

STRUCTURE AND EVOLUTION OF THE rDNA INTERNAL TRANSCRIBED SPACER (ITS) REGION 2 IN THE SYMBIOTIC DINOFLAGELLATES (*SYMBIODINIUM*, DINOPHYTA)¹

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Internal transcribed spacer (ITS) regions of the eukaryotic rDNA operon are integral to the correct processing and maturation of rRNAs. To further understand the evolution of this region, we elucidated the secondary structure of ITS2 from representatives of the eight divergent clades of *Symbiodinium* Freud., a large genus of dinoflagellate endosymbionts occurring in association with zooxanthellate marine protists and invertebrates. *Symbiodinium* ITS2 molecules folded into one of two distinct conformations. One conformation, the “four-fingered hand” model, has been described from a wide variety of eukaryotes, including free-living dinoflagellates. A monophyletic assemblage comprising several *Symbiodinium* clades shared an unusual conformation, a five-stem model previously known only from drosophilids, indicating that it arose in the common ancestor to this “superclade” of *Symbiodinium*. Several conserved features were identified in the ITS2 secondary structures, including a pyrimidine–pyrimidine bulge and a highly conserved 11 bp sequence motif, that correspond to known processing sites in other eukaryotes. Lastly, the ITS2 structural data are discussed in the context of *Symbiodinium* evolution, phylogenetics, and ecology.

Key index words: dinoflagellate; ITS2; ribosomal; secondary structure; *Symbiodinium*

Abbreviations: CBC, compensatory base change; ETS, external transcribed spacer; ITS, internal transcribed spacer

The organization of the rDNA operon is similar among all eukaryotes and typically includes three of the four ribosomal RNAs (18S, 5.8S, and 25–28S) separated by external and internal transcribed spacer

(ITS) regions (Hillis and Dixon 1991). Specifically, the 5.8S rRNA is separated from 18S, the SSU rRNA, by the first of two ITSs (ITS1), and from 25–28S, the LSU rRNA, by the second ITS (ITS2). The SSU and LSU, in turn, are flanked on their outer ends by two external transcribed spacers (ETSs): 5'-ETS and 3'-ETS, respectively. The three rDNAs, as well as the internal and external spacers, are transcribed by RNA polymerase I into a single precursor molecule (the 35–45S pre-rRNA), which undergoes a series of processing steps resulting in mature and fully functional rRNA. These alterations include excision of the transcribed spacer regions; nucleotide modifications, such as methylation and pseudouridylation; terminal additions of nucleotides; and further cleavages and trimming of the precursor molecule (Perry 1976, Venema and Tollervey 1999).

The proper folding of ITS rDNA is essential for the correct processing of rRNA transcripts (Musters et al. 1990a, Liu and Schardl 1994, van Nues et al. 1995, Michot et al. 1999, Côté and Peculis 2001). Even minor modifications to these spacers can inhibit or prevent the formation of mature products (Musters et al. 1990a, b, Beltrame and Tollervey 1992, van der Sande et al. 1992, Beltrame et al. 1994, Henry et al. 1994, van Nues et al. 1994). For example, specific positions within ITS2 have been identified as sites where processing reactions occur that generate the 7S pre-rRNA and the mature LSU rRNA (Venema and Tollervey 1999). Nucleotides surrounding processing sites are important as substitutions either decrease or eliminate LSU rRNA maturation (van Neus et al. 1995, Peculis and Greer 1998) or lead to decreased cell growth rates (van Neus et al. 1995). Furthermore, mutations disrupting the secondary structures in the ITS regions may also reduce or eliminate the production of precursor molecules and mature rRNA products (van Neus et al. 1995, Côté and Peculis 2001). The importance of ITS secondary structure in rRNA processing is supported by the fact that while the primary sequence of these regions can be quite variable, the secondary

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structures they form are relatively well conserved across eukaryotes (Mai and Coleman 1997, Coleman et al. 1998, Michot et al. 1999, Coleman 2003). Given this fact, the secondary structures of both ITS regions have been successfully used as aids in sequence alignment for phylogenetic reconstructions of distantly related taxa (Baldwin 1992, Schlötterer et al. 1994, Weekers et al. 2001, Coleman and Vaquier 2002, Oliverio et al. 2002, Young and Coleman 2004).

