Evolution of Scleractinian Corals Inferred from Molecular Systematics

Sandra L. Romano* and Stephen R. Palumbi

Scleractinian corals have a continuous fossil record from the mid-Triassic, but taxonomic difficulties have impeded an understanding of their evolution. A molecular phylogenetic analysis of mitochondrial 16S ribosomal RNA showed departures from previous hypotheses of coral evolution. Families clustered into two major groups that do not correspond to morphologically based suborders. These clades differed in their 16S ribosomal DNA sequence by 29.4 percent, which suggests evolutionary divergence before the appearance of scleractinian skeletons 240 million years ago. Together, these fossil and molecular data suggest multiple origins of the scleractinian skeleton, and the great morphological diversity of present-day scleractinians may be a reflection of these multiple origins.

Reef-building scleractinian corals support some of the world's most diverse marine communities (*1*). Their responses to environmental variation contribute to our understanding of global and regional climate change (*2*), sea-level fluctuations (*3*), and anthropogenic effects on nearshore communities (*4*). Corals are also central to our understanding of biogeographic patterns (*5*). The 240-million-year fossil record of corals is well studied, and a great deal is known about the biology of this order of anthozoans. However, difficulties in understanding skeletal variability (*6*, *7*), skeletal homologies (*8*, *9*), and fossil taxonomy (*10*) frustrate understanding of the evolution of these cnidarians. As a result, relations among coral families and their suborders are frequently not known, limiting our understanding of the varied roles that corals play in modern reef ecosystems. Molecular phylogenetics can yield additional information about evolutionary processes where fossil information is incomplete, and it can provide an alternative means to test the timing and topology of evolutionary divergence inferred from paleontological data. Here, we conducted a molecular phylogenetic analysis of scleractinian corals that shows differences from morphological or paleontological views and implies that the coral skeleton evolved more than once.

Molecular analysis of corals has been hampered by technical problems of DNA purification and the presence in some corals of abundant intracellular algae (zooxanthellae). We obtained initial coral sequences from an azooxanthellate coral by means of the polymerase chain reaction (PCR) with universal primers for the mitochondrial 16S ribosomal RNA gene (16S rDNA) (*8*, *11*–*13*). Coral-specific primers (*14*) designed from these initial sequences were used to produce sequences from 34 species of coral in 14 families (Table 1) that (i) are homologous to 16S rDNA sequences from other metazoan mitochondria, (ii) form a monophyletic group with other anthozoans and cnidarians, and (iii) have secondary structures similar to those predicted from homologous mitochondrial and bacterial genes (*8*).

Phylogenetic analysis of the 16S rDNA data supported traditional groupings within genera and families (*15*). In all five genera from which two or more species were analyzed, congeners formed a monophyletic cluster. In 9 of 10 families from which more than one genus was sampled, genera grouped monophyletically. By contrast, phylogenetic analysis strongly supported groupings of families that are placed traditionally in different suborders (for example, Poritidae and Oculinidae) (Fig. 1, bootstrap values 87 to 100%). Moreover, some families that are considered to be closely related morphologically were found by phylogenetic analysis to have only distantly related mitochondrial DNAs (mtDNAs) (for example, Acroporidae and Pocilloporidae).

Among the 34 species in our analysis, two distinct clades emerged whose 16S rDNA sequences differ by an average of 29.4%. This result is similar to the 16S rDNA sequence divergence between orders of holometabolous insects (24 to 31% different) that appeared in the fossil record between 200 million and 300 million years ago (Ma) (*16*). Confamilial coral genera that appeared in the Eocene (35 to 50 Ma) differ by only 2.5 to 3% (*8*), and sister families that appeared in the mid-Cretaceous (100 Ma) are no more than 9% different. Thus, the 29.4% divergence of the major clades probably represents divergence of soft-bodied forms before the appearance of coral skeletons at 240 Ma. This finding supports previous hypotheses that the Scleractinia are not descendants of the reef-forming rugose corals and that scleractinian skeletons evolved more than once (*10*). Up to nine suborders of Scleractinia appeared in the mid-Triassic with no known common ancestor (*9*, *17*, *18*). Fossil and molec-

