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Variation in Coding (NADH Dehydrogenase Subunits 2, 3, and 6) and Noncoding Intergenic Spacer Regions of the Mitochondrial Genome in Octocorallia (Cnidaria: Anthozoa)

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Abstract: Low rates of evolution in cnidarian mitochondrial genes such as *COI* and *16S rDNA* have hindered molecular systematic studies in this important invertebrate group. We sequenced fragments of 3 mitochondrial protein-coding genes (NADH dehydrogenase subunits *ND2*, *ND3* and *ND6*) as well as the *COI-COII* intergenic spacer, the longest noncoding region found in the octocoral mitochondrial genome, to determine if any of these regions contain levels of variation sufficient for reconstruction of phylogenetic relationships among genera of the anthozoan subclass Octocorallia. Within and between the soft coral families Alcyoniidae and Xeniidae, sequence divergence in the genes *ND2* (539 bp), *ND3* (102 bp), and *ND6* (444 bp) ranged from 0.5% to 12%, with the greatest pairwise distances between the 2 families. The *COI-COII* intergenic spacer varied in length from 106 to 122 bp, and pairwise sequence divergence values ranged from 0% to 20.4%. Phylogenetic trees constructed using each region separately were poorly resolved. Better phylogenetic resolution was obtained in a combined analysis using all 3 protein-coding regions (1085 bp total). Although relationships among some pairs of species and genera were well supported in the combined analysis, the base of the alcyoniid family tree remained an unresolved polytomy. We conclude that variation in the NADH subunit coding regions is adequate to resolve phylogenetic relationships among families and some genera of Octocorallia, but insufficient for most species - or population-level studies. Although the *COI-COII* intergenic spacer exhibits greater variability than the protein-coding regions and may contain useful species-specific markers, its short length limits its phylogenetic utility.

Key words: Alcyoniidae, mitochondrial DNA, *ND2*, *ND3*, *ND6*, octocoral, phylogenetics.

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INTRODUCTION

Over the past several decades, the mitochondrial genome has been one of the primary sources of characters used for low-level molecular systematic studies of animals. Because animal mitochondrial genes typically evolve 5 to 10 times faster than nuclear DNA, they have proved to be invaluable sources of information for construction of species-level

phylogenies and for studies of the phylogeography of populations (e.g., Avise, 1994). Unfortunately, attempts to use mitochondrial genes for similar studies of cnidarians have been unsuccessful owing to a pronounced lack of sequence variation in this group. Recent studies of the cnidarian large (*16S*) and small (*12S*) subunit ribosomal RNA and cytochrome B (*cytB*) genes have suggested that the cnidarian mitochondrial genome evolves at rates 10 to 20 times slower than in other animal groups (Romano and Palumbi, 1997; van Oppen et al., 1999; Chen and Yu, 2000).

As a result of these apparently slower rates of evolution, mitochondrial genes have not been phylogenetically informative at low taxonomic levels in cnidarians. Studies of *16S* and *12S* ribosomal DNA and the protein-coding genes cytochrome oxidase I (*COI*), *cytB*, and *ATPase-6* have revealed levels of sequence divergence that are typically less than 1% among congeneric species and less than 6% among confamilial genera (Best and Thomas, 1993; France et al., 1996; Romano and Palumbi, 1997; Medina et al., 1999; van Oppen et al., 1999; Fukami et al., 2000; France and Hoover, 2002). Noncoding regions appear to be similarly invariant; sequence divergence in an intergenic spacer (IGS) region present in the mitochondrial DNA of scleractinian corals is less than 3.5% among congeners (Márquez et al., 2002). Consequently, phylogenetic resolution has been limited to the level of orders (France et al., 1996), families (Romano and Palumbi, 1996), or occasionally genera (Fukami et al., 2000). Using a combined analysis of *16S rDNA* and cytochrome oxidase III (*COIII*), Geller and Walton (2001) were, however, able to resolve some intrageneric relationships among sea anemones. Among the anthozoans, members of the subclass Octocorallia exhibit especially low levels of divergence in the *16S rDNA* and *COI* regions, suggesting that rates of mitochondrial gene evolution in this group may be even slower than in other cnidarians (France et al., 1996; France and Hoover, 2002).

Nuclear ribosomal gene sequences that have been examined to date have proved equally unsuitable for low-level phylogenetic studies of cnidarians. Nuclear *18S rDNA* and *28S rDNA* sequences have been used to resolve relationships among the subclasses and orders of cnidarians, but are too invariant to resolve relationships among families or genera (Chen et al., 1995; Berntson et al., 2001; Won et al., 2001). The internal transcribed spacer (*ITS*) regions, however, evolve too rapidly to resolve intergeneric relationships. For instance, McFadden et al. (2001) found more than 25% sequence divergence among species within the alcyonacean genus *Alcyonium*, and similar or higher levels of divergence in *ITS* have been reported within other anthozoan genera (Chen and Miller, 1996; van Oppen et al., 2002). This hypervariability, combined with considerable length variation, frequently makes unambiguous alignment of *ITS* sequences from different genera impossible (Chen et al., 1996; McFadden et al., 2001).

Recent publication of the complete mitochondrial genome of the alcyonacean soft coral *Sarcophyton glaucum* (Beaton et al., 1998; Pont-Kingdon et al., 1998) has facilitated the search for more rapidly evolving mitochondrial gene regions that might allow resolution of genus- and species-level relationships among the Octocorallia. France and Hoover (2001) reported levels of sequence divergence among octocoral families of up to 12.5% in NADH dehydrogenase subunits *ND3* and *ND4L*, twice the variation found in *16S rDNA* (France et al., 1996). Their preliminary data further suggest that the *msh1* gene may evolve 2 times faster than either *ND3* or *ND4L*. This gene appears to be a homologue of the bacterial DNA mismatch repair gene *mutS* (Pont-Kingdon et al., 1995; Culligan et al., 2000), and its presence in the mitochondrial genome has been proposed to explain the slow rate of mtDNA evolution in octocorals (France and Hoover, 2001). To date, *msh1* has been found in a variety of octocoral mitochondrial genomes and in the nuclear genomes of some other eukaryotes, but it is not known to occur in other cnidarians (Culligan et al., 2000; France and Hoover, 2001).