One approach to understanding how the ITS regions evolve is to compare secondary structures derived from closely related organisms. Based on this, a common secondary structure, the four-helix model with a preserved central core (known as the “four-fingered hand” model), has been recovered for the ITS2 of the major animal groups, terrestrial plants, the higher fungi, and protists (Wesson 1992, Mai and Coleman 1997, Morgan and Blair 1998, Joseph et al. 1999, Michot et al. 1999, Yotsukura et al. 1999, Coleman and Vaquier 2002, Oliverio et al. 2002, Goertzen et al. 2003, Gottschling and Plotner 2004, Young and Coleman 2004). To extend these previous studies, we have chosen the endosymbiotic dinoflagellate genus *Symbiodinium* as a model to explore patterns of structural evolution in ITS2 because large amounts of primary sequence data (~120 unique variants) are available for comparative analyses, and the phylogenetics of the group are relatively well resolved, allowing comparative analyses in a phylogenetic framework. *Symbiodinium* is a genus of unicellular symbionts occurring in association with a variety of marine protists and invertebrates (Glynn 1996, Pochon et al. 2001, Lobban et al. 2002). Phylogenies based on nuclear and organellar [i.e., chloroplast (cp) and mitochondrial] DNA sequences support the existence of eight principal *Symbiodinium* clades, denoted A–H (Coffroth and Santos 2005, Pochon et al. 2006), while intracladal resolution has revealed appreciable numbers of differentiated ITS sequences marking ecologically distinctive populations in the clades (LaJeunesse 2002, LaJeunesse et al. 2003, 2004a, b).

In this study, the secondary structures of ITS2 from members of the eight major *Symbiodinium* clades, A–H, were elucidated and compared. Previously, Gottschling and Plotner (2004) proposed secondary structural models of ITS2 from the Caliciodinelloideae (Peridiniaceae) and other dinoflagellates, including a single *Symbiodinium* isolate belonging to clade C (*Symbiodinium* sp. 1591, GenBank accession number AJ291519). Interestingly, unlike the free-living dinoflagellates, *Symbiodinium* was observed to deviate from the “four-fingered hand” model because one of the major ITS2 helices (helix III) is divided into two distinct hairpins (Gottschling and Plotner 2004). However, whether this alternative ITS2 conformation is confined to the particular *Symbiodinium* isolate utilized by Gottschling and Plotner (2004) or whether it extends to other members of the genus was not determined. Here, we report that the secondary structure of *Symbiodinium* ITS2 takes one of two distinct conformations. For some isolates, the

ITS2 secondary structure is consistent with the model proposed by Gottschling and Plotner (2004), while for other isolates, the “four-fingered hand” model described from free-living dinoflagellates and other eukaryotes is recovered. Furthermore, these alternative conformations are not scattered randomly across the *Symbiodinium* phylogeny; instead, the unique structural difference in ITS2 noted by Gottschling and Plotner (2004) is confined to a monophyletic assemblage of clades.

MATERIALS AND METHODS

Symbiodinium ITS2 sequences. The majority of ITS2 sequences used in this study (Table 1) were generated in previous studies (LaJeunesse and Trench 2000, LaJeunesse 2002, LaJeunesse et al. 2003, LaJeunesse et al. 2004a, LaJeunesse et al. 2004b, LaJeunesse 2005) by PCR-denaturing gradient gel electrophoresis (DGGE) analyses. The brightest band in a PCR-DGGE profile (cf. LaJeunesse 2002), presumed to be the numerically dominant sequence copy in the array, was excised from the gel, reamplified, and directly sequenced. Direct sequencing was used by Pochon et al. (2001) to generate sequences of clades G and H populations. For *Symbiodinium* clades A, B, and C, six divergent sequences per clade were haphazardly chosen from larger intracladal alignments constructed with Clustal X (Thompson et al. 1997) under default settings. Based on these alignments, pair-wise uncorrected (e.g., *p*) distances revealed large differences between the sequences: clade A = 4.3%–41.9%; clade B = 1%–21.9%; and clade C = 1%–4%. Along with substitutions, sequences within these clades also differed from each other by insertion-deletion (indel) mutations of 1–41 bp. For *Symbiodinium* clades D–H, a single sequence was chosen from each to serve as a representative for that particular clade (Table 1). The sequences and alignments utilized

TABLE 1. Information on the *Symbiodinium* ITS2 sequences employed in this study.

<i>Symbiodinium</i> clade/species	ITS2 type/isolate name	ITS2 length (bp)	ITS2 %GC	GenBank accession number
Clade A	A1	162	42	AF333505
<i>S. pilosum</i>	A2	162	46.3	AF333506
R. K. Trench et R. J. Blank	A3	158	45.6	AF333507
<i>S. linucheae</i>	A4	162	42.6	AF333509
(R. K. Trench et Thinh)	“Cryptic A”	162	46.3	AF184948
LaJeunesse	“Mediterranean A”	163	46.6	DQ865210
Clade B	B2	171	48	AF333512
<i>S. muscatinei</i>	B4	200	54	AF333510
LaJeunesse	B11	203	51.7	DQ865211
et R. K. Trench	B19	197	51.3	DQ865212
	B21	193	50.8	DQ865213
	Pk702	201	50.3	AF360575
Clade C	C22	190	47.9	AY239373
	C31	192	49	AY258496
	C39	181	49.2	AY258484
	C49	236	47.5	AY589752
	C67	195	50.2	AY686647
	C69	182	50	AY589773
Clade D	D4	191	51.9	DQ865214
Clade E	E1	192	54.7	AF334659
Clade F	F1	195	46.2	AF333517
Clade G	1582	207	55.1	AJ291537
Clade H	Fr1	169	52.6	AJ291513

in this study are available from <http://www.auburn.edu/~santosr/sequencedatasets.htm>.

