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Molecular Evolution of a Portion of the Mitochondrial *16S* Ribosomal Gene Region in Scleractinian Corals

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Abstract. Relationships among families and suborders of scleractinian corals are poorly understood because of difficulties 1) in making inferences about the evolution of the morphological characters used in coral taxonomy and 2) in interpreting their 240-million-year fossil record. Here we describe patterns of molecular evolution in a segment of the mitochondrial (mt) *16S* ribosomal gene from taxa of 14 families of corals and the use of this gene segment in a phylogenetic analysis of relationships within the order. We show that sequences obtained from scleractinians are homologous to other metazoan 16S ribosomal sequences and fall into two distinct clades defined by size of the amplified gene product. Comparisons of sequences from the two clades demonstrate that both sets of sequences are evolving under similar evolutionary constraints: they do not differ in nucleotide composition, numbers of transition and transversion substitutions, spatial patterns of substitutions, or in rates of divergence. The characteristics and patterns observed in these sequences as well as the secondary structures, are similar to those observed in mt *16S* ribosomal DNA sequences from other taxa. Phylogenetic analysis of these sequences shows that they are useful for evaluating relationships within the order. The hypothesis generated from this analysis differs from traditional hypotheses for evolutionary relationships among the Scleractinia and suggests that a reevaluation of evolutionary affinities in the order is needed.

Key words: Scleractinia — Mitochondrial *16S* ribosomal RNA — Secondary structure models — Phylogenetic analysis

Introduction

The phylum Cnidaria is a group of simple, multicellular organisms that diverged from near the base of the metazoan tree. In addition to morphological differences that have long been used to show an ancient origin of Cnidaria, molecular research on cnidarians within the past 10 years has shown that they have characteristics not observed in more derived phyla. Work by Warrior and Gall (1985) and Bridge et al. (1992) has shown that members of the classes Hydrozoa and Scyphozoa have linear mitochondrial DNA (mtDNA) as opposed to the circular mtDNA found in members of the class Anthozoa and most other invertebrates. France et al. (1996) have shown that a specific segment of the mitochondrial 16S ribosomal gene in anthozoans is larger than seen in most animals as well as being highly variable in size among anthozoan orders and subclasses. Wolstenholme (1992) has shown that the mitochondrial genome of the anthozoan *Metridium senile* has only two tRNAs in contrast to the 22 found normally and has two introns, a phenomenon not observed in the animal mitochondrial genome. The mitochondrial genome of another anthozoan, *Sarcophyton glaucum,* has been determined to contain a homologue of MutS, a component of the bacterial MutSLH mismatch repair pathway (Pont-Kingdon et al. 1995). These authors suggest that a mismatch repair mecha-

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nism, unknown to date in any other animals, exists in the mitochondrial genome of this anthozoan. These analyses have provided a new perspective on molecular evolution in relatively primitive animals.

In addition to expanding our understanding of molecular evolution in metazoans, molecular techniques have proved to be powerful tools for the study of systematics and, can help address evolutionary questions where other methods have failed. Recently, molecular systematics has been used to study higher level relationships among the Cnidaria (Christen et al. 1991; Hori and Satow 1991; Wainright et al. 1993; Bridge et al. 1992; 1995; Chen et al. 1995; France et al. 1996). These systematic studies have been valuable contributions to resolving long-standing controversies about relationships within the Cnidaria.

The Scleractinia (hard corals) comprise an anthozoan order of polyp animals that secrete a calcium carbonate skeleton. They are primary contributors to the construction of coral reefs in the tropics and are found throughout the world's oceans. The order has a fossil record from the mid-Triassic, 240 mya. Relationships among families and suborders of scleractinians are poorly understood because of difficulties 1) in making inferences about the evolution of the highly variable morphological characters used in coral taxonomy and 2) in interpreting their 240-million-year fossil record. The use of molecular techniques for the study of scleractinians has only recently become feasible for two main reasons. First, rapid methods for amplification of the large amounts of pure DNA necessary for sequencing require only small amounts of animal tissue via the Polymerase Chain Reaction (PCR). This is especially important for corals because the animal is only a thin veneer over the skeleton making it difficult to obtain large quantities of animal tissue. Second, many corals live in association with intracellular dinoflagellate symbionts (zooxanthellae). Thus, separation of coral and algal genomes is crucial. The specificity of primer-annealing in PCR makes it possible to amplify only animal DNA from intact associations of corals and their endosymbionts although careful verification of sequences obtained in this manner is necessary.

Although McMillan and coworkers (1991) have used nuclear DNA sequences to study relationships among closely related species in the family Acroporidae, little other molecular systematic work has addressed relationships within the reef-building corals. Here we describe a molecular phylogenetic approach to studying the evolution of relationships within the Scleractinia. We have refined molecular techniques for the PCR amplification of a segment of the mitochondrial large ribosomal RNA gene of corals. The seven extant suborders of corals are thought to have diverged from each other 240 mya based on fossil evidence (Veron 1995) and this gene region has been found useful in studies of relationships ranging from within species to among kingdoms (e.g., Cedergren et al. 1988; DeSalle et al. 1987; Miyamoto et al. 1989). We have analyzed patterns of molecular evolution in the sequences from these amplified gene segments and used these sequences in phylogenetic analyses of family and suborder level relationships among the Scleractinia. This analysis suggests an entirely new perspective on the evolution of the group and shows the utility of a molecular phylogenetic approach for systematic study of scleractinians.

Methods

DNA Extractions

Total genomic DNA was extracted from living, frozen, or ethanol-preserved coral colonies. For some living and frozen colonies, tissue was scraped from approximately 4 cm^2 of skeleton, or mesenteries from 1 to 10 polyps were removed. When tissue removal was not possible by this method, pieces of living and frozen colonies were placed into a solution of 100 mM EDTA and 20 mM Tris (pH 7.5) with a drop of dimethylpolysiloxane antifoam ("Foam Fighter," Crescent Research Chemicals, Phoenix, AZ) to decrease the formation of mucus. The coral fragments were incubated at 4°C and periodically agitated for 2–3 h. The slurry of cells obtained from this procedure was centrifuged at 1500 *g* for 10 min. The resulting pellet or tissue scraped from the skeleton was suspended in 1 ml of 100 mM EDTA, 1% SDS, 10 mM Tris, pH 7.5 to which 1 μg of proteinase K was added. The solution was incubated at 65°C overnight, with periodic agitation. After incubation, DNA was phenol extracted from the supernatant and subsequently precipitated with ethanol (Palumbi et al. 1991; Romano 1995).

Extractions highly enriched in mtDNA were carried out separately using a modified protocol (Beckman et al. 1993, M.F. Smith personal communication 1993) for the ProMega Wizard Minipreps system. For these extractions, approximately 1 cm^2 of tissue plus skeleton or the mesenteries from 1 to 5 polyps was used.

