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Skeleton Loss in Scleractinia

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Hexacorallia includes the Scleractinia, or stony corals, characterized by having an external calcareous skeleton made of aragonite[1], and the Corallimorpharia, or mushroom corals[9], that lack such a skeleton. Although each group has traditionally been considered monophyletic, some molecular phylogenetic analyses have challenged this, suggesting that skeletal features are evolutionarily plastic[2-4], and reviving notions that the scleractinian skeleton may be ephemeral[5,6] and that the group itself may be polyphyletic[7,8]. Nevertheless, the most comprehensive phylogenetic study of Hexacorallia supported scleractinian monophyly (REF), and so this remains controversial. In order to resolve this contentious issue, we sequenced the complete mitochondrial genome sequences of nine scleractinians and four corallimorpharians and performed phylogenetic analysis that also included three outgroups (an octocoral and two sea anemones). Our data provide the first strong evidence that Scleractinia is paraphyletic and that the Corallimorpharia is derived from within the group, from which we conclude that skeletal loss has occurred in the latter group secondarily. It is possible that a driving force in such skeletal loss could be the high levels of CO_2 in the ocean during the mid-Cretaceous, which would have impacted aragonite solubility[10]. We estimate from molecular divergence measures that the Corallimorpharia arose in the mid-Cretaceous, approximately 87 million years ago (Ma), supporting this view. These data also permit us to date the origin of Scleractinia to 265 Ma, narrowing the gap between the group's phylogenetic origin and its earliest fossil record.

Unlike most metazoan groups with mineralized skeletons, Scleractinia appears relatively late in the fossil record, during the Middle Triassic, roughly 240 Ma[11,12].

However, like most mineralized groups, the early fossil history of scleractinians is quite diverse, with numerous higher taxa represented[1]. This explosive appearance, some ten million years after the great Permian-Triassic extinction event, which was responsible for the disappearance of the Palaeozoic coral groups, is seen as one of the main pieces of evidence in favour of the idea that scleractinians evolved independently from soft bodied ancestors[8]. Assuming a polyphyletic origin for Scleractinia from within the soft bodied hexacorallian groups (i.e., Actiniaria, Ceriantharia, Corallimorpharia, and Zoanthidea) seems particularly reasonable in light of a widely cited molecular-clock estimate of at least 300 Ma for the divergence of extant scleractinians[13] because it would help explain a rather lengthy period of hidden history for the group spanning the entire Permian[8].

On the other hand, lack of completeness in the fossil record obviously implies that the earliest fossil appearance of a group should fall sometime after its phylogenetic origin[14]. We have been able to date the origin of Scleractinia to roughly 265 million years ago during the Permian. This substantially shrinks the length of hidden scleractinian history and thereby weakens the argument that Scleractinia must have originated prior to the advent of a mineralized skeleton. Indeed, recent phylogenetic analyses have consistently found that the only soft bodied group of extant hexacorallians that has a close relationship to extant scleractinians is Corallimorpharia[9,15,16].

Our mitochondrial genome comparisons confirm the existence of two major groups of scleractinians, known as the short (robust) and long (complex) clades because of size differences in mitochondrial rDNA[2,17] (Fig. 1). Surprisingly, these comparisons also unambiguously indicate that the long clade scleractinians are more closely related

to tropical corallimorpharians than they are to the short clade of scleractinians (Fig. 1). In light of these findings, Scleractinia should be redefined to include Corallimorpharia so that the former taxon refers to a clade. More interesting than this systematic conclusion, however, is the inference that a calcified skeleton was likely lost during the ancestry of Corallimorpharia, rather than the alternative that the Scleractinia arose from the soft-bodied corallimorpharians.

Our estimate for the origin of Corallimorpharia is 87 Ma, a time when Cretaceous oceans were typified by high CO_2 levels. It has been suggested that such high levels would have lowered the solubility of aragonite and thereby providing a selective force favouring skeletal loss[10]. Cretaceous reefs were dominated by rudist bivalves rather than corals, which has been attributed to a more favourable biomineralization mechanism under less saturated water conditions[10]. Experimental data on phylogenetically diverse corals supports this notion by showing that skeletal growth is reduced when the ambient carbonate-ion concentration is decreased (Marubini et al, 2003). Therefore, our estimate for the origin of Corallimorpharia is consistent with a scenario of lower calcium carbonate saturation in the Cretaceous.

