Zoological Studies **43**(4): 759-771 (2004)

Zoological Studies

Secondary Structure and Phylogenetic Utility of the Ribosomal Internal Transcribed Spacer 2 (ITS2) in Scleractinian Corals

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(Accepted June 28, 2004)

Chaolun Allen Chen, Chau-Ching Chang, Nuwei Vivian Wei, Chien-Hsun Chen, Yi-Ting Lein, Ho-E Lin, Chang-Feng Dai and Carden C. Wallace (2004) Secondary structure and phylogenetic utility of the ribosomal internal transcribed spacer 2 (ITS2) in scleractinian corals. *Zoological Studies* **43**(4): 759-771. In this study, we examined the nucleotide characteristics, the secondary structure, and phylogenetic utility of the ribosomal internal spacer 2 (ITS2) from 54 species of scleractinian corals, representing 25 genera and 11 families of both the complex and robust clades previously defined through molecular phylogenetic analyses. The lengths and nucleotide contents of the ITS2 were highly variable among corals. The ITS2 of *Acropora* is significantly shorter than those of other corals. Dinucleotide or tetranucleotide microsatellites were identified in the genera *Acropora*, *Cyphastrea*, *Favites*, *Goniastrea*, *Hydnophora*, *Montipora*, *Madracis*, and *Porites*. Three distinct types of secondary structures with the smallest free energy values were predicted using the computer software, Mfold. A standard 4 domains were observed in 17 species of corals, while 23 species has a modified 5 domains with domain I divided into 2 subdomains. These 2 types of secondary structures were observed across 11 coral families. The 3rd type, 5 domains with domain III divided into 2 subdomains, was only seen in the genus *Acropora*. Among the domains, domain II is highly conserved and is flanked by conserved sequence motifs in adjacent stems. The motif, 5'-CRCGGYC-3', and its compensatory bases were highly conserved in both the complex and robust clades of scleractinian corals. The robust-clade phylogeny constructed using ITS2 data produced a concordant tree to those based on mitochondrial and nuclear genes. The comparative analysis indicated that the extremely high ITS intragenomic divergence of *Acropora* is an exception rather than the rule for the evolutionary history of scleractinian corals. Despite the atypical and unusual pattern of molecular evolution in the genus *Acropora*, data of the ITS2 are still applicable, with adequate adjustment of secondary structures, to the primary sequence alignment of different levels of phylogenetic analyses, from populations to genera, in scleractinian corals. http://www.sinica.edu.tw/zool/zoolstud/43.4/759.pdf

Key words: Internal transcribed spacer 2, Secondary structure, Conserved domain, Scleractinian corals, Phylogenetic utility.

DNA sequences of the 2 internal transcribed spacers (ITS1 and ITS2) of the ribosomal RNA (rRNA) transcription unit have proven useful in resolving phylogenetic relationships of closely related taxa and in distinguishing species in fungal, plant, and animal taxa due to their relatively rapid evolution rates (Baldwin 1992, Schlotterer et al. 1994, Mai and Coleman 1997, Weekers et al. 2001, Oliverio et al. 2002). In addition, the transcript folding structure of the ITS provides some signals that guide the ribosomal coding regions when they are processed into small, 5.8S, and large ribosomal RNA (van der Sande et al. 1992, van Nues et al. 1995). The potential to predict the folding structure has enhanced the role of ITS in phylogenetic studies, since it is important to guide reliable sequence alignment based on secondary structures (Michot et al. 1999).

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Many methods have been applied to infer the secondary structure of ITS, including electron microscopy (Gonzales et al. 1990), chemical and structure probing (Yeh and Lee 1990), site-directed mutagenesis (van der Sande et al. 1992, van Nues et al. 1995), and very commonly used computer software prediction programs (e.g., Mfold) which utilize minimum free energy values (Zuker and Steigler 1981). Based on those predictions, a secondary structure for the ITS2 with 4 domains (I~IV) has been proposed for green algae, flowering plants, fruit flies (*Drosophila* spp.), parasitic flatworms, gastropods, and the mouse (Schlötterer et al. 1994, Mai and Coleman 1997, Morgan and Blair 1998, Joseph et al. 1999, Michot et al. 1999, Coleman and Vacquier 2002, Oliverio et al. 2002, Gottschling and Plötner 2004). A highly conserved sequence is situated around a central loop and at the apex of a long stem in the 3'-half (Joseph et al. 1999).

The ITS region and 5.8S gene have been extensively used to reconstruct phylogenetic relationships among scleractinian corals (Hunter et al. 1997, Lopez and Knowlton 1997, Odorico and Miller 1997, Medina et al. 1999, van Oppen et al. 2000 2002, Diekmann et al. 2001, Forsman et al. 2003, Lam and Morton 2003, Marquez et al. 2003, Fukami et al. 2004). The results indicate that individual coral colonies host a high degree of intragenomic variation, and coral ITS phylogenies in several cases are polyphyletic among closely related congeners (Odorico and Miller 1997, Medina et al. 1999, van Oppen et al. 2000 2002, Diekmann et al. 2001, Marquez et al. 2003, but see Forsman et al. 2003, Lam and Morton 2003, Vollmer and Palumbi 2004). A high degree of intragenomic variation may result in unreliable sequence alignments that can generate incorrect phylogenies (Li 1997). Using the secondary structure to guide alignment of ITS DNA sequences may assist in reducing errors in phylogenetic constructions. For example, the variation in ITS2 was estimated to be as high as 40% (*p*-distance) at the interspecific level for *Acropora* spp. (Odorico and Miller 1997, van Oppen et al. 2001 2002), which is beyond the level which can be used to produce a reliable alignment (Li 1997). In contrast, the interspecific variation was relatively small (< 8%) among species of *Madracis* spp. in the Caribbean (Diekmann et al. 2001). Both studies lead to the conclusion that evolutionary patterns of potentially hybridizing corals are consistent with reticulation (Odorico and Miller 1997, Medina et al. 1999, van Oppen et al. 2000 2002, Diekmann et al. 2001, Marquez et al. 2003). However, this conclusion should be viewed with caution, since the ITS can be either highly or only slightly variable among different lineages of scleractinian corals and since mechanisms between maintaining and homogenizing ITS variations are complicated (Hillis and Dixon 1991, Vollmer and Palumbi 2004). Recent analyses of ITS regions have revealed that the phylogenetic signature of recent introgressive hybridization is obscured in the Caribbean *Acropora* because they share ancient rDNA lineages that predate the divergence of the species (Vollmer and Palumbi 2004).