Determination of *Symbiodinium* ITS2 secondary structure. To recognize common structural elements in *Symbiodinium* ITS2, individual sequences were initially superimposed onto the secondary structure of *Symbiodinium* sp. 1591 (Gottschling and Plotner 2004). Areas of high-sequence conservation in ITS2 between and among the clades served as reference points. Less-conserved regions, due to compensatory nucleotide changes or length differences, were folded by eye to conserve general secondary structural characteristics. For these, regions of pairing were identified both by the phylogenetic comparative method (e.g., mutual comparison of sequences in an alignment; Mai and Coleman 1997) and by complementary base changes that would preserve nucleotide interactions (Coleman 2003). Energy levels (e.g., $\Delta G = \text{kcal} \cdot \text{mol}$ at 25°C) of the final structures, which are indicative of their stability, were calculated using the software package mFOLD v.3.4 (Jaeger et al. 1989, Zuker 2003).

RESULTS

General characteristics of *Symbiodinium* ITS2. For *Symbiodinium*, the length of ITS2 varied from 158 to 236 bp and had a GC content averaging 49% (Lajeunesse 2001; Table 1). The length and GC content of *Symbiodinium* ITS2 fell within the values reported for a wide variety of taxa, ranging from 100 bp in corals (Odorico and Miller 1997) to 1089 bp in mice (Michot et al. 1993), with GC contents ranging from 25% in *Drosophila* (Schlötterer et al. 1994) to 74% in mice (Michot et al. 1993).

Secondary structure of *Symbiodinium* ITS2. The consensus ITS2 secondary structure proposed for *Symbiodinium* is shown in Fig. 1. Following the nomenclature of Gottschling and Plotner (2004), the model contained four major helices, designated I–IV, separated by single-stranded regions of the central core. The single-stranded regions were divided into

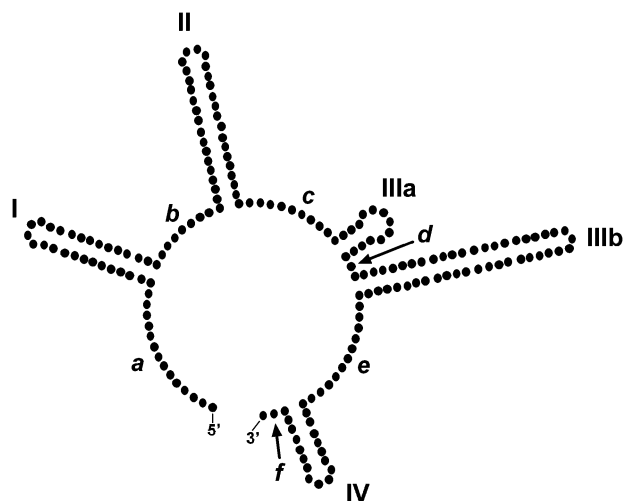


FIG. 1. Proposed consensus *Symbiodinium* internal transcribed spacer (ITS2) secondary structure. Lowercase letters (a–f) denote single-stranded regions within the structure. Roman numerals (I–IV) represent helices. Details for each region within and among the *Symbiodinium* clades are summarized in Table 1.

approximately five distinct areas (denoted a–c, e–f in Fig. 1) of varying lengths: region a (at the 5' end of ITS2) = 5–17 bp, region b = 0–8 bp, region c = 8–21 bp, region e = 3–17 bp, and region f (at the 3' end of ITS2) = 0–7 bp (Table 2). Although there were exceptions, the structure of the helices typically consisted of a base-paired stem capped by an apical loop of unpaired nucleotides. The helices showed distinct size classes: helix I (in paired bases) = 3–15 bp, helix II = 10–16 bp, and helix IV = 4–8 bp (Table 2). While helix III was generally the longest of the four (9–26 bp), its exact structure varied between the *Symbiodinium* clades. For *Symbiodinium* clades A, D, E, and G, helix III was comprised of a single stem (labeled helix IIIb in Fig. 1; see also supplementary material). In contrast, *Symbiodinium* clades B, C, F, and H possessed an additional stem-loop structure (labeled helix IIIa) of two to five paired bases located 5' of helix IIIb (Fig. 1 and supplementary material), resulting in a five-stem model for these clades. This helix is thought to derive from a division of helix III into two distinct hairpins: IIIa and IIIb (Gottschling and Plotner 2004). Owing to the presence of helix IIIa, these four *Symbiodinium* clades had an additional region of single-stranded nucleotides (denoted d in Fig. 1) ranging in size from 0 to 8 bp (Table 2) separating the two helices. The proposed *Symbiodinium* ITS2 secondary structures were all highly stable; energy levels (ΔG) varied between -35.6 and $-63.9 \text{ kcal} \cdot \text{mol}$ at 25°C (Table 2) and were consistent with the values reported for free-living dinoflagellates (Gottschling and Plotner 2004).