In this study, we examine levels of variability in several other regions of the octocoral mitochondrial genome, specifically the *ND2*, *ND3*, and *ND6* subunits of NADH dehydrogenase and the IGS between cytochrome oxidase subunits I and II. This spacer is the longest noncoding region found in the *S. glaucum* mitochondrial genome, and has been suggested to be the putative control region (Beaton et al., 1998). Because noncoding control and IGS regions typically evolve more rapidly than protein-coding genes, we speculated that the *COI-COII* IGS might harbor greater variation than any other region of the octocoral mitochondrial genome. We chose also to examine *ND2* and *ND6* because comparisons between the mitochondrial genomes of *S. glaucum* and the zoantharian *Metridium senile* indicated greater amino acid sequence divergence in these subunits than in most other protein-coding regions (Beaton et al., 1998; Pont-Kingdon et al., 1998). Because our primary interest lies in constructing a phylogeny of species and genera within the soft coral family Alcyoniidae, we have limited our comparisons to members of this group and one outgroup, the family Xeniidae.

Table 1. Species of Alcyonacean Soft Corals Used for Sequencing[a]

Species	Abbr	Collection locale	Collector, Year
Family Alcyoniidae			
Alcyonium digitatum	ALDI	Isle of Man	CSM, 1992
Alcyonium sp. A	ALspA	Isle of Man	CSM, 1992
Alcyonium coralloides	ALCO	France	CSM, 1994
Alcyonium glomeratum	ALGL	France	CSM, 1994
Alcyonium sp. NZ2	ALNZ2	New Zealand	JS, 1999
Alcyonium variabile	ALVA	South Africa	JS, 1998
Alcyonium rudyi	ALRU	Washington, U.S.A.	CSM, 1992
Alcyonium sp. B	ALspB	Washington, U.S.A.	CSM, 1991
Klyxum simplex	KLSI	Guam	JS, 1998
Cladiella sp.	CLSP	Guam, Palau	JS, 1998–99
Sinularia gaweli	SIGA	Guam	JS, 1998
Sarcophyton trocheliophorum	SATR	Guam	JS, 1998
Lobophytum pauciflorum	LOPA	Guam	JS, 1998
Family Xeniidae			
Xenia sp.	XESP	Palau	JS, 1999
Asterospicularia randalli	ASRA	Guam	JS, 1998

[a]*Alcyonium* sp. A and B are undescribed species that have been included in a previous phylogeny of the genus *Alcyonium* (McFadden et al. 2001). *Alcyonium* sp. NZ2 is an undescribed species from New Zealand. Species identifications made by the collector: CSM, C.S. McFadden; JS, J. Starmer.

Materials and Methods

Samples were collected by hand using SCUBA (McFadden et al., 2001) (Table 1). Small pieces of tissue were either preserved in 95% ethanol or frozen in liquid nitrogen and kept at −70°C prior to DNA extraction. DNA was extracted from tissues using the CTAB protocol of Coffroth et al. (1992), with modifications described in McFadden et al. (2001). Prior to extraction, ethanol-preserved tissues were soaked in 2× CTAB buffer for 24 hours with several solution changes, and in some cases digestion with proteinase K was extended for up to 24 hours (e.g., Berntson et al., 2001).

We amplified 3 separate regions (approx. 1400 bp total) of the mitochondrial genome, including the complete IGS (putative control region) located between the *COI* and *COII* coding regions, approximately 80% of the *ND6* (3′ end) and 30% of the adjacent *ND3* (5′ end) coding regions, and 40% of the *ND2* gene (5′ end). Primers were anchored in adjacent gene regions to increase specificity and prevent amplification of symbionts such as zooxanthellae (Figure 1). Primers were designed using complete mitochondrial genome sequences of *S. glaucum* (GenBank accession numbers AF063191 and AF064823) (Beaton et al., 1998; Pont-Kingdon et al., 1998;) and *Metridium senile* (AF000023) (Beagley et al., 1998). The sequences of all primers used are given in Table 2.

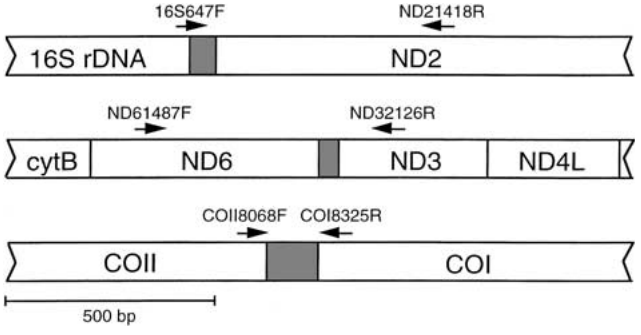

Figure 1. Schematic showing positions of the primers used to amplify 3 different regions of the soft coral mitochondrial genome. Shaded areas represent known or inferred noncoding spacer regions between genes.

PCR reactions were run with 30 to 50 ng DNA, 1.25 units *Taq* polymerase (Qiagen), 2.0 mM $MgCl_2$, 5 µl 10× polymerase chain reaction (PCR) buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl), 0.125 mM dNTPs, and 10 pmol for each primer, in a total reaction volume of 50 µl. Amplification conditions used for each primer pair are given in Table 2. Amplified DNA fragments of the expected size were purified by centrifugation using Ultra-Free-MC

Table 2. Primers used to amplify fragments of protein-coding and non-coding regions in the soft coral mitochondrial genome.

Primer[a]	Sequence	PCR protocol[b]
16S647F	5′-ACACAGCTCGGTTTCTATCTACCA-3′	51°C, 30:30:60, 30×
ND21418R	5′-ACATCGGGAGCCCACATA-3′	
ND61487F	5′-TTTGGTTAGTTATTGCCTTT-3′	50.5°C, 30:30:45, 35×
ND32126R	5′-CACATTCATAGACCGACACTTT-3′	
COII8068F	5′-CCATAACAGGACTAGCAGCATC-3′	47°C, 30:30:30, 40×
COI8325R	5′-TCCTTATGATTAGTAGAAAA-3′	
Internal sequencing primers[c]		
ND21059F	5′-CTCTTTATTGATTTTATTAGTGAT-3′	
ND21081R	5′-TCACTAATAAAATCAATAAAGAGC-3′	
ND61763F	5′-GGGCAATTGGAAGTCATCT-3′	
ND61878R	5′-AGGTGAATTTGGCTGCTTAG-3′	

[a]Primer names reflect their positions in the published *Sarcophyton glaucum* mitochondrial genome (GenBank accession nos. AF063191 (Pont-Kingdon et al., 1998) and AF064823 (Beaton et al., 1998)).