Our data also reveal interesting patterns in the evolution of mitochondrial genomes. Anthozoan genomes are quite divergent from bilaterian metazoan genomes in the lack of nearly all tRNAs and in the presence of introns. Whereas all scleractinians examined have a uniform mitochondrial gene order, the corallimorpharian mitochondrial genomes are arranged differently. We obtained complete sequences for three genera representing two of the four corallimorpharian families, *Discosoma* sp, *Ricordia florida*, and *Rhodactis* sp., as well as partial sequence for *Corynactis californica* representing a third family. The first three corallimorpharian species have a

unique shared gene order distinct from scleractinian corals (Fig. 2). Our phylogenetic analysis suggests that this is likely a derived trait shared by corallimorpharians. Even though we were only able to amplify a small fragment for *Corynactis*, we were able to determine that this lineage has a highly rearranged gene order. Resolution of the phylogenetic placement of this species will require additional mitochondrial sequence data.

An interesting feature of the mitochondrial molecule in Scleractinia is the clear trend for the expansion of the *nad5* intron. The case is most extreme in the corallimorpharians where most of the genome is inside this intron to the exclusion of the tryptophan tRNA.

Our findings represent the first strong evidence supporting the evolution of mushroom corals from scleractinians, raising important questions such as the role of molecular mechanisms of calcification and biomineralization in organisms where the skeleton is no longer present. Although a lineage of Cretaceous scleractinians was able to adapt to higher CO_2 levels in the ocean, it is not clear that modern corals will necessarily follow this same fate.

Methods

DNA extraction and amplification. Total DNA was extracted from each animal using the DNeasy kit (Qiagen) according to supplier's instructions. Mitochondrial DNA was amplified in two overlapping fragments of approximately equal size by long PCR with primers matching conserved regions of hexacorallian *rrnS* and *rrnL* (SOM). In several cases, one half was obtained with the hexacorallian primers and the second half was amplified with primers designed to match the sequence obtained.

Cloning. We sheared the randomly DNA to a size averaging 1.5 Kb by driving it repeatedly through a narrow aperture in a HydroShear device (Gene Machines). After enzymatic end repair and electrophoretic size selection, these fragments were ligated into pUC18 and transformed into *E. coli* DH10b to create plasmid libraries. These were plated and grown overnight, then colonies were robotically picked (Genetix) into 384-well plates of LB with 10% glycerol.

Sequencing. The 384 well glycerol plates then entered the production sequencing line at the Joint Genome Institute. Clones were amplified by rolling circle amplification (RCA) using Templiphi (Amersham Biosciences), then the product separated for forward and reverse sequencing reactions. Standard M13 -28 and M13 -40 primers were used with BigDye florescent terminators (ABI). These products were purified using solid phase reverse immobilization (SPRI) on magnetic beads and sequence was determined using an ABI 3730XL automated DNA sequencer. Detailed protocols are available at <http://www.jgi.doe.gov/sequencing/protocols/>.

Genome Assembly and annotation. Base calling, assembly, and consensus sequence determination were done using Phred, Phrap, and Consed (**REF**). Manual effort

verified sequence quality and annotated all genes using DOGMA (**REF**) while assuming conformity to the cnidarian mitochondrial genetic code.

Phylogenetic analysis. This included all mtDNAs reported here, plus three other anthozoan cnidarians with sequences available, *Sarcophyton glaucum* (Octocorallia), *Metridium senile*, and *Nematostella vectensis* (both Hexacorallia, Actiniaria). Amino acid alignments were generated using Clustal X (**REF**) for all protein encoding genes except *nad2*, since this gene is not available for Sarcophyton. Regions of unambiguous alignment were excluded, then all were concatenated into a single file. We performed maximum parsimony analysis in PAUP (100 random additions, TBR, 10,000 bootstrap replicates) (**REF**), minimum evolution in MEGA (**add parameters**) (**REF**), and Bayesian analysis in MrBayes 3.0 (prior = mixed amino acid models, likelihood settings= invariants and gamma, mcmc= 2 million generations, printfreq=1000, samplefreq=1000, burnin=500) (**REF**). Sarcophyton was designated as the outgroup to all other taxa.