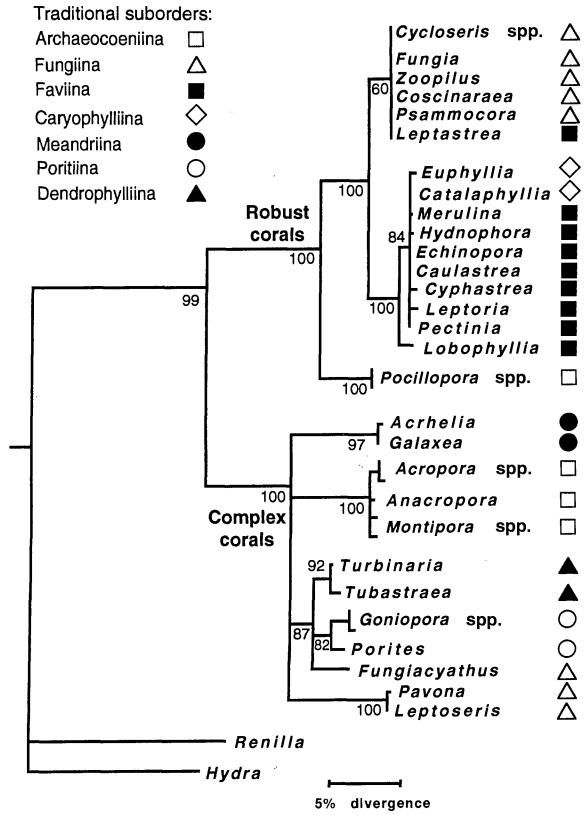

Fig. 1. Molecular phylogram [50% majority rule consensus of 112 most parsimonious reconstructions, generated with a heuristic search with the use of 10 random addition sequences in PAUP (*21*)] of relations among genera of 34 species of corals (Table 1) and two other cnidarians. This phylogram was generated on the basis of sequences from the mitochondrial 16S ribosomal gene region (*15*). The same topology was obtained with neighbor-joining analysis. The numbers on the branches represent values from 100 bootstrap replicates. *Leptastrea* is the only genus that does not group with other genera in the same family, even though it has similar morphological characteristics as well as the same mode of reproduction as other members of the Faviidae. All scleractinian sequences appear to be evolving under similar evolutionary constraints. They are similar to each other and to other metazoan 16S ribosomal sequences in terms of nucleotide composition, ratios of transition and transversion substitutions, spatial patterns of substitutions, rates of divergence, and secondary structure.

Kewalo Marine Laboratory and Department of Zoology, University of Hawaii, 41 Ahui Street, Honolulu, HI 96813, USA.

*To whom correspondence should be addressed. Present address: Laboratory of Molecular Systematics, MRC-534, MSC, National Museum of Natural History, Smithsonian Institution, Washington, DC 20560, USA.

ular evidence agree that at least two of these lineages survive today but disagree as to which families belong to the two ancient clades. The molecular data suggest that the surviving clades diverged before each had developed hard skeletons and that they represent independent experiments in skeletogenesis. An alternative hypothesis—that these two clades represent an ancient mitochondrial duplication, followed by independent assortment in modern families 200 million years later—is unlikely given the genetic economy of mtDNA (*8*).

Lineages derived from independent skeletal origins might be expected to display differences in architecture or skeletal organization. Published morphological data (*9*, *19*, *20*) suggest that there may be important differences between the two mitochondrially identified coral clades (Table 1). One clade, the "robust" corals (Fig. 1), consists largely of taxa with relatively solid, heavily calcified skeletons that result from the solid (septothecal or parathecal) construction of corallite walls. Colonies are largely platelike or massive (although there are some ramose genera). Many grow by intratentacular budding, which is thought to be related to coral shape (*20*).

The other clade consists of "complex" corals (Fig. 1). These corals, except for the Oculinidae, tend to be less heavily calcified, perhaps as a result of the relatively porous (synapticulothecal) construction of corallite walls. In addition, in all but one of the taxa in this clade, the septal walls are built from simple trabeculae that form a relatively porous and loose network of skeletal elements, resulting in a relatively light, complex architecture. Colonies often occur as ramose forms (growing as bushes, thickets, or tables) but also exhibit digitate, columnar, lamellate, and platelike forms. Four of the six families of complex corals grow by extratentacular budding, which may help form architecturally complex skeletons. This clade is exemplified by the genus *Acropora*, which has the greatest number of species and the widest range of growth forms of any coral genus (*9*).

