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Strategies for Amplification by Polymerase Chain Reaction of the Complete Sequence of the Gene Encoding Nuclear Large Subunit Ribosomal RNA in Corals

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Abstract: The nearly complete nuclear large subunit ribosomal RNA (LSU rRNA) gene in corals was amplified by primers designed from polymerase chain reaction (PCR) strategies. The motif of the putative 3′-terminus of the LSU rRNA gene was sequenced and identified from intergenic spacer (IGS) clones obtained by PCR using universal primers designed for corals. The 3′-end primer was constructed in tandem with the universal 5′-end primer for the LSU rRNA gene. PCR fragments of 3500 bp were amplified for octocorals and non-*Acropora* scleractinian corals. More than 80% of the *Acropora* LSU rRNA gene (3000 bp) was successfully amplified by modification of the 5′-end of the IGS primer. Analysis of the 5′-end of LSU rDNA sequences, including the D1 and D2 divergent domains, indicates that the evolutionary rate of the LSU rDNA differs among these taxonomic groups of corals. The genus *Acropora* showed the highest divergence pattern in the LSU rRNA gene, and the presence of a long branch of the *Acropora* clade from the other scleractinian corals in the phylogenetic tree indicates that the evolutionary rate of *Acropora* LSU rDNA might have accelerated after divergence from the common ancestor of scleractinian corals.

Key words: PCR, nuclear, large subunit ribosomal RNA gene, coral, *Acropora.*

INTRODUCTION

Eukaryotic ribosomal DNA (rDNA) is composed of tandemly repeated units (Figure 1) containing the coding regions for small subunit (SSU), 5.8S, and large subunit (LSU) ribosomal RNA separated by internal transcribed spacers (ITS1 and ITS2). The transcription units are separated from each other by an intergenic spacer (IGS), which comprises the signals for transcription initiation and termination (reviewed in Gerbi, 1985). Because different regions of the rDNA repeat units evolve at very different rates, phylogenetic analyses of these rDNA sequences provide an opportunity to investigate organismal evolution (reviewed in Hillis and Doxon, 1991). Of these accumulated molecular data (Van de Peer et al., 1994; reviewed in Van de Peer et al., 1997), complete or nearly complete SSU rDNA has been utilized extensively for studies of relationships among all different life forms (Cedergren et al., 1998; Van de Peer et al., 1990), evolution of metazoans (Field et al., 1988; Wain-

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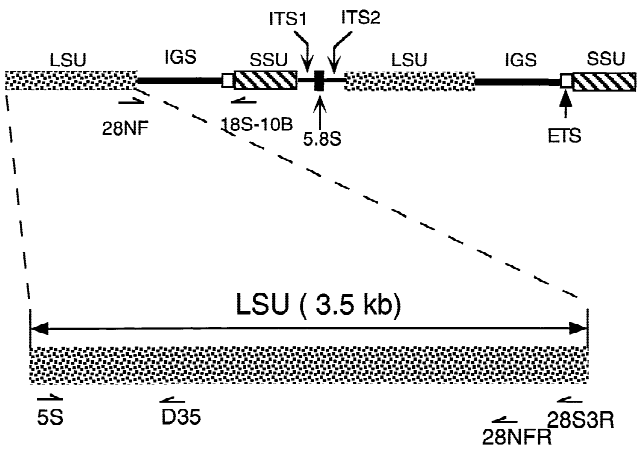

Figure 1. Primer localities and the basic organization of a typical rRNA cistron in a eukaryotic nuclear genome. The coding regions for mature RNA (SSU, 5.8S, and LSU), external transcribed spacer (ETS), intergenic spacer (IGS), and internal transcribed spacers (ITS) are shown.

right et al., 1993; Smothers et al., 1994; Aguinaldo et al., 1997; Kim et al., 1999), and higher hierarchies within a phylum (e.g., Steiner and Müller, 1996; Winnepenninckx et al., 1996; Berntson et al., 1999).

On the contrary, application of complete or nearly complete LSU rDNA sequences to phylogenetic approaches is growing slowly (Zardoya and Meyer, 1996; Mallatt and Sullivan, 1998). The longer chain length of LSU rDNA (>3000 bp) has retarded the acquisition of sequences, and larger differences in length and variability of divergent (D) domains (Hassouna et al., 1984) within LSU rDNA have frustrated attempts to assemble a good alignment of all sequences. Nonetheless, analyses of 225 representative sequences from 38 eukarya, 16 archaea, 64 bacteria, 31 plastids, and 76 mitochondria have been attempted (De Rijk et al., 1995). The comparative conclusions suggest that the impact of the species set on the topology of the resulting consensus tree is much lower using LSU than using SSU rDNA, indicating that LSU rDNA is a better molecule for studying wide-range relationships (De Rijk et al., 1995).

Corals, including soft corals (octocorallians) and hard corals (scleractinians), represent the basal group (i.e., class Anthozoa) in the evolution of the phylum Cnidaria (Bridge et al., 1995; Odorico and Miller, 1997a; Kim et al., 1999). External and internal evolutionary relationships of these two groups have been examined using different molecules, and the results are controversial (Chen et al., 1995; France et al., 1996; Romano and Palumbi, 1996; Veron et al., 1996; Berntson et al., 1999). Analysis of 225 bp of the 5′-end of LSU rDNA from a variety of anthozoans indicates that Octocorallia is the sister group of Scleractinia, supporting the 3-subclass relationship (Ceriantipatharia [Ocotocorallia, Scleractinia]) within the class Anthozoa corals (Chen et al., 1995), and a monophyly of scleractinians (Veron et al., 1996). A portion of mitochondrial LSU (16S) rRNA in 29 species of deep sea anthozoans, however, supports a 2-subclass organization (Hexacorallia and Octocorallia) of the class Anthozoa (France et al., 1996). However, analyses of the same segment of mtLSU rDNA sequences in 34 species of scleractinian corals find no support for the current grouping at the suborder level. These results suggest multiple origins of the scleractinian skeleton, and the great diversity of present-day scleractinians may reflect these multiple origins (Romano and Palumbi, 1996, 1997). Although an analysis of a large set of SSU rDNA sequences has recently been attempted for 40 anthozoan species, the limited phylogenetic information in SSU rDNA ultimately confines its application to the higher hierarchy within the class Anthozoa (Berntson et al., 1999). In order to resolve the internal or external evolutionary relationship of corals, it is clear that more sets of comparative molecular data, such as complete LSU rDNA, must be examined.