As noted above, base-paired stems capped by apical loops of unpaired nucleotides were a general feature of the proposed *Symbiodinium* ITS2 secondary structures. Along with this, two cases were identified where the apical loop of a particular helix also possessed bifurcating stem structures, similar to that reported for ITS2 helix III of the free-living dinoflagellate *Calciodinellum operosum* (Gottschling and Plotner 2004). This feature was recovered from the secondary structures of *Symbiodinium* ITS2 type C49 and clade G isolate 1582, where it was localized to helices II and IIIb, respectively (Fig. 2, A and B). Support for the distinct structure of helix IIIb in *Symbiodinium* clade G can be found in mutational differences between unique isolates. Specifically, comparison of *Symbiodinium* clade G isolates 1582 and 1584 (GenBank accession number AJ291539; Pochon et al. 2001) revealed eight positions scattered across helix IIIb and the associated bifurcating stem structures where substitutions have occurred in the sequences (Table 3). Although mutations at two positions (139 and 140) occurred in the loop of helix IIIb and were therefore not involved in base pairing, four positions (114, 142, 150, and 152) exhibited covarying and compensatory base changes (CBCs; Coleman 2003) that preserved the pairing of nucleotides either in the main section of stem IIIb or one of its bifurcating stems (Table 3; Fig. 2C). Additionally, two other positions (120 and 159) can be classified as

TABLE 2. Structural characteristics for the *Symbiodinium* ITS2 secondary structures proposed in this study.

<i>Symbiodinium</i> clade/species	ITS2 type/isolate name	ITS2 region										ΔG (25°C; kcal·mol ⁻¹)		
		<i>a</i>	I ^a	<i>b</i>	II ^a	<i>c</i>	IIIa ^a	<i>d</i>	IIIb ^a	<i>e</i>	IV ^a		<i>f</i>	
Clade A	A1	12	9+4	7	10+5	10	NP	NP	19+8	12	5+3	2	-51.7	
<i>S. pilosum</i>	A2	15	8+7	7	11+6	10	NP	NP	19+4	8	5+3	2	-49.1	
	A3	11	9+4	7	10+5	10	NP	NP	19+8	10	4+4	2	-53.8	
	A4	12	9+4	7	10+5	10	NP	NP	19+8	12	5+3	2	-50.9	
<i>S. linucheae</i>	“Cryptic A”	14	8+4	7	12+5	13	NP	NP	18+8	11	4+5	2	-47.2	
	“Mediterranean A”	16	9+4	7	12+4	11	NP	NP	18+6	9	4+4	2	-47.2	
Clade B	B2	17	15+4	7	14+4	8	2+3	1	9+10	3	6+5	1	-36.4	
<i>S. muscatinei</i>	B4	17	12+4	7	10+5	9	4+6	1	24+5	11	7+4	2	-63.4	
	B11	17	13+8	7	14+4	9	4+6	1	20+3	11	7+4	2	-63.7	
	B19	17	12+4	7	14+4	9	4+6	1	20+3	11	7+4	2	-63.6	
	B21	17	12+4	7	10+8	9	4+6	1	19+3	11	7+4	2	-49.2	
	Pk702	17	12+4	7	14+4	9	4+6	1	19+3	11	7+4	2	-62.4	
	Clade C	C22	13	11+5	6	11+7	17	5+4	8	11+5	10	8+6	6	-43.9
	C31	13	8+6	6	13+3	17	5+4	0	18+5	7	8+6	6	-49.3	
C39	13	11+5	6	11+7	17	5+4	0	15+4	7	8+6	6	-49		
C49	13	11+5	6	13+ ^b	17	5+4	0	18+5	7	8+6	6	-63.9		
C67	13	11+5	6	11+7	17	5+4	0	18+5	7	8+6	6	-53		
C69	13	11+5	6	11+7	17	5+4	2	11+8	10	8+6	6	-46.4		
Clade D	D4	9	8+8	7	10+6	21	NP	NP	26+6	11	5+4	3	-50.6	
Clade E	E1	15	7+5	8	16+8	18	NP	NP	17+4	17	7+6	0	-49.6	
Clade F	F1	12	10+7	7	10+8	18	5+3	0	17+7	7	7+6	6	-47.7	
Clade G	1582	14	9+3	8	12+4	18	NP	NP	18+ ^b	10	6+5	7	-37.8	
Clade H	Fr1	5	3+7	0	11+7	17	5+4	0	18+5	7	7+6	6	-35.6	

Lowercase letters (*a–f*) denote single-stranded regions within the structure, while Roman numerals (I–IV) represent stems (see Fig. 1 for consensus secondary structure). NP, specific single-stranded region or stem not present in particular *Symbiodinium* ITS2 secondary structure.

