DNA sequences of the 2 internal transcribed spacers (ITS1 and ITS2) of the ribosomal RNA (rRNA) transcriptions 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 transcribed 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).
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 monophilies, 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′-GGTACCCTTTGTACACACCGC-3′ and 2SS: 5′-GCTTTGGGCGGC-AGTCCCAAGCAAACCGGACTC-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). Nucleotide sequence analysis 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, WI, 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 longicyathus 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)2-6 and (GT)2-7 in Acropora (Isopora) brueggmanni, A. cuneata, A. palifera, and A. togianesis; (AG)5 in Anacropora sp. and Montipora spp.; (CA)3-6 in Madracis spp.; (GC)5 in Goniastrea spp. and Hydnophora exesa; and (TG)4 in Cyphastrea japonica and Favites abdita. The tetrancuotide repeats of (CCAT)4 and (AGCA)5-7 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.

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<td>Medina et al. (1999)</td>
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Fig. 1. Representation of the proposed secondary structure of scleractinian corals. (A) Type I, \textit{Anacropora} sp. with 4 putative domains; (B) type II, \textit{Acantherastrea echinata} with 5 putative domains, with domain I divided into 2 subdomains (I\textsubscript{a} and I\textsubscript{b}); and (C) type III, \textit{Acropora cervicornis} with 5 putative domains, with domain III divided into 2 subdomains (III\textsubscript{a} and III\textsubscript{b}). Minimum free energies (ΔG) are indicated.
(A) Robust clade

Faviidae/Merulinidae/Mussidae

Cyphastrea  Favites  Platygyra  Oulastrea  Cladocora  Goniastrea  Montastrea  Hydnophora  Acanthera astrea

Siderastreidae

Pseudosiderastrea  Psammocora

Pocilloporidae

Stylophora  Seriatopora  Pocillopora

(B) Complex clade

Acroporidae

Acropora  Isopora  Anacropora  Montipora  Astreopora  Alveopora  Porites

Astrocoeniidae

Oculinidae  Dendrophylliidae  Fungiacyathidae

Madracis  Styloceneiella  Galaxea  Tubastrea  Fungiacyathus

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 (Ia and Ib); and type III, a 5-domain model with domain III divided into 2 subdomains (IIIa and IIIb) (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 flanked 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, Merulinidae, 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).

**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 astronconeid, 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
Cyphastrea microsatellites were also observed in the genera, Cyphastrea, Favites, Goniastrea, Hydophora, 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 analyses. For example, a study on the species boundaries of 5 Caribbean gous regions and subsequently influence phyloge-
tically increase the difficulties of aligning homolo-
studies of corals. Short repeated sequences even-
region has had great impacts on phylogenetic
corals, different lineages of scleractinian corals. ITS2 microsatellites evolved independently in the M
mosa, 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 speci-
ation 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 mono-
phyly as seen in previous reports (data not
shown). Similar concerns about ITS microsatel-
lites for phylogenetic analyses were also discus-
ded 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 pre-
dicted 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
coral is probably due to limitations of the computer
program, Mfold. The Mfold program generates mul-
tiple free-energy diagrams, and a "consensus" model is inferred from the structural features com-
mmon 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 signifi-
cantly, experimentally derived RNA structures fre-
etly exhibit suboptimal free-energy conformations
(Gutell et al. 1994). Identifying covariation sites is
recognized to improve the stability of the sec-
condary 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 gen-
erally agree with structural features generated by
thermodynamic criteria (Goertzen et al. 2003), indi-
cating 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 adja-
cent regions of domains I and III found in all of the
scleractinian corals studies indicate that these con-
erved 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 mono-
phyletic within the suborder Faviini (except for
Oulastrea), but relationships among these families
are apparently not monophyletic (Romano and
2004). For example, both Fukami et al. (2004) and
the present study showed that Montastrea annu-
laris, 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 place-
ment of these 2 genera needs to be re-examined
(Romano and Cairns 2000, Chen et al. 2002). Our
data did not group Psammocora and Pseudo-
siderastrea in a monophyletic group, which indi-
cates that generic relationships within the family
Siderastreidae should also be reconsidered.

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

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