In the present study we therefore applied polymerase chain reaction (PCR) strategies to (1) identify the motif of the 3′-terminus of LSU rDNA from several species of corals, (2) redesign the primers to amplify the nearly complete LSU rDNA with a size of over 3000 nucleotides, and (3) reveal the molecular evolutionary pattern of LSU rDNA in corals using the sequences obtained in this study. Analysis of the 5′-end and 3′-end of sequences confirmed the successful amplification of complete or nearly complete LSU rDNA. A preliminary analysis of the 5′-end of LSU rDNA indicated that the evolutionary rate of LSU rDNA apparently differs among the major taxonomic groups of corals, with the genus *Acropora* showing the highest divergence in the LSU rRNA gene.

Materials and Methods

Coral Samples

Taxa, collecting locations, and DNA sources of corals used in the present study are summarized in Table 1. We collected sperm materials of *Acropora muricata, A. digitifera, A. cytherea, Echinophyllia orpheensis, Platygyra sinensis, Favia favus,* and *Favites abdita* on nights of predicted coral spawn-

Table 1. Taxonomic Information, Collecting Locations, and DNA Sources of the Corals Used in This Study

Taxon	Collecting sites	DNA source	Identification reference
Scleractinia			
Acropora muricata	Penghu, Taiwan	Sperm	Wallace (1999)
Acropora digitifera	Penghu, Taiwan	Sperm	Wallace (1999)
Acropora cytherea	Nanwan Bay, Taiwan	Sperm	Wallace (1999)
Porites cylindrica	Green Island, Taiwan	Tissue	Vernon and Pichon (1980)
Pavona catcus	Orpheus Island, Australia	Tissue	Vernon and Pichon (1980)
Galaxea fascicularis	Penghu, Taiwan	Sperm	Vernn and Pichon (1980)
Echinophyllia orpheensis	Nanwan Bay, Taiwan	Sperm	Vernon and Pichon (1980)
Platygyra sinensis	Penghu, Taiwan	Sperm	Veron et al. (1977)
Favia favus	Penghu, Taiwan	Sperm	Veron et al. (1977)
Favites abdita	Nanwan Bay, Taiwan	Sperm	Veron et al. (1977)
Tubastrea aurea	Nanwan Bay, Taiwan	Tissue	Vernon and Pichon (1980)
Octocorallia			
Junceella fragilis	Green Island, Taiwan	Tissue	Dai (1989)
Virgularia juncea	Penghu, Taiwan	Tissue	Rho and Song (1976)

ing in southern Taiwan in 1997 (Dai et al., 1992) and in the Penghu Islands in 1998 and 1999 (C.A. Chen et al., unpublished data). For *Porites cylindrica, Tubastrea aurea, Junceella fragilis,* and *Virgularia juncea,* a small fragment on the tip of a coral branch was clipped from each colony and placed in a labeled bag. Both sperm and tissues were frozen in liquid nitrogen or dry ice for transfer to the laboratory. All samples were stored at −70°C prior to analysis. DNA of *Pavona catcus* was a gift from Dr. David Miller, Department of Biochemistry and Molecular Biology, James Cook University, Australia.

DNA Extraction, Amplification, Cloning, and Sequencing

DNA extraction was modified from methods described by Chen and Yu (2000). IGS rDNA was initially amplified to determine the approximate position of the 3′-terminus of the 28S rRNA coding sequence. PCR of the IGS rDNA was modified after Smith et al. (1997) and Chen et al. (2000), using the "universal" primer pairs 28NF, 5′-GATTATGACTGAACGCCTCTAAGTCAGAAT CC-3′, and 18S-10B, 5′-TTACCATCGACAGTTGATAGGGCAGA-3′ (Figure 1). PCR was performed in a PC-9606 thermal sequencer (Corbett Research) using the following thermal cycle: 1 cycle at 95°C (1.5 minutes); 4 cycles at 94°C (30 seconds), 60°C (1 minute), 72°C (3.5 minutes), and 30 cycles at 94°C (30 seconds), 57°C (1 minute), 72°C (3.5 minutes). The amplification reaction used 50 to 200 ng of template and BRL *Taq* polymerase in a 50-μl volume reaction using the buffer supplied with the enzyme and under the conditions recommended by the manufacturer. The PCR products were electrophoresed in a 0.8% agarose (FMC Bioproduct) gel in 1× TAE buffer to check the yield. The amplified DNA was extracted once with chloroform, precipitated with ethanol at −20°C, and resuspended in water. PCR products were cloned using the pGEM-T system (Promega) under the conditions recommended by the manufacturer. The nucleotide sequences were determined for both ends of PCR products of at least 3 clones form each sample using an ABI 377 Genetic Analyzer. (The sequences obtained in this study have been submitted to GenBank under accession numbers listed in the legends to Figures 3 and 5.)

Sequence Alignment and Phylogenetic Analysis

DNA sequences were initially aligned using CLUSTAL W 1.7 (Thompson et al., 1994), followed by manual editing using SEQAPP 1.9 (Gilbert, 1994). The divergent (D) domains of both ends of 28S rDNA sequences in corals were determined by comparing predicted secondary structures to the corresponding rRNA database (De Rijk et al., 1998). In order to examine the molecular evolutionary pattern of LSU rDNA in corals, we performed the following analyses.

First, variability in 60-bp sliding windows plotted throughout the 5′-end of LSU rDNA sequences, including

the D1 and D2 domains (between 596 bp in *Acropora muricata* and 772 bp in *Galaxea fascicularis*), was performed for the 8 taxa using MEGA 1.01 (Kumar et al., 1993).

Second, because size differences in the D2 domain introduced a significant number of gaps for *Acropora* corals (see Results and Discussion), we analyzed the first 352 bp of eight 5′-ends of LSU rDNA obtained in this study for nucleotide composition differences. Each sequence was examined for nucleotide composition to the frequency distribution assumed in the maximum likelihood model; the transition-to-transversion ratio was estimated from the sequences, and maximum-likelihood (ML) distances were computed under the Hasegawa-Kishino-Yano (HKY) model using the package PUZZLE 4.01 (Strimmer and von Haeseler, 1996).

Third, we aligned the first 205 bp of the 5′-end of LSU rDNA to those of 19 published coral LSU rDNA sequences available in GenBank for phylogenetic inferences. Phylogenetic analyses were performed using the maximum-likelihood and the neighbor-joining methods. The maximum-likelihood analysis, the best suited for modeling the heterogenous substitution pattern, was implemented in the package PUZZLE 4.01. The ML distances and branch lengths were computed under the HKY model (Hasegawa et al., 1985). The neighbor-joining option was performed based on the Kimura 2-parameter distance with among-site rate variation determined using PAUP 4b3.ppc (Swofford, 1999). Robustness of the phylogenies was assessed by the 1000 bootstrap option using neighbor-joining tree construction in PAUP 4b3.ppc.