Polymerase Chain Reaction

A 1:100 dilution of genomic DNA extractions or undiluted mtDNA-enriched extractions were used as template for PCR. PCR products and sequences were initially obtained from the azooxanthellate coral *Tubastraea coccinea* in order to develop mtDNA primers that were specific for coral DNA (Table 1) and that could eventually be used with samples from zooxanthellate corals. Target segments of mtDNA from *T. coccinea* were obtained using primers 16Sg-5′ and 16S1–3′ (Table 1) designed for the 3′ end of the mtDNA *16S* ribosomal gene region of the azooxanthellate hydroid *Hydra vulgaris*. Reactions were carried out in 100 μl, with 0.5 μM of each primer, 0.8 μm dNTPs, 1% DMSO in PCR buffer (Palumbi et al. 1991), 1 unit of *Taq* polymerase and 1-μl template. PCR conditions used were 10 cycles of 30 s at 94°C, 30 s at 45°C, and 45 s at 72°C, followed by 30 cycles of 30 s at 94°C, 30 s at 50°C, and 1 min at 72°C. The PCR product from these reactions (a band of approximately 600 base pairs) was gel purified (Palumbi et al. 1991; Romano 1995) and used in a subsequent PCR reaction. This second PCR product was purified and concentrated, used as template in double-strand sequencing reactions, and electrophoresed on an 8% acrylamide gel with a gradient buffer system (Palumbi et al. 1991).

The *T. coccinea* sequence was aligned with sequences from *Hydra*

Table 1. Primers used in amplifying and sequencing mitochondrial ribosomal *16S* sequences from scleractinian corals

Primer name[a]	Primer sequence	Notes
16Sg-5′	5′-TCGACTGTTTACCAAAAACATAGC-3′	Primer 1 in Cunningham and Buss 1993; this primer is the equivalent of primer 16Sar-5′ (Palumbi 1996) with an additional three nucleotides on the 3′ end
16Sl-3′	5′-TTTAAAGGTCGAACAGACC-3′	Cunningham personal communication 1990; the position of this primer is five bases 5′ relative to 16Sbr-3′ (Palumbi 1996)
16Sd-5′	5′-GGTGAGACCTGCCCAATGGTT-3′	Primer specific for coral tissue designed from sequence from the azooxanthellate coral *T. coccinea*
16Sc-3′	5′-AACAGCGCAATAACGTTTGAGAG-3′	Primer specific for coral tissue designed from sequence from the azooxanthellate coral *T. coccinea*
T3-long-L	5′-ATTAACCCTCACTAAAGGGAAC-3′	Vector primer
M13-H	5′-CATTTTGCTGCCGGTCA-3′	Vector primer
M13-reverse	5′-GAATTCAACAGCTATGACCATG-3′	Vector primer for sequencing
16Se-3′	5′-CGCCTTTAAAAAAGTAAC-3′	Designed specifically for sequencing
16Sf-5′	5′-CTACATCCAAATTGTTAGAC-3′	Designed specifically for sequencing
16Si5-5′	5′-AATAGTTTTTAAATGTGGTG-3′	Designed specifically for amplification of long sequences

[a] 5′ designates a primer that anneals at the 5′ end of the sequence; 3′ designates a primer that anneals at the 3′ end of the sequence; L designates a primer on the light strand; H designates a primer on the heavy strand

(Cunningham and Buss 1993), the frog *Xenopus* (Roe et al. 1985), and a sea urchin, *Strongylocentrotus purpuratus* (Jacobs et al. 1988) to design primers [16Sd-5′ and 16Sc-3′ (Table 1)] specific for coral tissue. These coral specific primers were used in subsequent PCR reactions with all other coral samples. Reactions were carried out as above with PCR conditions of 40 cycles of 30 s at 94°C, 30 s at 50°C, and 1 min at 72°C.

Amplifications of ITS from both genomic and mtDNA-enriched samples of *T. coccinea* were carried out using primers provided by C. Hunter (Dept. of Botany, University of Hawaii). Conditions for these amplifications were the same as described above.

PCR assays were run to determine if β sequences (see below) were a result of PCR artifacts. To test the hypothesis that both long and short sequences are present in all samples, 29 samples were amplified with the long-sequence-specific primer pairs 16Sf-5′ and 16Se-3′, and 16Si5-5′ and 16Se-3′ (Table 1). PCR amplification conditions were as described above. In addition, PCR amplifications were carried out with mixes of long- and short-sequence templates to determine the sensitivity of long-sequence amplification in the presence of short sequences. Four different combinations of long- and short-sequence templates were mixed in ratios of 9:1, 4:1, 3:2, 2:3, and 1:4.

Cloning

Due to problems encountered in consistently obtaining good DNA template for sequencing, double-strand PCR products were ligated into pBluescript II KS-(Stratagene; using the modified protocol of Marchuk et al. 1991; Palumbi and Baker 1994). The ligation product was used to transform competent *Escherichia coli* cells (Stratagene Bacterial Strain XL1-Blue MRF′; Romano 1995). The transformed cells were grown overnight. Colonies containing target segment inserts were identified by PCR assay of white colonies (Palumbi and Baker 1994). PCR assays were carried out (Romano 1995) using vector primers T3-long-L and M13-H (Table 1). Cells from white colonies were added directly to the PCR cocktail (prepared as described above). PCR conditions were 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. Single-stranded DNA from colonies containing the target segment was obtained with helper phage, then precipitated, and resuspended in water. This template was used in subsequent sequencing reactions.

Sequencing

All sequencing reactions were carried out using the Sanger dideoxy chain termination method with USB dITP labelling mix, Sequenase Version 2.0 T7 DNA Polymerase, and ATP-^{35}S. The manufacturers' protocol was used with slight modifications as described in Romano (1995). Primers used for sequencing included vector primer M13-reverse, 16Sg-5′, 16Sc-3′, 16Sd-5′, and primers designed specifically for sequencing, 16Se-3′, and 16Sf-5′ (Table 1).

For phylogenetic analyses, sequences were first aligned among themselves and then to outgroups using the GCG subroutine Pileup (Genetics Computer Group 1993). Two separate analyses of aligned sequences were carried out using PAUP (Swofford 1993). In the first, two scleractinian 16S sequences (*Tubastraea coccinea* and *Cyphastrea ocellina*) were aligned with the published sequences of *Hydra vulgaris* (Cunningham and Buss 1993), *Strongylocentrotus purpuratus* (Jacobs et al. 1988), *Locusta migratoria* (Uhlenbusch et al. 1987), and human (Anderson et al. 1981). Other cnidarian sequences, provided by Diane Bridge (1994; Bridge et al. 1995), used in the analysis included two sequences from another order in the class Anthozoa, the octocorals *Renilla* and *Leptogorgia;* two sequences from the class Scyphozoa, *Aurelia* and *Pelagia;* and two sequences from the class Hydrozoa, *Liriope* and *Physalia.* The most parsimonious reconstruction of relationships among these 12 sequences was found with a branch and bound search in PAUP using only variable positions and designating the human sequence as the outgroup. The significance of these groupings was measured with 1000 bootstrap replicates.

In the second phylogenetic analysis, all scleractinian sequences were aligned with *Hydra vulgaris* and *Renilla.* The most parsimonious hypothesis for the relationship among these taxa was generated with a heuristic search using a random addition sequence in PAUP, using only variable nucleotide positions, ignoring insertion/deletions, and with *Hydra vulgaris* designated as the outgroup. One hundred bootstrap replicates were carried out on these data. A neighbor joining analysis was also carried out on these sequences using PHYLIP (Felsenstein 1993).