[b]PCR protocol indicates annealing temperature; seconds at 94°C: at annealing temp: at 72°C, and number of cycles.

[c]Internal sequencing primers for the ND2 and ND6 regions were required only when using ABI373 automated sequencer.

100,000 NMWL filtration units (Millipore Corp.), and 5 to 100 ng of DNA was used in a cycle-sequencing reaction according to the manufacturer's protocol (ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit, PE Applied Biosystems). Products were run on either an ABI373A or an ABI3100 automated sequencer. All samples were sequenced in both directions using the same primers as the initial PCR amplification and, when necessary, additional internal primers (Table 2).

LaserGene software (DNASTAR Inc.) was used to proofread DNA sequences and to translate coding regions to amino acid sequences based on the cnidarian mitochondial genetic code (Beaton et al., 1998; Pont-Kingdon et al., 1998). Nucleotide and amino acid sequences were aligned using CLUSTALW Version 1.4 (Thompson et al., 1994) and adjusted by eye. We used PAUP* Version 4.0b10 (Swofford, 2002) to estimate pairwise distances among species and to construct phylogenetic trees using both maximum parsimony and maximum likelihood criteria. Maximum parsimony trees were generated using a heuristic search with TBR branch swapping (default parameters), and support for nodes was determined from 1000 bootstrap replicates. The program Modeltest Version 3.06 (Posada and Crandall, 1998) was used to find the best-fit model of DNA substitution for each alignment, and maximum likelihood analyses were run using those recommended likelihood parameters and the heuristic search option (simple sequence addition). Support for maximum likelihood trees was generated from 100 bootstrap replicates. Phylogenetic trees were constructed using nucleotide sequences from (1) the *ND2* coding region only; (2) the *ND6* coding region only; (3) the *COI-COII* IGS only; and both (4) nucleotide and (5) amino acid sequences from the combined data set of all protein-coding regions (*ND2, ND6* and *ND3*). *Xenia* sp. was specified as the outgroup for all analyses.

Results

We obtained sequences for the complete *COI-COII* IGS (106–122 bp), 444 bp (148 amino acids) of the 3′ end of the *ND6* coding region, 102 bp (34 amino acids) of the 5′ end of the *ND3* coding region, the IGS between *ND6* and *ND3* (35–55 bp), 539 bp (179 amino acids) of the 5′ end of the *ND2* coding region, and an estimated 159 bp of the 3′ end of the *16S rDNA*. Sequences for each of these gene regions were obtained for 15 species in 2 families (Table 1); all sequences have been deposited in GenBank (accession numbers AF529375– AF529389 and AF530482–AF530513). We sequenced 2 individuals of all species except *Lobophytum pauciflorum*, *Xenia* sp., and *Asterospicularia randalli*, species for which only a single specimen was available. Virtually no intraspecific variation was found in any of the gene regions. The 2 individuals of *Sinularia gaweli* differed by a single nucleotide substitution at the 3′ end of the *16S rDNA* fragment (see Figure 2), and the 2 *Alcyonium glomeratum* differed by a single silent nucleotide substitution in the *ND6* coding region.

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                                    800
SAGL    GGAAAACAAA AGGCTTAGGG ATTAA-TAAG GTGCC----- ---------- ----------
ALDI    ·········· G··T····· ·········· ··T··-ACGAA AGTG····AC CTTCTCATAT
ALspA   ·········· G··T····· ·········· ··T··-ACGAA AGTG····AC CTTCTCATAT
ALCO    ·········· G··T····· ·········· ····-ACTTT TGTG··ACAC CTTCTCATAT
ALGL    ·········· G··T····· ·········· ····-ACTTT CGTG··GCAC CTTCTCATAT
ALNZ2   ·········· G··T····· ·········· ·C···ACTTT TGTG··GCAC CTTCTCATAT
ALVA    ·········· G··T····· ·········· ····-ACTTT CGTG··GCAC CTTCTCATAT
ALRU    ·········· G··T····· ·········· ·····ACGAA AGTGGTGCAC CTTCTCATAT
ALspB   ·········· ···T····· ·····C··· ·····ACGAA AGTGGCGCAC CTTCTCATAT
KLSI    ·········· ···T·CG··· ·········· ·····AC··· ·····CGCAC CTTCTCATAT
CLSP    ·········· ···T··G··· ·········· ·····ACAAC AGTGGCGCAC CTTCTCATAT
SIGA    ·········· ···T····· ·········· ·····ACGAA AGTGGCGTAT CTTCTCATAT
SATR    ·········· ···T····· ·········· ·········· ·········· ··········
LOPA    ···G····· ···T····· ·····G·C·· ···T····· ·········· ··········
XESP    ·········· ···T····· ·····C··· ·····ACGAA AGTGGCGTGC CTTTTCATAT
ASRA    ·········· ···T····· ·····C··· ·····ACGAA AGTGGCGTGC CTTTTCATAT

           811                          838
SAGL    ---ATGTGGG TGCATAGCCC CTGGCATACT ATGGAATTAA CACTAGGGCT CATCACACTA
ALDI    ACC··· ·········· ····T····· ·········· ·········· ·····T····
ALspA   ACC··· ·········· ····T····· ·········· ·········· ·····T····
ALCO    ACC··· ·········· ····T····· ·········· ·········· ·····T····
ALGL    ACC··· ·········· ····T····· ·········· ·········· ·····T····
ALNZ2   ACC··· ·········· ····T····· ·····G··· ·········· ·····T····
ALVA    ACC··· ·········· ····T····· ·········· ·········· ·····T····
ALRU    GCC··· ·········· ····T····· ·········· ·········· ·····T····
ALspB   GCC··· ·········· ····T····· ·········· ·········· ·····T····
KLSI    GCC·G····· C··T····· ····TC··C· ·····G··· ·········· ·····T····
CLSP    ACC·G····- C··T····G· ····T····· ·····G··· ·········· ·····T····
SIGA    GCC··· ·········· ·····M·· ·········· ·········· ·····T····
SATR    ·········· ·········· ·····T· ·········· ·········· ·····T····
LOPA    ·········· ·········· ·····T· ·········· ·········· ·····T····
XESP    GCC··· ·········· ····T···T· ·········· ·········· ·····T····
ASRA    GCC··· ·········· ····T···T· ·········· ·········· ·····T····
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Figure 2. Numbers above the sequences indicate nucleotide positions relative to the published *Sarcophyton glaucum* (SAGL) sequence (GenBank AF064823). Beaton et al. (1998) proposed position 811 as the start of the *ND2* coding sequence and placed the 3′ end of the *16S rDNA* between positions 800–820. We suggest instead that the *ND2* coding region starts at position 838 (see text). For species abbreviations see Table 1.