Results

The IGS region of the 3′-end of LSU rDNA was successfully amplified in several coral species (Figure 2) using primers designed from previous studies (Smith et al., 1997; Chen et al., 2000). As evidenced by the general characteristics of the IGS region of rDNA (reviewed in Gerbi, 1985), the sizes of amplified fragments were polymorphic within individuals (e.g., *Virgularia juncea* and *Porites cylindrica* in Figure 2) and extremely variable among coral species as a result of the presence or absence of tandem subrepeats within the IGS in several coral species (C.A. Chen et al., unpublished data). In *Junceella fragilis,* reproduction is dominated by asexual fragmentation (Walker and Bull, 1983; Vermeire, 1994). Sequence analysis of IGS in *J. fragilis* indicated that only a segment of tetra nucleotide simple sequence repeats, (C[A/G]GA)$_n$, was found in the 5′-end of IGS (Figure 3), and distinctive subrepeats were not observed in the entire region (Chen et al., 2000). The alignment showed a clear line between conserved blocks and the remaining highly variable IGS sequences at 251 bp (Figure 3). The BLAST search in the GenBank and secondary structure comparison indicated that the motif 5′-CTTGTTCTAAGATTTGT-3′ represented the putative 3′-terminus of LSU rDNA of corals.

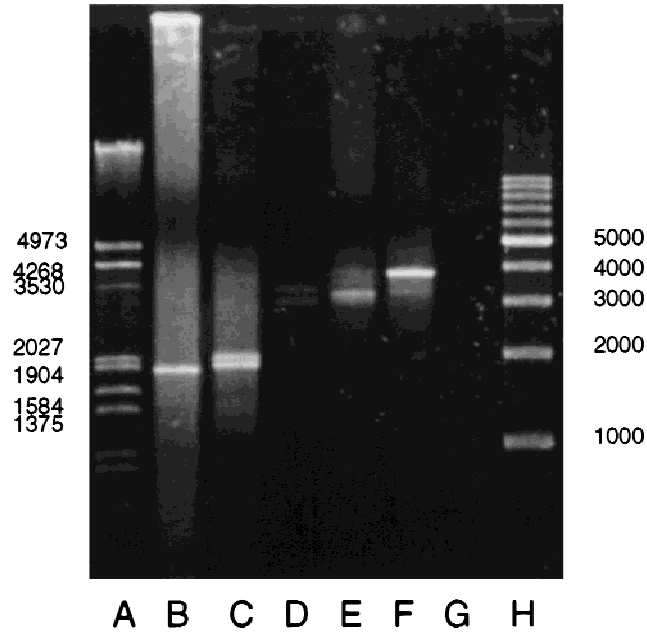

Figure 2. Agarose (0.8%) gel electrophoresis of PCR products representing the IGS region from a variety of corals. Lane A, λ cut by *Hin*dIII and *Eco*RI, DNA standard size markers; lane B, *Junceella fragilis;* lane C, *Virgularia juncea;* lane D, *Porites cylindrica;* lane E, *Favites abdita;* lane F, *Echinophyllia orpheensis;* lane G, negative control; lane H, 1-Kb ladder, Waston, DNA standard size marker.

We designated the 3′-end primer of LSU rDNA based on the alignment in Figure 3. The primer 28S3R, in conjunction with the 5′-end primer 5S (Table 2), can successfully amplify the entire region of LSU rDNA for both octocorals and non-*Acropora* scleractinian corals with PCR products of about 3500 bp (Figure 4). In order to amplify the LSU rDNA of *Acropora,* the reverse complement of 28NF, 28NFR, was modified (Table 2). *Acropora* LSU rDNA could be successfully amplified with PCR products of 3000 bp (Figure 4). These results imply that the 3′-end of *Acropora* LSU rDNA is probably divergent compared with those of other corals. This high divergence between *Acropora* and other corals was further revealed by the sequence and phylogenetic analyses of the 5′-end of LSU rDNA (see below).

