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Molecular Characterization of Nuclear Small Subunit (18S)-rDNA Pseudogenes in a Symbiotic Dinoflagellate (*Symbiodinium*, Dinophyta)

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ABSTRACT. For the dinoflagellates, an important group of single-cell protists, some nuclear rDNA phylogenetic studies have reported the discovery of rDNA pseudogenes. However, it is unknown if these aberrant molecules are confined to free-living taxa or occur in other members of the group. We have cultured a strain of symbiotic dinoflagellate, belonging to the genus *Symbiodinium*, which produces three distinct amplicons following PCR for nuclear small subunit (18S) rDNA genes. These amplicons contribute to a unique restriction fragment length polymorphism pattern diagnostic for this particular strain. Sequence analyses revealed that the largest amplicon was the expected region of 18S-rDNA, while the two smaller amplicons are *Symbiodinium* nuclear 18S-rDNA genes that contain single long tracts of nucleotide deletions. Reverse transcription (RT)-PCR experiments did not detect RNA transcripts of these latter genes, suggesting that these molecules represent the first report of nuclear 18S-rDNA pseudogenes from the genome of *Symbiodinium*. As in the free-living dinoflagellates, nuclear rDNA pseudogenes are effective indicators of unique *Symbiodinium* strains. Furthermore, the evolutionary pattern of dinoflagellate nuclear rDNA pseudogenes appears to be unique among organisms studied to date, and future studies of these unusual molecules will provide insight on the cellular biology and genomic evolution of these protists.

Key Words. Culture, truncated genes, zooxanthellae.

DINOFLAGELLATES are an important group of unicellular protists common to aquatic environments, being major photosynthetic and heterotrophic members of the plankton as well as the causative agents of 'red tides' and fish kills. In addition, dinoflagellates exhibit a diversity of lifestyles, ranging from free-living to parasitic and mutualistic symbioses. The taxonomy and phylogenetics of the dinoflagellates have traditionally relied on diagnostic morphological characters, such as number and structure of thecal plates (Fensome et al. 1996). Analyses of nuclear ribosomal DNAs (rDNAs), however, have demonstrated the shortcomings of employing morphological characters for inferring relationships within this group. In some cases, phylogenies based on morphological characters do not necessarily delineate related groups of dinoflagellates (Saunders et al. 1997; Wilcox 1998; Zardoya et al. 1995). In other cases, diversity has been uncovered in genera that are depauperate in morphologically distinguishing characteristics (Rowan and Powers 1991a). These revelations, along with ease, speed, and reproducibility, have made nuclear rDNAs analysis the technique of choice for molecular ecological and evolutionary studies of extant dinoflagellates (Daugbjerg et al. 2000; Saldarriaga et al. 2001).

Analyses of dinoflagellate rDNAs have also discovered unexpected, but interesting, molecules. Some isolates of the toxin-producing free-living dinoflagellate *Alexandrium catenella* have been found to produce two distinct polymerase chain reaction (PCR) amplicons for nuclear large subunit (28S)-rDNA (Yeung et al. 1996). Sequence analysis revealed that the larger amplicon was the expected region of *A. catenella* 28S-rDNA while the smaller amplicon was a 28S-rDNA gene that had suffered an 87-bp deletion (Yeung et al. 1996). A similar situation has been observed in American isolates of the free-living dinoflagellate *Dinophysis acuminata* (Rehnstam-Holm, Godhe, and Anderson 2002). In both cases, additional mutational differences were apparent between the full-length and aberrant amplicons (Rehnstam-Holm, Godhe, and Anderson 2002; Yeung et al. 1996), suggesting that they were not artifacts of the PCR but instead represent 28S-rDNA pseudogenes, or non-functional copies of the molecule. Full-length nuclear small subunit (18S)-rDNA pseudogenes have also been documented from the toxin-producing free-living dinoflagellate *A. fundyense* (Scholin, Anderson, and Sogin 1993). However, it is not known whether nuclear rDNA pseudogenes are confined to the free-living dinoflagellates or extend to other members of the group.