Variation in the *ND6* and *ND3* Coding Regions

Pont-Kingdon et al. (1998) reported the total length of the *S. glaucum ND6* gene to be 558 bp (186 amino acids). Of the 444 bp sequenced here, 377 nucleotide positions were invariant, 67 (15.1%) were variable, and 18 (26.9%) of the substitutions were nonsynonymous. There were 37 nucleotide substitutions and 12 amino acid substitutions that were parsimony-informative. Pairwise distances (uncorrected *p*) among species ranged from 0.5% to 6.6%, with the lowest values occurring among species in the genus *Alcyonium* and between the 2 xeniid species (Table 3). The largest values were found among comparisons between the 2 families. We found no indels in the coding region, but some discrepancy among species in the apparent location of the stop codon. All of the alcyoniid species we sequenced have a TAG in the position equivalent to nucleotides 1954, 1955, and 1956 of the *S. glaucum* sequence (AF063191). The 2 xeniid species, however, have a CAG at this position, but a TAA (the only other stop codon known to be used in the cnidarian mitochondrial code [Beaton et al., 1998]) at positions 1976, 1977, and 1978. The location of this stop codon suggests that the xeniid ND6 subunit is 7 amino acids longer than that of the alcyoniids.

We also obtained sequence for the first 102 nucleotides (34 amino acids) of the *ND3* coding region, a gene that is 354 bp long in *S. glaucum* (Pont-Kingdon et al., 1998). We found 85 invariant nucleotide positions and 17 (16.7%) that were variable, 7 (41.2%) of which had nonsynonymous substitutions. There were 10 nucleotide substitutions and only 3 amino acid substitutions that were parsimony-informative. Pairwise distances (uncorrected *p*) ranged from 0% to 11.8%, with the smallest distances between *Alcyonium* species and the largest between families (Table 3).

Table 3. Pairwise Genetic Distances (uncorrected *p*) Between Species of Alcyoniid and Xeniid Soft Corals

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1 *Alcyonium digitatum*	***	2.8	5.6	3.8	2.9	3.8	2.9	2.9	14.4	13.4	3.8	11.3	13.1	9.9	11.5
	***	0.0	3.2	3.2	3.9	3.2	2.6	3.9	7.6	8.0	4.2	3.9	7.1	5.2	5.2
2 *Alcyonium* sp. A	0.4	***	6.6	4.8	3.9	4.8	3.9	3.85	15.3	16.3	4.8	12.3	13.1	10.9	12.5
	0.5	***	3.2	3.2	3.9	3.2	2.6	3.9	7.6	8.0	4.2	3.9	7.1	5.2	5.2
3 *Alcyonium coralloides*	1.9	2.2	***	3.6	2.7	3.6	2.7	2.7	11.7	11.0	4.5	10.9	12.6	7.8	9.6
	1.1	1.6	***	1.3	1.3	1.3	5.1	6.4	7.5	9.1	7.3	3.1	6.3	8.3	8.3
4 *Alcyonium glomeratum*	2.4	2.8	1.3	***	0.9	1.8	0.9	0.9	13.5	14.6	2.7	10.9	12.6	8.8	10.6
	1.0	1.5	0.6	***	1.3	0.0	4.5	5.7	6.8	8.5	6.7	3.1	6.3	7.6	7.6
5 *Alcyonium* sp. NZ2	2.0	2.4	1.7	2.2	***	0.9	0.0	0.0	13.5	13.7	1.8	9.8	11.6	7.7	9.5
	2.0	2.5	2.0	1.7	***	1.3	5.1	6.4	7.4	9.1	7.3	3.9	7.0	8.2	8.2
6 *Alcyonium variabile*	1.7	2.0	1.3	1.9	1.5	***	0.9	0.9	11.7	14.6	0.9	9.1	10.8	8.8	10.6
	1.8	2.3	1.4	1.0	2.5	***	4.5	5.7	6.8	8.5	6.7	3.1	6.3	7.6	7.6
7 *Alcyonium rudyi*	2.6	3.0	2.6	2.8	2.8	2.4	***	0.0	13.5	13.7	1.8	9.8	11.6	7.7	9.5
	1.1	1.6	1.6	1.2	2.3	2.0	***	2.5	7.2	9.1	4.1	3.1	4.6	5.0	5.0
8 *Alcyonium* sp. B	2.0	2.4	2.0	2.6	2.2	1.9	1.3	***	13.5	13.7	1.8	9.8	11.6	7.7	9.5
	1.1	1.6	1.8	1.7	2.7	2.5	0.9	***	6.6	8.4	4.1	3.9	5.5	3.8	3.8
9 *Klyxum simplex*	4.3	4.6	3.5	4.5	3.9	4.1	3.5	2.6	***	13.6	13.4	18.6	20.4	14.2	15.8
	3.6	4.1	4.1	3.7	4.3	4.5	2.9	3.4	***	3.4	7.5	8.0	11.1	8.5	8.5
10 *Cladiella* sp.	5.2	5.6	4.5	5.4	5.0	5.0	4.5	3.5	2.4	***	15.5	17.0	19.7	13.4	15.3
	3.4	3.8	3.8	3.5	4.1	4.3	2.7	3.2	2.9	***	9.3	7.8	11.1	10.3	10.3
11 *Sinularia gaweli*	3.0	3.3	3.0	2.8	2.8	2.8	3.0	2.8	4.5	5.6	***	9.8	11.6	9.6	11.3
	2.9	3.4	2.9	3.0	3.2	3.8	2.3	2.7	4.3	4.1	***	1.2	4.2	4.1	4.1
12 *Sarc. Trocheliophorum*	4.6	5.0	4.6	4.5	4.5	4.5	4.3	4.1	5.9	6.1	3.5	***	7.1	11.3	12.9
	4.3	4.7	4.3	4.4	5.0	4.7	3.6	4.1	5.2	5.4	2.7	***	3.1	1.6	1.6
13 *Lobophytum pauciflorum*	4.6	4.6	4.3	4.5	4.1	4.1	4.6	4.1	5.9	6.5	3.9	2.4	***	16.1	17.8
	4.1	4.5	4.1	4.2	4.7	4.5	3.4	3.8	5.0	4.7	2.5	1.6	***	4.6	4.6
14 *Xenia* sp.	5.0	5.4	3.9	4.6	4.8	4.8	4.1	4.0	5.8	6.3	4.8	5.9	6.5	***	1.9
	4.3	4.3	5.0	4.9	5.0	5.7	3.8	4.1	6.1	5.9	5.4	5.9	6.1	***	0.0
15 *Asterospicularia randalli*	5.2	5.6	4.1	4.8	5.0	5.0	4.3	4.1	5.9	6.9	5.4	6.1	6.7	0.6	***
	4.8	4.8	5.4	5.3	5.4	6.1	4.3	4.5	6.6	6.3	5.9	6.3	6.5	0.5	***
	5.9	6.9	4.9	3.9	4.9	3.9	4.9	5.9	4.9	6.9	11.8	9.8	8.8	1.0	***

