Proceedings of the 11th International Coral Reef Symposium, Ft. Lauderdale, Florida, 7-11 July 2008
Session number 26

Coral systematics inferred from the gene galaxin: Exploring phylogenetic relationships using a putative determinant of skeletal morphology

H.H. Wirshing and A.C. Baker

Division of Marine Biology and Fisheries, Rosenstiel School of Marine and Atmospheric Science, University of Miami, 4600 Rickenbacker Causeway, Miami, FL 33149, USA

Abstract. Molecular phylogenies and morphological datasets may not entirely agree, and often fail to accurately reconcile many phylogenetic uncertainties. In corals, one way of resolving this disagreement is to complement the existing sets of genetic markers by identifying and analyzing genes directly responsible for generating morphology. Since traditional taxonomy in corals has been dependent almost entirely on their biomineralized skeletal morphology, the use of genes controlling these processes may help identify cases where morphological taxonomy has led to different conclusions about scleractinian evolution. To test this approach, we used the cDNA sequence of the protein galaxin, isolated from the organic matrix of *Galaxea fascicularis*, to amplify a ~363bp DNA fragment from 18 scleractinian species in 8 genera. Translated amino acid sequences revealed minimal within-species sequence variation within *G. fascicularis* collected from 5 different geographic locations. Phylogenetic reconstruction found three well-supported clades, one containing members of the Acroporidae (Complexa), one comprising members of the Oculinidae and Euphyllidae (Complexa), and one comprised of genera from the Siderastreidae, Faviidae and Agariciidae (Complexa and Robusta). These preliminary results illustrate an approach which may help better understand the morphological variation upon which traditional taxonomy has been based.

Key words: coral, systematics, phylogenetics, taxonomy, galaxin, DNA, scleractinia.

Introduction

Uncertainties in scleractinian taxonomy are rooted in the inability to accurately decipher morphological homologies. Formal taxonomic descriptions of scleractinians have utilized a variety of morphological features ranging from septal microstructure (Wells 1956) to corallite and colony morphology (Veron 1995, 2000) to create taxonomic classifications that imply the diversification of the group. Molecular DNA approaches provide an opportunity to independently test these taxonomic hypotheses. Over the last ~12 years, the systematic relationships of scleractinian corals have been challenged by phylogenetic reconstructions based on nuclear and mitochondrial DNA (Romano and Palumbi 1996, Chen et al. 2002, Fukami et al. 2004, 2008). These studies recovered phylogenies that do not agree with many of the traditional taxonomic assignments within the Scleractinia, particularly at the subordinal and family levels.

At least two major themes have arisen concerning the evolution of the scleractinia based on DNA evidence. The first theme asserts the scleractinian lineage is comprised of two major clades – the "robust" and "complex" corals (Romano and Palumbi 1996, 2000, Chen et al. 2003). The "robust" corals consist of heavily calcified corals as a result of a dense construction of their corallite walls (e.g. septothecal or parathecal). In contrast, the "complex" corals contain many corals that build more lightly calcified skeletons due to a relatively porous skeletal assembly of their corallite walls (e.g. synapticulothecal). A second theme states that there is pervasive morphological convergence of many Atlantic and Pacific scleractinian genera (Fukami et al. 2004, 2008). Many Atlantic genera are more closely related to each other than they are to their Pacific congeners and *vice versa*. This implies that many Atlantic and Pacific lineages each experienced their own intra-basin radiations and converged on similar morphological features, detected by examination of their corallite wall architecture.

Other studies have narrowed the link between molecular phylogenies and the biomineralization patterns of corallite microstructures in order to determine homology among taxonomically questionable groups. Benzoni et al. (2007) examined septal micro-features of the centers of calcification of *Psammocora* spp. of debatable taxonomic affinity. Cuif et al. (2003) demonstrated that the growing

edges and numerous granules and spines related to septal growth, once considered unimportant ornaments, are important features of the initial skeletal framework and contain valuable phylogenetic information.

