Coral Reef Genomics: Developing tools for functional genomics of coral symbiosis

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ABSTRACT

Symbioses between cnidarians and dinoflagellates in the genus *Symbiodinium* are widespread in the marine environment. The importance of this symbiosis to reef-building corals and reef nutrient and carbon cycles is well documented, but little is known about the mechanisms by which the partners establish and regulate the symbiosis. Because the dinoflagellate symbionts live inside the cells of their host coral, the interactions between the partners occur on cellular and molecular levels, as each partner alters the expression of genes and proteins to facilitate the partnership. These interactions can examined using high-throughput techniques that allow thousands of genes to be examined simultaneously. We are developing the groundwork so that we can use DNA microarray profiling to identify genes involved in the *Montastraea faveolata* and *Acropora palmata* symbioses. Here we report results from the initial steps in this microarray initiative, that is, the construction of cDNA libraries from 4 of 16 target stages, sequencing of 3450 cDNA clones to generate Expressed Sequenced Tags (ESTs), and annotation of the ESTs to identify candidate genes to include in the microarrays. An understanding of how the coral-dinoflagellate symbiosis is regulated will have implications for atmospheric and ocean sciences, conservation biology, the study and diagnosis of coral bleaching and disease, and comparative studies of animal-protist interactions.

INTRODUCTION

The field of genomics has generated new tools for studying biology at the cellular and molecular levels. For example, high-throughput methods for examining gene expression (DNA microarrays) have allowed biologists to collect information about the activity levels of thousands of genes at once, thus permitting the identification of genes and pathways that operate in a particular scenario. While developed primarily for biomedical questions, these approaches are successfully being applied to ecological and environmental questions. We are interested in developing cDNA microarrays to study

interactions between corals and their endosymbiotic dinoflagellates. In particular, we are interested in identifying genes and pathways that are involved in establishing and regulating the symbosis.

Significance of coral-algal symbioses: Scleractinian corals are key organisms in the formation of the most phylogenetically diverse of marine ecosystems, coral reefs. Reef-building scleractinian corals and shallow water octocorals are characterized by their mutualistic symbiosis with dinoflagellate algae (zooxanthellae). By mechanisms still poorly understood, the zooxanthellae contribute to their hosts' nutrition and high rates of calcification (e.g., Muscatine and Cernichiari 1969; Lewis and Smith 1971; Muscatine and Porter 1977; Chalker and Taylor 1978; Falkowski et al. 1984; Muscatine et al 1984). These are vulnerable ecosystems, however, because coral health is so highly dependant on a functioning symbiosis. Disruption of the coral-zooxanthellae symbiosis can have fatal consequences for the host, thus coral survival is highly correlated with a stable symbiotic relationship. Understanding the host-symbiont interactions gains a new sense of urgency under the present and increasing threat of global warming.

Bleaching is often characterized as a stress response of the host (Glynn 1993, Brown 1997); but is increasingly clear that the zooxanthellae may play an active role in the disruption of the symbiosis, when responding to stresses such as extremes in temperature or light (Rowen et al 1997; Perez et al 2001, Iglesias-Prieto et al 1992, Hoegh-Guldberg 1999, Fitt et al 2001, Jones and Hoegh-Guldberg, 2001). It is critical that we begin to understand in more detail the events that occur to cause disruption in the symbiotic interaction. However, to do this, we need to understand what is involved in the stable and functioning symbiosis.

It is now well established that the genus *Symbiodinium* is highly speciose, with at least 6 clades containing hundreds of distinct genotypes. This diversity carries over to the symbiosis; molecular approaches have shown that some hosts can simultaneously harbor more than one symbiont genotype (Rowan and Knowlton, 1995, Rowan et al 1997, Coffroth et al 2001), and that a host's complement of symbionts can change over time. For example corals recovering from bleaching became populated with a different genotype than they had before bleaching (Baker 2001, Baker et al. 2004, Lewis and

Coffroth 2004, Rowan 2004, Toller et al, 2001). This tremendous diversity and apparent flexibility in host-symbiont specificity suggests that there may be complex and environmentally-regulated processes leading to the specificity of a particular host-symbiont combination.