Secondary Structure

The secondary structure for two sequences (one long sequence, *Tubastraea coccinea,* and one short sequence, *Fungia scutaria*) was deter-

Table 2. Base composition, substitutions, and distance among all sequences and within major clades

	Among all short sequences	Among all long sequences	Among all sequences	Among sea urchins[a]
G (%)	19.9	24.1	22.0	22.0
A (%)	35.3	31.9	33.5	33.0
T (%)	32.7	29.9	31.0	26.2
C (%)	12.1	14.1	13.0	18.8
Transition substitutions (%)	54.8	61.9	58.9	45.2
Transversion substitutions (%)	45.2	38.1	41.1	54.8
Distance[b] (mean %)	7.65	7.5	18.2	22.0

[a] *Stongylocentrotus purpuratus* and *Paracentrotus lividus*, genera from two families thought to have diverged from each other 60 mya (Smith et al. 1992a)

[b] Jukes Cantor corrected genetic distance

mined manually by comparison with published hypothesized secondary structures from other metazoans. The scleractinian sequences were first joined to Okimoto's (1994) alignment of mt 16S ribosomal sequences including *Escherichia coli*, (Maly and Brimacombe 1983), *Paramecium tetraurelia* (Seilhamer et al. 1984), *Saccharomyces cerevisiae* (Sor and Fukuhara 1983), *Mus musculus* (Bibb et al. 1981), *Paracentrotus lividus* (Cantatore et al. 1989), *Drosophila yakuba* (Clary and Wolstenholme 1985), *Caenorhabditis elegans*, and *Ascaris suum*. Secondary structures were then drawn using this alignment along with comparison to proposed models of mt *16S* ribosomal RNA secondary structure of these organisms as well as those of *Locusta migratoria* (Uhlenbusch et al. 1987), *Polyamia neoyavapai* (Fang et al. 1993), mouse (Glotz et al. 1981), and human (Anderson et al. 1981). Stems were required to be two base pairs or longer. G-C, G-T, A-T, and G-A bonds were allowed.

Results

Extractions and PCR

Genomic extractions of scleractinian samples yielded between 0.75 and 250 μg DNA. Sequences were obtained from 34 species of Scleractinia, representing 29 genera in 14 of 24 extant families (Genbank accession numbers L75922-L76024, L76132) from all seven extant suborders recognized in the latest revision of the group (Veron 1995). PCR with scleractinian samples resulted in the amplification of DNA segments ranging in size from 406 to 565 base pairs, corresponding to positions 5085–5528 of the mtDNA sequence of *Strongylocentrotus purpuratus* (Jacobs et al. 1988) and positions 13355–13010 of the mtDNA sequence of *Drosophila yakuba* (Clary and Wolstenholme 1985). When aligned with *Renilla* and *Hydra*, these sequences comprise 577 sites, of which 262 are constant, 210 informative, and 105 uninformative. The alignments used in sequence analyses are available from S. Romano.

Sequence Composition, Alignment, and Size Variation

All of the scleractinian sequences are similar to each other in base composition (Table 2). These sequences are on average 46.1% different from the two anthozoan outgroups. There are 16 insertion/deletion events among the scleractinian sequences which were not used in phylogenetic analyses.

In the most parsimonious reconstruction of the relationships among all of the scleractinian sequences, with *Renilla* and *Hydra* as outgroups, the scleractinian sequences fall into two major clades (Fig. 1) which are on average 29.4% (range 20.3–32.2) different from each other. These two major clades can be defined by sequence length as a result of insertion/deletion events. One clade is characterized by shorter segments that range in size from 406 to 425 bp and differ from each other by a mean of 7.6% (Table 2). The other clade is characterized by longer segments that range in size from 536 to 565 bp and that are a mean of 7.5% different from each other (Table 2).

Conservation of segment size within genera and families was confirmed by amplification of mtDNA-enriched samples extracted using ProMega Wizard Minipreps from 31 species of corals, including 10 species of corals not previously sampled and which were not sequenced. These 10 taxa included two genera from the Pocilloporidae, six different species of *Acropora*, and two different *Porites* species. Amplification of all mtDNA-enriched samples generated segments of the same size as those from other members of the family or genus previously amplified and sequenced.

Characteristics of Short and Long Clades

The segments from the short and long clades were examined in a variety of ways that suggest that they are 1) mitochondrial in origin, 2) under similar selective constraints, and 3) homologous to each other. One sequence from each of these clades was used in a phylogenetic analysis with mt *16S* sequences from cnidarians (Fig. 2). In this analysis, both sequences are more closely related to each other than to any other taxa (Fig. 2). The base composition of each clade is similar to the other and does not differ from all of the scleractinian sequences taken together (Table 2).

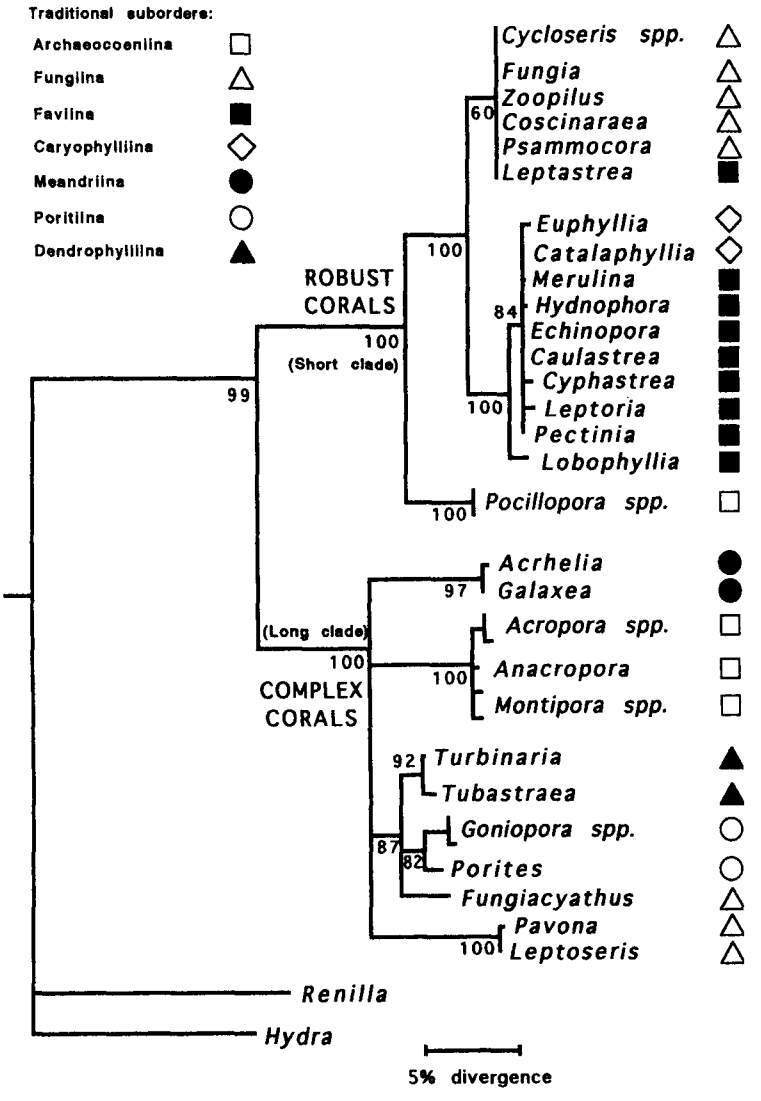


Fig. 1. Molecular phylogram (50% majority rule consensus of 112 most parsimonious reconstructions generated with a heuristic search using 10 random addition sequences in PAUP) of relationships among genera of 34 species of corals based on sequences from the mt *16S* ribosomal gene region. The numbers on the branches represent values from 100 bootstrap replicates. Reprinted with permission from Romano and Palumbi (1996).