Associations between invertebrates and symbiotic dinoflagellates belonging to the genus *Symbiodinium* Freudenthal (Taylor 1974) are a common feature of tropical marine environments. Collectively known as zooxanthellae, these dinoflagellates play an important role in their host's nutrition and physiology (reviewed in Davies 1993). Additional smaller amplicons in 18S-rDNA PCRs have been reported for *Symbiodinium* from some coral hosts (Loh, Carter, and Hoegh-Guldberg 1998; Rowan and Powers 1991b). We have also observed this phenomenon from *Symbiodinium* populations *in hospite*; in these cases, one or two smaller amplicons are apparent following PCR and contribute additional bands to the diagnostic restriction fragment length polymorphism (RFLP) pattern. Unfortunately, the identity of these additional amplicons and the possibility that they represent nuclear rDNA pseudogenes in the *Symbiodinium* genome has not been established. In this report, we provide the first molecular evidence of 18S-rDNA pseudogenes in symbiotic dinoflagellates by characterizing the additional 18S-rDNA amplicons produced by a cultured *Symbiodinium* strain from the Ryukyu Islands, Japan.

MATERIALS AND METHODS

Biological materials and nucleic acid extraction. Individual anemones (n = 45), tentatively identified as *Entacmaea quadricolor* (Fautin, D., pers. commun.), were haphazardly collected from a water table at the Sesoko Station, Okinawa Prefecture, Ryukyu Islands, Japan, in late July–early August, 2000. Oral disks, approximately 1.0 cm in diam., were severed from each anemone and one-third (n = 16) were used to establish *Symbiodinium* cultures by the methods of Santos, Taylor, and Coffroth (2001). The remaining cells, as well as the other oral disks (n = 29), were preserved in 95% ethanol for later molecular analysis. *Symbiodinium* cultures were maintained as described for *Aiptasia pallida* zooxanthella cultures in Santos, Taylor, and Coffroth (2001) and sampled monthly over a six-month period. Total nucleic acids from live and preserved materials were extracted and quantified according to the methods of Coffroth et al. (1992).

Amplification and RFLP analysis of *Symbiodinium* 18S-rDNA. *Symbiodinium* 18S-rDNA was amplified using the di-

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noflagellate biased primer set ss5 and ss3z (Rowan and Powers 1991b), digested with TaqI and separated in 2% 0.5× Tris-borate (TBE) agarose gels to generate RFLPs. RFLP analysis of 18S-rDNA 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 2001; LaJeunesse and Trench 2000), with each clade probably comprised of many species (Rowan 1998). RFLPs were compared to cloned standards to assess *Symbiodinium* cladal identity.

Characterization of novel *Symbiodinium* 18S-rDNA amplicons. To determine the identity of novel amplicons and how they contribute to the RFLP, 18S-rDNA PCR products were first purified in 2% 1× modified Tris-acetate-EDTA (TAE) (0.04 M Tris-acetate, 0.0001 M EDTA final concentration) agarose gels. Bands of interest were cut from the gel, recovered from the excised agarose blocks by centrifugation using Spin-X® 0.22 μm CA centrifuge tube filters (Costar Corporation, Corning, NY), and cloned using the TOPO TA Cloning® kit (Invitrogen, Carlsbad, CA). Prior to sequencing, clones were screened by PCR with primers ss5 and ss3z for the presence of appropriately sized inserts. Those chosen for sequencing were grown overnight in 1.5 ml Luria-Bertani (LB) broth and plasmids isolated by alkaline extraction (Birnboim 1983). Two clones for each novel amplicon were sequenced in both directions using 5′-IRD800 M13 labelled primers (LI-COR Biotechnology Division, Lincoln, NE) and the SequiTherm EXCEL™ II DNA Sequencing Kit-LC (Epicentre Technologies, Madison, WI) according to the suppliers' recommendations. Sequences were deposited into GenBank under accession numbers AF396619–AF396622.

Reverse transcription (RT)-PCR of *Symbiodinium* 18S-rDNA. To determine if the novel amplicons were being transcribed into RNA, total RNA was extracted from cultured materials with the MasterPure™ RNA Purification Kit (Epicentre Technologies, Madison, WI). Reverse transcription (RT)-PCR using primers ss5 and ss3z was done with the MasterAmp™ High Fidelity RT-PCR Kit (Epicentre Technologies, Madison, WI) under the manufacturer's suggested conditions. Reactions products were separated in 2% 0.5× TBE agarose gels as described above for RFLPs.