Family-level phylogenies among scleractinian corals have been inferred using mitochondrial ribosomal RNA genes, and results indicated that 2 major clades, i.e., "robust" and "complex", can be defined according to the skeletal morphology (Romano and Palumbi 1997, Romano and Cairns 2000, Chen et al. 2002). Fukami et al. (2004) examined 3 mitochondrial and nuclear protein-coding genes and demonstrated that several major families in the robust clade, such as the Faviidae, Mussidae, and Merulindae, do not form monophylies, suggesting a deep divergence between Pacific and Atlantic coral faunas. Whether a rapidly evolving region such as ITS2 rDNA also exhibits a similar phylogeny as seen in the robust clade deserves further investigation. In this study, we examined the secondary structure and phylogenetic utility of ITS2 DNA sequences from 54 species of scleractinian corals, representing 25 genera and 11 families. We addressed the following questions: (1) Does the ITS2 secondary structure in scleractinian corals fit the canonical "4-domain model" as seen in other eukaryotes? (2) Are there conserved regions of the secondary structures that can be identified between the 2 clades of scleractinian corals? (3) What is the improvement in the DNA sequence alignment based on guidance by the ITS2 secondary structure? (4) And, what is the resolution of ITS2 DNA sequences in inferring the higher-level phylogeny of scleractinian corals?

MATERIALS AND METHODS

Coral samples and ITS2 DNA sequences retrieved from the database

ITS2 DNA sequences were obtained from 2 sources: (1) DNA sequencing of coral collected from reefs in Taiwan and Togian I., Sulawesi, Indonesia by the authors (C.A.C. and C.C.W.),

respectively, and (2) available sequences from GenBank. Taxonomic information, collection sites, clades, and GenBank retrieval information are listed in table 1.

Molecular methods

DNA extraction, PCR, cloning, and DNA sequencing are described in our previous work (Chen et al. 2000 2002 2003 2004). Target segments containing the ITS1-5.8S-ITS2 region were amplified using the "anthozoan-universal" primer pairs, 1S: 5'-GGTACCCTTTGTACACACCGCCCGTCGCT-3' and 2SS: 5'-GCTTTGGGCGGCAGTCCCAAGCAACCCGACTC-3', as described in Odorico and Miller (1997). Nucleotide sequence analysis concentrated on the ITS2 region, as this region is more typically used in the secondary structure prediction than is the ITS1 region. In addition, the ITS1 region in several scleractinian corals has shown great differences in length and thus cannot be used to produce a reliable alignment for further analysis (Odorico and Miller 1997). PCR was performed in a PC-9606 thermal sequencer (Corbett Research, Sydney, NSW, Australia) using the following thermal cycle: 1 cycle of 95°C for 4 min; 4 cycles of 94°C for 30 s, 50°C for 1 min, and 72°C for 2 min; followed by 30 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 2 min. The amplification reaction used 50~200 ng of template and BRL *Taq* polymerase in a 50-µl reaction volume, using the buffer supplied with the enzyme, under conditions recommended by the manufacturer. The PCR products were electrophoresed in a 1% agarose (FMC Bioproduct, Rockland, ME, USA) gel in 1X TAE buffer to assess the yield. Amplified DNA was extracted once with chloroform, precipitated with ethanol at –20°C, and resuspended in TE buffer. PCR products were cloned using the pGEM-T system (Promega, Madison, MI, USA) under conditions recommended by the manufacturer. Nucleotide sequences were determined for complementary strands of at least 2 clones from each sample using an ABI 377 Genetic Analyzer. The sequences obtained were submitted to GenBank under the accession numbers listed in table 1.

Sequence alignment, folding of sequences into a putative secondary structure, and phylogenetic analysis

DNA sequences were initially aligned using CLUSTAL X (Thompson et al. 1994), and default gap and extension penalties were used followed by manual editing with SeqApp 1.9 (Gilbert 1994). Alignments were then adjusted by eye following the guidance of the predicted secondary structure (see below). Sequences were folded with the RNA secondary structure prediction subroutine, Mfold (Zuker 2003), in SeqWeb vers. 2.1 (Wisconsin package, Wisconsin, USA). Default values were used to fold the ITS2 rDNA. About 20 bases of flanking sequences (5.8S and 28S rRNA) were included because these 2 coding regions have been shown to be important for the folding of ITS2 sequences. Structures inferred by Mfold were examined for common stems, loops, and bulges. Uncorrected pairwise *p*-distances (Li 1997) were calculated for the alignments from the default options of CLUSTAL X and after guidance of ITS2 secondary structures for the genus *Acropora* and the families of the Faviidae, Mussidae, and Merulinidae, respectively. Student's *t*-test was used to examine the statistical significances of the *p*-distance matrices before and after readjustment. In order to assess the phylogenetic utility of ITS2, the *p*-distances at the interspecific level were calculated for those genera or subgenera, including *Acropora*, *Isopora*, *Montipora*, *Monastrea*, *Goniastrea*, and *Madracis*, for which ITS2 sequences were available for more than 3 species. Phylogenetic analyses based on the maximum-parsimony (MP), neighbor-joining (NJ), and maximum likelihood (ML) algorithms were performed for the robust clade using PAUP* 4.0b10 (Swofford 2002).

