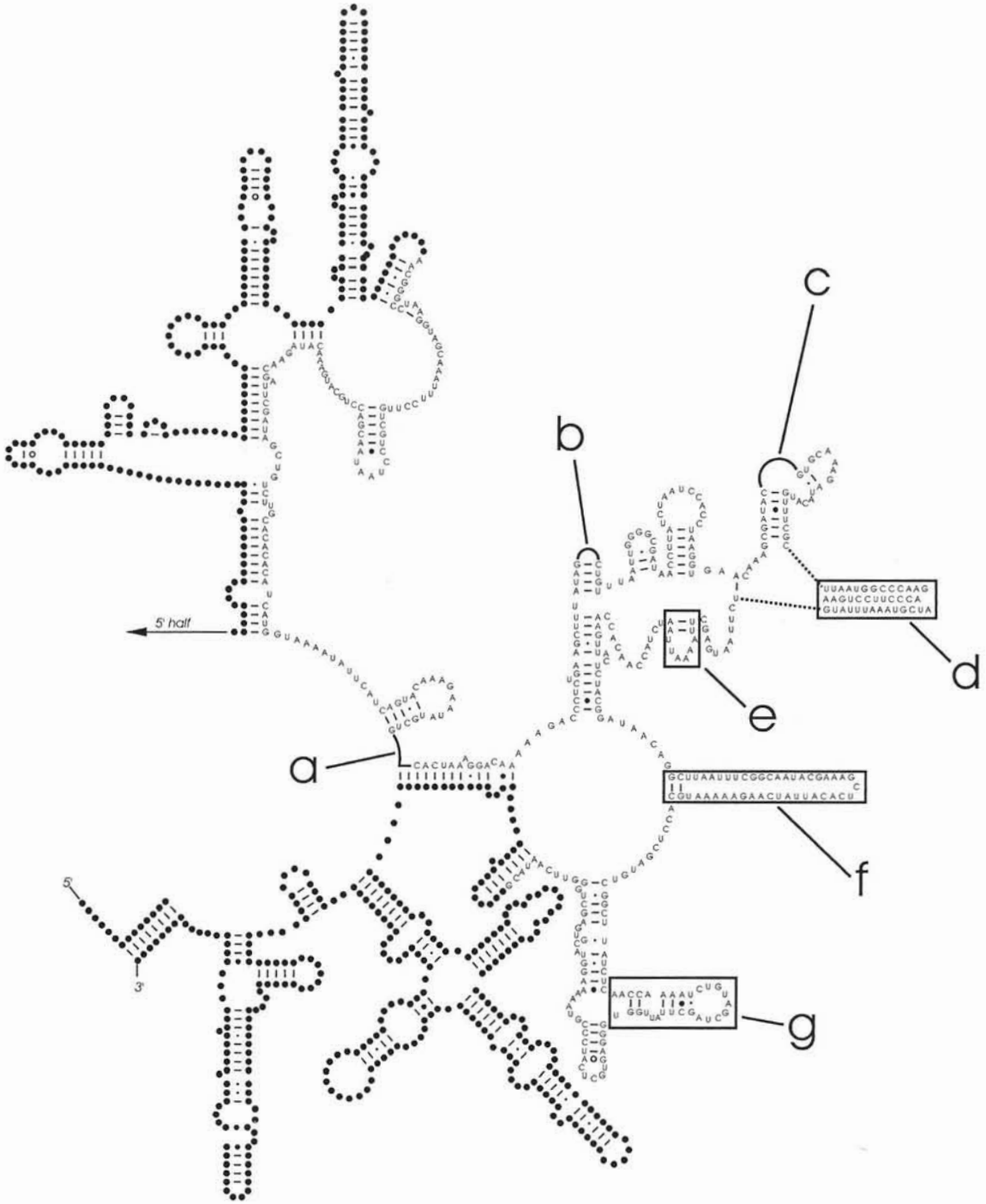

Fig. 1. Proposed consensus secondary structure of the *Symbiodinium* chloroplast 23S-rDNA domain V. Filled circles outside domain V represent nucleotides whose sequence is unknown. Secondary structure formed by filled circles represents a consensus secondary structure for cp23S-rDNA based on other eukaryotic cp23S-rDNA. Letters a–g represent areas discussed in the text and in Table 2. Base pairing is indicated as follows: standard canonical pairs by lines (–), wobble pairs by dots (·), A·G pairs by open circles (○), and other noncanonical pairs by filled circles (e.g. C●A) (Schnare *et al.* 1996).