All of these studies assume that the molecular markers used can serve as accurate proxies for the evolution of their taxonomic characters, and can thus be tied to morphological changes in those taxa. In many cases, this assumption has been validated and accurate corroborations between molecular phylogenies and coral morphologies have elucidated many novel evolutionary relationships (e.g. Romano and Palumbi 1996, Fukami et al 2004, 2008). However, many systematic relationships still remain unresolved because of the inability of the molecular markers used to unequivocally resolve all of the systematic uncertainties within the Scleractinia. An approach to solving this problem is to augment the current regime of scleractinian molecular markers by identifying and analyzing genes directly responsible for generating morphology. Unlike most organisms (whose taxonomy is based on many phenotypic characteristics that collectively can not be pinned to a single genetic function), coral taxonomy is almost exclusively based on their skeletal (calcium carbonate) morphology that is genetically controlled by the biomineralizing machinery of their genomes. This characteristic offers the possibility to utilize these molecular "drivers" (genes responsible for calcification/morphology) in concert with existing molecular markers in order to potentially identify areas where morphological taxonomy may have led to different conclusions concerning scleractinian evolution.

This study represents an initial look at the utility of the gene galaxin, a region that encodes for a protein isolated from the organic matrix (a consortium of proteins that forms the scaffolding for biomineralized structures) of the coral *Galaxea fascicularis* (Fukuda et al. 2003), for phylogenetic reconstruction.

Material and Methods

Samples were collected by coring a 1cm x 1cm fragment from individual coral colonies. Tissue and skeletal material were stored in either 95% EtOH or saline DMSO. Total genomic DNA was extracted by established protocols (see Rowan and Powers 1991). Primers Gal16(F) 5'-GGAGCTACCCAGTTATGCTG-3' and Gal13(R) 5'-TATGCTAGCCCAGCACAGGAT-3' were designed from the published cDNA sequence of the gene galaxin (Fukuda et al. 2003) and were used to amplify all *Galaxea* spp., *Euphyllia* spp., *Diploria labyrithiformis*, *Pachyseris speciosa*, and *Acorpora cervicornis* samples. Primer Gal13mc(R) 5'-TATGCAAGTCCTGCGCAAGAT-3', designed from the galaxin sequence of *Montipora capitata* (Accesion # EU022118) was used with primer Gal16(F) (see above) to amplify all *Montipora* spp., *Siderastrea* spp., and *Solenastrea bournoni* (Table 1). PCR (polymerase chain reaction) was conducted using Promega GoTaq® DNA polymerase and provided buffers. Thermocycler reaction parameters were: initial denaturing step at 94°C for 3 min., followed by at total of 35 rounds of 94°C for 1min., 50°C for 1min., and 74°C for 1min. A final extension step of 72°C for 7min completed the reaction. PCR amplifications were confirmed by running products on 2% agarose gels. All successful amplifications were directly sequenced on an ABI 3730 sequencer and forward and reverse sequence contigs were edited with the software ContigExpress in Vector NTI Advance™ 10 (www.Invitrogen.com).

Primer Pair	Species (with ID)	Accesion #
Gal 16(F)/ Gal 13(R)	*Galaxea fascicularis* (AS301)	TBA
	Galaxea fascicularis (MLD197)	TBA
	Galaxea fascicularis (WA270)	TBA
	Galaxea fascicularis (MA142)	TBA
	Galaxea fascicularis (JP581)	TBA
	Galaxea astreata (GA33)	TBA
	Euphyllia ancora (JP163)	TBA
	Euphyllia glabrescens (JP603)	TBA
	Euphyllia yaeyamaensis (JP655)	TBA
	Diploria labyrinthiformis (B56)	TBA
	Pachyseris speciosa (MLD1)	TBA
	Acropora cervicornis (B38)	TBA
Gal 16(F)/ Gal 13mc(R)	*Montipora faveolata* (JP426)	TBA
	Montipora mollis (WA219)	TBA
	Montipora caliculata (AS237)	TBA
	Montipora monasteriata (AS216)	TBA
	Montipora grisea (AS219)	TBA
	Montipora verrucosa (JP 447)	TBA
	Siderastrea siderea (KL101)	TBA
	Siderastrea radians (KL55)	TBA
	Solenastrea bournoni (KL86)	TBA

Table 1: Coral species amplified, and GenBank Accesion #'s, with custom oligonucleotide markers designed from galaxin cDNA sequences of *G. fascicularis* (Fukuda et al. 2003) and *M. capitata* (Accesion # EU022118). ID codes contain geographic location and collection number. AS=American Samoa, MLD=Maldives, WA=Western Australia, MA=Mauritius, JP=Japan, GA=Gulf of Aqaba, B=Belize, KL=Key Largo.