***Establishment of the host-symbiont relationships*:** When corals (scleractinian and octocorals) reproduce, most species produce eggs without zooxanthellae (Szmant 1986, Harrison and Wallace 1990). The larvae or the newly settled polyps are faced with acquiring zooxanthellae from the environment. The larvae of some species can acquire zooxanthellae upon development of a functioning mouth and gastric cavity (Schwarz et al 1999, Schwarz et al 2002), and newly settled hard corals and gorgonians can acquire zooxanthellae within a week when maintained on the reef as well as when cultured in the laboratory (Coffroth et al 2001, Szmant in prep). Some corals brood their larvae to an advanced planula stage and most of these acquire their zooxanthellae from the parental colony before they are released (Szmant 1986, Harrison and Wallace 1990).

This pattern of corals producing asymbiotic larvae, with each new generation acquiring zooxanthellae *de novo*, presents us with an ideal experimental system with which to study the development of the symbiosis at the molecular level. We can rear large quantities of larvae and experimentally infect them with symbionts, to ask questions about which genes are activated or repressed when the symbiosis is initiated.

***The Central Dogma*:** Every individual organism responds to changes in its internal or external environment by altering the suites of proteins that are used to respond to the changes. Although it is currently impossible to build a comprehensive picture of how biochemical pathways are modified in response to variables, there are methods to study each of the steps that alter protein synthesis and behavior. Currently, the most accessible stage of protein regulation that can be examined is the gene expression step, because microarray technology has become increasingly available to the larger scientific community. Examining the gene transcript levels present in a complete mRNA sample at any specific time can give a reasonable approximation of which proteins are active. By capturing the mRNA transcripts that are generated when genes are expressed, the

researcher can compare the populations of mRNA under different conditions, to identify genes that are upregulated or downregulated. It is this approach we are are employing to study the coral symbiosis.

Molecular interactions and regulation of the symbiosis: Microarray technology is a relatively new and powerful technique that permits a genome-wide assessment of gene expression in a time series (Schena et al 1995), making it an ideal tool for examining complex interactions between organisms (reviewed in Gibson, 2002). This approach will allow us to effectively look at snapshots of the transcriptional state of genes as the symbiosis is initiated and as it matures. Microarray analysis can thus clarify the physiological process of symbiosis by relating the transcriptome to a time course that marks the initiation, maturation and decline of the symbiosis. An additional advantage to using a gene expression approach is that the large number of Expressed Sequenced Tags (EST) data will generate a first glance at genomic composition for these organisms that will benefit any other genomic study undertaken for these species in the future. It is likely that both a coral genome and a symbiont genome will be sequenced in the next few years, and this EST project will be of use in annotating those genomes.

One of our main goals is to gain a comprehensive understanding of the transcriptome involved in the symbiotic relationship based on both the host and symbiont gene expression profiles. Evidence from model organisms as diverse as yeast and mouse has demonstrated a marked shift in gene expression profiles under different metabolic states correlated with environmental conditions (De Risi et al 1997, Chu et al 1998, Lee et al 2000). In the case of anthozoan hosts and their symbionts, we will test the hypothesis that their tight mutualistic relationship is highly dependant on suites of genes that are differentially expressed during different stages of the symbiosis, as well as under varying environmental regimes.

Experimental approach

Our goal is to gain an understanding of how coral-algal symbioses are set up at a molecular level, from each partner's perspective. Our two working goals are: first, to

generate sequence data from transcriptomes of several stages of coral, symbiont, and symbiosis development and to perform a thorough annotation of each gene identified from this sequencing effort. This will serve as a valuable resource to the entire coral research community, as well as to other research groups that are interested in comparative genomics. Our second goal is to perform microarray expression profiling to identify genes and cellular pathways involved in host-zooxanthella symbioses. To develop the microarrays, we are constructing multiple cDNA libraries representative of different ontogenetic stages involved in the initiation and maintenance of symbiosis. Based on the sequence information obtained under Goal 1, individual clones from each library will be chosen for the microarrays, with the ultimate goal of creating as comprehensive a selection of genes as possible from each partner. The resulting microarrays will then be employed in a time course study, to identify genes that are differentially expressed under different stages of symbiosis. They will also be employed to study other questions in coral/dinoflagellate biology, for example response to environmental stress.