These morphological comparisons are limited, however, because no detailed cladistic analysis of coral skeletons is available. Ancestral and derived character traits are unknown, and homologies among skeletal elements are uncertain. In addition, the morphological generalizations listed above have important exceptions. A comprehensive comparison of coral skeletons is required to discern whether these exceptions are attributable to parallel skeletal evolution in the two divergent mtDNA clades or

Table 1. Coral species sampled and their biological attributes. Attributes listed are for the genus represented by each species (*7*, *9*, *17*, *19*, *20*). The molecular topology for relations among taxa is shown on the left. Codes are as follows: Families and suborders [based on traditional morphological analyses (*9*)]: Fun, Fungiidae; Sid, Siderastreidae; Fav, Faviidae; Car, Caryophylliidae; Mer, Merulinidae; Pec, Pectiniidae; Mus, Mussidae; Poc, Pocilloporidae; Ocu, Oculinidae; Acr, Acroporidae; Den, Dendrophylliidae; Por, Poritidae; Fuc, Fungiacyathidae; Aga, Agariciidae; Fung, Fungiina; Favi, Faviina; Cary, Caryophylliina; Arch, Archaeocoeniina; Mean, Meandriina; Dend, Dendrophylliina; Pori, Porit-ina. Source codes: P, Palau; F, Fiji; H, Hawaii; G, Guam; I, imported corals from unknown locations in the Indo-Pacific confiscated in Hawaii; S, Solomon Islands; E, Eastern Pacific. Calcification: H, heavily calcified; L, lightly calcified. Trabeculae: C, compound; S, simple. Corallite walls: A, absent; E, septothecal; P, parathecal; Y, synapticulothecal; Y-E, initially synapticulothecal, secondarily septothecal. Corallum shape: R, ramose; M, massive (including low incrusting forms); P, platelike (including foliaceous, discoidal, and laminar forms); C, columnar. Colony formation: S, solitary; C, cerioid; P, plocoid; A, phaceloid; M, meandroid; D, dendroid. Budding: E, extratentacular; I, intratentacular.

Species	Family	Suborder	Source	Calcification	Trabeculae	Corallite wall	Corallum shape	Colony formation	Budding
Fungia (Cycloseris) fragilis	Fun	Fung	H	H	C	Y-E	P	S	E
Fungia (Cycloseris) vaughani	Fun	Fung	H	H	C	Y-E	P	S	E
Fungia scutaria	Fun	Fung	H	H	C	Y-E	P	S	E
Zoopilus echinatus	Fun	Fung	F	H	C	Y-E	P	C	I
Coscinaraea sp.	Sid	Fung	S	H	C	Y	MP	C	I
Psammocora stellata	Sid	Fung	H	H	S	AY	MPC	P	I
Leptastrea bottae	Fav	Favi	H	H	S	EP	MR	P	E
Euphyllia ancora	Car	Cary	P	H	S	E	R	M	I
Catalaphyllia jardinei	Car	Cary	I	H	S	E	M	M	I
Merulina scabricula	Mer	Favi	F	H	C	EP	PC	M	I
Hydnophora rigida	Mer	Favi	P	H	S	EP	P	M	I
Echinopora lamellosa	Fav	Favi	F	H	S	EP	PR	P	E
Caulastrea furcata	Fav	Favi	F	H	S	EP	R	A	I
Cyphastrea ocellina	Fav	Favi	H	H	S	EP	M	P	E
Leptoria phrygia	Fav	Favi	G	H	S	E	M	M	I
Pectinia alcicornis	Pec	Favi	P	H	C	A	PR	M	I
Lobophyllia hemprichii	Mus	Favi	P	H	S	EP	MP	AM	I
Pocillopora damicornis	Poc	Arch	H	H	S	A	R	P	E
Pocillopora meandrina	Poc	Arch	H	H	S	A	R	P	E
Acrhelia horrescens	Ocu	Mean	F	H	S	EP	R	D	E
Galaxea fascicularis	Ocu	Mean	G	H	S	EP	PM	P	E
Acropora cytherea	Acr	Arch	G	L	S	Y	R	P	E
Acropora humilis	Acr	Arch	G	L	S	Y	R	P	E
Anacropora sp.	Acr	Arch	P	L	S	Y	R	P	E
Montipora digitata	Acr	Arch	P	L	S	Y	RMP	P	E
Montipora capitata	Acr	Arch	H	L	S	Y	RMP	P	E
Turbinaria peltata	Den	Dend	I	H	S	Y	PM	P	E
Tubastraea coccinea	Den	Dend	H	L	S	Y	RM	D	E
Goniopora stokesii	Por	Pori	P	L	S	Y	RMC	C	E
Goniopora sp.	Por	Pori	P	L	S	Y	RMC	C	E
Porites compressa	Por	Pori	H	L	S	Y	RMP	C	E
Fungiacyathus marenzelleri	Fuc	Fung	E	L	C	E	P	S	E
Pavona varians	Aga	Fung	H	L	S	YA	MP	C	I
Leptoseris incrustans	Aga	Fung	H	L	S	YA	MP	C	I

to unusual mitochondrial evolution.