PCR primer mismatch and novel *Symbiodinium* 18S-rDNA amplicons. To determine if sequence heterogeneity and primer mismatch was responsible for production of novel amplicons, the V1–V4 region (Dams et al. 1988) of 18S-rDNA was sequenced for our previously undescribed symbiont as well as two *Symbiodinium* clade D cultures. These two isolates, which do not produce additional amplicons, were obtained from the scleractinian corals *Acropora* sp. (isolate A002) and *Acropora bruegemanni* (isolate A024) and maintained as previously described. Region V1-V4 was amplified using the primer ss5M13 (5′–CACGACGTTGTAAAACGACGGTTGATCCTG CCAGTAGTCATATGCTTG–3′) and an internal primer biased toward *Symbiodinium* 18S-rDNA, ssE21.6M13 (5′–GGATAA-CAATTTCACACAGGCTAGAAACCAACAAAATAGAACT-GAGGTC–3′), under the conditions described in Rowan and Powers (1991b). Amplicons were gel-purified and sequenced in both directions directly from PCR products as described above. Sequences were deposited into GenBank under accession numbers AF396623–AF396625.

Sequence comparisons to other *Symbiodinium* isolates. To characterize the molecular divergence between our undescribed symbiont and cultures A002 and A024, the D1–D3 regions of 28S-rDNA, the entire internal transcribed spacer (ITS)-rDNA region and Domain V of chloroplast large subunit (cp23S)-rDNA were sequenced directly from PCR products as described previously. Primers and conditions to amplify these rDNAs are given in Santos et al. (2002a). Sequences were manually aligned and comparisons between isolates made by visual inspection. Sequences were deposited into GenBank under the following accession numbers: 28S-rDNA (AF396626–AF396628), ITS-rDNA (AF396629–AF396631), and cp23S-rDNA (AY035426; AY035428–AY035429). These sequences, along with those from the V1–V4 regions of 18S-rDNA, represent approximately 3,000 nucleotides from each *Symbiodinium* isolate (approximately 2,300 and 700 nucleotides from the nuclear and chloroplast genomes, respectively).

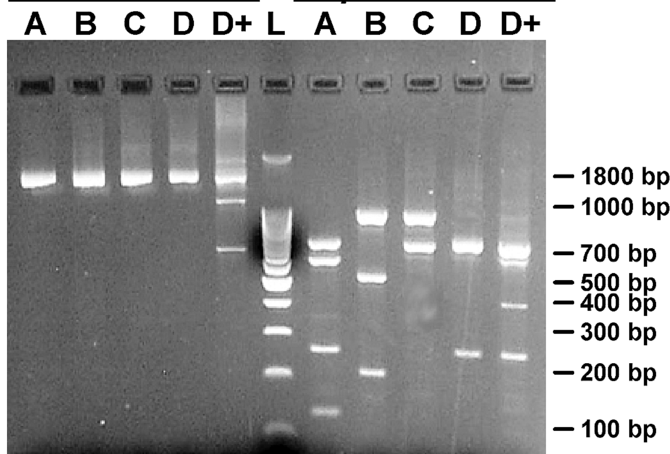

Fig. **1**. 18S-rDNA PCR amplifications and TaqI RFLPs of *Symbiodinium*. Letters above lanes represent *Symbiodinium* clade. DNA template for all PCR amplifications were extracted from *Symbiodinium* cultures. L = 100-bp DNA size ladder.

RESULTS

Novel 18S-rDNA amplicons and their contributions to a unique RFLP pattern. Either one or three amplicons were observed in *Symbiodinium* 18S-rDNA amplifications from cultures or preserved materials of *E. quadricolor*. The largest amplicon, present in all samples, was of the predicted size for *Symbiodinium* 18S-rDNA (approx. 1,800 bp). For 15 of the 45 (33%) *Symbiodinium* isolates, in addition to the 1800-bp amplicon, products of approximately 1,100 bp and 700 bp were also observed (example in Fig. 1, Lane D+). RFLP analysis revealed that samples possessing only the 1,800-bp amplicon belonged to *Symbiodinium* clade C while an uncharacterized RFLP was associated only with samples that produced the additional amplicons. When the uncharacterized RFLP was compared to the RFLPs of the recognized *Symbiodinium* clades, it was found to be most similar to the *Symbiodinium* clade D (Fig. 1). We assigned the designation ''D+'' to our unique RFLP since the differences between the D+ and *Symbiodinium* clade D RFLP are the presence of two additional fragments in D+ that are absent in *Symbiodinium* clade D (Fig. 1).