RESULTS

ITS2 rDNA in scleractinian corals

In the scleractinian corals surveyed, the ITS2 region varied in length from 104 bp in *Acropora longicyanthus* to 369 bp in *Pocillopora damicornis*, whereas the GC content ranged from 44% in *Porites lutea* to 74% in *Astreopora myriophthalma* (Table 1). Short dinucleotide simple sequence repeats (microsatellites) were identified in several coral species: $(GA)_{2\text{-}6}$ and $(GT)_{2\text{-}7}$ in *Acropora* (*Isopora*) *brueggmanni*, *A. cuneata*, *A. palifera*, and *A. togianesis*; $(AG)_5$ in *Anacropora* sp. and *Montipora* spp.; $(CA)_{3\text{-}6}$ in *Madracis* spp.; $(GC)_5$ in *Goniastrea* spp. and *Hydnophora exesa*; and $(TG)_4$ in *Cyphastrea japonica* and *Favites abdita*. The tetranucleotide repeats of $(CCAT)_4$ and $(AGCA)_{5\text{-}7}$ were respectively identified in *A. humilis*

Table 1. Taxonomic information, collection site, clades, length, GC content, microsatellites, and secondary structure types of the ribosomal internal transcribed spacer 2 (ITS2), and GenBank accession numbers of the scleractinians used in this study. [a]: Complex (C) and robust (R) clades were assigned by Romano and Cairns (2000) and Chen et al. (2002); [b]: Types of secondary structure, I: four-domain model; II: five-domain model with domain I divided into two subdomains; III: five-domain model with domain III divided into two subdomains

Taxon	Collection site	Clade[a]	Size (bp)	GC (%)	Microsatellite	Secondary structure type[b]	Accession no.	Reference	Identification
Acroporidae									
Acropora humilis	Penghu Is., Taiwan	C	131	51	$(CCAT)_4$	III	AY722741	this study	Wallace (1999)
Acropora muricata	Penghu Is., Taiwan	C	107	51		III	AY722743	this study	Wallace (1999)
Acropora cytherea	Magnetic Is., GBR	C	116	53		III	ACU82723	Odorico and Miller (1997)	
Acropora formosa (=*muricata*)	Magnetic Is., GBR	C	111	48		III	AFU82730	Odorico and Miller (1997)	
Acropora hyacinthus	Magnetic Is., GBR	C	114	49		III	AHU82719	Odorico and Miller (1997)	
Acropora longicyathus	Magnetic Is., GBR	C	104	54		III	ALU82733	Odorico and Miller (1997)	
Acropora valida	Magnetic Is., GBR	C	112	52		III	AVU82727	Odorico and Miller (1997)	
Acropora cervicornis	Bonaire, Invisibles	C	128	54		III	AF239046	van Oppen et al. (2000)	
Acropora palmata	Bonaire, Redslave	C	124	52		III	AF239081	van Oppen et al. (2000)	
Acropora prolifera	Bonaire, Redslave	C	129	52		III	AF239119	van Oppen et al. (2000)	
Acropora (*Isopora*) *brueggemanni*	Togian Is., Sulawesi	C	140	64	$(GA)_{2-6}$, $(GT)_{2-7}$	III	AY722737	this study	Wallace (1999)
Acropora (*Isopora*) *cuneata*	Togian Is., Sulawesi	C	136	68	$(GA)_{2-6}$, $(GT)_{2-7}$	III	AY722738	this study	Wallace (1999)
Acropora (*Isopora*) *palifera*	Togian Is., Sulawesi	C	136	69	$(GA)_{2-6}$, $(GT)_{2-7}$	III	AY722744	this study	Wallace (1999)
Acropora (*Isopora*) *togianensis*	Togian Is., Sulawesi	C	141	67	$(GA)_{2-6}$, $(GT)_{2-7}$	III	AY722745	this study	Wallace (1999)
Anacropora sp.	Togian Is., Sulawesi	C	177	53	$(AG)_{4-5}$	I	AY722747	this study	Veron (2000)
Astreopora listera	Kenting, Taiwan	C	197	71		I	AY722742	this study	Veron (2000)
Astreopora sp.	Kenting, Taiwan	C	194	74		I	AY722748	this study	Veron (2000)
Montipora aequituberculata	Yahliu, Taiwan	C	177	51	$(AG)_{4-5}$	I	AY722772	this study	Veron (2000)
Montipora angulata	Penghu Is., Taiwan	C	181	52	$(AG)_{4-5}$	I	AY722773	this study	Veron (2000)
Montipora molis	Penghu Is., Taiwan	C	180	51	$(AG)_{4-5}$	I	AY722776	this study	Veron (2000)
Montipora peltiformis	Penghu Is., Taiwan	C	177	50	$(AG)_{4-5}$	I	AY722777	this study	Veron (2000)
Montipora taiwanensis	Penghu Is., Taiwan	C	177	53	$(AG)_{4-5}$	I	AY722778	this study	Veron (2000)
Montipora tuberculosa	Penghu Is., Taiwan	C	177	53	$(AG)_{4-5}$	I	AY722779	this study	Veron (2000)
Montipora venosa	Yehliu, Taiwan	C	181	52	$(AG)_{4-5}$	I	AY722780	this study	Veron (2000)
Astrocoeniidae									
Madracis decactis	Curacao, N. Antilles	C	207	65	$(CA)_{3-6}$	II	AF251875	Diekmann et al. (2001)	
Madracis formosa	Curacao, N. Antilles	C	211	64	$(CA)_{3-6}$	II	AF251891	Diekmann et al. (2001)	
Madracis mirabilis	Curacao, N. Antilles	C	209	65	$(CA)_{3-6}$	II	AF251849	Diekmann et al. (2001)	
Madracis pharensis	Curacao, N. Antilles	C	209	65	$(CA)_{3-6}$	II	AF251925	Diekmann et al. (2001)	
Madracis senaria	Curacao, N. Antilles	C	206	66	$(CA)_{3-6}$	II	AF251904	Diekmann et al. (2001)	

Table 1. (Cont.)