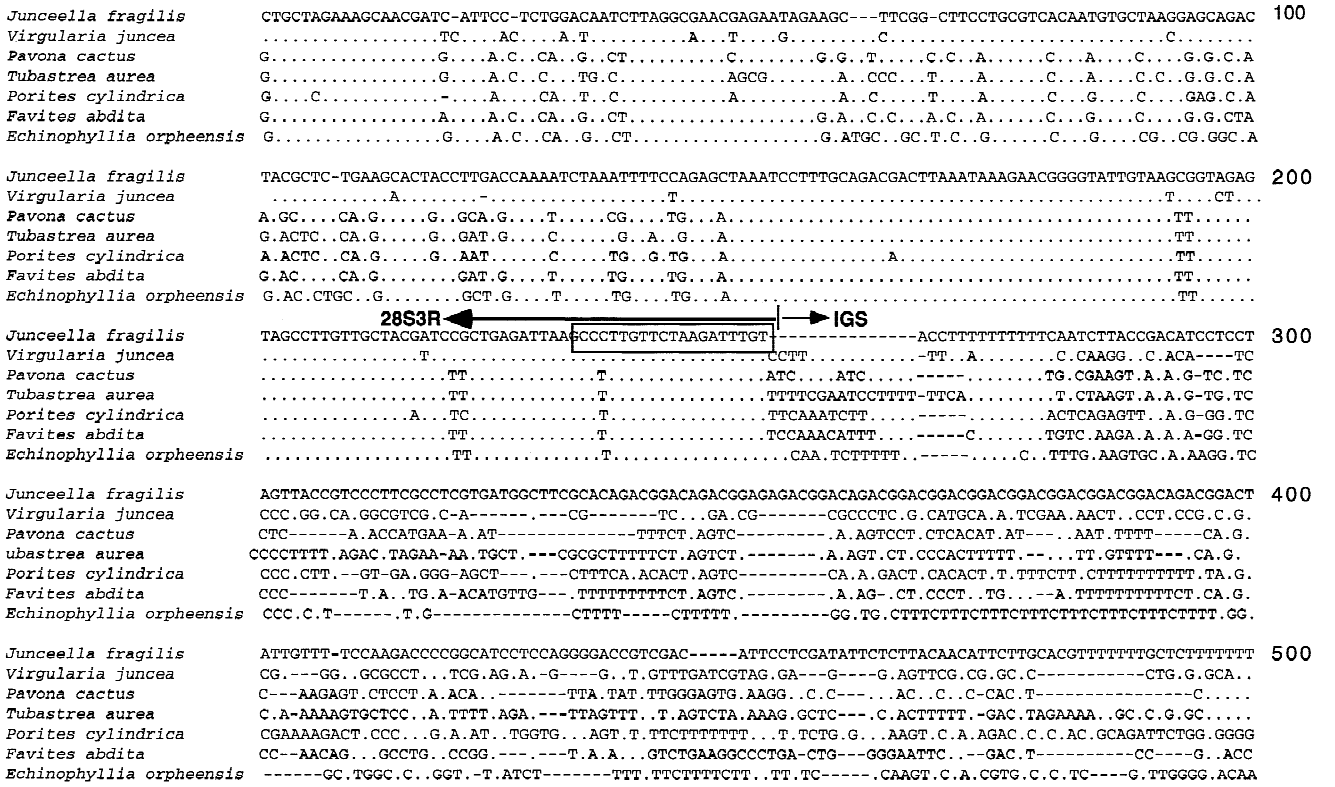

Figure 3. Sequence alignment of the region at the 3′-end of LSU rDNA and parts of the 5′-end of IGS from a variety of corals. Dots indicate identity with the *Junceella fragilis* sequence, and dashes indicate gaps introduced to optimize the alignment. The putative motif of the 3′-terminus of the 28S rRNA coding sequence and the locality of the primer are indicated by an open box and boldface arrows, respectively. GenBank accession numbers: *Junceella fragilis,* AF154670; *Virgularia juncea,* AF263349; *Pavona cactus,* AF263350; *Tubastrea aurea,* AF263351; *Porites cylindrica,* AF263352; *Favites abdita,* AF263353; and *Echinophyllia orpheensis,* AF263354.

Table 2. Primer Sequences Used and Designed in This Study

Primer	Sequence	Location in the28S/18S rDNA of mouse
5S*	5′-GCCGACCCGCTGAATTCAAGCATAT-3′	21–45
D35†	5′-GAGTTTCCTCTGGCTTCACCCTATT-3′	1365–1380
28NF‡	5′-GATTATGACT GAACGCCTCT AAGTCAGAAT CC-3′	4331-4352
28NFR§	5′-GGATTCTGAC TTAGAGGCGT TCAGTCATAA TC-3′	4331–4352
18S-10B‡	5′-TACCATCGAC AGTTGATAGGGCAGA-3′	278–312
28S3R§	5′-CAAATCTTAG AACAAGGGCTTAATCTCAG-3′	—

*PCR primer adapted from Chen et al. (1995).
†Sequencing primer designed in this study.
‡PCR primer adapted from Smith et al. (1997).
§PCR primer designed in this study.

We sequenced and assessed the molecular evolutionary pattern of the 5′-end of PCR fragments. The results indicate that the PCR products we obtained represent the nuclear LSU rDNA of corals. The alignments, including the D1 and D2 domains, of 8 species of corals are presented in Figure 4. The 5′-end of LSU rDNA of corals showed moderate to high bias of G+C contents in their nucleotide composition, ranging from 51% in D1 of *Junceella fragilis* to 71.8% in D2

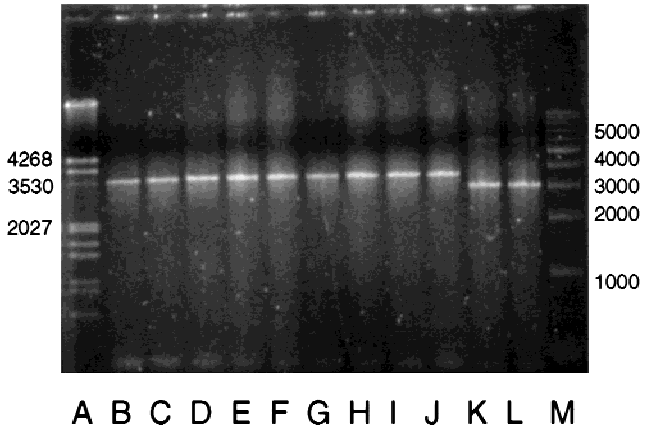

Figure 4. PCR products amplified by 5S and 28SFR (lanes B–J) and 28SNFR (lanes K, L) from a variety of corals. Lanes A and M represent DNA standard markers (corresponding with lanes A and H, respectively, in Figure 2). Lane B, *Junceella fragilis;* lane C, *Virgularia juncea;* lane D, *Pavona catcus;* lane E, *Porites cylindrica;* lane F, *Favites abdita;* lane G, *Echinophyllia orpheensis;* lane H, *Favia favus;* lane I, *Platygyra sinensis;* lane J, *Tubastrea aurea;* lane K, *Acropora muricata;* and lane L, *Acropora digitifera.*

Table 3. Size (bp) and G + C Content of Two Divergent Domains (D1 and D2) in the 5′-End of LSU rDNA

Taxon	5′-End of LSU rDNA	D1 (% G + C)	D2 (% G + C)
Octocorallia			
Junceella fragilis	760	157 (51)	410 (62.7)
Virgularia juncea	769	158 (51.8)	418 (65.3)
Scleractinia			
Acropora digitifera	604	162 (68.6)	246 (69.1)
Acropora muricata	596	155 (70.4)	246 (69.1)
Acropora cytherea	616	161 (71.6)	260 (65.7)
Galaxea fascicularis	772	157 (71.4)	422 (71.8)
Platygyra sinensis	759	157 (58)	409 (67)
Favites abdita	756	157 (57.3)	406 (65.5)

of *Galaxea fascicularis* (Table 3). Sequence length ranged from 596 bp in *Acropora muricata* to 772 bp in *Galaxea fascicularis* with a similar size of the D1 domain (155–162 bp). However, D2 domains were highly variable in size, with the shortest D2 found in both *A. digitifera* and *A. muricata* (246 bp), and the longest (422 bp) in *Galaxea fascicularis* (Table 3). Size differences in D2 domains introduced significant numbers of gaps for *Acropora* corals, and led to an alignment of up to 846 bp among the 8 taxa (Figure 5).

Analysis of the 5′-end of LSU rDNA, including the D1 and D2 divergent domains, indicated that the divergence rate of the LSU rDNA differs between each taxonomic group of corals. First, the variability of aligned sequences was plotted for a series of overlapping 60-bp windows along the sequence from the 5′-end, including the D1 and D2 domains (Figure 6). The results of this analysis give a visual image of regional variability along the length of the sequences (Kumar et al., 1993). The following comparisons were carried out: among 3 species of *Acropora,* between 2 genera of Faviidae corals, between 2 genera of octocorals, between *Acropora* spp. and the other scleractinian genera, between *Acropora* spp. and *Junceella fragilis,* and between *Acropora* spp. and *Virgularia juncea.* In the 6 comparisons, the sequence divergences of both the D1 and D2 domains was high between in *Acropora* species (Figure 6, A), and even higher than that of 2 Faviidae corals at the generic level (Figure 6, B). Between 2 octocorallian genera, representing 2 families, the divergence was moderate (Figure 6, C). However, comparisons between *Acropora* spp. and the remaining taxa all showed extremely high divergences (Figure 6, D–F), with the highest one located at the 5′-end of the D2 domain among scleractinian genera (Figure 6, D).