DNA sequences were translated into amino acid sequences using GeneDoc (Nicholas and Nicholas 1997) and aligned using MUSCLE (Multiple Sequence Comparison by Log-Expectation) (Edgar 2004). ProtTest (Abascal et al. 2005) was used to determine the model of amino acid substitution that best fit the alignment. Phylogenetic reconstruction was performed using Bayesian Maximum Likelihood (MB) in MrBayes v3.1.2 (Huelsenbeck and Ronquist 2001). Tree space was explored using 4 Markov chains for 1,000,000 generations with trees sampled every 100 generations. Posterior probabilities were calculated by setting the "burn-in" to 2500 (25% of

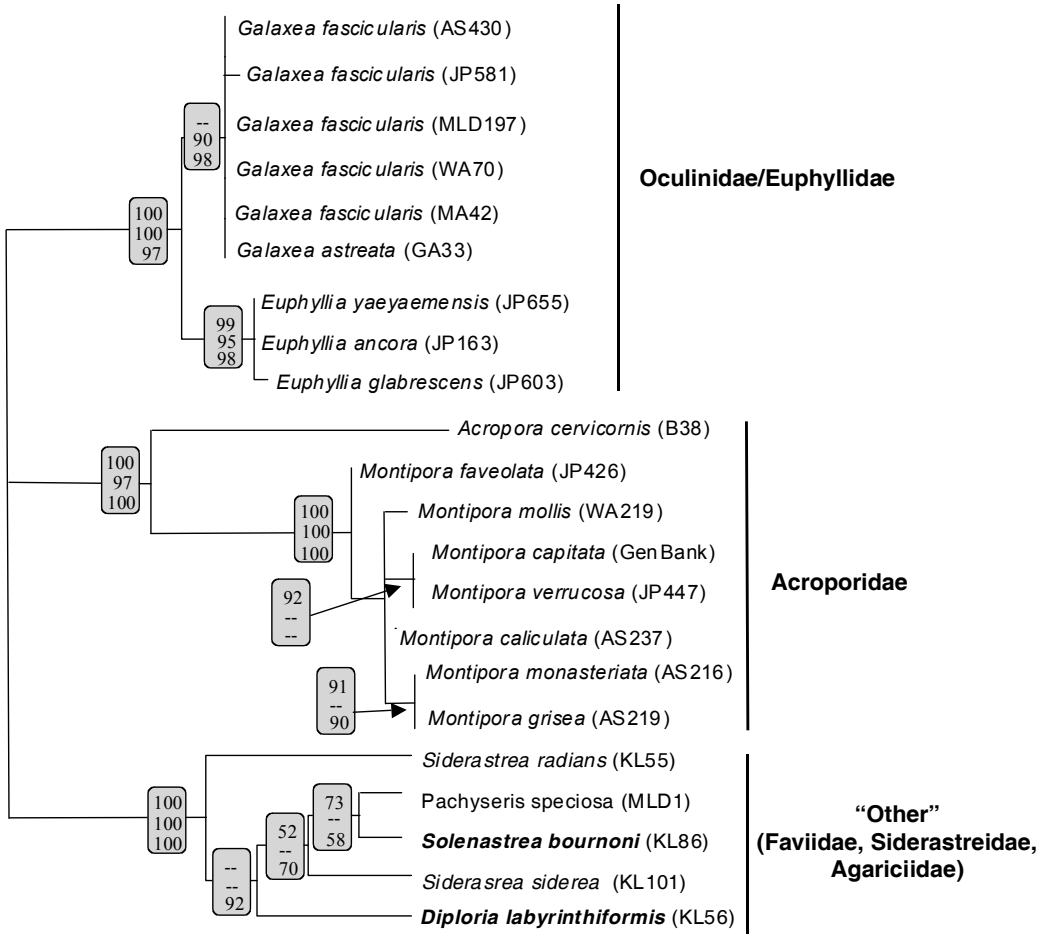

Figure 1: A cladogram depicting relationships recovered by Bayesian Maximum Likelihood (MB), Maximum Parsimony (MP), and Minimum Evolution (ME). Posterior probabilities (MB) and bootstrap values for MP and ME >50 for each node are shown in boxes from top to bottom, respectively. Vertical lines on right highlight clades that correspond to familial groups recovered (Oculinidae/Euphyllidae and Acroporidae) or where relationships were uncertain ("other"). "Robust" corals are in bold. "Complex" corals are in normal font. "--" indicates node <50 or not following branching topology for that method.

sampled trees), and values were generated from the remaining 7500 trees. Maximum Parsimony (MP), with branch swapping set to TBR (tree bisection-reconnection) and starting trees gained by stepwise addition (random), and Minimum Evolution (ME) (default parameters for amino acid sequences) were performed using PAUP* v4.0b10 (Swofford 2002). 1000 bootstrap replicates were performed for each method.

An appropriate outgroup to root the phylogenetic trees was not available, as the gene galaxin has not been found outside the Scleractinia. As a result, all trees were rooted with midpoint rooting in PAUP*. MrBayes does not allow for midpoint rooting, so no root was used.

Results

The sequenced PCR fragments, after editing, were 363bp in length except for the *Monitpora* spp. and *Acropora cervicornis*, which were 357bp. Translated amino acid sequences were, thus, 121 and 119 amino acids in length. Two indels, of one amino acid each, were located at the 9th and 37th positions in the alignment (data not shown). ProtTest found the model Dayhoff + G to best fit the alignment with both Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC) frameworks.