The *ND6* and *ND3* coding regions were separated by an IGS region that ranged from 35 to 55 bp in length. Relative to *S. glaucum*, 6 species had a 12-bp insertion at the 3′ end of this IGS; species with this insertion included both xeniids, *Sinularia gaweli*, and 3 of 7 species of *Alcyonium*. In addition, *Cladiella* sp. had a deletion of 8 bp near the 5′ end of the region. When gaps were coded as fifth characters, 32 (58.2%) of the 55 positions in this IGS were variable.

Variation in the *ND2* Coding Region

Beaton et al. (1998) identified position 811 in the *S. glaucum* mitochondrial genome (AF064823) as the start of the *ND2* coding sequence. Our sequence alignments suggest instead that the *ND2* coding region is initiated at position 838. Sequences for *Klyxum simplex* and *Cladiella* sp. are missing the start codon at position 811, whereas all 15 species have an in-frame ATG at position 838 (Figure 2). Alternatively, *K. simplex* and *Cladiella* sp. may simply have an *ND2* subunit that is 9 amino acids shorter than that of the other species. Placement of the initiation codon for *ND2* at position 838 of the *S. glaucum* sequence suggests that this coding region is 1347 bp (448 amino acids) in length, rather than the 1374 bp (457 amino acids) estimated by Beaton et al. (1998). Of the 539 nucleotides we sequenced, 457 were invariant, 82

(15.2%) were variable, and 17 (20.7%) of the substitutions were nonsynonymous. There were 52 nucleotide substitutions and 5 amino acid substitutions that were parsimony-informative. Pairwise distances (uncorrected *p*) ranged from 0.4% to 6.9%, with the smallest distances among *Alcyonium* species and the largest between members of the two families (see Table 3).

Beaton et al. (1998) were unable to identify the 3′ terminus of the *16S rDNA* coding region, but suggested that it was located between positions 800 and 820 of the *S. glaucum* genome (AF064823). We found little variation in the first 95 nucleotide positions we sequenced in this region (8 variable positions [8.4%], 4 of them parsimony-informative). Starting at position 800 of the *S. glaucum* genome, however, variability increased greatly, in particular the occurrence of indels (Figure 2). Both *Sarcophyton* species and *Lobophytum pauciflorum* share a 28-bp deletion in this region, and 8 other species have indels ranging from 2 to 8 bp. *Cladiella* sp. has a 4-bp deletion immediately downstream of the position inferred by Beaton et al. (1998) to be the start codon for the *ND2* gene. We speculate that the region between approximately positions 800 and 838 of the *S. glaucum* genome may be a noncoding intergenic region separating the *16S rDNA* and *ND2* coding regions. Homology throughout this region was low, and sequences could not be aligned with certainty. When gaps were coded as fifth characters, 47 (72.3%) of the positions in the 65-bp alignment were variable and 36 (55.4%) were parsimony-informative. Pairwise distances among species ranged from 0% to 24%, with the largest distances separating *K. simplex* and *Cladiella* sp. from *L. pauciflorum* and *S. trocheliophorum*, the 2 species with the large deletion (Table 3).

Variation in the *COI-COII* IGS Region

The 111-bp sequence separating the *COI* and *COII* coding regions is the largest noncoding region found in the *S. glaucum* mitochondrial genome, and was inferred by Beaton et al. (1998) to be the control region. This region varied from 106 to 122 bp among the species we sequenced, although most of this length variation was accounted for by a 10-bp insertion unique to *K. simplex*. Coding gaps as fifth characters, 53 (43.4%) of 122 positions were variable and 27 (22.1%) were parsimony-informative. Pairwise distances (uncorrected *p*) ranged from 0% to 20.4%, with the largest distances separating *K. simplex* and *Cladiella* sp. from *S. trocheliophorum* and *L. pauciflorum* (Table 3).

Phylogenetic Inference

Phylogenetic trees constructed separately for the *ND2* and *ND6* coding regions and the *COI-COII* IGS all had low resolution and produced a polytomy of alcyoniid genera (not shown). The only species that were united with moderately high bootstrap support in all of these analyses were (1) *Alcyonium digitatum* with *Alcyonium* sp. A; (2) *K. simplex* with *Cladiella* sp.; (3) *S. trocheliophorum* with *L. pauciflorum*; and (4) all alcyoniid taxa. The position of the genus *Sinularia* as a sister taxon to the *Sarcophyton-Lobophytum* clade was well supported by *ND2* and *ND6* but not by the *COI-COII* IGS. Combined analysis of nucleotide sequences from all 3 protein-coding regions (*ND2*, *ND6*, and *ND3*, 1085 bp total) produced trees with better resolution and higher bootstrap support than any of the analyses of individual regions (Figure 3). Both maximum parsimony and maximum likelihood analyses united a majority of the *Alcyonium* species with relatively high bootstrap support, but the basal relationships among alcyoniid genera remained largely unresolved (Figure 3). Combined analysis of ND2, ND6, and ND3 amino acid sequences (360 amino acids) produced a tree that was poorly resolved and supported only those 4 nodes listed above (tree not shown).