Further comparisons of patterns of sequence evolution in the short and long clades also support the hypothesis that sequences in both clades are homologous to each other. The percentage of transition and transversion substitutions between the clades was similar (Table 2). Overall, transitions accumulate more quickly than transversions in both clades (Fig. 3). Spatial patterns of nucleotide substitution along the sequenced region in the two clades show substantial similarities. The genetic distance between pairs of sequences was calculated for a series of overlapping 60 bp windows along the sequences, starting at the 5′ end. Distance for each successive window was calculated by shifting the 60 bp window three base pairs downstream, until the entire sequence had been compared. The results of this analysis give a visual image of regional variability along the length of the two sequences. Such comparisons were carried out between two of the most different sequences within each of the major clades (Figs. 4a,b), between two of the most different sequences among the two major clades (Fig. 4c), and between two sequences from two

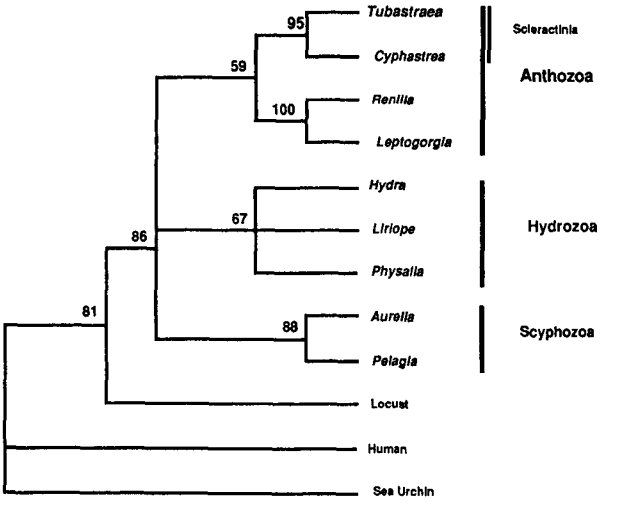


Fig. 2. Cladogram (50% majority rule consensus of two most parsimonious reconstructions generated with a branch and bound search using PAUP) of relationships among two scleractinians, seven cnidarians (Bridge et al. 1995), sea urchin (Jacobs et al. 1988), and human (Anderson et al. 1981). The numbers on the branches represent values from 1000 bootstrap replicates.

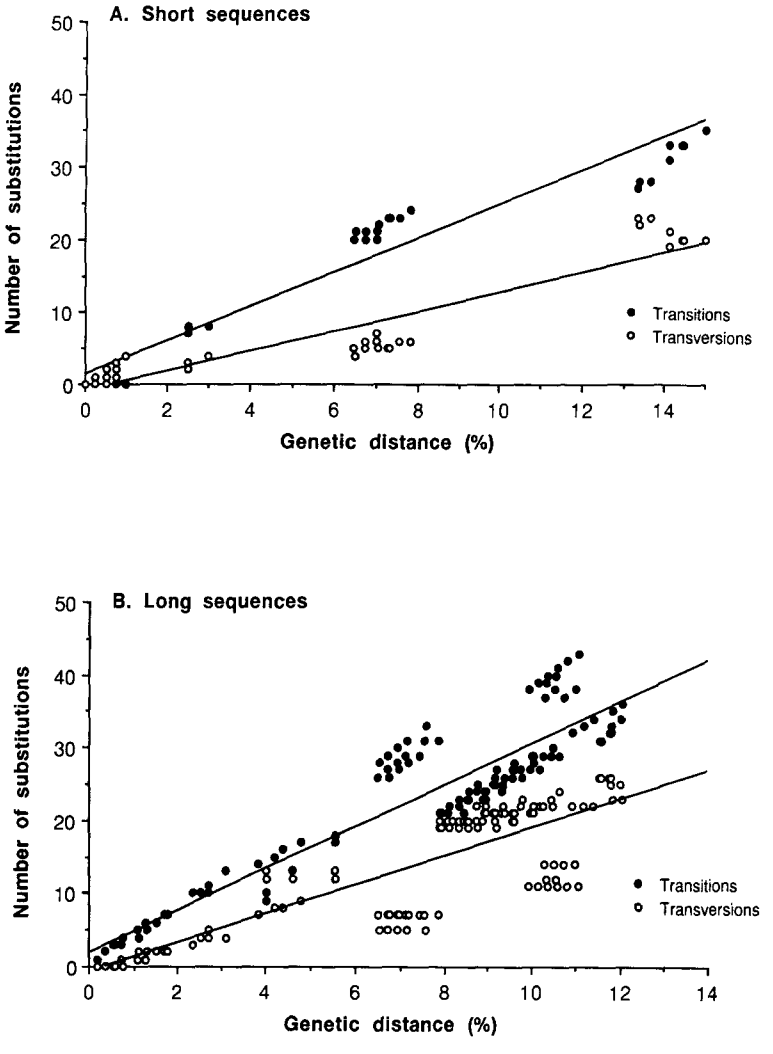


Fig. 3. Relationship between genetic distance and numbers of transition and transversion substitutions for all possible pairwise comparisons of (**A**) short sequences (slope for transitions = 2.3, transversions = 1.4; ratio of slopes = 1.6) and (**B**) long sequences (slope for transitions = 2.9, transversions = 2.0; ratio of slopes = 1.4).

different species of sea urchins (Fig. 4d). In all of these comparisons, variability along the sequences is similar (Fig. 4). The first gap seen in the graph comparing short and long sequences (Fig. 4c) indicates the region where the large insertion/deletion occurs between the two major sequence clades. The second gap in this graph is an area where there is so much variability that the sequences are poorly aligned.

When comparing distantly related short (Fig. 4a) or long (Fig. 4b) sequences, the same regions tend to show higher sequence variability. These regions of sequence variability also correspond to regions of variability in secondary structure. It is in these regions that major differences in secondary structure between the two clades, and more minor differences within clades, are found. Comparison of the same *16s* mtDNA gene region from two genera of sea urchins [from two different families thought to have diverged approximately 60 mya (Smith et al. 1992a)] results in a similar trimodal pattern. This conserved pattern of variability along the length of the *16S* sequence in urchins and both coral clades suggests that these sequences are evolving under similar evolutionary constraints, and that these constraints are similar across phyla.