Cloning and RFLP analysis of the 1,100-bp and 700-bp amplicons revealed their contributions to the overall D+ RFLP. The 1,100-bp amplicon possessed a single TaqI restriction site and was digested into fragments of approximately 700 and 400 bp. Since the 700-bp fragment co-migrates with the 700-bp fragments produced from digestion of the full length (1,800-bp) *Symbiodinium* clade D 18S-rDNA amplicon, it does not visibly contribute to the D+ RFLP. The 400-bp fragment, on the other hand, visibly contributes to the D+ RFLP (Fig. 1). The 700-bp amplicon was not digested with TaqI, suggesting

Table **1**. Identification of *Symbiodinium* isolates from *Entacmaea quadricolor* following one and six months in culture. Cladal identification is based on TaqI RFLPs in 18S-rDNA. Numbers in parentheses indicate percentage of 16 cultures.

	Symbiodinium clade C RFLP only	Unknown RFLP only	*Symbiodinium* clade C & unknown RFLPs	Crash[a]
One month in culture	11 (68.7%)	1 (6.3%)	2 (12.5%)	2 (12.5%)
Six months in culture	3 (18.8%)	9 (56.3%)	2 (12.5%)	0

[a] Cultures that died out within the first month of establishment.

that it lacks restriction sites for this enzyme. However, the 700-bp amplicon migrates slightly faster than the 700-bp fragments produced by the digestion of the full length *Symbiodinium* clade D 18S-rDNA amplicon, suggesting that it is smaller than the estimated 700 bp. Thus, it contributes to the overall D+ RFLP as the lower band of the doublet that migrates at approximately 700 bp (Fig. 1).

***Symbiodinium* cultures and D+.** For the *Symbiodinium* isolates used to establish cultures, a *Symbiodinium* clade C RFLP was recovered from most (11 of 14, 78.5%) inocula (Table 1). The D+ RFLP was recovered from a single initial inoculum and in two cases, in combination with the *Symbiodinium* clade C pattern (Table 1). Over a six-month period, D+ succeeded *Symbiodinium* clade C in a number of the cultures (Table 1 and Fig. 2). In some cases, D+ was detected (arrows in Fig. 2a, lane #4) at the start of culturing. However, for most cultures that underwent a succession, the presence of D+ was not readily apparent at the outset of culturing (Fig. 2). These culturing experiments demonstrate the stability of the D+ RFLP over time.

Sequence analyses of novel 18S-rDNA amplicons. Replicate clones for the 1,100-bp amplicon of D+ were identical in sequence and had an exact length of 1,116 bp. Clones of the 700-bp amplicon were 99% identical (differing by a transition and one insertion deletion (indel)) and had an exact length of either 638 or 639 bp. Computer-simulated TaqI digestion (Sequencher™ 3.0.1, Gene Codes Corp, Ann Arbor, MI) of the 1,116-bp amplicon produced fragments of 707 and 409 bp, while the 638/639-bp amplicon lacked TaqI restriction sites, which is consistent with the restriction digestions discussed earlier. Sequence comparisons between the 1,100-bp and 700-bp amplicons revealed 99% similarity across their overlapping regions. BLAST (Altschul et al. 1997) searches to Genbank using the 1,100-bp and 700-bp amplicon sequences found best matches to *Symbiodinium* clade D 18S-rDNA (AF238261 and AF238262; submitted to GenBank by Toller, Rowan, and Knowlton, 2001, as *Symbiodinium* clade E harboured by members of the *Montastraea annularis* species complex) with a similarity of approximately 98.6% across the overlapping regions. These data rule out the possibility that the 1,100-bp and 700-bp amplicons result from non-*Symbiodinium* contamination and demonstrate that they are some form of *Symbiodinium* clade D 18S-rDNA.