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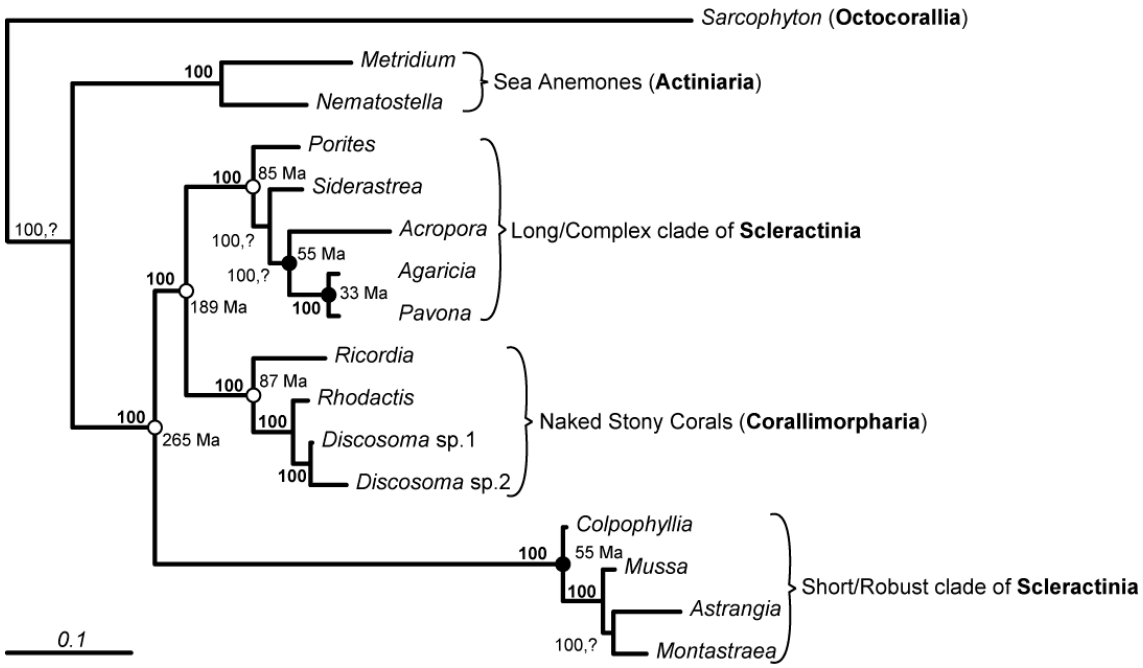
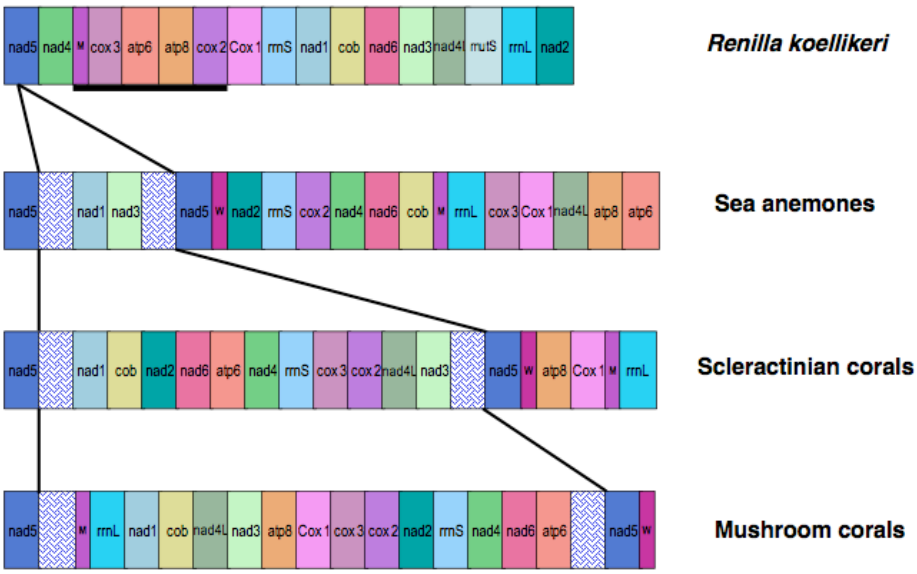
Figure 1. Phylogenetic relationships among sampled scleractinians and corallimorpharians. Bayesian posterior probabilities and maximum parsimony bootstrap values are shown at each node. Single numerals in boldface apply to both methods. Estimated divergence dates are shown for nodes indicated with open circles. Fixed divergence dates based on earliest fossil appearances are shown at nodes indicated with closed circles.

Figure 2. Consensus gene order of cnidarian mitochondrial genomes. Patterned boxes represent non-coding regions of the *nad5* intron. Black lines highlight intron expansion in the different anthozoan genomes.


Sarcophyton (**Octocorallia**)
Metridium
Nematostella
Sea Anemones (**Actiniaria**)
Porites
Siderastrea
Acropora
Agaricia
Pavona
Long/Complex clade of **Scleractinia**
Ricordia
Rhodactis
Discosoma sp.1
Discosoma sp.2
Naked Stony Corals (**Corallimorpharia**)
Colpophyllia
Mussa
Astrangia
Montastraea
Short/Robust clade of **Scleractinia**
100
100,?
85 Ma
55 Ma
33 Ma
189 Ma
87 Ma
265 Ma
0.1


nad5 nad4 M cox 3 atp6 atp8 cox 2 Cox 1 rrnS nad1 cob nad6 nad3 nad4l rrutS rrnL nad2
Renilla koellikeri
nad5 nad1 nad3 nad5 W nad2 rrnS cox 2 nad4 nad6 cob M rrnL cox 3 Cox 1 nad4L atp8 atp6
Sea anemones
nad5 nad1 cob nad2 nad6 atp6 nad4 rrnS cox 3 cox 2 nad4L nad3 nad5 W atp8 Cox 1 M rrnL
Scleractinian corals
nad5 M rrnL nad1 cob nad4L nad3 atp8 Cox 1 cox 3 cox 2 nad2 rrnS nad4 nad6 atp6 nad5 W
Mushroom corals