Taxon	Collection site	Clade[a]	Size (bp)	GC (%)	Microsatellite	Secondary structure type[b]	Accession no.	Reference	Identification
Stylocoeniella guentheri	Kenting, Taiwan	C	234	60		II	AY722791	this study	Veron (2000)
Dendrophylliidae									
Tubastrea aurea	Penghu Is., Taiwan	C	211	56		II	AY722796	this study	Veron (2000)
Fungiacyathidae									
Fungiacyathus sp.	Ilan, Taiwan	C	187	60		II	AY722757	this study	Veron (2000)
Oculinidae									
Galaxea fascularis	Penghu Is., Taiwan	C	248	71		I	AY722764	this study	Veron (2000)
Poritidae									
Alveopora sp.	Kenting, Taiwan	C	165	64		I	AY722746	this study	Veron (2000)
Porites lutea	Penghu Is., Taiwan	C	233	44	$(AGCA)_{5-7}$	I	AY722755	this study	Veron (2000)
Faviidae									
Cladocora sp.	Kaohsiung, Taiwan	R	196	59		II	AY722752	this study	Veron (2000)
Cyphastrea japonica	Penghu Is., Taiwan	R	208	63	$(TG)_4$	II	AY722749	this study	Veron (2000)
Favites abdita	Penghu Is., Taiwan	R	232	63		I	AY722755	this study	Veron (2000)
Goniastrea aspera	Penghu Is., Taiwan	R	226	68		II	AY722759	this study	Veron (2000)
Goniastrea sp.	Penghu Is., Taiwan	R	228	65		II	AY722762	this study	Veron (2000)
Goniastrea palaunesis	Penghu Is., Taiwan	R	230	67		II	AY722766	this study	Veron (2000)
Montastrea annularis	Key Largo, Florida	R	209	62		II	AF013731	Medina et al. (1999)	
Montastrea faveolata	Key Largo, Florida	R	209	63		II	AF013733	Medina et al. (1999)	
Montastrea franksi	Key Largo, Florida	R	209	64		II	AF013734	Medina et al. (1999)	
Montastrea curta	Penghu Is., Taiwan	R	252	64		II	AY722774	this study	Veron (2000)
Oulastrea crispata	Penghu Is., Taiwan	R	191	60		II	AY722781	this study	Veron (2000)
Platygyra sinensis	Hong Kong, China	R	222	68		II	AF481901	Lam and Morton (2003)	
Merulinidae									
Hydnophora exesa	Penghu Is., Taiwan	R	246	64		II	AY722769	this study	Veron (2000)
Mussidae									
Acanthastrea echinata	Penghu Is., Taiwan	R	228	67		II	AY722739	this study	Veron (2000)
Pocilloporidae									
Seriatopora hystrix	Kenting, Taiwan	R	236	55		II	AY722794	this study	Veron (2000)
Pocillopora damicornis	Penghu Is., Taiwan	R	369	61		I	AY722785	this study	Veron (2000)
Stylophora pistillata	Penghu Is., Taiwan	R	226	58		II	AY722795	this study	Veron (2000)
Siderastreidae									
Psammocora contigua	Penghu Is., Taiwan	R	197	61		II	AY722782	this study	Veron (2000)
Pseudosiderastraea tayami	Kaohsiung, Taiwan	R	197	64		I	AY722789	this study	Veron (2000)