Discussion

Variation in Protein-Coding Regions

The relatively low levels of variability in the *ND2*, *ND3*, and *ND6* regions examined here suggest that these mitochondrial protein-coding genes will provide only limited phylogenetic resolution below the level of octocoral families. Although sister relationships among several species and genera were strongly supported by the data, the base of the alcyoniid family tree formed an unresolved polytomy. The distinction of the families Xeniidae and Alcyoniidae, however, was well supported by analyses of both *ND2* and *ND6* coding regions, separately and combined, suggesting that these genes may prove useful for resolving relationships among families of octocorals. Sánchez et al. (2003) used *ND2* and *ND6* sequences in a combined analysis with the more variable *msh1* gene to resolve relationships of genera within the octocoral families Plexauridae and Gorgoniidae. Although this combined analysis produced a

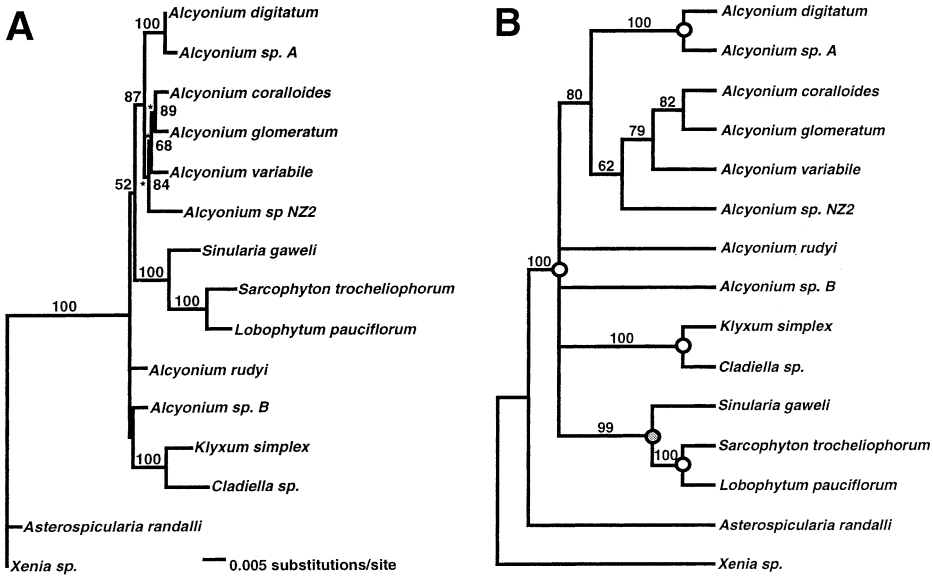

Figure 3. Phylogenetic trees generated by combined analysis of nucleotide sequences for the *ND2*, *ND6*, and *ND3* protein-coding regions (1085 bp) of alcyoniid soft corals. **A**: Maximum likelihood tree (−lnL = 2775.3) based on best-fit model of evolution estimated by Modeltest (HKY+G, substitution model: $Ti/Tv = 4.4909$; γ distribution shape parameter = 0.2659). Bootstrap values (percentage 100 replicates) are shown for nodes with more than 50% support. **B**: Maximum parsimony tree. Strict consensus of 7 equally most-parsimonious trees, with bootstrap values shown for nodes with more than 50% support (1000 replicates). White circles indicate nodes that were also well supported (bootstrap values >70%) by individual analyses of the *ND2* (539-bp), *ND6* (444-bp), and *COI-COII* IGS (122-bp) regions. Shaded circle indicates node supported by *ND2* and *ND6* but not by the *COI-COII* IGS.

strongly supported phylogeny of genera, the relationships among species within most genera still could not be resolved using these mitochondrial genes.

All 3 of the protein-coding regions we examined (*ND2*, *ND6*, and *ND3*) exhibited similar levels of variation (15%–17% of nucleotides variable, 8%–10% parsimony-informative), although in most cases pairwise distances among species were slightly greater for the *ND3* fragment than for either *ND2* or *ND6* (Table 3). France and Hoover (2001) have previously reported on the variation in approximately 228 bp (68 amino acids) of the 3′ end of the *ND3* gene. In a comparison of octocoral families, they found that 19.3% of nucleotide positions were variable (10.5% of them parsimony-informative) and pairwise distances among families ranged from 0.4% to 12.5%. They found very similar values for the complete *ND4L* subunit (294 bp). Because their analysis was conducted at the level of families rather than genera, and they examined only a single genus within each family, it is difficult to compare their data directly with ours. The average divergence between the 2 xeniid species and the alcyoniids in our study was approximately 5% to 6% for all 3 protein-coding regions, near the low end of the range of interfamilial distances reported by France and Hoover. However, we chose the Xeniidae as an outgroup specifically because of their apparently close relationship to the Alcyoniidae, so a low interfamilial distance between these 2 families is not necessarily indicative of lower variability in the *ND2* and *ND6* genes relative to *ND3* and *ND4L*.

We found somewhat greater differentiation among congeneric species in our study than was reported by France and Hoover (2001) for *ND3* and *ND4L*. Distances among *Alcyonium* species ranged from 0% to 2.9% for *ND2* and *ND6* and up to 3.9% for the *ND3* 5′ fragment. In contrast, all of France and Hoover's intrageneric distances were less than 1.0%, with the exception of one species of *Corallium* that differed from congeners by up to 1.8%. This greater apparent intrageneric variability in the *ND2* and *ND6* genes may simply be a reflection of the genus we chose to use for interspecific comparisons. *Alcyonium* is a morphologically diverse genus that is greatly in need of revision (Alderslade, 2000; Williams, 2000). Three of the species included here, *Alcyonium rudyi*, *A. variable*, and *Alcyonium* sp. B, belong to other genera that are currently being revised or established (McFadden and Hochberg, 2003; G.C. Williams, personal communication). In addition, a previous molecular phylogenetic analysis based on the highly variable nuclear ribosomal internal transcribed spacer (*ITS*) regions suggested that species such as *A.*

coralloides and *A. glomeratum* belong to clades that are genetically very distinct from the type species, *A. digitatum* (McFadden et al., 2001). It is therefore not surprising that interspecific divergence values in this genus are high relative to those among species of better-defined genera such as *Corallium*.

The differences between our study and that of France and Hoover (2001) in taxonomic scope and the specific taxa examined make it difficult to accurately assess differences in variability among the various NADH dehydrogenase subunits. Nonetheless, the ranges of intergeneric and interfamilial variation documented here for *ND2* and *ND6* appear to be fairly comparable to the values reported for *ND3* and *ND4L*. Further preliminary results of ours support France and Hoover's (2001) conclusion that the NADH dehydrogenase subunits exhibit greater variability than *COI*, but are less variable than the *msh1* gene (C.S. McFadden, unpublished data).