METHODS

Study organisms

We are using two scleractinian corals, *Montastraea faveolata* and *Acropora palmata,* and the dominant symbiont strain from each species as our study organisms. These two species are the major reef-building corals of the Caribbean and are therefore extremely important to the ecology of the Caribbean. For many reasons, they provide an excellent model system for studying the symbiosis. First, they produce azooxanthellate eggs and larvae that can be reared under laboratory conditions. Second, spawning of these species is predictable, producing copious amounts of material to generate thousands of larvae (Szmant 1986, 1991). Third, we can experimentally infect the larvae with selected strains of *Symbiodinium*. Together, these aspects of the experimental system sets the stage for generating sufficient material to proceed with the microarray studies.

Collection of Material

Gametes from the two coral species were collected as described in Szmant et al. (1997). Conical nets suspended over spawning colonies collected the positively buoyant gamete bundles. Gamete bundles from multiple colonies (and in the case of *A. palmata*, from multiple reefs) were combined within an hour of release to obtain cross-fertilization among different genets (these hermaphroditic species do not self-fertilize). Sperm concentrations were not measured but the gametes were kept concentrated in a ratio of 20 % gamete bundles to 80 % seawater to maintain high sperm concentrations. After one hour, sperm were washed out with several rinses of clean filtered seawater. Batches of fertilized eggs were put into 4 L plastic bins for culture at concentrations of about 2-3 thousand embryos per liter. Water was changed 2-3 times per day or whenever it became cloudy.

M. faveolata larvae reach a swimming planula stage by 48 hrs after fertilization, while those of *A. palmata* take ca. 60 hrs. Settlement begins about 2 days later.

Infection studies

We are currently in the process of isolating the dominant strain of *Symbiodinium* in *A. palmata* and *M. faveolata* so that our cDNA libraries will represent the host in association with its dominant strain. Future studies will examine how different host-symbiont combinations influence gene expression patterns. Currently we have 6 and 54 isolates from *A. palmata* and *M. faveolata*, respectively that we are genotyping using microsatellites. These represent members of clade A, B and C for *A. palmata* and clades A, B, C and D for *M. faveolata* (data not shown). Zooxanthella isolations and characterizations followed standard methods (Santos et al 2001, Coffroth et al. 2001).

Target stages for cDNA library construction

Ultimately, we will create a total of 16 cDNA libraries that will represent a range of stages in both host and symbiont development and symbiosis: these 16 target stages

are described in Table 1. This paper describes cDNA libraries from 4 of the target stages: 1) *A. palmata* eggs 2) *M. faveolata* eggs, 3) *M. faveolata* embryos and 4) *M. faveolata* adult tissue.

Stage	Source of RNA	*M. faveolata*	*A. palmata*
NS	Coral Eggs	**Completed**	**Completed**
NS	Coral Embryos	**Completed**	In progress
NS	Coral Larvae	In progress	In progress
S	Coral Larvae	In progress	In progress
S	Coral adult colony	**Completed**	In progress
NS	*Symbiodinium* grown in culture	In progress	In progress
S	*Symbiodinium* isolated from larvae	In progress	In progress
S	Native *Symbiodinium* isolated from adult colony	In progress	In progress

Table 1. Target stages of symbiosis for which cDNA libraries will be created. Status of each library is indicated for both host species and the respective strains of *Symbiodinium* used to infect the host. NS = non-symbiotic, S = symbiotic.

cDNA library construction

Total RNA was isolated from tissue samples using Qiazol reagent (Qiagen), according to manufacturer's instructions, and passage through a 21G syringe to lyse the cells. To remove residual phenol or other contaminants, the RNA was purified using an RNEasy clean up kit (Qiagen). Total RNA was quantified using an Agilent Bioanalyzer. To construct the cDNA libraries, we chose to use the Clontech SMART cDNA Library Construction Kit with the pDNR-lib vector because the kit is designed to create directionally cloned cDNA libraries from very small amounts of starting RNA (nanogram to microgram amounts of total RNA). Briefly, RNA was reverse transcribed to cDNA using PowerScript Reverse Transcriptase, using kit primers SMART IV Oligonucleotide and CDS III/3' PCR primer. The cDNA was PCR-amplified using the Advantage 2 PCR kit, using the MSART 5' PCR III primer and CDS III/3' PCR primer, using between 18 and 26 cycles, depending on the starting amount of RNA. The polymerase was removed with Proteinase K, and then the amplified cDNA was digested with the restriction enzyme *Sfi*I, which produces different 5' and 3' overhanging sequences to allow for