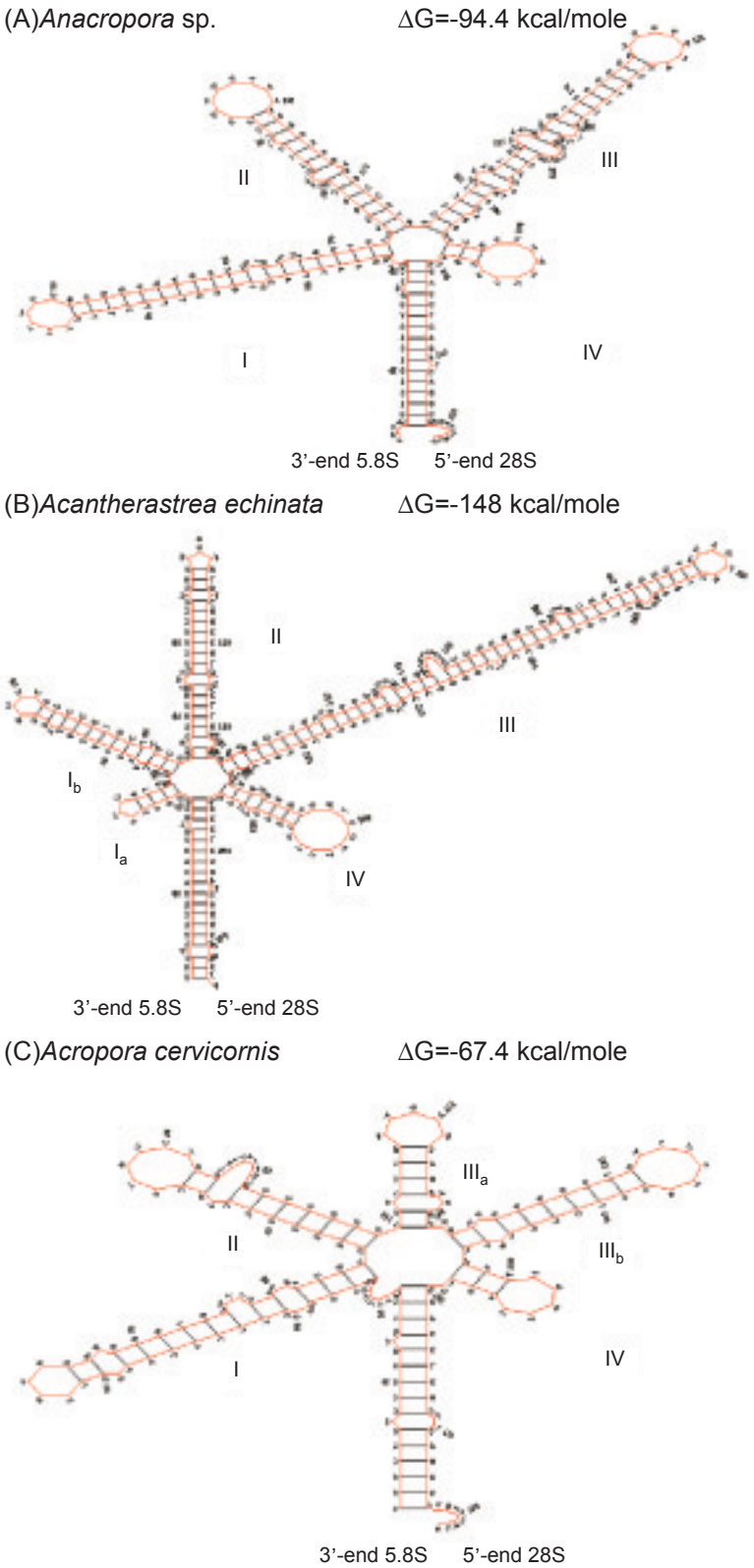

Fig. 1. Representation of the proposed secondary structure of scleractinian corals. (A) Type I, *Anacropora* sp. with 4 putative domains; (B) type II, *Acantherastrea echinata* with 5 putative domains, with domain I divided into 2 subdomains (Ia and Ib); and (C) type III, *Acropora cervicornis* with 5 putative domains, with domain III divided into 2 subdomains (III_a and III_b). Minimum free energies (ΔG) are indicated.

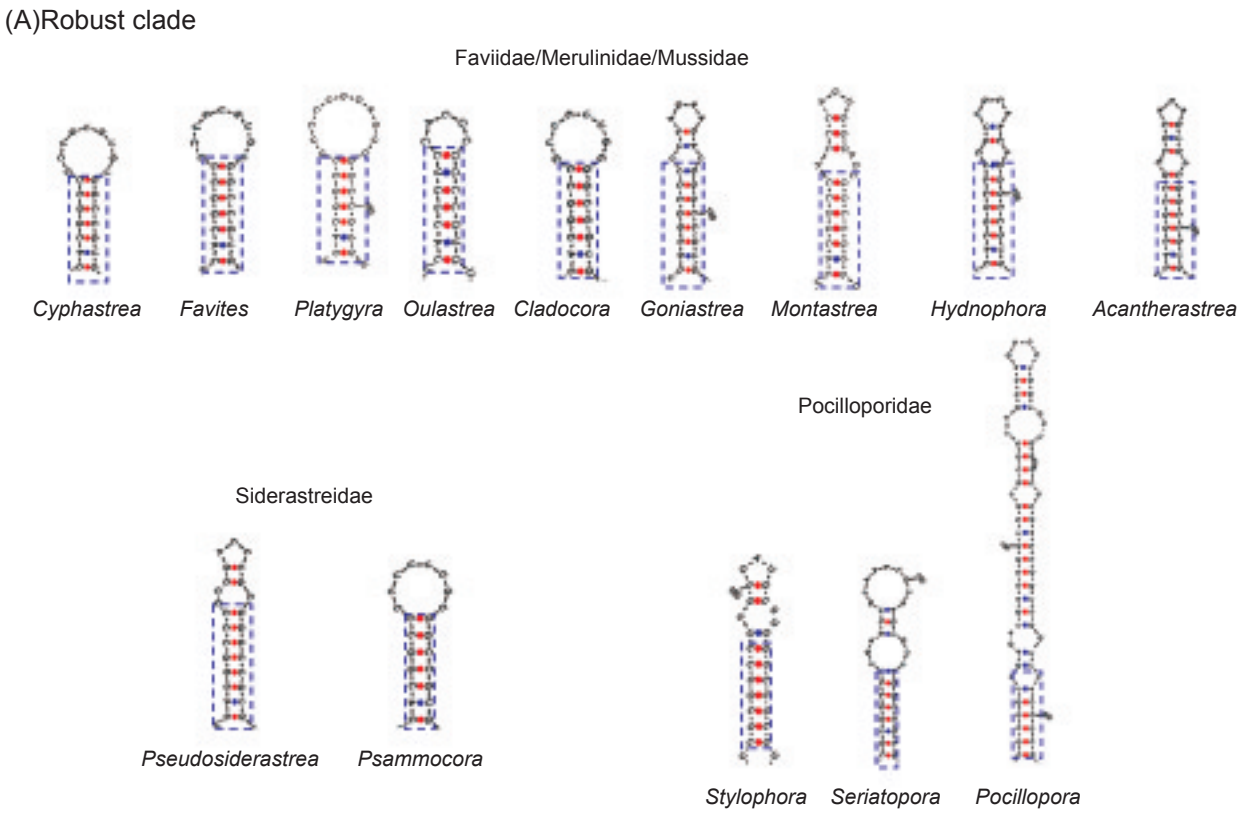

Fig. 2. Structures of domain II representing scleractinian corals of (A) the robust and (B) complex clades. Conserved pyrimidine-pyrimidine pairings are marked by a dashed-line box.

and *Porites lutea* (Table 1).