17–103 bp, whereas that in the free-living dinoflagellates is comparable with that in other eukaryotes (Table 2). Secondary structural analysis of this region in *Symbiodinium* using Mfold (http://mfold.burnet.edu.au/) suggests that it forms a stem loop (data not shown), which is consistent for this region in other 23S-rDNAs (Gerbi 1996; Schnare *et al.* 1996).

AREA D (POSITIONS 2296–2322): This area contains no ES regions in the 23S-rDNA (Gerbi 1996) and is nearly identical in size among *Symbiodinium,* free-living dinoflagellates and other plastid-harbouring eukaryotes (Table 2). For *Symbiodinium,* however, this region cannot be folded into the typical secondary structure (with two stem loops) found in other

Table 2. Nucleotide lengths (bp) for locations highlighted in Fig. 1.

	a	b	c	d	e	f	g
Symbiodinium clade A	14	96–106	17–20	39	10	45	28
Symbiodinium clade B	4	102–142	28–34	39	10	45	28
Symbiodinium clade C	14–15	86–87	30–34	39	10	45	28
Symbiodinium clade D	16	112	103	40	10	45	28
Free-living dinoflagellates	3	138–195	27–28	37–40	10	42–43	28–194
Other eukaryotes[1]	3	159–165	5 or 27	40	27–28	42	29

[1] *Astasia longa* (Euglenozoa), *Chlamydomonas reinhardtii* (Chlorophyta), *Cyanophora paradoxa* (Glaucocystophyceae), *Euglena gracilis* (Euglenozoa), *Plasmodium falciparum* (Apicomplexa), *Zea mays* (Tracheophyta).

cp23S-rDNA molecules. Attempts to fold this region into these two stem loops results in numerous nucleotide mispairings. Secondary structural analysis of this region in *Symbiodinium* using Mfold (http://mfold.burnet.edu.au/) results in a single stem loop containing several bulges (data not shown). However, detailed comparative studies are required to deduce if a single stem loop is the secondary structure of this region in *Symbiodinium.*

AREA E (POSITIONS 2396–2421): This area contains two ES regions (ES 34–35) in the 23S-rDNA (Gerbi 1996). In *Symbiodinium,* as well as in the free-living dinoflagellates examined here, this stem loop is approximately half the size of those found in the cp23S-rDNA of other plastid-harbouring eukaryotes (Table 2).

AREA F (POSITIONS 2455–2496): This area is located in the universal core secondary structure of 23S-rDNA (Gerbi 1996). In *Symbiodinium,* three additional nucleotides are present in this stem loop, compared with other plastid-harbouring eukaryotes, and an additional nucleotide is found in *Heterocapsa* (Table 2). Because of the presence of this 3 bp insertion in *Symbiodinium,* the exact base pairings in this stem loop remain to be determined.

AREA G (POSITIONS 2518–2546): This area is also located in the universal core secondary structure of the 23S-rDNA (Gerbi 1996; Schnare *et al.* 1996). There is a single nucleotide difference between *Symbiodinium* and some of the free-living dinoflagellates, compared with other plastid-harbouring eukaryotes (Table 2). However, the cp23S-rDNA of the free-living dinoflagellate *Protoceratium reticulatum* possesses an insertion of 166 nucleotides that has not been found in any other cp23S-rDNA genes to date (Zhang *et al.* 2000).