Secondary Structure

The secondary structures hypothesized for both short and long sequences are similar to each other and to models proposed for other organisms (Fig. 5). The majority of stems and loops are conserved among both groups of sequences. There are two major differences between the short and long sequences. First, the region in the long sequence between nucleotides 250 to 400 that forms five stems and loops (Fig. 5b) is missing in the short sequence and is replaced by a region consisting of a single loop (Fig. 5a). Second, the region between nucleotides 475 and 540 in the long sequence comprises three stems and loops (Fig. 5b), while there is only one stem and loop in this region in the short sequence (Fig. 5a). These two regions also differ in *Escherichia coli* (Gutell et al. 1994, Fig. 2), and *Cenorhabditis elegans* (Okimoto et al. 1994, Fig. 5). The nucleotides in this region are not considered

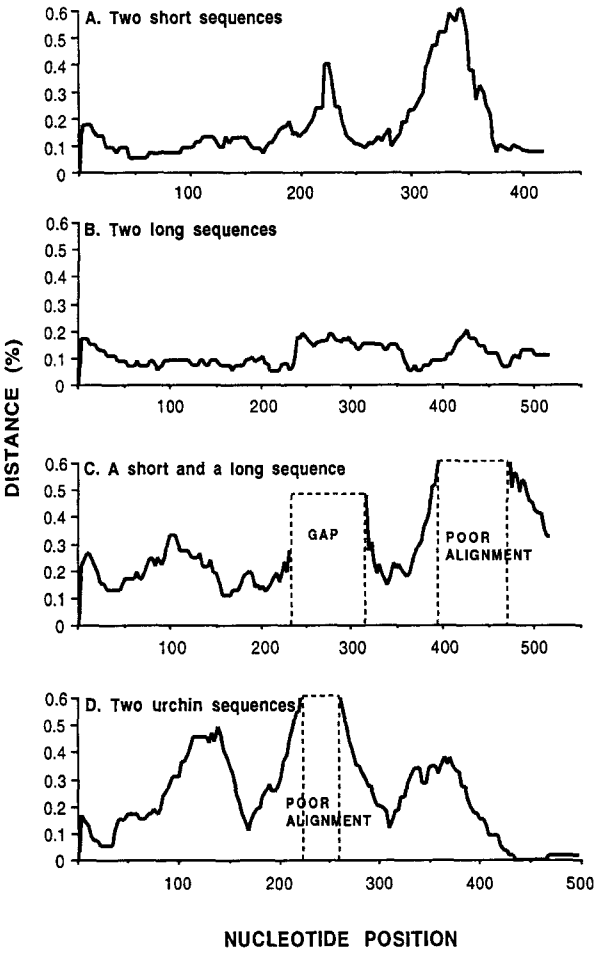


Fig. 4. Genetic distance in 60-bp windows shifted every three nucleotide positions for **(A)** two short sequences (*Pocillopora meandrina* versus *Catalaphyllia jardinei*), **(B)** two long sequences (*Acropora humilis* versus *Pavona varians*), **(C)** a short and a long sequence (*Fungia scutaria* versus *Tubastraea coccinea*), and **(D)** two sea urchin sequences (*Paracentrotus* [Cantatore et al. 1989] versus *Strongylocentrotus* [Jacobs et al. 1988]).

to be conserved (Okimoto et al. 1994). Regions of conserved nucleotides are all very similar in secondary structure.

β *Sequences*

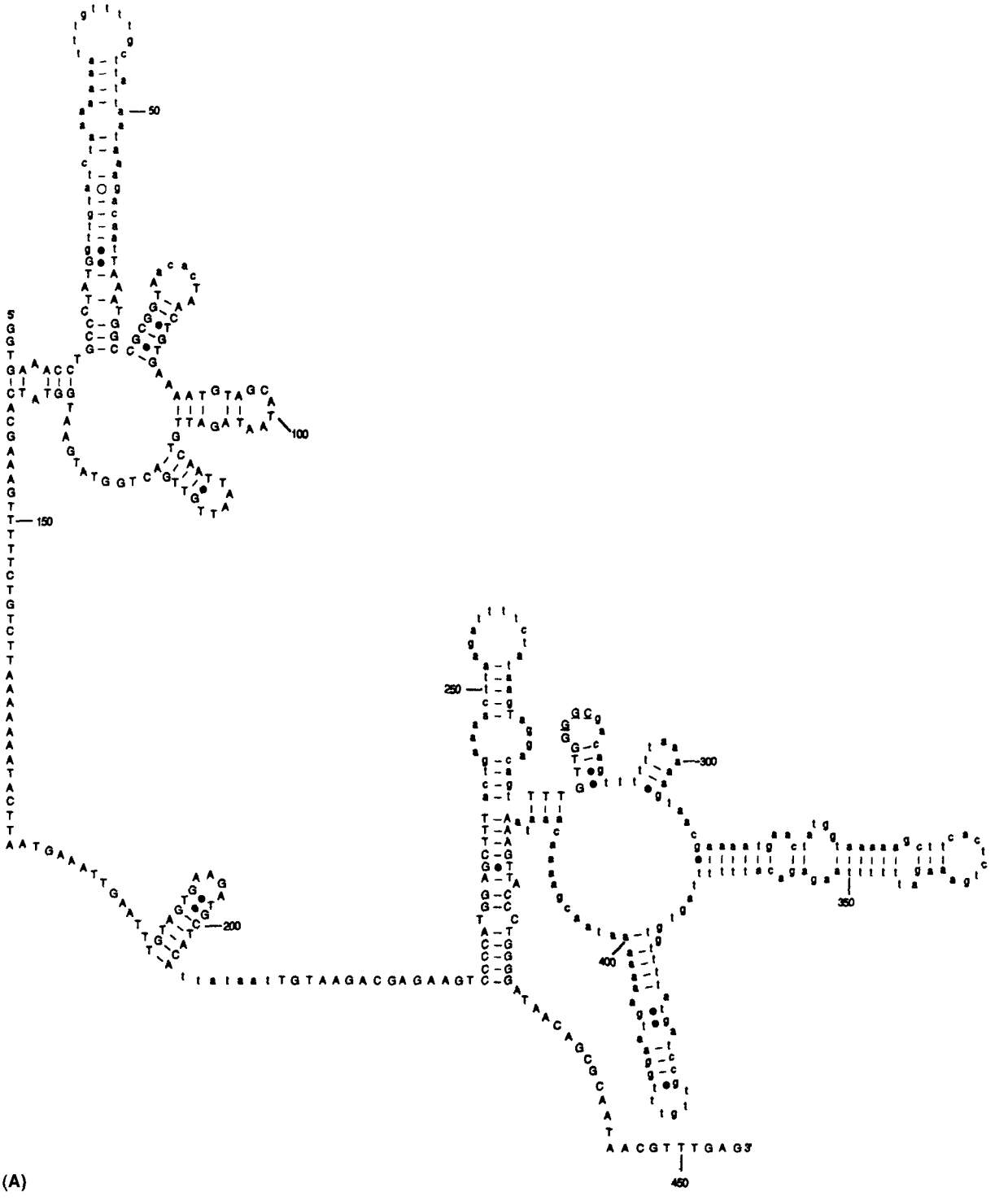
To examine the possibility that sequences from either the short or long clades are not of mitochondrial origin (e.g., a nuclear pseudogene, Gellissen and Michaelis 1987), segment size was compared from amplifications of both total genomic DNA and mtDNA-enriched fractions from 17 species. In 14 of these, amplifications from mtDNA-enriched samples generated the same size segments as from total genomic DNA. Amplification of a mtDNA-enriched sample from one of these templates (*T. coccinea*) with nuclear ribosomal ITS primers failed, although amplification of genomic DNA was successful using these same primers. In three species, *Turbinaria peltata, Euphyllia ancora,* and *Echinopora lamellosa,* different size segments were generated from different extractions (Table 3). One of each of these pairs of sequences has been given the designation β, and these β sequences will be discussed in detail below. Repeated DNA extractions gave similar, multiple amplification products (Table 3) showing that contamination was an unlikely source of β sequences. Amplification of the expected size fragment tended to occur from mtDNA-enriched samples (10 of 12 observations) whereas amplifications from total genomic DNA were more likely to give the "wrong" size product (nine of 12 observations). These results suggest that both the short and long segments are generated from mtDNA but that β sequences may represent nuclear pseudogenes, jumping PCR products, or some other unknown phenomenon.