Second, we analyzed the nucleotide composition focusing on 352 bp of the 5′-end of LSU rDNA. In this region, nucleotide frequencies did not differ significantly among coral species (χ^2-test, $P < 0.05$); the transition-to-transversion ratio estimated from the data set was 1.37 ± 0.24. Pairwise comparisons of ML distances (Table 4) reflected similar patterns from the 60-bp sliding windows analyses. Among *Acropora* species, the ML genetic distances were relatively small with a mean of 0.0338 (0.01926–0.04425), but higher than that between *Platygyra sinensis* and *Favites abdita* (0.02554) at the generic level. The distances between *Acropora* and the other 3 genera of scleractinian corals were dramatically higher (0.27172–0.35346), and are comparable to those between *Acropora* and the 2 octocorals (0.393–0.47391).

Third, phylogenetic analyses of 205 bp of the 5′-end of LSU rDNA sequences using maximum likelihood produced the same topology as neighbor-joining criteria (Figure 7). The branch length of the *Acropora* clade (0.418789) estimated with the HKY model was significantly longer than that of the scleractinian clade (0.19564), indicating that the evolutionary rate of *Acropora* LSU rDNA had accelerated after divergence from the common ancestor of scleractinian corals.

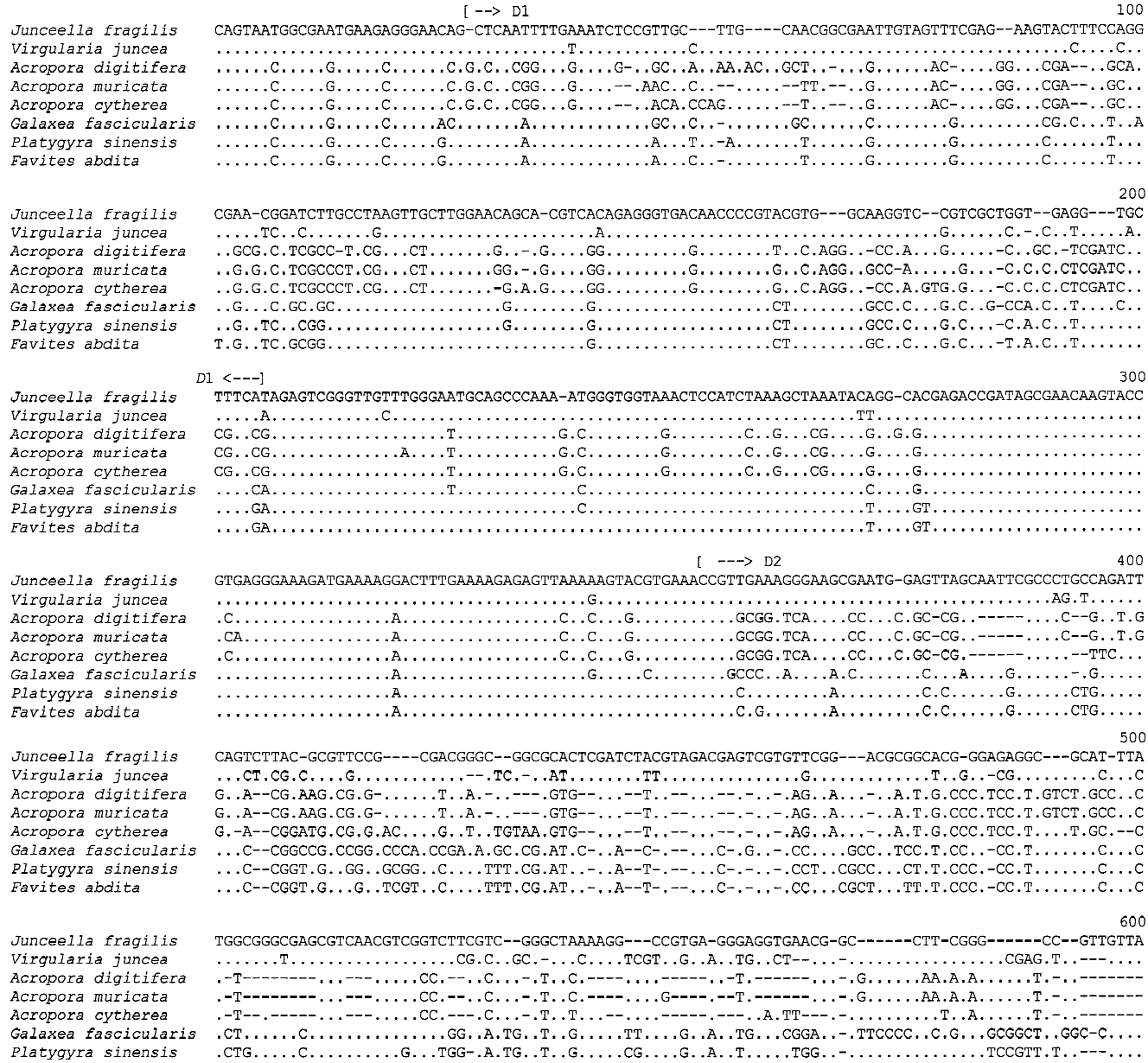
[--> D1
100
Junceella fragilis CAGTAATGGCGAATGAAGAGGGAACAG-CTCAATTTTGAAATCTCCGTTGC---TTG----CAACGGCGAATTGTAGTTTCGAG--AAGTACTTTCCAGG
Virgularia juncea
Acropora digitifera
Acropora muricata
Acropora cytherea
Galaxea fascicularis
Platygyra sinensis
Favites abdita
200
Junceella fragilis CGAA-CGGATCTTGCCTAAGTTGCTTGGAACAGCA-CGTCACAGAGGGTGACAACCCCGTACGTG---GCAAGGTC--CGTCGCTGGT--GAGG---TGC
Virgularia juncea
Acropora digitifera
Acropora muricata
Acropora cytherea
Galaxea fascicularis
Platygyra sinensis
Favites abdita
D1 <---]
300
Junceella fragilis TTTCATAGAGTCGGGTTGTTTGGGAATGCAGCCCAAA-ATGGGTGGTAAACTCCATCTAAAGCTAAATACAGG-CACGAGACCGATAGCGAACAAGTACC
Virgularia juncea
Acropora digitifera
Acropora muricata
Acropora cytherea
Galaxea fascicularis
Platygyra sinensis
Favites abdita
[---> D2
400
Junceella fragilis GTGAGGGAAAGATGAAAAGGACTTTGAAAAGAGAGTTAAAAAGTACGTGAAACCGTTGAAAGGGAAGCGAATG-GAGTTAGCAATTCGCCCTGCCAGATT
Virgularia juncea
Acropora digitifera
Acropora muricata
Acropora cytherea
Galaxea fascicularis
Platygyra sinensis
Favites abdita
500
Junceella fragilis CAGTCTTAC-GCGTTCCG----CGACGGGC--GGCGCACTCGATCTACGTAGACGAGTCGTGTTCGG---ACGCGGCACG-GGAGAGGC---GCAT-TTA
Virgularia juncea
Acropora digitifera
Acropora muricata
Acropora cytherea
Galaxea fascicularis
Platygyra sinensis
Favites abdita
600
Junceella fragilis TGGCGGGCGAGCGTCAACGTCGGTCTTCGTC--GGGCTAAAAGG---CCGTGA-GGGAGGTGAACG-GC------CTT-CGGG------CC--GTTGTTA
Virgularia juncea
Acropora digitifera
Acropora muricata
Acropora cytherea
Galaxea fascicularis
Platygyra sinensis
Favites abdita
700
Junceella fragilis TAACCTCG---CGAGCGTATGGCTCGACGAAGACC-GTGGA-----GTCGCGAC--------GCGTGCTT--CCCTCCGGGGGGGCTGGTGTCCTGTT--
Virgularia juncea
Acropora digitifera
Acropora muricata
Acropora cytherea
Galaxea fascicularis
Platygyra sinensis
Favites abdita
800
Junceella fragilis --CTGTTGGTCCGGTTGTCGACCGTGGCGGACGGCACGCCTGTGCGCTCGAACTGCTGCCGGTC-AACAGGGCGGTGAT-CACACACTACGCGTCGAGGA
Virgularia juncea
Acropora digitifera
Acropora muricata
Acropora cytherea
Galaxea fascicularis
Platygyra sinensis
Favites abdita