Chloroplast rDNAs show strong conservation in the secondary structure across their domains, including domain V of cp23S-rDNA (Harris *et al.* 1994). However, previously undocumented changes in the typically conserved secondary structures of cp23S-rDNA genes are reported here for the symbiotic dinoflagellates. Members of *Symbiodinium* possess a unique insertion that has no homologue in other eubacterial or chloroplast 23S-rDNA genes (area a, Fig. 1), whereas the ES regions (areas b and c, Fig. 1) of *Symbiodinium* are the most variable in size of any known plastid-harbouring organism (Table 2). For the free-living dinoflagellates, Zhang *et al.* (2000) found a unique 166 bp insertion in *P. reticulatum* cp23S-rDNA (area g, Fig. 1) and Tengs *et al.* (2000) found that *Gymnodinium galatheanum* (Braarud) Taylor cp16S-rDNA contains at least one insertion with no apparent homologue in any known cp16S-rDNA sequence. Our data, along with the observations of Tengs *et al.* (2000) and Zhang *et al.* (2000), suggest that unique insertions are common in cp-rDNAs of dinoflagellates. In the case of *Symbiodinium,* how this unique insertion affects the overall secondary struc-

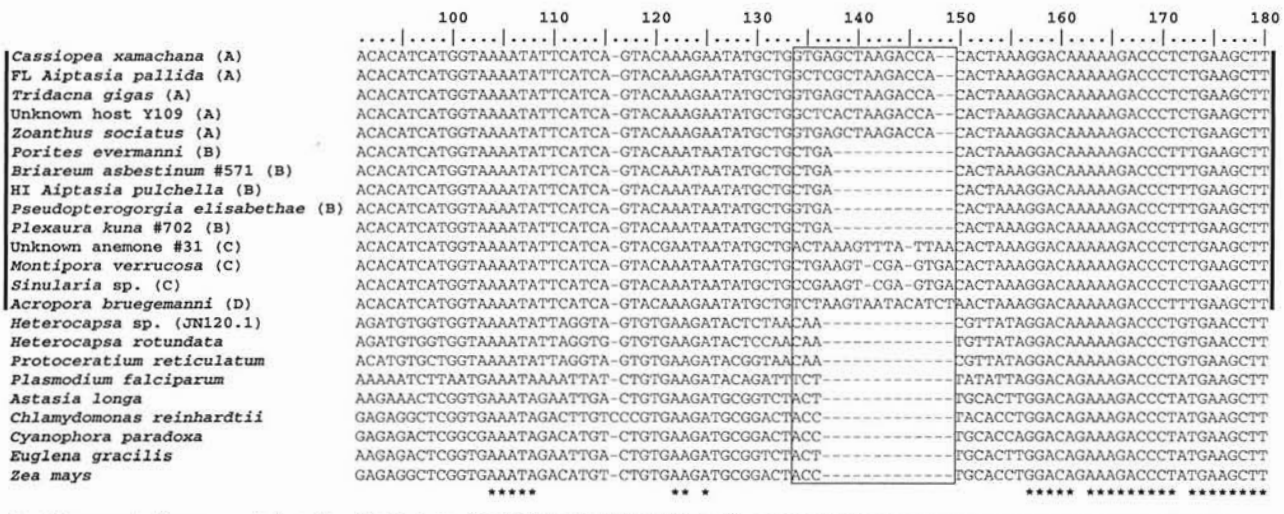

Fig. 2. Alignment of representative *Symbiodinium,* free-living dinoflagellate and other eukaryotic cp23S-rDNA sequences, showing a novel nucleotide insertion in *Symbiodinium.* Numbered scale above nucleotides designates the position in the full alignment. Brackets (vertical black lines) surround *Symbiodinium* sequences. Boxed region indicates nucleotides present in area a of Fig. 1. Asterisks (*) under nucleotides designate residues that are identical in all sequences. The host from which *Symbiodinium* was isolated is used to designate cultures. The letter in parentheses following the host name indicates the *Symbiodinium* clade.

ture of the cp23S-rDNA molecule is unknown and awaits sequence information for the entire molecule. However, both the *Symbiodinium* and *P. reticulatum* insertions, along with the 3 bp insertion in loop f of Fig. 1, occur in the universal core secondary structure of cp23S-rDNA. These data demonstrate that the universal core secondary structure of cp23S-rDNAs is not as constrained as was previously thought. Additional cp23S-rDNA sequences from *Symbiodinium* and other dinoflagellates are required to construct more detailed secondary structures for these organisms. These data will contribute to the elucidation of the chloroplast ribosome and the organellar genome evolution in the dinoflagellates, as well as in other plastid-harbouring organisms.

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