Phylogenetic Analyses

Parsimony analysis verified that the coral sequences reported here clustered with other Cnidaria, and that the Cnidaria as a whole form a monophyletic group. In the topology generated in this analysis (Fig. 2), the Scleractinian sequences group together. They are most closely related to two other anthozoan taxa from the order Octocorallia. These results show that the sequences reported here are derived from coral mitochondrial genes, not from zooxanthellae or coral nuclear genes (but see Discussion of alternative hypotheses).

The evolutionary hypothesis for the relationships among the Scleractinia and in relation to two cnidarian outgroups, whether generated by parsimony or neighbor-joining analysis, is the same (Fig. 1). The Scleractinia sequences fall into two major clades that are characterized by an insertion/deletion occurring just after position 250 (leading to the "long" and "short" designations mentioned above) which corresponds to INDEL#1 in France et al.'s (1996) alignment of anthozoan sequences. Taxa with short sequences, shown on the upper branch of the topology, fall into three major groups. One group, representing two species in the family Pocilloporidae, is rather distinct (14%) from other members of this clade. Taxa with long sequences, shown on the lower branch of the topology, separate into four groups that are approximately equally different from each other.

All species and genera placed in the same family by morphological criteria (Veron 1995) grouped together, with one exception. This exception was one genus (*Leptastrea*) in the Faviidae that grouped with the family Siderastreidae in the molecular topology. Sequence length is conserved within genera and families. The same size segment was always amplified from the six genera for which more than one species was assayed (four congeneric pairs, three species of *Porites* and eight species of *Acropora*), including ten species that were not se-



(A)

Fig. 5. Hypothesized secondary structure for **(A)** the short sequence of *Fungia scutaria* and **(B)** the long sequence of *Tubastraea coccinea*. Capital letters indicate conserved core nucleotides. Underlined letters are the hypothesized A–P binding site.

quenced. Except for the three species from which both size segments were amplified, the same size segment was also always amplified from genera in the same family, including two genera that were not sequenced (Fig. 1). Although existing generic and family designations are broadly supported by Fig. 1, relationships above this taxonomic level are unexpected.

Discussion

Evidence from amplification of genomic DNA and mtDNA-enriched samples, phylogenetic analysis, and comparisons with other animals supports the hypothesis that all scleractinian sequences reported here are *16S* mtDNA sequences from the coral animal. Sequences am-

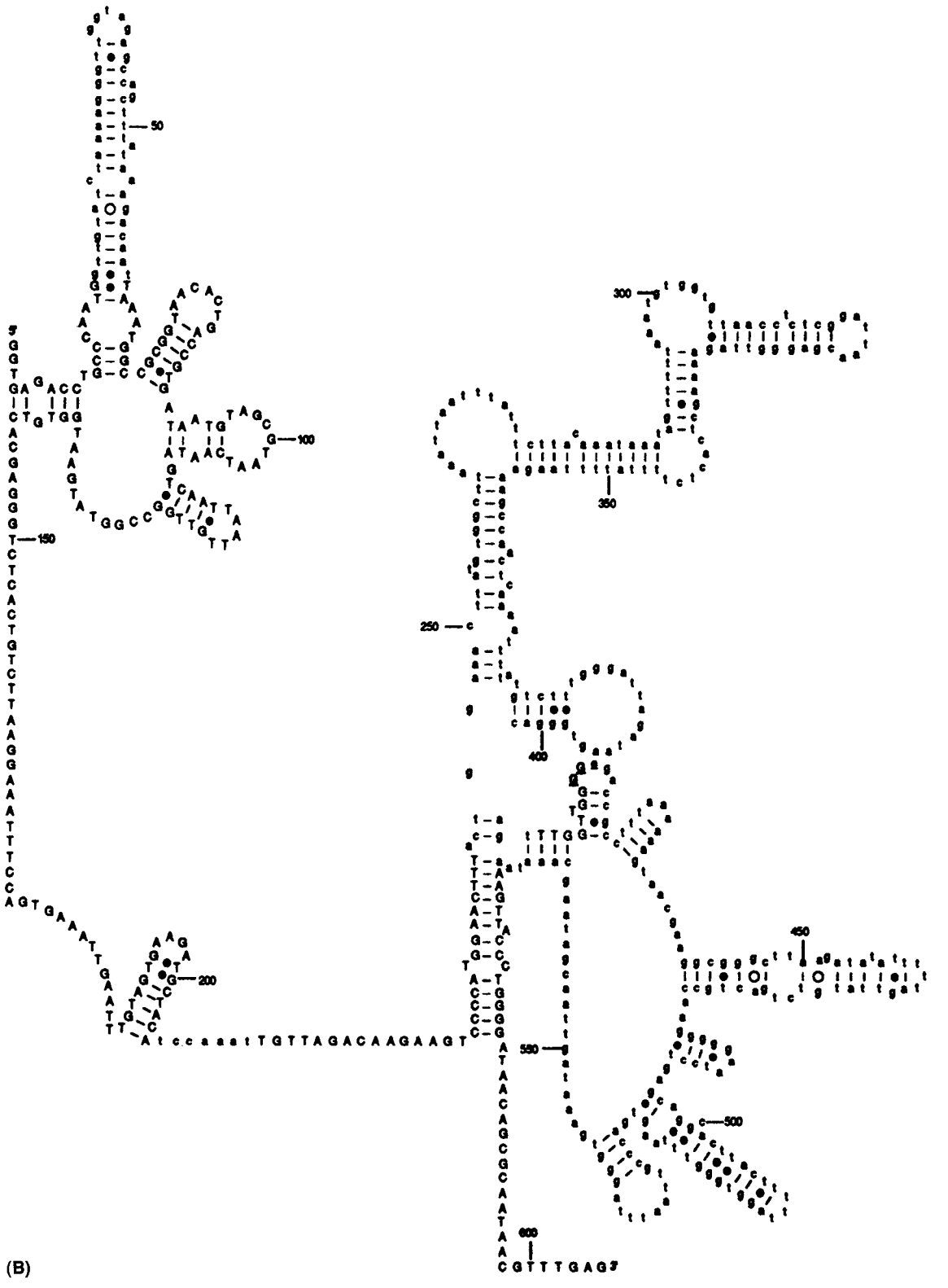


Fig. 5. Continued.

plified from both azooxanthellate and zooxanthellate scleractinians appear to be homologous to 16S sequences from other metazoans (Fig. 2).

The nucleotide composition of the sequences from both major scleractinian clades does not differ greatly from each other or from the nucleotide composition of other marine invertebrates such as sea urchins (Table 2). The approximately equal number of transition and transversion substitutions among both clades of scleractinian sequences is also observed in sea urchins (Table 2), nem-

Table 3. Amplification of short and long sequences from genomic and mtDNA extractions of three species of corals

Coral species	Clade of other family members	Amplification of short sequences (no. replicates)[b]		Amplification of long sequences (no. replicates)[b]	
		from Genomic DNA	from mtDNA	from Genomic DNA	from mtDNA
Turbinaria peltata	Long	2[a]	0	2	4
Euphyllia ancora	Short	0	3	4[a]	2[a]
Echinopora lamellosa	Short	1	3	3[a]	0

[a] Amplification of incorrect fragment size as determined from comparison to other genera in the same families
[b] numbers shown are the number of independent amplifications that gave rise to PCR products of the long or short type

atodes (Okimoto et al. 1992), and *Drosophila* (DeSalle et al. 1987).