The deep split in coral mitochondrial lineages traces evolutionary events that predate skeleton formation and are thus invisible in the fossil record. Combined molecular and traditional analyses suggest that there was repeated evolution of the scleractinian skeleton in early seas. Understanding the selective scenario that led to such major convergent events may help to illuminate the evolutionary and ecological basis for the diversity of scleractinians and the complex ecosystems they support.

REFERENCES AND NOTES

1. J. H. Connell, *Science* **199**, 1302 (1978).
2. S. V. Smith, *Annu. Rev. Ecol. Syst.* **23**, 89 (1992); M. T. McCulloch, M. K. Gagan, G. E. Mortimer, A. R. Chivas, P. J. Isdale, *Geochim. Cosmochim. Acta* **58**, 2747 (1994).
3. D. R. Stoddart, *Prog. Phys. Geogr.* **14**, 521 (1990).
4. J. B. C. Jackson *et al.*, *Science* **243**, 37 (1989); T. P. Hughes, *ibid.* **265**, 1547 (1994).
5. T. F. Dana, *Mar. Biol.* **33**, 355 (1975); B. R. Rosen, in *Analytical Biogeography*, A. A. Myers and P. S. Giller, Eds. (Chapman & Hall, New York, 1988), pp. 23–55.
6. A. B. Foster, *Paleontogr. Am.* **54**, 58 (1984).
7. J. E. N. Veron and M. Pichon, *Aust. Inst. Mar. Sci. Monogr. Ser.* **1**, 1 (1976).
8. S. L. Romano, thesis, University of Hawaii, Manoa (1995).
9. J. E. N. Veron, *Corals in Space and Time: The Biogeography and Evolution of the Scleractinia* (Univ. of New South Wales Press, Sydney, 1995) and references therein.
10. W. A. J. Oliver, *Paleobiology* **6**, 146 (1980).
11. Initial work with *Tubastraea coccinea* used mtDNA primers 16Sar and 16Sbr (*12*) and a hydroid primer [primer 1 (*13*)].
12. S. R. Palumbi *et al.*, *The Simple Fool's Guide to PCR* (Special Publication, Department of Zoology, University of Hawaii, Honolulu, 1991).
13. C. W. Cunningham and L. W. Buss, *Biochem. Syst. Ecol.* **21**, 57 (1993).
14. Genomic DNA extractions were from living, frozen, or ethanol-preserved colonies (*8*, *12*). Highly enriched mtDNA was from the ProMega Wizard Minipreps system (*8*) [K. B. Beckman, M. F. Smith, C. Orrego, *Promega Notes* **43**, 10 (1993)]. Coral-specific primers (16Sd-L, 5′-GGTGAGACCTGCCCA-ATGGTT-3′, and 16Sc-H, 5′-AACAGCGCAATA-ACGTTTGAGAG-3′) amplified a 406- to 565-base pair (bp) segment (*8*), which was cloned and sequenced (*8*) with primers 16Sd-L, 16Sc-H, 16Se-H (5′-CGCCTTTAAAAAAGTAAC-3′), and 16Sf-L (5′-CTACATCCAAATTGTTAGAC-3′). Sequences have been deposited in GenBank (accession numbers L75992–L76024 and L76132). Replicate PCR sequences revealed most corals to be homoplasmic. However, three were heteroplasmic with infrequent amplifications of sequences from the opposite major clade. These results were repeatable from different DNA extractions and different colonies and were not found to result from contamination (*8*). The source of heteroplasmy of corals is unknown; hypotheses for the source include hybridization and unusual modes of mtDNA transmission (*8*).
15. Scleractinian sequences were aligned to each other and to sequences from a hydrozoan (*13*) and *Renilla* [D. Bridge, C. W. Cunningham, R. DeSalle, L. W. Buss, *Mol. Biol. Evol.* **12**, 679 (1995)], an anthozoan in the order Octocorallia. This alignment (577 bp, of which 315 bp are variable and 210 bp are informative) includes 16 insertion-deletion events, which are not used in phylogenetic analyses, that map onto clades observed on the phylogenetic tree.
16. D. P. Pashley and L. D. Ke, *Mol. Biol. Evol.* **9**, 1061 (1992).
17. T. W. Vaughan and J. W. Wells, *Geol. Soc. Am. Spec. Pap.* **44**, 1 (1943).
18. E. Roniewicz and E. Morycowa, *Sonderdr. Cour. Forschungsinst. Senckenberg* **164**, 233 (1993).
19. S. D. Cairns, in *Biology of the Antarctic Seas XI*, L. S. Kornicker, Ed. (American Geophysical Union, Washington, DC, 1982), vol. 34, pp. 1–74; J.-P. Chevalier and L. Beauvais, in *Traité de Zoologie: Anatomie, Systématique, Biologie*, P.-P. Grassé, Ed. (Masson, Paris, 1987), vol. 3, pp. 679–753; B. W. Hoeksema, *Zool. Verh.* **254**, 1 (1989).
20. J. W. Wells, in *Treatise on Invertebrate Paleontology: Coelenterata*, R. C. Moore, Ed. (Geological Society of America and Univ. of Kansas Press, Lawrence, KS, 1956), part F, pp. F328–F443.
21. D. L. Swofford, *Phylogenetic Analysis Using Parsimony (PAUP)*, version 3.1.1 (Illinois Natural History Survey, Champaign, IL, 1993).