Putative secondary structures, conserved motifs, and improvements in the ITS2 alignment

The putative secondary structures of the 54 species from the 25 genera and 12 families of scleractinian corals could be categorized into 3 types: type I, the standard 4-domain model; type II, a 5-domain model with domain I divided into 2 subdomains (I_a and I_b); and type III, a 5-domain model with domain III divided into 2 subdomains (III_a and III_b) (Fig. 1). The 20 bp of the 5.8S and 28S rDNA that was immediately adjacent to the 5'-end and 3'-end of the ITS2 apparently forms canonical bonds with each other. Among the 54 ITS2 secondary structures constructed, 17 belonged to type I, 23 were of type II, and 14 were of type III (Table 1). Type I and II structures were observed in all 12 families of scleractinian corals. In contrast, type III was only seen in the genus *Acropora*. Among the domains, while stem-loop IV was the most-variable domain, stem II was highly conserved and was flanking by a conserved sequence motif in the adjacent stems I and III. The motif, 5'-CRCG-GYC-3', and its compensatory bases in stem II were highly conserved in corals of both the robust and complex clades (Fig. 2).

Alignment of the ITS2 primary sequences was considerably improved based on adjustment of the secondary structure. Conserved stems identified in the secondary structural domains provided consistent bases for correcting the alignment of variable loop regions for the phylogenetic analysis (data not shown). The ITS2 uncorrected *p*-distances guided by the secondary structure were significantly smaller than those derived from the default options of CLUSTAL X. In the complex clade, the mean *p*-distance was 0.2717 ± 0.09756 before manual readjustment, which was significantly larger than that (0.2353 ± 0.09583) after guidance by the secondary structure for species in the subgenus *Acropora* (paired *t*-test = 5.688, $p < 0.001$). However, a reliable alignment could not be obtained among the lineages within the complex clade due to the extremely high divergence between *Acropora* and other corals (see below). In the robust clade, a similar trend could be observed in the families of the Faviidae, Merilinidae, and Mussidae which means that *p*-distances (0.27569 ± 0.07609) were significantly decreased after readjustment using the secondary structure (0.24848 ± 0.06634, paired *t*-test = 9.438, $p < 0.001$). However, attempts to align the ITS2 between the robust and complex clades were unsuccessful due to the extremely high divergence among clades (see below).

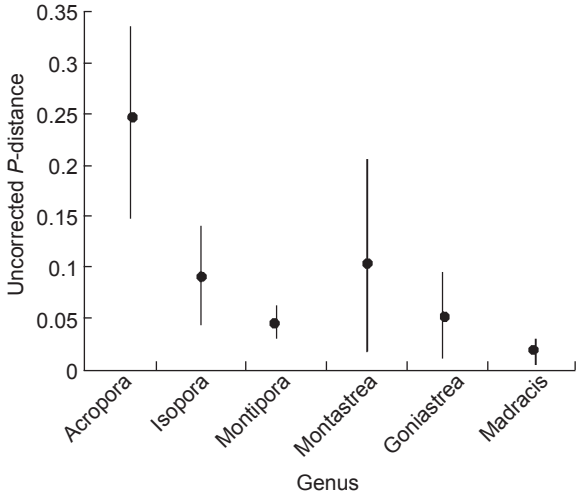

Fig. 3. Interspecific variability (uncorrected *p*-distances) in different genera of scleractinian corals. Means and standard deviations are indicated by closed dots and bars, respectively.

Phylogenetic utility of ITS2 in scleractinian corals

The interspecific *p*-distances of ITS2 varied considerably in different genera and subgenera of scleractinian corals (Fig. 3). In the 6 genera examined, the highest value was observed in the subgenus *Acropora* with a mean *p*-distance of 0.2353. This value is close to the level of intergeneric variation within the robust clade. Variation within the genus *Madracis* was the smallest with a mean *p*-distance of 0.0134. *p*-distances among *Acropora* and other corals of the complex clade were so high (> 0.6) that intergeneric alignment was not reliable for phylogenetic reconstruction.

In order to assess the phylogenetic utility of ITS2, we focused only on an analysis of the robust clade, since it was not possible to produce a reliable alignment within the complex clade or between the 2 clades. Taxa were selected based on the phylogenetic trees published in Romano and Cairns (2000) and Chen et al. (2002). Two species of the complex, *Tubastrea aurea* and *Fungiacyathus* sp., were used as outgroups. Of the 334 characters in the ITS2 alignment, 207 (61.98%) were variable and 138 (41.32%) were parsimony-informative for the robust clade. Phylogenetic construction using the MP, NJ, and

ML algorithms produced identical topologies (Fig. 4). Removal of the large insertions and deletions (indels) at the hypervariable domains (e.g., domain IV) did not affect the topology of the phylogenetic tree (data not shown). Parsimony analysis revealed a single MP tree with a tree length of 564, a consistency index of 0.628, a retention index of 0.53, and a rescaled consistency index of 0.33. The ML analysis of ITS2 yielded a topology of a -ln likelihood of 3345.17. The tree showed that the generic relationship based on the ITS2 did not correspond with the family tree based on skeletal morphology (Veron 2000), except for the astroconeid, *Styloceneiella guentheri*, and *Madracis formosa*. Using *Tubastrea aurea* and *Fungiacyathus* sp. as outgroups, genera of the Faviidae (except for *Oulastrea* and *Cladocora*) were grouped with *Hydnophora* (Merulinidae) and *Acantherastrea* (Mussidae) with high bootstrapping support (Fig. 4).