Patterns of Molecular Evolution of Scleractinian Sequences

A comparison of patterns of molecular evolution in sequences belonging to the two major scleractinian clades demonstrates that sequences from both clades are evolving under evolutionary constraints similar to those observed in other metazoan mt *16S* ribosomal DNA sequences. Transitions outnumber transversions in comparison of species within genera and genera within families. In more distant comparisons, these ratios decline to approximately 1:1. High transition biases are typical of mtDNA evolution, and are thought to reflect a lack of efficient mismatch repair mechanisms in mitochondria. Transition biases are known to be lower in the *16S* gene region than at silent sites of protein coding regions (DeSalle et al. 1987). In insects, fewer transitions may be related to the high AT bias shown by mtDNA in these groups that is thought to be the result of functional constraints (DeSalle 1992). The low number of transitions along with the relatively low transition transversion bias in corals may be the result of functional constraints in the ribosomal DNA.

The spatial pattern of nucleotide substitutions is also similar in each clade (Fig. 4a, b) and similar to other mtDNA sequences. In comparisons of short and long sequences, the region where a gap occurs between the sequences and the region where the sequences align poorly are the regions of highest variability in comparisons of sequences within each clade (Fig. 4a,b). Homologous regions are also highly variable when different species of sea urchins are compared (Fig. 4d). The regions of highest variability are also the regions where differences in secondary structure are observed in both clades, as well as in *C. elegans* and *16S*-like genes in *E. coli* (Fig. 5). These comparisons are further evidence that evolutionary constraints on sequence evolution of coral 16S sequences are similar to those acting in other taxa.

Secondary Structure

The secondary structures hypothesized for short and long sequences are similar to secondary structure models for *Escherichia coli* (Gutell et al. 1994, Fig. 2), and nematodes (Okimoto et al. 1994, Fig. 5), as well as to models for other metazoans. Although there are size differences among the scleractinian sequences themselves and in comparison to *E. coli* and nematodes, the number of conserved core nucleotides (as defined by Okimoto et al. 1992) among all of these taxa is very similar (*Fungia* 235, *Tubastraea* 233, *C. elegans* 238, *E. coli* 237). The secondary-structure elements and nucleotides that in *E. coli* are associated with tRNA binding to the ribosomal A and P sites (Moazed and Noller 1989) are also found in the scleractinian secondary structure models (Fig. 5).

The differences among secondary structure models among metazoa are due to insertions/deletions that occur outside of the conserved core nucleotides (Fig. 5). Size variation in this region of the mt *16S* gene is known from insects with divergence times of up to 250 mya (Derr et al. 1992; Fang et al. 1993; Pashley and Ke 1992). The regions where these insertions/deletions occur among metazoa are the same regions that differ among the Scleractinia (Fig. 5). Given that the scleractinian genes for which secondary structures are shown here are 29.4% different in nucleotide sequence, such differences as are seen between these two taxa are perhaps not surprising. This size variation appears to be largely the result of an insertion/deletion event that took place early in the evolutionary history of the Scleractinia. In addition, variation is known in three-dimensional rRNA structure (Gutell et al. 1994). It is possible that differences in secondary structure of coral short and long *16S* genes affect the functioning of ribosomes, but little is understood about correlations of function with ribosomal secondary structure (Gerbi 1985). For example, it has been demonstrated that protein synthesis is not affected by deletion of a large stem/loop region of the *16S* rRNA of *Escherichia coli* (Gravel et al. 1989). Because there is relatively little known about ribosomal tertiary structure and function, there is no reason to conclude that the large

deletion in the short sequences would result in nonfunctional ribosomes. In every other respect, both short and long sequences are similar to other functional ribosomal sequences. Study of the entire mt *16S* ribosomal DNA from these organisms would also help in evaluating the significance of the different structures in corals.

Evolutionary Rates of the Scleractinia

An attempt to estimate rates of evolution of these ribosomal sequences was made using relatively closely related genera and families with the same first date of appearance in the fossil record (Table 4). We estimate the rate to be about 0.10% divergence/million years (myr) or less. This rate is similar to the divergence rates of mt *16S* sequences observed among insects (DeSalle et al. 1987) though much lower than the rate of approximately 0.5 to 1% per myr observed in bovids (Gatesy et al. 1992). The estimate of this rate could be improved using taxa whose divergence times are known precisely. Further studies of mt *16S* DNA sequences from a much wider variety of taxa will be necessary to determine whether this region is evolving at different rates in different taxa. DeSalle et al. (1987) suggested that insect *16S* sequences were evolving slowly because of constraints imposed by a high AT content. Coral sequences do not have such a highly skewed nucleotide bias, and the reasons for their slow evolutionary rate are unknown. Comparison of *COI* sequences from coral genera (Best and Thomas 1993) also show unexpectedly low differences, suggesting that low rates of substitution may be a general feature of coral mtDNA.

The amplification of two different sequences from three species is unexpected. For each species, one sequence falls clearly in the same phylogenetic position as other members of its family. The second sequence (β) is most similar to corals in another suborder. One possible hypothesis for these β sequences is that they are the result of heteroplasmy occurring in these taxa, possibly due to a hybridization event in the past. Pacific corals live in close association with each other. They are often broadcast spawners (Richmond and Hunter 1990) that spawn once a year in synchrony (Harrison et al. 1984; Harrison and Wallace 1990; Richmond and Hunter 1990), and so, a natural opportunity for hybridization exists. In fertilization experiments, hybridization has been observed between closely related and distantly related species of the genera *Acropora, Montipora,* and *Platygyra* (Willis et al. 1992). Hybridization was also observed among favids and acroporids, families from both major clades (Willis et al. 1992). The hybrids within genera were able to complete development and continue growing for at least 3 years. In these experiments, successful development of hybrids between families across clades was not observed.

It is difficult to imagine that hybridization alone could account for these observations if mtDNA is maternally transmitted in corals as is the case for most organisms studied (Avise 1986; Wilson et al. 1985). However, unusual modes of mtDNA inheritance have been observed in other invertebrates (Zouros et al. 1994). It is possible that mitochondrial transmission in corals is different from what is known for other animals.

In this study, only one colony from one location was sampled for the majority of species analyzed. In all cases, sequences of the same size were generated from species in the same genus, and from genera in the same family. It is possible that further sampling of more individuals in each species from a wider variety of locations and of more species and genera will demonstrate that β sequences are more common than observed here. Some evidence for this hypothesis is that long sequences were amplified from two additional taxa from which only short sequences had previously been amplified. The sequence from *Leptastrea,* the only example of a genus not grouping with other family members (although it grouped in the same major clade), may be another example of one of these β sequences. β sequences do not seem to be a result of PCR artifacts. For the majority of genomic samples from which only short sequences were amplified, no amplification of long sequences was possible (Table 4). Further investigation will be necessary to characterize this phenomenon.