All three methods of phylogenetic reconstruction found three major clades with high statistical support – *Galaxea* spp.-*Euphyllia* spp., *Montipora* spp.-*A. cervicornis*, and *Siderastrea* spp.-*Pachyseris speciosa*-*Solenastrea bournoni*-*Diploria labyrinthiformis* (Fig. 1). The phylogenetic relationship among them was unresolved. The *Montipora* spp.-*A. cervicornis* clade corresponds to generic memberships within the family Acroporidae. The *Galaxea* spp.-*Euphyllia* spp. clade unites genera from the families Oculinidae and Euphyllidae. And a third clade joins species from four genera and three families (Faviidae-Siderastreidae-Agariciidae).

The *G. fascicularis* from five different geographic locations (Table 1) were invariant in their amino acid sequences except for one change (threonine to serine) in the Japan individual at position 64 in the alignment (data not shown). Among congeners, the relationships among the three species of *Euphyllia* were unresolved. The *Montipora* spp. did, however, show some structuring, although statistical support was inconsistent among all phylogenetic reconstruction methods. For example, *M. capitata* and *M. verrucosa* grouped as supported sister species with MB (92) only, and *M. monasteriata* and *M. grisea* fromed a clade supported by MB (91) and ME (90) (Fig. 1).

The "Faviidae-Siderastreidae-Agariciidae" clade contained unexpected relationships, most of which were not supported by high bootstraps values or posterior probabilities (Fig. 1). For example, *S. radians* and *S. siderea,* two well-known morphological sister species, did not group monophyletically.

Discussion

The organic matrix of corals is a consortium of proteins that is intimately associated with the calcification process in many organisms including corals (Simkiss and Wilbur 1989). Cuif and Dauphin (2005) describe the calcification procedure in corals as a biochemically driven process where genetically controlled coral fibers, of which sulfated acidic proteoglycans are likely to play a significant role, guide calcification in repeated cycles. Similarly, Meibom et al. (2008) state that the morphology of the coral skeleton is related to the genetically defined positions and distributions of their centers of calcification. It is, therefore, plausible to assume that much of the evolutionary history of the coral skeleton, including convergences, may be found in the genes involved in the biomineralization process of the coral skeleton. It is still unknown, however, to what degree (or if at all) organic matrix proteins, including galaxin, play in that process.