^a*X* + *Y*, where *X* is the length of a helix in paired bases and *Y* represents the number of unpaired nucleotides at apex (i.e., loop) of the helix.

^bSingle loop not present since bifurcation occurs within the helix (see Fig. 2 for examples of *Symbiodinium* ITS2 with bifurcating stems).

hemi-CBCs (Coleman 2003) because they varied between one of two nucleotides, both of which form a pairing and maintain the proposed secondary structure (Table 3; Fig. 2C).

In addition to the general conservation of ITS2 secondary structure within and among the *Symbiodinium* clades, regions of high sequence and positional homology were also identified. The secondary structures recovered for the ITS2 sequences used in this study are presented as supplementary material (Figs. S1–S4); in these figures, we have highlighted in gray nucleotides a specific region of conservation present in all of the structures. This region, which corresponds to an 11 bp sequence located on the 3' side of helix II, typically extended to the base of the stem and into the single-stranded region *c* (Fig. 1). Alignment of this region (Fig. 3) from a representative set of the *Symbiodinium* clades revealed two positions where nucleotide identity was identical across all sequences, four positions where the nucleotide identity was conserved in all but one sequence, and two positions where nucleotide identity varied between one of two residues. Previous studies have identified this region, which occurs 78–130 bp from the 5' end of ITS2, as a processing site in yeast and rats (Wesson 1992). In *Symbiodinium* clades A–G, this potential processing site occurred in approximately the same position (60–120 bp from the 5' end of the molecule); for clade H,

the reduced length of single-stranded regions *a* and *b* (Fig. 1) and helix I relative to the other clades placed it 40–50 bp from the 5' end of ITS2. Another feature of the ITS2 secondary structure from *Symbiodinium* clades A, C, D, and H (and present in most clade B structures) was the presence of a pyrimidine–pyrimidine bulge in the stem of helix II (boxed nucleotide[s] in supplementary material, Figs. S1–S4). This bulge is a nearly universal feature of ITS2 secondary structures and is thought to play a role in excision of the molecule during rRNA processing (Mai and Coleman 1997).

DISCUSSION

Secondary structure of ITS2 in Symbiodinium. The existence of either a four- or five-stem configuration in *Symbiodinium* demonstrates that proper processing of the rRNA precursor molecule can occur in the presence of subtle, but distinct, differences in the overall structure of ITS2. The common “four-fingered hand” model observed for *Symbiodinium* clades A, D, E, and G was proposed for yeast (Joseph et al. 1999), green algae and flowering plants (Mai and Coleman 1997, Coleman 2003, Denboh et al. 2003, Goertzen et al. 2003), trematodes and monogeneans (Morgan and Blair 1998), abalone (Coleman and Vaquier 2002), muricid neogastropods (Oliverio et al. 2002), *Drosophila* (Schlötterer et al. 1994, Young and