DISCUSSION

While the ITS2 region presents a dramatic range of length variations among scleractinian corals, its size remains relatively homogenous within each of the major groups. *Acropora* has the shortest ITS2 not only among scleractinian corals but also in metazoans and eukaryotes (Odorico and Miller 1997). This is an atypical but unique feature of *Acropora*, since lengths of the ITS2 from other genera in the Acroporidae are comparable to those of other scleractinians. Nevertheless, some species of *Acropora*, such as *A. humilis*, *A. togianensis* (this study), *A. aspera*, *A. pulchra*, *A. florida* (Marquez et al. 2003), *A. cervicornis*, *A. palmata*, and *A. prolifera* (van Oppen et al. 2000, Vollmer and Palumbi 2004), as well as the subgenus *Isopora* possess significantly longer ITS2 regions than others due to the occurrence of dinucleotide or tetranucleotide simple sequence repeats (i.e., microsatellites). Different compositions of

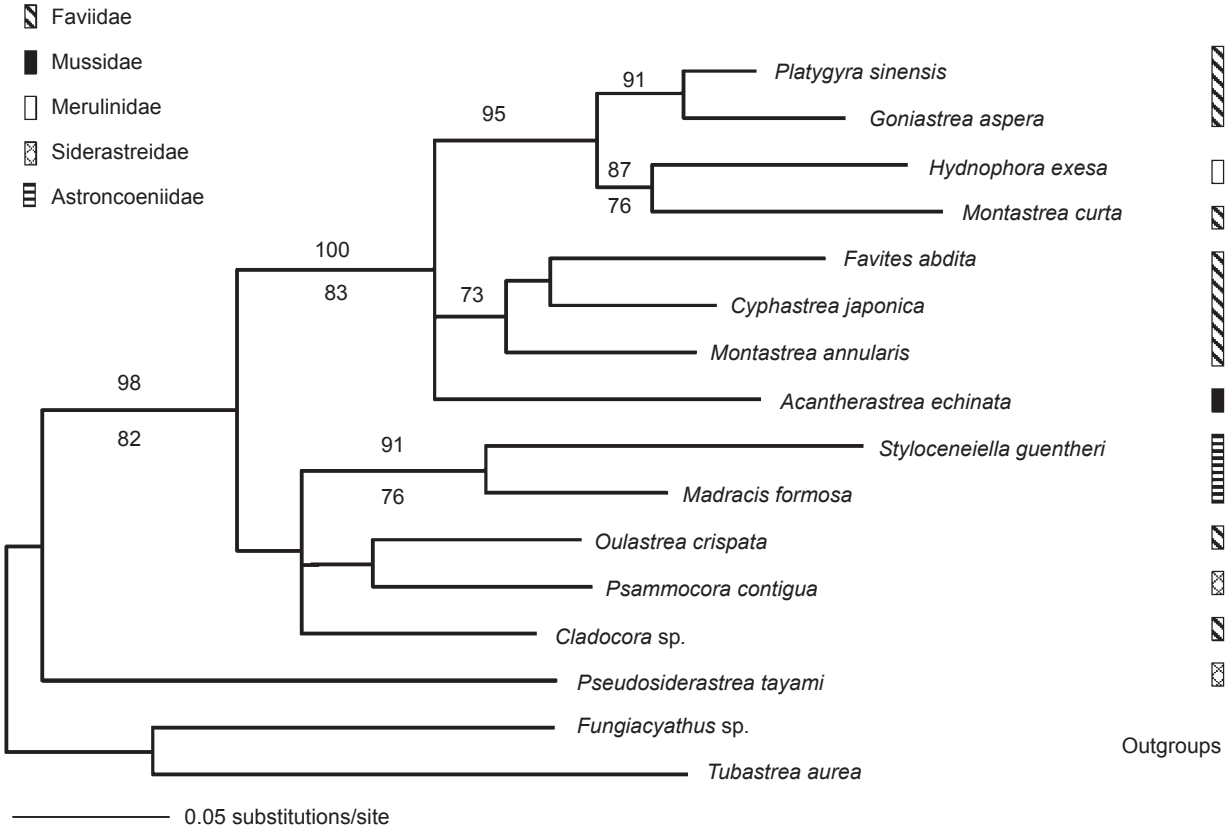

Fig. 4. Phylogenetic analysis of the robust-clade ITS2 derived from the Neighbor-joining (NJ), maximum parsimony (MP), and maximum likelihood (ML) methods. The 3 algorithms produced the same tree topology. The tree is presented with bootstrapping support labeled above (NJ) and below (MP) the branches (< 50% not shown). Scleractinian families are coded by bars with different shading.

microsatellites were also observed in the genera, *Cyphastrea*, *Favites*, *Goniastrea*, *Hydnophora*, *Madracis*, *Montipora*, and *Porites*, indicating that ITS2 microsatellites evolved independently in the different lineages of scleractinian corals.

The occurrence of microsatellites in the ITS region has had great impacts on phylogenetic studies of corals. Short repeated sequences eventually increase the difficulties of aligning homologous regions and subsequently influence phylogenetic reconstructions. For example, a study on the species boundaries of 5 Caribbean *Madracis* corals, *M. mirabilis*, *M. senaria*, *M. decactis*, *M. formosa*, and *M. pharensis*, using the ITS1-5.8S-ITS2 region indicated that *M. senaria* and *M. mirabilis* formed monophyletic groups, while the other 3 formed paraphyletic groups (Diekmann et al. 2001). Those results suggested a reticulate speciation through repeated introgressive hybridizations in *Madracis*. Nevertheless, this conclusion should be accepted with extreme caution, since careful alignment and inclusion/exclusion of CA repeats (Table 1) at the 3'-end of ITS2 can generate very different phylogenetic relationships. *Madracis senaria* and *M. mirabilis* no longer formed a monophyly as seen in previous reports (data not shown). Similar concerns about ITS microsatellites for phylogenetic analyses were also discussed in freshwater crayfish (Harris and Crandall 2000) and ants of the genus *Strumingenys* (Hung et al. 2004).