Aligning the 1,100-bp and 700-bp amplicons to the full-length *Symbiodinium* clade D sequences from Genbank revealed that single, long tracts of the gene were missing from each amplicon (Fig. 3a–c). For the 700-bp amplicon, the nucleotides corresponding to positions 99–1,152 of the gene were found to be absent (Fig. 3b). This missing region is situated approximately 70 bp and 513 bp from the 3′ ends of primers ss5 and ss3z, respectively. Thus, the discontinuous nature of the 700-bp amplicon strongly suggests that it is not an artifact or chimera generated by primer-template mismatch during PCR. The 1,100-bp amplicon, on the other hand, lacked the region corresponding to positions 30–606 (Fig. 3c). Given that position

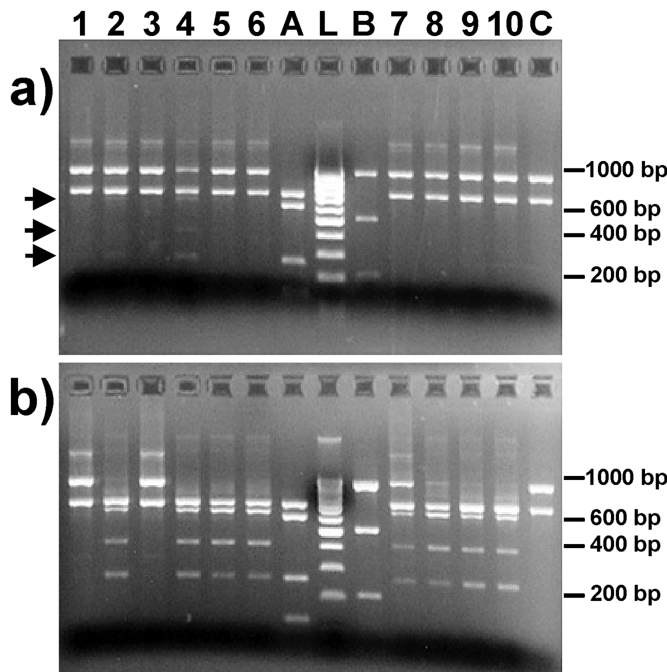

Fig. **2**. 18S-rDNA TaqI RFLPs obtained from Okinawan *Entacmaea quadricolor* zooxanthella cultures at initial inoculation (**a**) and following six months of culturing (**b**). Numbers 1–10 above lanes designate individual cultures. A = *Symbiodinium* clade A RFLP standard; B = *Symbiodinium* clade B RFLP standard; C = *Symbiodinium* clade C RFLP standard; L = 100 bp DNA size ladder. Arrows indicate the presence of the uncharacterized RFLP in the initial inocula of culture # 4 (lane # 4).

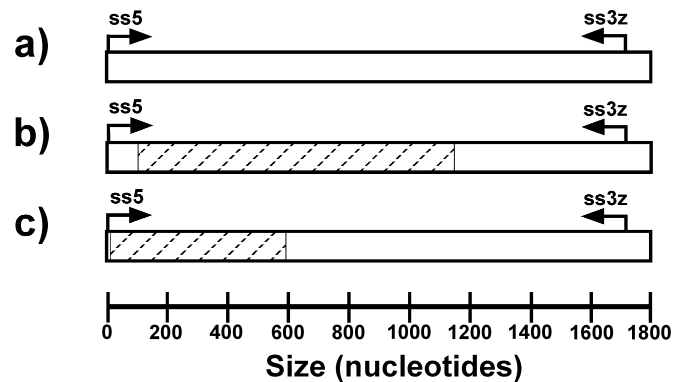

Fig. **3**. Schematic of full length (**a**) *Symbiodinium* clade D 18S-rDNA relative to the 700-bp (**b**) and 1,100-bp (**c**) 18S-rDNA amplicons of D+. Arrows marked as ss5 and ss3z designate annealing sites for each PCR primer. Broken diagonal strips represents areas which are missing from the smaller amplicons relative to the full length 18S-rDNA.

29 is the nucleotide at the 3′ end of primer ss5, the continuous nature of the 1,100-bp amplicon suggests that it could have resulted from primer-template mismatch during PCR.