directional cloning into the pDNR-lib vector. To minimize cloning incomplete or degraded transcripts, we preferentially selected cDNA >500bp, by first passing the *Sfi*I-digested cDNA over CHROMA SPIN-400 columns, and then cutting out a >500bp smear from a 1.1% agarose gel. The size-selected cDNA was ligated to the pDNR-lib vector. Electrocompetant cells were transformed with the vector, grown overnight in liquid suspension and then plated onto Teknova LB agar plates with 30µg/ml chloramphenicol. Depending on the library, between 2 and 5 plates were chosen for sequencing.

From each plate chosen for sequencing, 384 clones were robotically picked from the plate and processed for Rolling Circle Amplification (RCA) to amplify the vector. These were then sequenced from both 5' and 3 ' ends, on ABI 3730 Sequencers.

Sequence Assembly and Analysis

EST clusters and consensus sequences were generated using the Joint Genome Institute's Prototype EST Analysis Pipeline. All results are preliminary. Vector sequence was trimmed from each EST and then ESTs were clustered based on NCBI blastn similarities (Altschul 1990). Blast hits considered significant cover at least 150 bases with 96% identity. All ESTs sharing significant similarities were clustered together. Additionally, ESTs from the same cDNA clone, but without significant similarity, are placed in the same cluster. Clusters containing a single EST are allowed: ESTs with no significant similarities to any other EST are given their own cluster ID number and are referred to as "singletons."

After ESTs are placed in clusters, the ESTs making up each cluster were assembled into a consensus sequence using phrap (Green 1996) with default parameters. Due to splice variants or sequencing errors, phrap may produce more than one consensus sequence for each cluster. All consensus sequences are then compared to nr, using NCBI blastx with default parameters.

(Note to EDITOR: we are in the process of submitting all of the EST sequences to GenBank and would like to have the accession numbers listed here, once we receive them.)

Categorization of ESTs into larger-order biological functions

Our goal is to comprehensively annotate the ESTs in order to select genes that may be involved in biological pathways in host-symbiont interactions, for example roles in immunity (i.e. host-symbiont recognition), calcification, and specific metabolic pathways. To begin developing ways to group genes into pathways and higher-order biological categories, we performed a tblastx search against a TIGR database of human ESTs, each of which has a unique ID number that connects the EST to an assigned biological function (EGAD, The Expressed Gene Anatomy Database; http://www.tigr.org/tdb/egad/egad.shtml). The sequences in our libraries that matched EGAD sequences were sorted into biological categories and summed. We then calculated the fraction of EGAD matches that fall into each higher order category.

RESULTS

cDNA library sequence assembly and annotation

We have constructed four cDNA libraries from the following tissues, *A. palmata* eggs, *M. faveolata* eggs, *M. faveolata* 60 hour old embryos, and *M. faveolata* adult. We sequenced 3450 clones from both the 5' and 3' ends, to generate 6995 ESTs that assembled into 2144 clusters (i.e., two or more ESTs that overlap for part or all of their length, see methods) and 1552 singletons (i.e., a single EST sequence that does not match or overlap with any other EST sequence from that cDNA library, see methods). A summary of the cDNA libraries is shown in Table 2.

We performed blastx searches against the non-redundant (nr) databases at GenBank to 1) assign putative identities to the genes in our libraries (Table 3), and 2) to determine the how many of the sequences in our libraries may represent "novel" sequences (Table 2). To identify which sequences may represent "novel" sequences (i.e., almost certainly do not match any other sequence in the nr databases) we assigned a cutoff E value of 0.1 (Table 2). The E value represents a statistical measure of the number of times one might expect to see a query sequence match another sequence in the

NCBI nr databases merely by chance (for example, an E value of $1e^{-6}$ has a one in a million chance of mistakenly matching with another sequence).