The secondary structure of coral ITS2 does not always conform to the 4-domain model as predicted in green algae, flowering plants, fruit flies (*Drosophila* spp.), parasitic flatworms, gastropods, and the mouse (Michot et al. 1999, Schlötterer et al. 1994, Mai and Coleman 1997, Morgan and Blair 1998, Joseph et al. 1999, Coleman and Vacquier 2002, Oliverio et al. 2002, Gottschling and Plötner 2004). The difficulties of arriving at a common ITS2 secondary structure for scleractinian corals is probably due to limitations of the computer program, Mfold. The Mfold program generates multiple free-energy diagrams, and a "consensus" model is inferred from the structural features common to all. It has been argued that even closely related taxa with very similar primary sequences can result in/exhibit vastly different structures (Hershkovitz and Zimmer 1996). More significantly, experimentally derived RNA structures frequently exhibit suboptimal free-energy conformations (Gutell et al. 1994). Identifying covariation sites is recognized to improve the stability of the secondary structure. Covaration analysis based on a large-scale comparison of 340 sequences of Asteraceae ITS suggested that 20% of ITS1 and 38% of ITS2 nucleotide position are involved in base pairing to form helices (Goertzen et al. 2003). Interestingly, the ITS2 secondary structure model of Asteraceae based on covariation analysis generally agree with structural features generated by thermodynamic criteria (Goertzen et al. 2003), indicating that even without considering covariations the computer-based secondary structure model should still be regarded as a reliable "backbone" for further readjustment of primary alignment in phylogenetic analysis. A future study examining ITS sequences from a large data set of closely related taxa may be suitable for examining the covariations of ITS2 in more detail for scleractinian corals. In addition, the most-conserved feature of the scleractinian ITS2 in domain II and the adjacent regions of domains I and III found in all of the scleractinian corals studies indicate that these conserved sequences are likely to play an important role in folding the secondary structure of coral ITS2. Similar conservation was also observed among different genera of hard ticks which have a 5-domain ITS2 secondary structure (Hlinka et al. 2002).

The robust clade phylogeny constructed using the ITS2 is concordant with the phylogenies based on mitochondrial and nuclear ribosomal genes and protein-coding genes which show that the Faviidae, Merulindae, and Mussidae are monophyletic within the suborder Faviina (except for *Oulastrea*), but relationships among these families are apparently not monophyletic (Romano and Cairns 2000, Chen et al. 2002, Fukami et al. 2004). For example, both Fukami et al. (2004) and the present study showed that *Montastrea annularis*, a major Caribbean reef builder, is grouped with *Cyphastrea japonica* and forms a paraphyletic relationship with its Pacific congener, *M. curta*. *Oulastrea crispata* is clustered with a siderastreid, *Psammocora contigua*, and forms a trichotomic relationship with another Faviid, *Cladocora* sp. The affinity of both *Oulastrea* and *Cladocora* to the family Faviidae has been questioned, and placement of these 2 genera needs to be re-examined (Romano and Cairns 2000, Chen et al. 2002). Our data did not group *Psammocora* and *Pseudosiderastrea* in a monophyletic group, which indicates that generic relationships within the family Siderastreidae should also be reconsidered.

ITS rDNA sequences are the most frequently used DNA markers for studying scleractinian evolution (Hunter et al. 1997, Lopez and Knowlton

1997, Medina et al. 1997, Odorico and Miller 1997, van Oppen et al. 2000 2002, Diekmann et al. 2001, Rodriguez-Lanetty and Hoegh-Guldberg 2002, Vollmer and Palumbi 2004), especially on the topics of reticulate evolution among closely related species. However, the phylogenetic utility of the ITS has been questioned (van Oppen et al. 2002, Vollmer and Palumbi 2004). This is due to the extremely high levels of variability in the ITS which have been observed within and among several *Acropora* species (Odorico and Miller 1997, van Oppen et al. 2002, Marquez et al. 2003). Debates on interspecific hybridization versus incomplete lineage sorting as an explanation for the high ITS intragenomic variation have been overwhelmed by evidence from *Acropora* species (van Oppen et al. 2000 2001 2002, Vollmer and Palumbi 2002 2004, Marquez et al. 2003, Miller and van Oppen et al. 2003). Vollmer and Palumbi (2004) concluded that nuclear rDNA should be abandoned as a species- and population-level phylogenetic marker due to its complicated and undistinguishable characteristics of molecular evolution. We argue that this conclusion should be treated with great caution, since *Acropora* has several atypical and unusual characteristics that are significantly distinct from other scleractinian corals. First, *Acropora* has the shortest ITS not only among scleractinian corals but also among metazoans (Odorico and Miller 1997). For the other scleractinian corals, the length of the ITS is compatible among genera. The mechanism by which *Acropora* species possess such a short sequence is still unknown. Second, even though it has the shortest DNA sequences, *Acropora* ITS2 forms a unique but stable 5-domain secondary structure, which differs from that of other scleractinian corals. Third, ITS sequence divergence within and among *Acropora* spp. is the highest observed so far. Except for *Acropora*, variations in the ITS2 are moderate, and it can reliably be aligned even across different genera of scleractinians to produce robust phylogenies. These characteristics strongly indicate that high ITS intragenomic divergence of *Acropora* may be an exception rather than the rule for the evolutionary history of scleractinian corals. In contrast, our analyses strongly indicate that ITS rDNA in scleractinian corals, with careful readjustment under guidance of the secondary structure, is still applicable to different levels of phylogenetic analyses from populations to genera.

Acknowledgments: Many thanks to the staff of the Penghu Aquarium, a facility of the Taiwan Fishery Research Institute, for providing hospitality during our field trips. We thank members of the Evolution and Ecology Discussion Group, Research Center for Biodiversity (RCBAS), Institute of Zoology, Academia Sinica (IZAS), and 2 anonymous referees for constructive comments. This is Evolution and Ecology Group, IZAS contribution no. 32. This work was supported by grants from RCBAS, IZAS, and the National Science Council, Taiwan to the senior author.

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