Variation in Non-coding Regions

As expected, we found greater variation in the non-protein-coding regions we sequenced (*COI-COII* IGS, 3′ end of 16S *rDNA*, and *ND6-ND3* IGS) than in any of the protein-coding genes. Unfortunately, the short length of the highly variable *16S rDNA* 3′ end and the *ND6-ND3* IGS, combined with large indels (and consequent alignment difficulties) in these regions, limits their phylogenetic utility. Although some of the indels in these regions appear to be phylogenetically informative (e.g., see Figure 2), those in the *ND6-ND3* IGS suggest species affinities that are not supported by data from the protein-coding regions. The *COI-COII* IGS is the longest of the noncoding intergenic spacers in the octocoral mitochondrial genome, but phylogenetic trees constructed using this region alone were much less resolved than those constructed using the *ND2* or *ND6* coding regions. The low phylogenetic resolution provided by this noncoding region is probably due to its length and the smaller absolute number of parsimony-informative characters it contains. Despite their apparent limitations for phylogenetic inference, variations in these 3 noncoding regions might, however, provide useful diagnostic markers for detection of species boundaries. For instance, several substitutions in the *COI-COII* IGS distinguish *A. digitatum* and *Alcyonium* sp. A, sister taxa that are virtually indistinguishable on the basis of allozyme frequencies and *ITS* sequences (McFadden et al., 2001).

Conclusions

In conclusion, no region of the octocoral mitochondrial genome is likely to contain sufficient variation to be useful for studies of species-level phylogenetics or intraspecific phylogeography. Although the NADH dehydrogenase subunits are more variable than either the *16S rDNA* or *COI* coding regions, they do not appear to be variable enough to provide much phylogenetic resolution below the level of families or well-separated genera. Noncoding regions of the genome are more variable than protein-coding regions, but are too short to offer sufficient characters for phylogenetic reconstruction. We suggest that the best approach to genus-level systematics in the Octocorallia will be combined analyses of several of these NADH dehydrogenase subunits, along with *msh1*, which appears to be somewhat more variable (France and Hoover, 2001; Sánchez et al., 2003). Unfortunately, the application of molecular approaches to the study of phylogenetic relationships among species and populations of octocorals must await the identification of more rapidly evolving nuclear DNA regions, perhaps intron sequences (e.g., Hatta et al., 1999; van Oppen et al., 2000, 2001; Márquez et al., 2002). Alternatively, amplified fragment length polymorphism (AFLP) markers have recently been used to examine the intrageneric relationships of several, diverse groups of organisms (e.g., Kardolus et al., 1998; Giannasi et al., 2001; Buntjer et al., 2002). Although thus far AFLPs have only been used to study species boundaries and intraspecific genetic variation in anthozoans (Lopez et al., 1999; Barki et al., 2000), the potential use of these markers for phylogenetic reconstruction in this group should be explored.

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References

Alderslade, P. (2000). Four new genera of soft corals (Coelenterata: Octocorallia), with notes on the classification of some established taxa. *Zool Med Leiden* 74:237–249.

Avise, J.C. (1994). Molecular Markers, Natural History and Evolution. New York, N.Y.: Chapman & Hall.

Barki, Y., Douek, J., Graur, D., Gateño, D., and Rinkevich, B. (2000). Polymorphism in soft coral larvae revealed by amplified fragment-length polymorphism (AFLP) markers. *Mar Biol* 136:37–41.

Beagley, C.T., Okimoto, R., and Wolstenholme, D.R. (1998). The mitochondrial genome of the sea anemone *Metridium senile* (Cnidaria): introns, a paucity of tRNA genes, and a near-standard genetic code. *Genetics* 148:1091–1108.

Beaton, M.J., Roger, A.J., and Cavalier-Smith, T. (1998). Sequence analysis of the mitochondrial genome of *Sarcophyton glaucum:* conserved gene order among octocorals. *J Mol Evol* 47:697–708.

Berntson, E.A., Bayer, F.M., McArthur, A.G., and France, S.C. (2001). Phylogenetic relationships within the octocorallia (Cnidaria: Anthozoa) based on nuclear 18S rRNA sequences. *Mar Biol* 138:235–246.

Best, B.A., and Thomas, W.K. (1993). Anthozoan mt DNA: a fundamentally different rate of evolution. *Am Zool* 43:99A.

Buntjer, J.B., Otsen, M., Nijman, I.J., Kuiper, M.T.R., and Lenstra, J.A. (2002). Phylogeny of bovine species based on AFLP fingerprinting. *Heredity* 88:46–51.

Chen, A.C., and Miller, D.J. (1996). Analysis of ribosomal ITS1 sequences indicates a deep divergence between *Rhodactis* (Cnidaria: Anthozoa: Corallimorpharia) species from the Caribbean and the Indo-Pacific/Red Sea. *Mar Biol* 126:423–432.

Chen, A.C., and Yu, J.-K. (2000). Universal primers for amplification of mitochondrial small subunit ribosomal RNA-encoding gene in scleractinian corals. *Mar Biotechnol* 2:146–153.

Chen, A.C., Odorico, D.M., ten Lohuis, M.J., Veron, J.E.N., and Miller, D.J. (1995). Systematic relationships within the Anthozoa (Cnidaria: Anthozoa) using the 5′-end of the 28S rDNA. *Mol Phylogenet Evol* 4:175–183.

Chen, A.C., Willis, B.L., and Miller, D.J. (1996). Systematic relationships between tropical corallimorpharians (Cnidaria: Anthozoa: Corallimorpharia): utility of the 5.8S and internal transcribed spacer (ITS) regions of the rRNA transcription unit. *Bull Mar Sci* 59:196–208.

Coffroth, M.A., Lasker, H.R., Diamond, M.E., Bruenn, J.A., and Bermingham, E. (1992). DNA fingerprints of a gorgonian coral: a method for detecting clonal structure in a vegetative species. *Mar Biol* 114:317–325.

Culligan, K.M., Meyer-Gauen, G., Lyons-Weiler, J., and Hays, J.B. (2000). Evolutionary origin, diversification and specialization of eukaryotic MutS homolog mismatch repair proteins. *Nucleic Acids Res* 28:463–471.

France, S.C., and Hoover, L.L. (2001). Analysis of variation in mitochondrial DNA sequences (ND3, ND4L, MSH) among Octocorallia (=Alcyonaria) (Cnidaria: Anthozoa). *Bull Biol Soc Wash* 10:110–118.