Library	# of ESTs	# of Clusters	Average length (# bp/cluster)	ESTs/ Cluster	# of Singletons	% of library with no blastx match
Ap eggs	4543	1210	820	3.77	774	42%
Mf eggs	399	105	560	3.80	91	73%
Mf embryos	471	185		2.55	149	
Mf adults	1482	644		2.30	558	

Table 2. Summary of EST Assembly and Cluster sequence novelty.

To examine some of the genes to which an almost certain identity could be assigned, we have drawn up a table showing the top ten blastx hits (based on significance). We excluded from this list any of the top blastx hits to mitochondrial genes and genes encoding ribosomal proteins. The top ten non-mitochondrial, non-ribosomal hits, based on E values, are shown for each library in Table 3.

Cluster ID (cluster length)	Organsim	blastx top hits (NCBI Definition line)	Accession	E value
Acropora palmata egg cDNA library				
161469 (1254 bp)	*Strongylocentrotus purpuratus*	Actin, cytoskeletal IIB	113243	e0.0
160964 (784 bp)	*Mus musculus*	similar to DYSKERIN	28530336	1e-177
161030 (1475 bp)	*Homo sapiens*	Serine/threonine protein phosphatase 2A	7387498	1e-174
161851 (1258 bp)	*Homo sapiens*	protein phosphatase 1, catalytic subunit	4506005	1e-172
161779 (1367 bp)	*Homo sapiens*	SH2 domain binding protein 1	7661950	1e-169
161342 (1407 bp)	*Homo sapiens*	chromosome 20 open reading frame 23; sorting nexin 23	31077079	1e-153
161186 (883 bp)	*Homo sapiens*	MAP/microtubule affinity-regulating kinase 2	30583523	1e-153
160953 (1231 bp)	*Xenopus laevis*	XNop56 protein	14799394	1e-149
162082 (1078 bp)	*Xenopus laevis*	CRM1/XPO1 protein	5690335	1e-148
161197 (1486 bp)	*Danio rerio*	ribonucleotide reductase M2 polypeptide	18859327	1e-147

***Montastraea faveolata* egg cDNA library**				
140530 (645 bp)	*Rattus norvegicus*	Adapter-related protein complex 2 (Alpha-adaptin C)	113337	1e-91
140517 (683 bp)	*Homo sapiens*	transcription factor ICBP90	6815251	1e-82
140495 (563 bp)	*Homo sapiens*	Coatomer delta subunit (Delta-coat protein)	1351970	3e-70
140526 (426 bp)	*Mus musculus*	zinc finger protein 403; dioxin inducible factor 3	23346591	7e-58
140523 (617 bp)	*Homo sapiens*	ubiquitin carrier protein E2 - human	345829	3e-51
140450 (1374 bp)	*Homo sapiens*	protein tyrosine phosphatase type IVA, member 1	4506283	3e-51
140540 (644 bp)	*Nymphicus hollandicus*	chromodomain helicase DNA binding protein 1	5917756	2e-50
140521 (714 bp)	*Mus musculus*	Ser/Arg-related nuclear matrix protein	7949115	1e-48
140493 (499 bp)	*Homo sapiens*	tumor necrosis factor type 1 receptor associated protein 2	687239	2e-48
140441 (447 bp)	*Mus musculus*	Kars protein	23270695	4e-42
***Montastraea faveolata* embryo cDNA library**				
140574 (622 bp)	*Rattus norvegicus*	dynein, cytoplasmic, heavy chain 1	31377489	4e-83
140672 (724 bp)	*Homo sapiens*	ubiquitin specific protease 7	4507857	5e-65
140723 (557 bp)	*Mus musculus*	Cysteinyl-tRNA-synthetase	11191800	3e-50
140553 (490 bp)	*Dictyostelium discoideum*	Fimbrin	1706804	2e-45
140689 (690 bp)	*Gallus gallus*	Transcriptional regulator Erg	3913600	2e-44
140704 (403 bp)	*Mus musculus*	5'-3' exoribonuclease 1	6756025	3e-38
140635 (318 bp)	Bovine viral diarrhea virus 2	polyprotein	5523975	9e-34
140660 (465 bp)	*Danio rerio*	Heat shock transcription factor 1a	8117742	8e-29
140545 (676 bp)	*Dictyostelium discoideum*	dynamin like protein	2689219	6e-24
140626 (249 bp)	*Homo sapiens*	Eukaryotic translation initiation factor 2 subunit 1 (eIF-2A)	124200	3e-19
***Montastraea faveolata* adult cDNA library**				
141223 (1279 bp)	*Sus scrofa*	cytosolic malate dehydrogenase	6226874	1e-127
140786 (619 bp)	*Rattus norvegicus*	Dynein heavy chain	729378	4e-83