<TBLTTL> Table 1. Primers used for long PCR –

Universal Primers

Name	Sequence	Reference
12sCrl538F	5'-CWGGTRTTGCATGGCCGTCGTCAATTT-3’	This contribution
12sCrl106R	5'-CCTAAGTYTYAGGGCGTCTGCTGGCACCTT-3’	This contribution
12SaiL	5’-AAACTAGGATTAGATACCCTATTAT-3’	Palumbi et al, 1991
16sbrH	5’-CCGGTCTGAACTCAGATCACGT-3’	Palumbi et al, 1991
16sCrl371F	5’- ATAAGYTTGACAGTTTKGTTGGGGCGA-3’	This contribution
16sCrl388F	5'-GTTGGGGCGACAGTTTKGTTGGGGCGA-3'	This contribution
16sCrl64R	5'-GTYAGTGTTACCGCRGCCATTWARYTRTC-3’	This contributionn
ANTMTSSU-R	5'-GTTCCCYYWCYCTYACYATGTTACGAC-3’	Chen & Yu, 2000

CobF	5'-GGWTAYGTWYTWCCWTGRGGWCARAT-3'	Boore & Brown, 2000
CobR	5'-GCRTAWGCRAAWARRAARTAYCAYTCWGG-3'	Boore & Brown, 2000
Cox3F	5'-TGGTGGCGAGATGTKKTNCGNGA-3'	Boore & Brown, 2000
Cox3R	5'ACWACGTCKACGAAGTGTCARTATCA-3'	Boore & Brown, 2000
HCO2198	5'-TAAACTTCAGGGTGACCAAAAAATCA-3'	Folmer et al, 1994
LCO1940	5'-GGTCAACAAATCATAAAGATATTGG-3'	Folmer et al, 1994

Species-Specific

Name	**Sequence**	**Reference**
Agar3352F	5'-CCCTCACCCTTACTATGTTACGACTTACTCC-3'	T.C.
Agar5050R	5'-GTTGAATCACTGTATGCTGGGAGGGCTTGGA-3'	T.C.
Agar2171F	5'-ACTAAACATAACCCCAGCAAAGAACCAA-3'	T.C.
Agar4854R	5'-GåGGGAAGTAAATAGTGGAAGAAAGGAGT-3'	T.C.
Astran184F	5'-CCTGCCCTATGGTTGTATCTA-3'	T.C.

Astran297R	5'-GATGAACAAACCAACCCTTAG-3'	T.C.
Colp2935F	5'-GCCCGTCGCCTCTACTGA-3'	T.C.
Colp3045R	5'-AATAAATAAAAGCATACC-3'	T.C.
Colp7653R	5'-GGTTGAGCAAATGGGAGTTCT-3'	T.C.
Colp7500F	5'-GATTACGCTACATTTTCACAG -3'	T.C.
Muss851F	5'-AAGTGCGTAGTTGTTTATTTA-3'	T.C.
Muss950R	5'-TATTAATAAGCAAAACAAACT-3'	T.C.
Pori2630F	5’-TTGAAGTGGACAGACAGACAGGGGGCGAATA-3'	T.C.
Pori5563R	5’-TAAAAACCAATAAAACGAAAAGACCAAAATA-3'	
Pav4398F	5’-TCCACTACACCCCCTTCTACTAATACG-3'	
Pav5514R	5’-GGTAAAAGGAAAGGGGGAGAGGAGGAAG-3'	
Pavon1453F	5’-GTACTCCAAAAGGCTCAAACCCACATTCATA-3'	
Pavon4489R	5'-TATGACCTCTTTTATGGGGGCTCCGACAACC-3'	
Sider2925F	5’-TCACAATAACAATCAATAAAAACATAATCTG-3'	
Sider5729R	5’-CGTAAGTAGCAGGGAGCGAAAGCGGAGGAGT-3'	
Rico80R	5’-TAAACCTTTTGGCAGCAG-3'	
Rico19705F	5’-CCCCTCCTAAATCACTCG-3'	

Rico11511F	5’-CACTATTACCCGCACAAG-3'	
Rico11902R	5’-TGGTTGCTGTGTCGGTAG-3'	