France, S.C., and Hoover, L.L. (2002). DNA sequences of the mitochondrial *COI* gene have low levels of divergence among deep-sea octocorals (Cnidaria: Anthozoa). *Hydrobiologia* 471:149–155.

France, S.C., Rosel, P.E., Agenbroad, J.E., Mullineaux, L.S., and Kocher, T.D. (1996). DNA sequence variation of mitochondrial large-subunit rRNA provides support for a two-subclass organization of the Anthozoa (Cnidaria). *Mol Mar Biol Biotechnol* 5:15–28.

Fukami, H., Omori, M., and Hatta, M. (2000). Phylogenetic relationships in the coral family Acroporidae reassessed by inference from mitochondrial genes. *Zool Sci* 17:689–696.

Geller, J.B., and Walton, E.D. (2001). Breaking up and getting together: evolution of symbiosis and cloning by fission in sea anemones (genus *Anthopleura*). *Evolution* 55:1781–1794.

Giannasi, N., Thorpe, R.S., and Malhotra, A. (2001). The use of amplified fragment length polymorphism in determining species trees at fine taxonomic levels: analysis of a medically important snake, *Trimeresurus albolabris*. *Mol Ecol* 10:419–426.

Hatta, M., Fukami, H., Wang, W., Omori, M., Shimoike, K., Hayashibara, T., Ina, Y., and Sugiyama, T. (1999). Reproductive and genetic evidence for a reticulate evolutionary history of mass-spawning corals. *Mol Biol Evol* 16:1607–1613.

Kardolus, J.P., van Eck, H.J., and van den Berg, R.G. (1998). The potential of AFLPs in biosystematics: a first application in *Solanum* taxonomy (Solanaceae). *Plant Syst Evol* 210:87–103.

Lopez, J.V., Kersanach, R., Rehner, S.A., and Knowlton, N. (1999). Molecular determination of species boundaries in corals: genetic analysis of the *Montastraea annularis* complex using amplified fragment length polymorphisms and microsatellite markers. *Biol Bull* 196:80–93.

Márquez, L.M., van Oppen, M.J.H., Willis, B.L., Reyes, A., and Miller, D.J. (2002). The highly cross-fertile coral species, *Acropora hyacinthus* and *Acropora cythera*, constitute statistically distinguishable lineages. *Mol Ecol* 11:1339–1349.

McFadden, C.S., and Hochberg, F.G. (2003). Biology and taxonomy of encrusting alcyoniid soft corals in the Northeastern Pacific Ocean with descriptions of two new genera (Coelenterata: Anthozoa: Octocorallia). *Invert Biol* 122:93–113.

McFadden, C.S., Donahue, R., Hadland, B.K., and Weston, R. (2001). A molecular phylogenetic analysis of reproductive trait evolution in the soft coral genus *Alcyonium*. *Evolution* 55:54–67.

Medina, M., Weil, E., and Szmant, A.M. (1999). Examination of the *Montastraea annularis* species complex (Cnidaria: Scleractinia) using ITS and COI sequences. *Mar Biotechnol* 1:89–97.

Pont-Kingdon, G., Okada, N.A., Macfarlane, J.L., Beagley, C.T., Wolstenholme, D.R., Cavalier-Smith, T., and Clark-Walker, G.D. (1995). A coral mitochondrial *mutS* gene. *Nature* 375:109–111.

Pont-Kingdon, G., Okada, N.A., Macfarlane, J.L., Beagley, C.T., Watkins-Sims, C.D., Cavalier-Smith, T., Clark-Walker, G.D., and Wolstenholme, D.R. (1998). Mitochondrial DNA of the coral *Sarcophyton glaucum* contains a gene for a homologue of bacterial mutS: a possible case of gene transfer from the nucleus to the mitochondrion. *J Mol Evol* 46:419–431.

Posada, D., and Crandall, K.A. (1998). Modeltest: testing the model of DNA substitution. *Bioinformatics* 14:817–818.

Romano, S.L., and Palumbi, S.R. (1996). Evolution of scleractinian corals inferred from molecular systematics. *Science* 271:640–642.

Romano, S.L., and Palumbi, S.R. (1997). Molecular evolution of a portion of the mitochondrial *16S* ribosomal gene region in scleractinian corals. *J Mol Evol* 45:397–411.

Sánchez, J.A., McFadden, C.S., France, S.C., and Lasker, H.R. (2003). Molecular phylogenetic analyses of shallow-water Caribbean octocorals. *Mar Biol* 142:975–987.

Swofford, D.L. (2002). *PAUP*: Phylogenetic Analysis Using Parsimony* (**and Other Methods*), *Version 4*.. Sunderland, Mass: Sinauer Associates.

Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22:4673–4680.

van Oppen, M.J.H., McDonald, B.J., Willis, B.L., and Miller, D.J. (2001). The evolutionary history of the coral genus *Acropora* (Scleractinia, Cnidaria) based on a mitochondrial and a nuclear marker: reticulation, incomplete lineage sorting, or morphological convergence?. *Mol Biol Evol* 18:1315–1329.

van Oppen, M.J.H., Willis, B.L., and Miller, D.J. (1999). Atypically low rate of cytochrome *b* evolution in the scleractinian coral genus *Acropora*. *Proc R Soc Lond B* 266:179–183.

van Oppen, M.J.H., Willis, B.L., Van Vugt, H.W.J.A., and Miller, D.J. (2000). Examination of species boundaries in the *Acropora cervicornis* group (Scleractinia, Cnidaria) using nuclear DNA sequence analyses. *Mol Ecol* 9:1363–1373.

van Oppen, M.J.H., Willis, B.L., Van Reede, T., and Miller, D.J. (2002). Spawning times, reproductive compatibilities and genetic structuring in the *Acropora aspera* group: evidence for natural hybridization and semi-permeable species boundaries in corals. *Mol Ecol* 11:1363–1376.

Williams, G.C. (2000). Two new genera of soft corals (Anthozoa: Alcyoniidae) from South Africa, with a discussion of diversity and endemism in the Southern African octocorallian fauna. *Proc Cal Acad Sci* 52:65–75.

Won, J.H., Rho, B.J., and Song, J.I. (2001). A phylogenetic study of the Anthozoa (phylum Cnidaria) based on morphological and molecular characters. *Coral Reefs* 20:39–50.