Here, we use only ~40% of the organic matrix protein sequence of galaxin across several coral taxa. We utilized translated amino acid sequences in order to examine any variation in the protein sequence that may account for morphological differences in coral skeletons. Our phylogenetic reconstruction employed only a small set of scleractinian species due to the inability to successfully amplify galaxin across divergent taxonomic groups. Our tree, however, recovered many traditional taxonomic groupings based on morphology as well as groupings based on studies using other DNA markers. For example, the *Montipora* spp.-*A. cervicornis* clade recovered corroborates the well-established morphological relationship between these two genera (e.g. Wells 1956), both of which are placed within the family Acroporidae. This relationship has also been verified by molecular phylogenies using both nuclear and mitochondrial DNA (Romano and Palumbi 1996, Chen et al. 2002, Fukami et al. 2008). Although not well supported by all of the reconstruction methodologies used, the pairing of four *Montipora* spp. (*M. capitata* with *M. verrucosa* and *M. monasteriata* with *M. grisea*) corroborates morphological similarities among these species. For example, both *M. capitata* and *M. verrucosa* share large coenosteum tuberculae forming verrucae and *M. monasteriata* and *M. grisea* approach each other morphologically with their coenosteum tuberculae and thecal papillae (Veron 2000).

The close relationship between the *Galaxea* spp. and *Euphyllia* spp. recovered is similar to that found by Fukami et al. (2008). Although *Galaxea* is in the family Oculinidae (Veron 2000), it likely shares a closer phylogenetic history with members of the family Euphyllidae (Fukami et al. 2008).

The "Faviidae-Siderastreidae-Agariciidae" clade contained unexpected groupings. The polyphyly of *S. radians* and *S. siderea* is an example. Given the poor statistical support for the branching topology within this clade, however, it is likely that insufficient sampling and/or the lack of phylogenetic information available at this locus resulted in the uncertain relationships found.

All of the taxa used in this analysis are considered "complex" corals except two species, *S. bournoni* and *D. labyrinthiformis* (see Romano and Palumbi 1996 and Fukami et al. 2008). Trends concerning the evolution of the "complex" and "robust' corals were, therefore, not possible. Our "Faviidae-Siderastreidae-Agariciidae" clade grouped two "robust" corals, *S. bournoni* and *D. labyrinthiformis*, with three "complex" coral species (Fig 1). The relationship of *P. speciosa* to these "robust" corals is inconsistent with Fukami et al. (2008). They recovered *P. speciosa* as a sister taxon to a clade containing *Galaxea* and *Euphyllia* spp. However, *Siderastrea* branches divergently among the "complex" corals (Fukami et al. 2008), and it is, therefore, likely that both *S. bournoni* and *D. labyrinthiformis* would share a closer phylogenetic affinity to the *Siderastrea* than to the other taxonomic groups in our phylogeny.

This study represents the first use of an organic matrix protein to reconstruct phylogenetic relationships among corals. There is still much that is not known about the gene galaxin or the biomineralization process in corals. For example, how many other genes are involved in the calcification process? And how exactly do organic matrix proteins influence morphology? The gene

galaxin is likely part of a gene family, and there are at least two galaxin-type genes indentified (D. Miller, unpub. data). It is still uncertain how these genes, and the proteins they encode, affect skeletal morphology and what they may reveal about the evolutionary history of the entire scleractinian lineage. Future work will involve complementing organic matrix loci with other established molecular markers. A complementary approach should help unravel the uncertainties of scleractinian evolution and possibly highlight areas where morphological taxonomy and molecular phylogenies have been confounding and/or add corroborative support to established relationships.

Acknowledgement

We thank Craig Starger for sequencing several of the samples, and Adrienne M.S. Correa and Rachel Silverstein for the samples from Western Australia. This work was supported by National Science Foundation BIO-OCE 0547169.

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