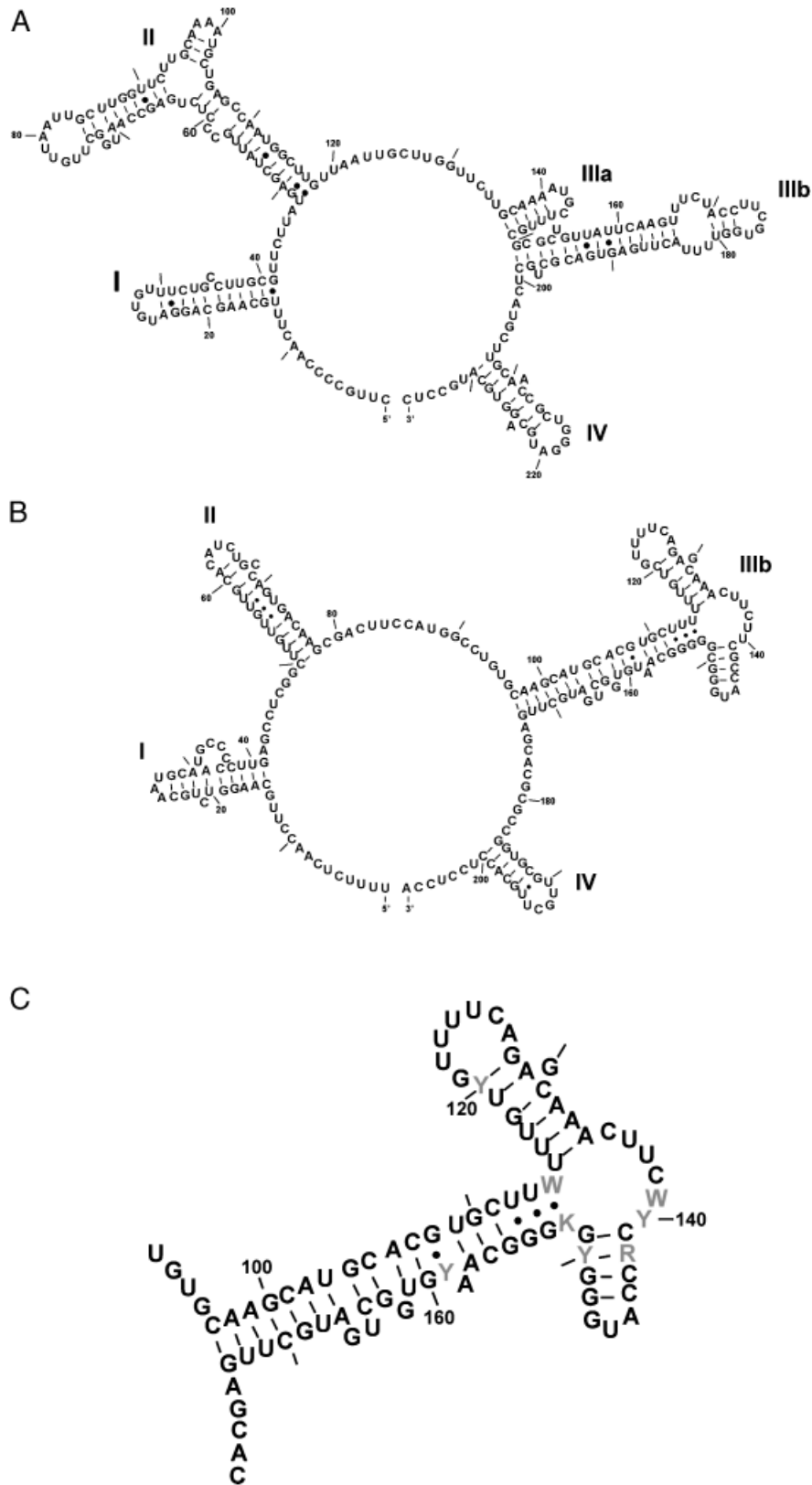


FIG. 2. *Symbiodinium* internal transcribed spacer (ITS2) structures with bifurcating stems. (A) *Symbiodinium* ITS2 type C49, with bifurcation in stem II. (B) *Symbiodinium* sp. 1582 (Pochon et al. 2001) with bifurcation in stem IIIb. (C) Stem IIIb of *Symbiodinium* clade G. Sites with nucleotide variability between *Symbiodinium* clade G isolates 1582 and 1584 (Pochon et al. 2001) are depicted in gray and summarized in Table 3.

TABLE 3. Mutations in stem IIIb of *Symbiodinium* clade G isolates 1582 and 1584 (Pochon et al. 2001).

Position in ITS2 (base #)	Clade G isolate		
	Isolate 1582	Isolate 1584	Pairs with nucleotide at position:
114	U	A	152
120	C	U	128
139	U	A	N/A (occurs in loop)
140	U	C	N/A (occurs in loop)
142	G	A	150
150	C	U	142
152	G	U	114
159	U	C	108

Compensatory mutations that preserve base pairing in stems are highlighted in bold. See Fig. 2 for location of mutations in the context of the secondary structure for stem IIIb.

Coleman 2004), mosquitoes (Wesson 1992), leaf beetles (Gómez-Zurita et al. 2000), vertebrates (Joseph et al. 1999, Michot et al. 1999), and in free-living dinoflagellates including the subfamily Calciodinelloideae (Gottschling and Plotner 2004). Thus, our data contribute to the growing body of evidence that the ITS2 region of eukaryotes folds into a (more or less) universally conserved secondary structure. For the remaining clades, the existence of subhelix IIIa (resulting in a five-stem model), first proposed by Gottschling and Plotner (2004) for a member of *Symbiodinium* clade C and reported here to occur in *Symbiodinium* clades B, C, F, and H, has, to the best of our knowledge, only been documented in drosophilids (where it is referred to as helix IIa; Young and Coleman 2004). Secondary structural analyses of ITS2 sequences seldom recover a five-stem configuration, suggesting that this apomorphy is rare among eukaryotes.

For two *Symbiodinium* isolates, the apical loop of a particular helix in the ITS2 secondary structure ter-

A1	ACUGGCAUGCU
“Med A”	ACUGGCAUGCG
“Cryptic A”	AUUGGCAUGCU
B2	AAUGGCUUGUU
B4	AGUGGCUUGUU
C22	AAUGGCUUGUU
C67	AAUGGCUUGUG
D4	CAUGGCUUGCU
E1	AGCGGCGCGCU
F1	AAUGGCUUAUU
G1582	AGUGACAAGCG
H (Fr1)	AAUGGCUUGUU
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	* * * * *
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FIG. 3. Conserved nucleotide tract identified from *Symbiodinium* internal transcribed spacer (ITS2) sequences. The tract is located 3' of the apical loop, and approximately at the base, of stem II. Marked positions are as follows: ***100% nucleotide conservation at a position; **11 of 12 sequences (>90%) share identical nucleotide at a position; * >25% of the 12 sequences possess one of two specific nucleotides at a position. *Symbiodinium* isolates are named as in Tables 1 and 2.