Alternative Hypotheses

The evidence presented here strongly supports the hypothesis that all of the scleractinian sequences are from mt *16S* ribosomal DNA and that there was an early split in the ancient corals that led to the two major clades of short and long sequences. However, the resulting split of coral families between long and short clades stands in strong contrast to traditional views of coral taxonomy at the subordinal level (Romano and Palumbi 1996). In addition, major differences in secondary structure along with the large amount of genetic distance among short and long sequences, and the appearance of β sequences suggest that alternative hypotheses about the evolution of short and long coral clades be considered.

Short and long sequences do not appear to be the result of contamination of PCR reactions. All results were consistent and repeatable: any given sample always produced amplification products of the same size. Negative controls included in sets of PCR reactions were always blank. In the three species where both size segments were amplified (Table 3), repeated extractions gave similar, multiple amplification products.

Another alternative hypothesis is that either the short or the long sequences are actually derived from a mitochondrial pseudogene in the nuclear genome. The pre-

Table 4. Divergence dates, genetic distance, and divergence rates calculated from mitochondrial *16S* sequences from various taxa

Taxa compared	Divergence dates (mya)	Genetic distance (%)	Divergence rate (%/myr)
Corals[a]			
Genera	30–60	0.7–2.5	0.02–0.04
Families	67–100	3.9–11.6	0.06–0.12
Insects[b]			
Drosophila species	2–60	0.4–3	0.05–0.2
Aedes albopictus versus *Drosophila* species	200	14.0	0.07
Bovids[c]			
Species: *Damaliscus dorcas* versus *D. hunteri*	2.5	6.6	2.6
Tribes: *Bos taurus* versus *Tragelaphus angasi*	11.5	15.5	1.3

[a] Coral genera compared: *Acropora* versus *Montipora* (40–50 mya), *Pavona* versus *Leptoseris,* (30–40 mya), *Porites* versus *Goniopora* (40–60 mya), *Caulastrea* versus *Leptoria* (40–60 mya); Coral families compared: Poritidae (100 mya), Dendrophylliidae (100 mya), Agariciidae (67 mya), Oculinidae (67–100 mya), Acroporidae (67 mya)

[b] *Drosophila* species compared: *sproati, pilimana, disjuncta, affinidisjuncta, mimica, stigma, clavisetae, funebris, yakuba;* data from DeSalle et al. 1987

[c] Data from Gatesy et al. 1992

dicted secondary structure for the short sequences, which appears modified relative to the long sequences and other secondary structure models, supports the hypothesis that the short sequences are pseudogenes. However, short sequences have the strongest pattern of spatial variation in sequence evolution. In addition, short sequences were the only PCR product obtained from 16 species. In none of these cases was there evidence of a second amplification product, although long sequences were subsequently amplified from two of these species using long-sequence specific primers. If the short sequence represents a pseudogene, then this would imply that at least 16 taxa have no functional copy of the *16S* ribosomal genes or that for these taxa, our universal and coral specific primers fail to amplify the functional gene.

Although mitochondrial pseudogenes have been observed in the nuclear genome of other organisms (Fukuda et al. 1985; Gellissen and Michaelis 1987; Jacobs et al. 1983; Lopez et al. 1994; Smith et al. 1992b; Tsuzuki et al. 1983; Zullo et al. 1991) they have characteristics that are generally very different from those observed in functional mtDNA sequences. Pseudogenes are not thought to have any evolutionary constraints (Li et al. 1985). For this reason, no patterns in spatial distribution of substitutions would be expected after a great length of time (e.g., over 200 million years for corals), transition/transversion ratios should be low and rates of evolution are expected to be relatively high (Li and Graur 1991). Because none of these predictions are supported by examination of either long or short coral sequences, we conclude that both types of coral sequences represent functional genes evolving under similar selective constraints.

If both long and short genes are functional, then the only remaining alternative hypothesis is that these two sequences are from a duplication of the mt *16S* ribosomal gene within the mitochondrial genome such as has been observed in lizards (Moritz and Brown 1986, 1987). Because of the large amount of divergence between the short and long sequences, this duplication would have had to occur over 300 mya. Subsequently, different coral families might have lost one or the other of the copies independently, shedding either the short or long copy at some point in time. This must have happened about the time of the evolution of most modern coral families because different families in the same suborders have different sizes. Because modern families formed in the Cretaceous but the long and short clades diverged at least 300 mya, this hypothesis requires that the two proposed functional copies of the *16S* gene must have persisted in the coral mitochondrial genome for 100–200 million years and then been lost in a short period of time. It seems unlikely that two copies of the same functional ribosomal genes would be maintained in the mitochondrial genome over this long time frame as the mitochondrial genome is known for its genetic economy (Attardi 1985).

In addition, under this hypothesis the phylogeny of suborders in the long clade should parallel the phylogeny of these suborders in the short clade. For example, species in the suborder Archaeocoeniina are the outgroups of other taxa in the short clade but not in the long clade. This is not the case.

In mussels of the genus *Mytilus,* two mitochondrial genotypes occur that differ by about 20% (Zouros et al. 1994). MtDNA inheritance is sex-specific in these organisms. Females have one mtDNA type whereas males have the other mtDNA type in gonad tissue. Because most corals are homoplasmic—having only short or long genes—this mechanism cannot operate in modern corals. It is possible that the short and long clades represent male and female mtDNA lineages that have long since ceased being sex specific. However, most corals are hermaphrodites and there is no evidence for the role of either gene clade in gonad-specific tissue specificity.

The hypothesis best supported by the data gathered in

this study is that both the short and the long sequences are from the mt *16S* ribosomal RNA gene region and are both functional. Divergence between these two clades represents divergence of two lineages of coral species not the duplication of two loci. This divergence took place long ago—estimates of *16S* divergence rate in recent families and genera suggest a date of at least 300 mya for the divergence of short and long clades.

Evolutionary Relationships Among the Scleractinia

The evolutionary hypothesis for the relationships among the Scleractinia (Fig. 1) generated from these data is robust. All groupings shown in Fig. 1 are supported by bootstrap values of 82% or higher while the two major clades on the tree are supported by bootstrap values of 100%. Genera within families, as determined by morphological taxonomy, group together on the molecular topology. By contrast, relationships among families and suborders hypothesized from morphological data are not supported by the molecular data. Chen et al. (1995) include nine species of Scleractinia in their analysis of relationships within the class Anthozoa based on 28S nuclear ribosomal sequences. Although these taxa represent only six families, the families fall into two major clades that correspond to the two major clades observed in our analysis. Grouping of families within each clade is also the same as occurs in our analysis.

The approximate rate of divergence of 0.10% or less suggests that the divergence time of the two major clades of Scleractinia is greater than 300 mya. This date is conservatively calculated and is earlier than estimates of 240 mya as the first appearance of the Scleractinia in the fossil record (Stanley 1981). As a result, it is possible that the divergence of the two major clades of the Scleractinia occurred before the acquisition of a calcium carbonate skeleton. If this is true, it implies the coral exoskeleton was invented at least twice.

This analysis illustrates the utility of a molecular phylogenetic approach to the systematic study of the Scleractinia. This region of the mt *16S* ribosomal gene is useful for studying family and suborder relationships within the order. It has provided a robust framework that will be useful for further examination of morphological evolution of corals (Romano and Palumbi 1996). A molecular approach to coral systematics does provide a useful alternative to morphologically based systematic study of the Scleractinia. This approach should be useful for further systematic study of the order at lower taxonomic levels.

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