We hypothesized that sequence heterogeneity in the V1–V4 region of 18S-rDNA, the most sequence variable portion of the molecule (Carlos, Baillie, and Maruyama 2000) and the region that primer ss5 anneals to, could lead to primer-template mismatch and production of the 1,100-bp amplicon. To test this hypothesis, the 18S-rDNA V1–V4 region was sequenced for D+ as well as *Symbiodinium* clade D isolates A002 and A024. Sequence analysis revealed that D+, A002 and A024 possessed identical nucleotide sequences across this region of 18S-rDNA. Although the V1–V4 sequences between the isolates were identical, it should be noted that these data do not completely rule out the possibility that primer-template mismatch generates the 1,100-bp amplicon. It is possible that rare heterogeneous copies of full-length 18S-rDNA in the D+ genome escaped detection since these data were generated directly from PCR products. In spite of this, the absence of sequence difference between D+ and the *Symbiodinium* clade D isolates strongly suggests that the 1,100-bp amplicon, like the 700-bp amplicon, is genuine and not an artifact generated by primer-template mismatch during PCR.

Are novel *Symbiodinium* 18S-rDNA amplicons transcribed? Reverse transcription (RT)-PCR of RNA isolated from D+, A002 and A024 resulted in a single 18S-rDNA amplicon of approximately 1,800 bp and RFLP analysis of this amplicon generated a *Symbiodinium* clade D pattern for all three isolates. Taken together, the lack of transcription into RNA, along with the single long stretches of missing nucleotides, strongly suggests that the 1,100-bp and 700-bp amplicons represent 18S-rDNA pseudogenes, or non-functional copies of the gene, in the D+ genome.

Inferring relationships between the isolates by sequence comparisons across various rDNAs. Sequence comparisons of regions from the nuclear 18S-, 28S- and ITS-rDNAs as well as the cp23S-rDNA gene revealed a two nucleotide difference (2/~ 3,000 bp = 0.0006% absolute difference) separating D+ from isolates A002 and A024. While isolates A002 and A024 were identical across all nucleotides that were examined, the differences between them and D+ were localized to the ITS-rDNA region, with a single transition in ITS1 and a second transition occurring in ITS2. These sequence data demonstrate that D+ and the *Symbiodinium* clade D isolates are distinct entities that probably diverged relatively recently.

DISCUSSION

Our documentation of 18S-rDNA pseudogenes in *Symbiodinium* is the first report of such aberrant molecules in the nuclear genome of symbiotic dinoflagellates and contributes to the growing list of rDNA pseudogenes reported from the dinoflagellates (Rehnstam-Holm, Godhe, and Anderson 2002; Santos et al. 2002b; Scholin, Anderson, and Sogin 1993; Yeung et al. 1996). For the free-living dinoflagellates, the presence of rDNA pseudogenes in a genome have facilitated their use as taxonomic and biogeographical markers for particular strains and populations (Rehnstam-Holm, Godhe, and Anderson 2002; Scholin and Anderson 1994; Yeung et al. 1996). Likewise, as this study demonstrates, pseudogenes also serve as an effective identifier of closely related, but unique, *Symbiodinium* isolates. Thus, the characterization of pseudogenes provides an additional tool for assessing genomic diversity within this dinoflagellate genus.

While internally deleted rDNA pseudogenes have been characterized from the genomes of several dinoflagellates, there has been little discussion regarding the mechanisms creating and maintaining these molecules. Dinoflagellate rDNA pseudogenes tend to have extensive deletions within the molecule while the remainder of the gene retains high sequence similarity to functional rDNA copies (Rehnstam-Holm, Godhe, and Anderson 2002; Yeung et al. 1996; this study). This is unexpected since a relaxation of functional constraints following inactivation of the molecule should lead to an accumulation of mutations in a rate and pattern typical of neutral evolution (Li, Gojobori, and Nei 1981). However, this is apparently not the case in the pseudogenes studied here or reported in the literature. Although we do not know the exact mechanism(s) responsible for this pattern in dinoflagellate pseudogenes, partial gene conversion between pseudogenes and functional rDNA copies could maintain sequence similarity, as it does in *Drosophila melanogaster* (Benevolenskaya et al. 1997). A second possibility is that these dinoflagellate rDNA pseudogenes have arisen recently, leaving little time for sequence divergence from functional copies.

In conclusion, the rDNA pseudogenes of *Symbiodinium* and other dinoflagellates appear to be unique molecules. The exact mechanism(s) that create and maintain these unusual pseudogenes, however, remain a mystery. Future studies that focus on the cellular and molecular aspects of rDNA pseudogenes in the dinoflagellates will provide answers to these questions as well as provide insight into the cellular biology and genomic evolution of these important single-celled protists.

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