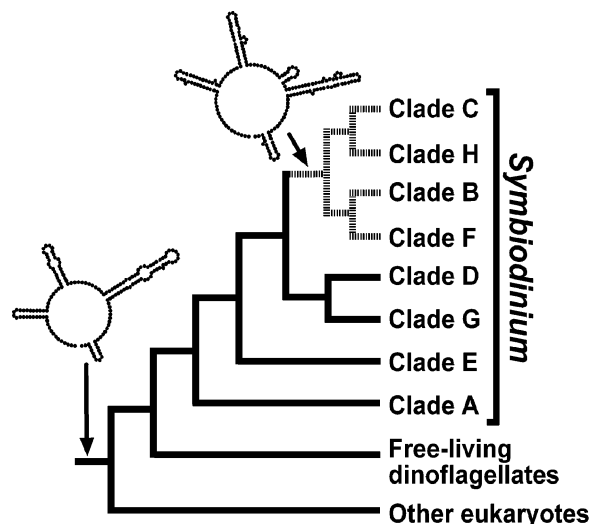


FIG. 4. Variation in the secondary structure of *Symbiodinium* internal transcribed spacer (ITS2) and its relationship with the cladal phylogeny for the genus. Hatched branches leading to *Symbiodinium* clades B, C, F, and H indicate these clades as possessing the five-stem model of ITS2 secondary structure. The phylogenetic relationships between the major clades of *Symbiodinium* are represented as a consensus cladogram from various studies and molecules (see Coffroth and Santos 2005 for additional details).

minated in bifurcating stems. For one of these, support for the secondary structure of bifurcating stems comes from CBCs between two unique *Symbiodinium* clade G isolates. It has been emphasized that CBCs and hemi-CBCs serve as strong evidence for a proposed secondary structure due to their role in preserving the pairings of helices (Coleman 2003). The secondary structures of the major helices are also supported by CBCs. For example, a 5bp motif (5'-GCAAG vs. CUUGC-3') identified in *Symbiodinium* sp. 1591 is thought to represent CBCs that maintain the structure of helix I (Gottschling and Plotner 2004). The fact that this motif also occurs at the base of helix I in members of clades B, C, D, and G (see supplementary material) further supports this hypothesis. Along the same lines, although a 6bp motif at the base of helix I exhibits sequence variability (e.g., GAGCAG/GGGCAG/GGGU GG-3') within *Symbiodinium* clade A, all of the substitutions are capable of pairing with their proposed complement (e.g., 5'-CUGCUC) and thus represent hemi-CBCs in helix I (see supplementary material). Similarly, the variable 5' partners (see supplementary material) to the highly conserved 11bp sequence located on the 3' side of helix II (Fig. 3) are another example of CBCs that preserve pairings and the structure of a helix. Thus, the occurrence of CBCs provides further support for the common structural features of *Symbiodinium* ITS2.

In our proposed *Symbiodinium* ITS2 secondary structural models, the helices belong to distinct size classes, with helix III being the longest of the base-paired regions, while helix IV is the shortest. This situ-

ation is consistent with that found in other eukaryotes (Coleman 2003). For example, in the calciodinelloid and other dinoflagellates, helix III is the longest paired region (18–33 bp), while helix IV is the shortest, with an average of 5 bp (Gottschling and Plotner 2004). Although the exact reason for this size heterogeneity is unknown, it is thought that the helices (along with other features) provide the docking and recognition signals required for processing of the precursor molecule into mature rRNAs (Hadjiolova et al. 1994, Lalev and Nazar 1999, Venema and Tollervey 1999).

Other evolutionarily conserved features were recovered from our structural analyses of *Symbiodinium* ITS2. These include a pyrimidine–pyrimidine bulge in helix II as well as the presence of an 11 bp motif located on the 3' side of the same helix. The pyrimidine–pyrimidine bulge, present in most *Symbiodinium*, is a nearly universal feature of ITS2 secondary structures (Coleman 2003) and is thought to have an important function in processing due to its sequence and positional conservation among high levels of nucleotide variability in the surrounding regions of helix II (Mai and Coleman 1997). Along with this, the 11 bp motif conserved across the *Symbiodinium* clades occupies a region at the base of helix II that has been definitively recognized as a processing site in yeast and rats (Wesson 1992) and as a putative processing site in *Xenopus*, sea urchins, mosquitoes, *Drosophila*, trematodes, and monogeneans (Wesson 1992, Morgan and Blair 1998). Given the correspondence to homologs identified across a wide array of taxa, these two features likely play similar roles in *Symbiodinium* ribosomal processing and biogenesis.