Figure 5.

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                                    D2<---]                    846
Junceella fragilis    CGTTGACGACCATACGGCTCTATCCGACCCGTCTTGAAACACGGACC
Virgularia juncea     .....................................................
Acropora digitifera   ---.TG.CG..--------GC...G.....................
Acropora muricata     ---.TG.CG..--------GC...G.....................
Acropora cytherea     ..A.TC..G..--------GC...G.....................
Galaxea fascicularis  .....G..GA....T..G..C.........................
Platygyra sinensis    T....G..GT....T..G.GC.........................
Favites abdita        T....G..GT....T..G.GC.........................
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Figure 5. Sequence alignment of the region at the 5′-end of LSU rDNA from a variety of corals. Dots indicate identity with the *Junceella fragilis* sequence and dashes indicate gaps introduced to optimize the alignment. GenBank accession numbers: *Junceella fragilis,* AF263355; *Virgularia juncea,* AF263356; *Acropora digitifera,* AF263357; *Acropora muricata,* AF263358; *Acropora cytherea,* AF263359; *Galaxea fascicularis,* AF263360; *Platygyra sinensis,* AF263361; and *Favites abdita,* AF263362.

Discussion

We have designed a set of primers that facilitates the amplification of nearly complete nuclear large subunit ribosomal RNA genes in both scleractinian and octocorallian corals. Although amplification of full-length LSU rDNA in the genus *Acropora* was not successful because of its high divergence compared with the other taxa, the 5S and 28S3R primers still show certain "universality" to corals. Further PCR tests obtained successful amplifications from 3 actiniarians and 2 corallimorpharians (data not shown), suggesting that this primer set is universal for at least 4 orders (Scleractinia, Octocorallia, Actiniaria, and Corallimorpharia) of the class Anthozoa. Over 80% of the LSU rRNA gene of *Acropora* spp. was still recovered in conjunction with the 5S primer and the reverse primer of 28NF, indicating the PCR strategies used in the present study provided an efficient approach to obtain the nearly complete LSU rDNA sequence for further phylogenetic analysis.

An interesting aspect of the eukaryotic LSU rRNA gene is the existence of 12 divergent domains (D1–D12) and the phylogenetic information that these domains provide (Michot and Bachellerie, 1987; Michot et al., 1990; Nunn et al., 1996). The D1 domain was applied to phylogenetic analyses of the Anthozoa (Chen et al., 1995), evolutionary relationships of scleractinian corals (Veron et al., 1996), and echinoderm phylogeny (Lafay et al., 1995; Littlewood et al., 1997; Smith et al., 1997). Phylogenetic trees inferred from the D2 domain have confirmed the multiple origins of anural development of anural and urodele ascidians (Hadfield et al., 1995). Domains D3 to D6 were used to uncover evolutionary relationships within the platyhelminths (Litvaitis and Rohde, 1999). Analysis of the D9 and D10 domains supports the monophyly of each copepod order, but this is in contrast to traditional morphology-based phylogenies of copepod orders (Braga et al., 1999). In our preliminary analysis, the 5′-end of LSU rDNA has shown distinctly different rates of divergence among the major taxonomic groups of corals. For example, the D1–D2 domains may be useful for phylogenetic analysis in the genus *Acropora* at the species level, but for the Faviidae corals, this region appears to be relatively conserved. These results indicate that caution should be taken in choosing divergent domains for phylogenetic analysis. Sequence and structural analysis of the remaining divergent domains of corals are in progress and may provide further information useful for elucidating the relationships of coral phylogeny at different levels.

The finding of a high divergence rate of the 5′-end of LSU rDNA in *Acropora* spp. is not surprising. Failure to amplify members of the *Acropora* group by 5S and 28S3R indicates that the 3′-end of the LSU rRNA gene in *Acropora* might also be divergent. The ITS sequences from *Acropora* have shown several unique characteristics, including one that is the shortest so far identified in any metazoan and among the shortest seen in eukaryotes, and the highly variable sequences which are consistent with a reticulate evolutionary history for the genus *Acropora* (Odorico and Miller, 1997b; C.A. Chen et al., unpublished data). These results imply that fast evolution might be a common feature in the cistron of ribosomal RNA units of *Acropora.* Further investigation of the nuclear SSU rRNA gene and IGS region in *Acropora* will confirm this hypothesis. In contrast to nuclear rRNA genes, their counterparts in the mitochondrial genome of the genus *Acropora* showed a slowly evolving pattern (Romano and Palumbi, 1997; Chen and Yu, 2000), and this pattern also applies to the mitochondrial protein-coding genes, cytochrome *b* (van Oppen et al., 1999; C.A. Chen et al., unpublished data) and cytochrome oxidase I (C.A. Chen et al., unpublished data). van Oppen et al. (1999) hypothesized that a functional mtDNA mis-