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Catalysis of Amide Proton Exchange by the Molecular Chaperones GroEL and SecB

Ralph Zahn, Sarah Perrett, Gun Stenberg, Alan R. Fersht*

Hydrogen-deuterium exchange of 39 amide protons of *Bacillus amyloliquefaciens* ribonuclease (barnase) was analyzed by two-dimensional nuclear magnetic resonance in the presence of micromolar concentrations of the molecular chaperones GroEL and SecB. Both chaperones bound to native barnase under physiological conditions and catalyzed exchange of deeply buried amide protons with solvent. Such exchange required complete unfolding of barnase, which occurred in the complex with the chaperones. Subsequent collapse of unfolded barnase to the exchange-protected folding intermediate was markedly slowed in the presence of GroEL or SecB. Thus, both chaperones have the potential to correct misfolding in proteins by annealing.

Molecular chaperones contribute to the folding, assembly, and transport of proteins (*1*). Two homo-oligomeric molecular chaperones have been identified in *Escherichia coli*: the tetradecameric chaperonin GroEL (*2*) and the tetrameric SecB (*3*). GroEL is composed of 57-kD subunits, each of which has three functional domains, arranged as a hollow cylinder of two stacked rings with sevenfold symmetry (*4*). The SecB protein is composed of 17-kD subunits (*3*, *5*). It has been proposed that GroEL acts as a "folding cage" (*6*), in which aggregation of incompletely folded proteins is prevented (*7*). It has also been proposed that chaperonins act as "unfoldases" (*8*), using protein-protein binding energy to reverse incorrect interactions in proteins (*9*). A correction mechanism implies that GroEL would be able to bind a fully unfolded protein. Amide proton exchange has been used to analyze the GroEL-bound state of cyclophilin A (*10*) and α-lactalbumin (*11*), and, in both instances, has shown that the secondary structure of the GroEL-bound substrate is markedly destabilized. However, these and other studies (*12*) could not clearly define to what extent a protein is unfolded in the complex with a chaperone.

Here, we used barnase as a model substrate to investigate the GroEL- and SecB-bound conformations by nuclear magnetic resonance (NMR). This technique is a powerful tool for investigating both the structure and dynamics of proteins with high resolution (*13*). However, although small peptides have been studied with magnetization transfer (*14*), the large molecular mass of most of the chaperones does not allow direct observation of complexed polypeptide chains by NMR because of line broadening when bound to the slowly tumbling chaperone. Amide proton exchange detected by NMR may be applied to provide detailed information on the secondary and tertiary structure of a protein complexed with a chaperone. Chaperone-mediated unfolding of a protein should result in an increase in rate constants of amide proton exchange (k_{ex}^{obs}). Barnase is a particularly suitable substrate for such studies for two reasons. First, the amide protons that exchange in response to local fluctuations in structure and those that require complete unfolding for exchange to occur have been identified (*15*, *16*). Exchange of 15 of the 39 protected amide protons of barnase (Fig. 1A) occurs only from the fully unfolded state; these are termed globally exchanging protons. Most of these protons are located in the central β sheet of barnase. Sixteen amide protons exchange predominantly in response to local fluctuations or "breathing" of the native structure; these are referred to as locally exchanging protons and are mostly situated in the α helices and loop regions. Eight protons exchange by a mixture of the two mechanisms. The second reason that barnase is an appropriate sub-

MRC Unit for Protein Function and Design, Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge CB2 1EW, UK.

*To whom correspondence should be addressed.