Evolutionary, phylogenetic, and ecological significance of Symbiodinium ITS2 secondary structure. The secondary structure of *Symbiodinium* ITS2 takes one of two distinct conformations: the “four-fingered hand” model for clades A, D, E, and G, or the five-stem model for clades B, C, F, and H. Overlaying these data on the currently accepted *Symbiodinium* cladal phylogeny (Coffroth and Santos 2005) demonstrates that these alternative conformations are not scattered randomly across the genus. Instead, the distinctive five-stem model of ITS2 secondary structure is a character state shared by the monophyletic assemblage of *Symbiodinium* clades B, C, F, and H (Fig. 4). This suggests that the “four-fingered hand” configuration is the ancestral state for the genus while the five-stem configuration arose in the last common ancestor of *Symbiodinium* clades B, C, F, and H (Fig. 4). Hence, the occurrence of helix IIIa in the ITS2 secondary structure of *Symbiodinium* clades B, C, F, and H represents a synapomorphy that supports their close relationship while grouping these clades into a larger phylogenetic entity or “superclade.”

Between *Symbiodinium* clades, the ITS2 region is difficult to align due to high levels of divergence (LaJeunesse 2001), and this has precluded its phylogenetic utility at the genus level. However, the application of ITS2 secondary structure information can

optimize sequence alignments for phylogenetic reconstruction of organisms in the same family or order (Coleman 2003). This is accomplished by identifying features inherent to the ITS2 secondary structure (i.e., helices, the pyrimidine–pyrimidine bulge, the putative processing site of helix II, and CBCs among a group of taxa) and aligning homologous regions before inferring the phylogeny. Such an approach has created well-resolved and strongly supported phylogenies for a variety of organisms (Morgan and Blair 1998, Joseph et al. 1999, Goertzen et al. 2003). To determine whether this is the case for *Symbiodinium*, the ITS2 sequences utilized in this study were aligned using the secondary structure as a guide, and the resulting alignment subjected to maximum-likelihood analysis as described in Santos et al. (2002). Overall, branching orders were consistent with the accepted *Symbiodinium* cladal phylogeny (Fig. 4); however, bootstrap support for the positions of clades D and F was below 50% (data not shown). Thus, although ITS2 possesses an appreciable phylogenetic signal, it does not resolve the *Symbiodinium* cladal phylogeny as well as nuclear 28S rDNA or cp 23S rDNA (Wilcox 1998, Santos et al. 2002, Pochon et al. 2006).

Along with providing insight into the significance of ITS2 structural conformations in posttranscription rRNA processing and additional support for current evolutionary relationships among divergent lineages within this ancient dinoflagellate group, our structural skeletons may be useful for analyzing intragenomic rDNA variants. For example, recovery of secondary structures consistent with what is predicted for the molecule offers a simple way to test the validity of novel sequences obtained following bacterial cloning. Preliminary analyses on isoclonal *Symbiodinium* cultures have recovered some ITS sequences that contain base changes in regions that destabilize the secondary structure. Secondary structural analyses appear to provide an objective method for identifying and removing suspect sequences to produce data sets that are more reflective of the actual genetic diversity of *Symbiodinium* populations under study (unpublished data). Future application of secondary structural information will greatly improve the quantification of “true” *Symbiodinium* diversity and ecological distributions when using ITS2 (or other ribosomal molecules) as a genetic marker.

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Supplementary Material

The following supplementary material is available for this article: Secondary structures for each *Symbiodinium* ITS2 sequence employed in this study are available as supplementary materials (Figs. S1–S4). Unless otherwise noted, ITS2 type nomenclature is based on LaJeunesse (2002).

Fig. S1. Proposed ITS2 secondary structures for *Symbiodinium* clade A. (A) ITS2 type A1. (B) ITS2 type “Mediterranean A.” (C) ITS2 type A3. (D) ITS2 type A4 (*S. linucheae*). (E) ITS2 type “Cryptic A.” (F) ITS2 type A2 (*S. pilosum*).

Fig. S2. Proposed ITS2 secondary structures for *Symbiodinium* clade B. (A) ITS2 type B2. (B) ITS2 type B11. (C) ITS2 type B19. (D) ITS2 type B21. (E) ITS2 type B4 (*S. muscatinei*). (F) Isolate Pk702 (Santos et al. 2001).

Fig. S3. Proposed ITS2 secondary structures for *Symbiodinium* clade C. (A) ITS2 type C22. (B) ITS2 type C31. (C) ITS2 type 39. (D) ITS2 type C49. (E) ITS2 type C67. (F) ITS2 type C69.

Fig. S4. Proposed ITS2 secondary structures for *Symbiodinium* clades D–H. (A) ITS2 type D4 (clade D). (B) ITS2 type E1 (clade E). (C) ITS2 type F1 (clade F). (D) Isolate 1582 (clade G; Pochon et al. 2001). (E) Isolate Fr1 (clade H; Pochon et al. 2001).

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