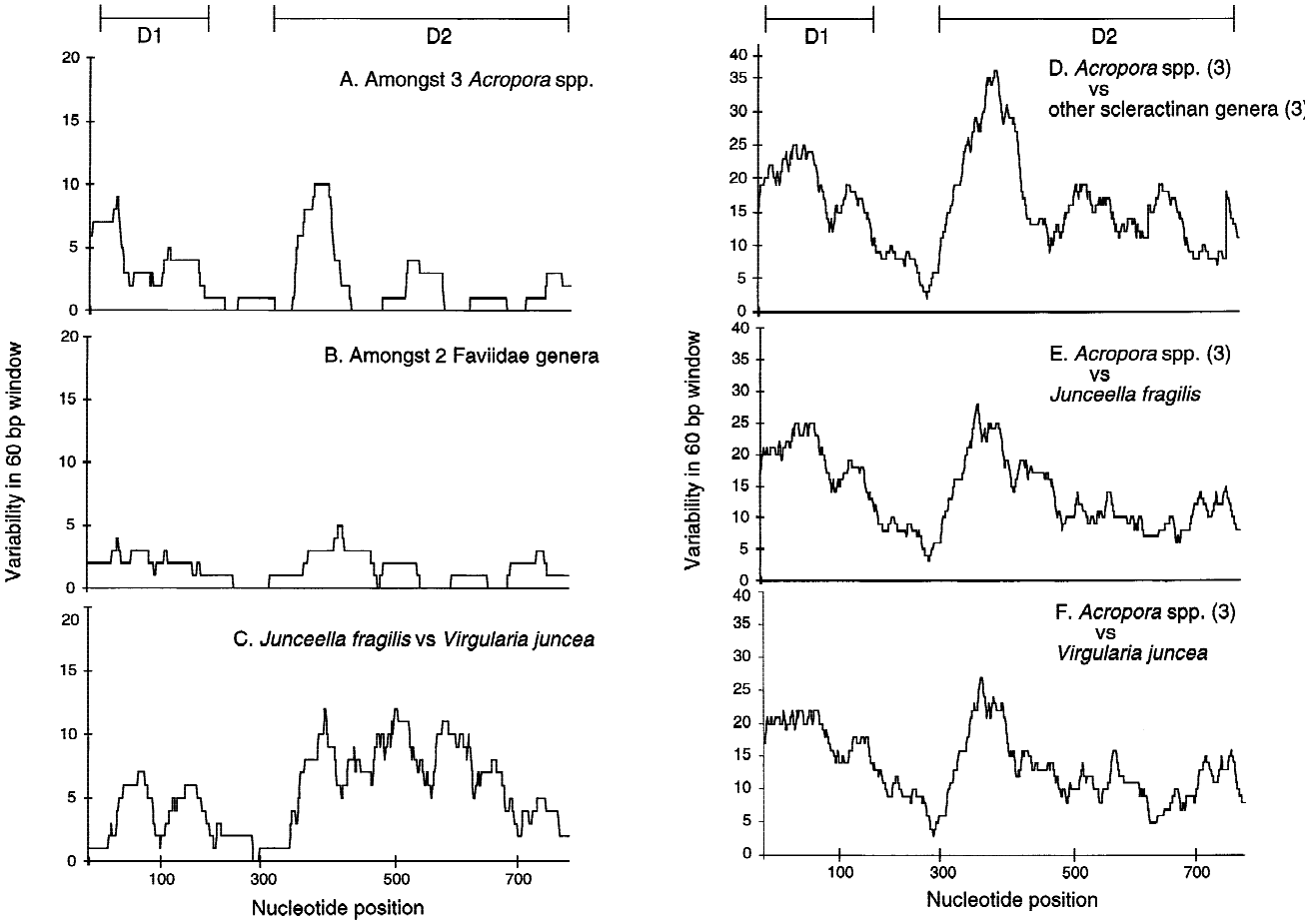

Figure 6. Variability in 60-bp sliding windows plotted throughout the 5′-end of the LSU rDNA region: among three *Acropora* spp. **(A);** among two Faviidae genera **(B),** *Junceella fragilis* versus *Virgularia juncea* **(C);** *Acropora* spp. versus other scleractinian genera **(D);** *Acropora* spp. versus *Junceella fragilis* **(E);** and *Acropora* spp. versus *Virgularia juncea* **(F).**

Table 4. Maximum-Likelihood Distances Calculated Under the HKY Model (Hasegawa et al. 1985) in the Pairwise Comparison of 352 bp of the 5′-End of LSU rDNA Among the Eight Taxa of Corals*

	JF	VJ	AD	AF	AC	PS	FA
Junceella fragilis (JF)							
Virgularia juncea (VJ)	0.05892						
Acropora digitifera (AD)	**0.45824**	**0.42089**					
Acropora muricata (AM)	**0.47391**	**0.43838**	0.04425				
Acropora cytherea (AC)	**0.45186**	**0.39300**	0.03661	0.01926			
Platygyra sinensis (PS)	0.15887	0.15895	**0.31076**	**0.31856**	**0.29188**		
Favites abdita (FA)	0.14666	0.14705	**0.34792**	**0.35346**	**0.31407**	0.02555	
Galaxea fascicularis (GF)	0.22140	0.20840	**0.30283**	**0.30742**	**0.27152**	0.09072	0.09906

*Boldface indicates the interordinal and interfamilial comparisons between *Acropora* and other corals.

match repair system (Pont-Kingdon et al., 1998) may account for this unique phenomenon, although the actual repair system has not yet been identified in the *Acropora* mitochondrial genome. Unfortunately, repair mechanisms appear to be extremely complex, and our knowledge of them is far from complete (Page and Holmes, 1998). Therefore whether the lack of a functional repair system or an inefficient repair system in the nuclear genome causes the