141158 (556 bp)	*Montastraea cavernosa*	cyan fluorescent protein	32188174	4e-78
140803 (568 bp)	*Branchiostoma belcheri tsingtaunese*	ubiquitin/ribosomal protein S27a fusion protein	18071662	3e-60
141101 (934 bp)	*Homo sapiens*	Pre-mRNA branch site protein p14	12585536	9e-48
140922 (594 bp)	*Mus musculus*	RING finger protein 7	37538006	8e-45
141186 (504 bp)	*Branchiostoma belcheri tsingtaunese*	cathepsin B	34979797	4e-42
140852 (544 bp)	*Hordeum vulgare* subsp. *vulgare*	14-3-3 protein homologue	22607	1e-41
140868 (655 bp)	*Morone saxatilis*	myosin heavy chain FM3A	10440888	9e-36
141190 (968 bp)	*Galaxea fascicularis*	galaxin	26106077	3e-35

Table 3. Top ten Blastx hits from each cDNA library.

To begin developing ways to group expressed genes into pathways and higher-order biological categories, we performed a tblastx search against a TIGR database (EGAD) of human ESTs each of which has a unique ID number that connects the EST to an assigned biological function. The sequences in our libraries that matched EGAD sequences were sorted into biological categories and summed. In this way, we were able to determine the fraction of each library that is composed of genes belonging to these higher order categories (Figure 1). The cDNA libraries generated from eggs of both *A. palmata* and *M. faveolata* contained a high proportion of genes belonging to such functional categories as gene/protein expression, metabolism and cell/organism defense. In the libraries from embryos and the adult tissue of *M. faveolata* gene/protein expression genes were most abundant (Figure 1).

DISCUSSION

This paper describes our initial efforts to develop the tools to examine coral-dinoflagellate symbiosis using cDNA microarrays. To date we have constructed 4 cDNA libraries from coral eggs, embryos, and adults. We have sequenced 3450 clones and have

annotated the corresponding ESTs using blastx to assign putative identities to the genes, and EGAD to assign larger-order biological function to each gene. From these preliminary annotation efforts, it is clear that the cDNA libraries show differences in gene expression (Figure 1), for example the embryo and adult libraries are enriched in members of the gene and protein expression categories. Ultimately we would like to develop other methods to categorize genes into more specific categories, for example a particular signal transduction pathway, or genes that play roles in innate immunity. This will require the development of additional computational tools to annotate ESTs for functional categories.

We are interested in gaining as comprehensive a collection of cDNAs as possible, from the hosts and their symbionts, so that our microarrays will be representative and non-redundant. The main challenge in developing cDNA libraries is that they often contain large amounts of ribosomal RNA and mitochondrial genes, so that unless these are somehow filtered out, the microarrays would contain large numbers of unwanted rRNA and mitochondrial cDNA spots. By sequencing large numbers of cDNAs, we can selectively avoid clones that represent rRNA and mitochondrial genes, and choose clones that represent genes that we wish to examine. However, we will not be able to examine every gene that is expressed at every target stage; even after sequencing almost 2300 clones from the *Acropora palmata* egg library, we were still uncovering sequences that represented new genes within that library. It would be impractical to sequence enough clones so that we could include in our microarrays all genes that are expressed at each target stage. Thus we will have to balance the effort and cost of sequencing with the goal of creating microarrays with a comprehensive collection of genes.

The next steps in this project will be to construct the rest of the cDNA libraries and then construct the microarrays. Eventually, we hope that the microarrays will provide a tool for the coral research community to examine a variety of questions. For example, we envision that the microarrays will be useful both for the study of coral symbiosis, as well as the study of coral disease, as many host genes involved in the establishment of the symbiosis may also play roles in the response to pathogenic organisms. We will also be able to use the microarrays to examine the symbiosis with different host-symbiont combinations, or under different environmental conditions.

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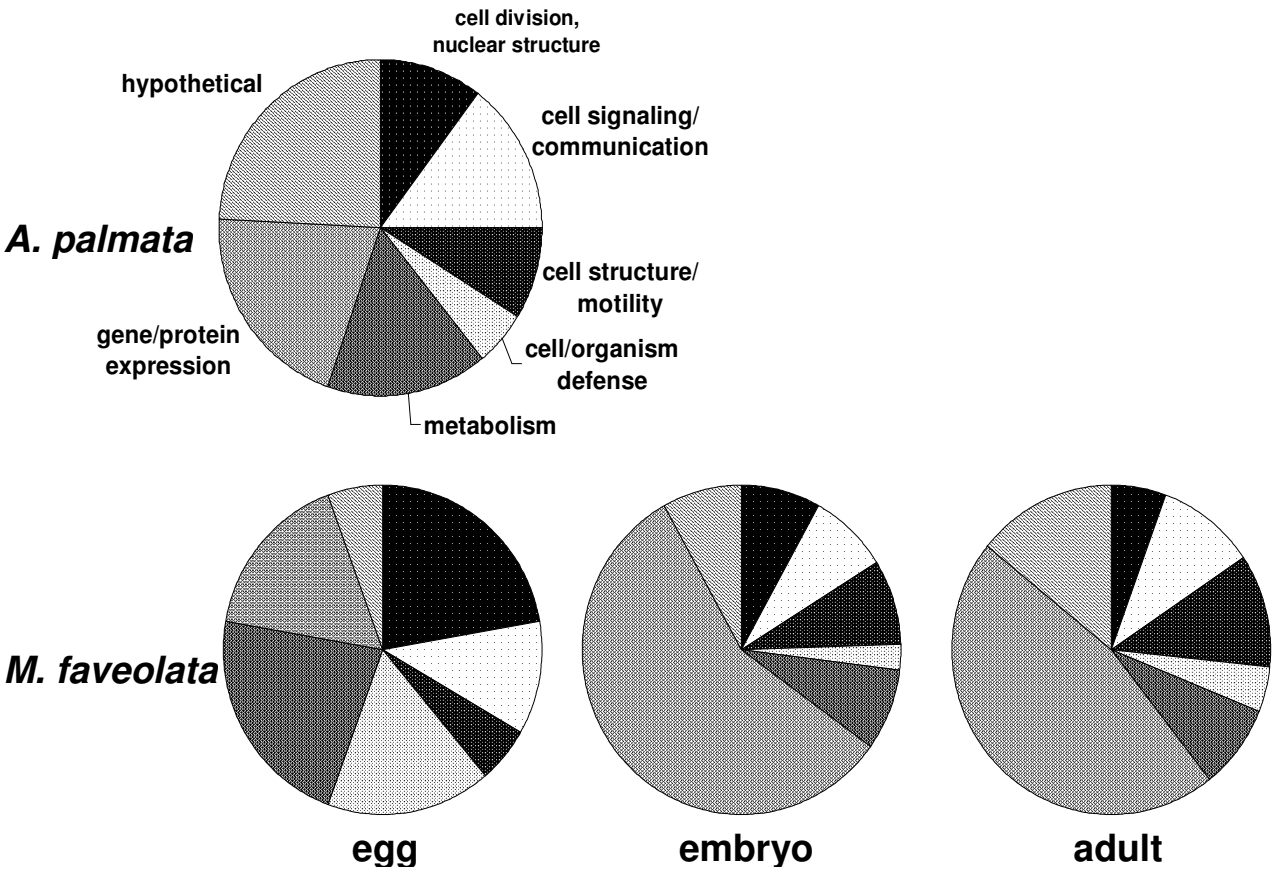

Figure 1. Relative abundance of genes belonging to larger-order biological processes in cDNA libraries representing two species and 3 developmental stages. Developmental stages are organized by column, and species are organized by row (top row, *Acropora palmata*; bottom row *Montastraea faveolata*). The notation shown for the *Ap* library is the same as for the *Mf* libraries.