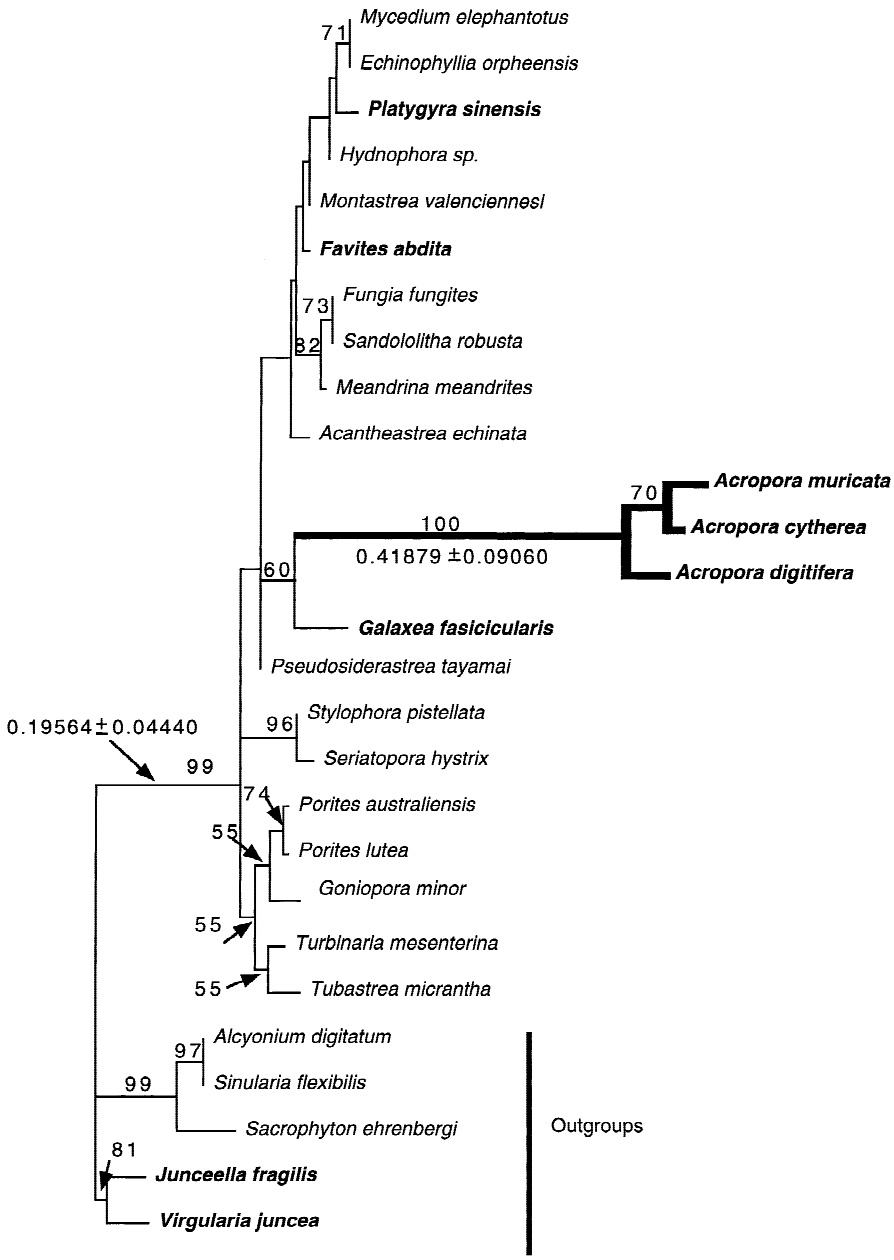

Figure 7. Phylogenetic analyses of 205 bp of the 5′-end of LSU rDNA sequences. The figure shows the tree inferred by the neighbor-joining method. Octocorallians assigned as outgroups are indicated by vertical bars. Branch lengths are proportional to evolutionary distances. A tree of the same topology was inferred by the maximum-likelihood method. Branch lengths and standard errors estimated by the maximum-likelihood method are indicated for those larger than 0.1. The numbers above or below the branches leading to the nodes represent the percentage of 1000 bootstrap pseudoreplications that support the node in the neighbor-joining analysis. Bootstrap values less than 50% are not shown. LSU rDNA sequences obtained from this study are shown in boldface. GenBank accession numbers of coral 5′-end LSU rDNA sequences were indicated as follows: *Alcyonium digitatum,* U69680; *Sinularia flexibilis,* U69681; *Sacrophyton ehrenbergii,* U69679; *Stylophora pistillata,* U65519; *Seriatopora hystrix,* U65520; *Pseudosiderastrea tayamai,* U65518; *Acanthastrea echinata,* U65524; *Hydnophora* sp., U65526; *Mycedium elephantotus,* U65527; *Montastrea valenciennesi,* U65525; *Turbinaria mesenterina,* U65513; *Tubastrea micrantha,* U65514; *Goniopora minor,* U65515; *Porites australiensis,* U65516; *Porites lutea,* U65517; *Fungia fungites,* U65523; *Meandrina meandrites,* U65521; and *Sandalolitha robusta,* U65522.

high variability of rRNA genes in the genus *Acropora* remains equivocal.

An alternative hypothesis for the high rDNA sequence divergence in the genus *Acropora* is repeated recombination and crossing-over among *Acropora* species through past hybridization events (Kenyon, 1997; Odorico and Miller, 1997a, 1997b). *Acropora* is the largest extant genus of reef-building coral, with 113 species currently described (Wallace, 1999). Most species of *Acropora* are broadcast spawners, releasing eggs and sperm synchronously during mass spawning events (Harrison et al., 1984; Harrison and Wallace, 1990; Richmond and Hunter, 1990; Dai et al., 1992; C.A. Chen et al., unpublished data). Multispecies gamete slicks therefore form at the water surface, where fertilization takes place. The opportunity for hybridization is very plausible. In supporting this scenario, in vitro hybridization experiments have been successfully carried out between species within a genus, including the genus *Acropora,* as well as between species of different genera (Willis et al., 1997). Counting the somatic chromosome number in 22 species of *Acropora,* Kenyon (1997) proposed that the ability to propagate clones through vegetative fragmentation plus the

opportunities for hybridization may have contributed to the development of polyploidy and rapid, sympatric speciation in the genus *Acropora.* Odorico and Miller (1997b) have demonstrated, at the molecular level, that the pattern of ITS variation is unusual. For example, 2 distinct ITS2 types were detected in both *A. hyacinthus* and *A. cytherea,* species known to hybridize in vitro with a high success rate (Willis et al., 1997); and a putative intermediate ITS2 form was also detected in *A. cytherea,* supporting the idea of reticulate evolution for the genus *Acropora.* Further investigation of sequence variation of the divergent domains within LSU rDNA from variety of *Acropora* spp. might provide potential evidence to support this scenario.

In conclusion, the nearly complete nuclear large subunit ribosomal RNA gene in corals was successfully amplified by primers designed by strategies of PCR. Analysis of the 5′-end of the LSU rRNA gene shows different divergence rates among the major taxonomic group of corals. The presence of a long branch of the *Acropora* clade from the other scleractinian corals in the phylogenetic tree indicates that the evolutionary rate of *Acropora* LSU rDNA accelerated after divergence from the common ancestor of scleractinian corals.

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