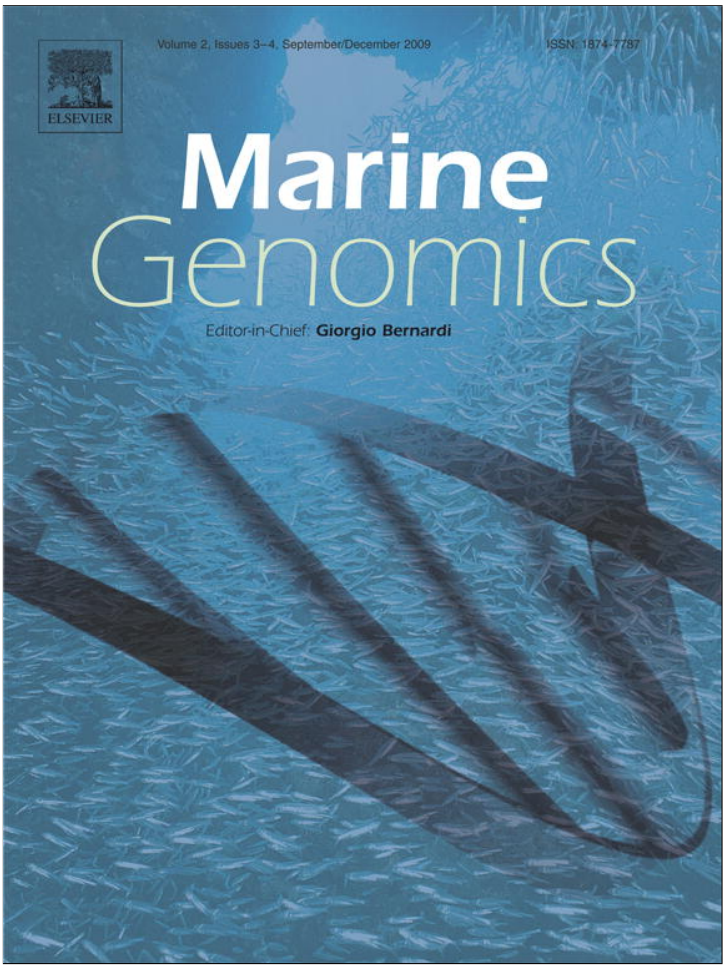

Marine Genomics 2 (2009) 149–159

Contents lists available at ScienceDirect

Marine Genomics

journal homepage: www.elsevier.com/locate/margen

Gene expression microarray analysis encompassing metamorphosis and the onset of calcification in the scleractinian coral *Montastraea faveolata*

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ARTICLE INFO

Article history:
Received 15 May 2009
Received in revised form 10 July 2009
Accepted 23 July 2009

Keywords:
Microarray
Coral
Calcification
Metamorphosis
Transcriptomics

ABSTRACT

Similar to many marine invertebrates, scleractinian corals experience a dramatic morphological transformation, as well as a habitat switch, upon settlement and metamorphosis. At this time, planula larvae transform from non-calcifying, demersal, motile organisms into sessile, calcifying, benthic juvenile polyps. We performed gene expression microarray analyses between planulae, aposymbiotic primary polyps, and symbiotic adult tissue to elucidate the molecular mechanisms underlying coral metamorphosis and early stages of calcification in the Robust/Short clade scleractinian coral *Montastraea faveolata*. Among the annotated genes, the most abundant upregulated transcripts in the planula stage are involved in protein synthesis, chromatin assembly and mitochondrial metabolism; the polyp stage, morphogenesis, protein catabolism and organic matrix synthesis; and the adult stage, sexual reproduction, stress response and symbiosis. We also present evidence showing that the planula and adult transcriptomes are more similar to each other than to the polyp transcriptome. Our results also point to a large number of uncharacterized adult coral-specific genes likely involved in coral-specific functions such as symbiosis and calcification.

1. Introduction

Reef-building corals belong to the class Anthozoa (Hexacorallia: Scleractinia), the most basal lineage within the phylum Cnidaria, which is considered the sister group to Bilateria (Dunn et al., 2008; Medina et al., 2001). Anthozoan cnidarians such as the anemone *Nematostella vectensis* (Darling et al., 2005) and the reef-building coral *Acropora millepora* (Miller and Ball, 2000) have emerged as informative taxa to study how variations of an ancestral developmental scheme have triggered the diversification of triploblastic metazoans. Besides their utility in understanding the evolution of developmental mechanisms (de Jong et al., 2006; Hayward et al., 2004; Knack et al., 2008), scleractinian corals also play an important ecological role, as their skeletons constitute the framework for coral reefs, one of the most productive ecosystems on earth (Gunderson, 2007).

Similar to other marine invertebrates, larvae of most scleractinian corals experience a change from a planktonic habitat to a benthic environment (i.e. settlement). This transition involves morphological modifications from a larval post-embryonic body into a juvenile/adult body plan. Metamorphic morphogenesis in scleractinian corals is a unique process that differs from the metamorphosis experienced by the anthozoan *N. vectensis* (Hand and Uhlinger, 1992). Coral settlement involves the transition from a free-swimming, non-calcifying, spindle-shaped planula larva, into a sessile, calcifying, juvenile polyp (Ball et al., 2002). At this time, the aboral planula ectoderm changes from a columnar organization to a squamous calcifying epithelium known as the calicoblastic ectoderm (Clode and Marshall, 2004; Vandermeulen, 1974). This newly formed epithelium, which is absent in other anthozoan cnidarians, secretes an organic matrix that controls the formation of the coral skeleton (Puverel et al., 2005) allowing the formation of corals reefs.

Due to their ability to examine the transcription levels of thousands of genes simultaneously, gene expression microarray analyses have been used to characterize gene expression profiles during key transitions in the life cycle of a diverse range of organisms (Arbeitman et al., 2002; Azumi et al., 2007; Baldessari et al., 2005; Hill et al., 2000; Wei et al., 2006). However, few studies have focused on the transcriptional changes

Abbreviations: cDNA, complementary DNA; EST, expressed sequence tag; RT, reverse transcription; aRNA, amplified RNA; BAGEL, Bayesian Analysis of Gene Expression Levels; GO, gene ontology; GOEAST, Gene ontology enrichment analysis software toolkit; qRT-PCR, quantitative real-time polymerase chain reaction; *HKG*, housekeeping gene; *DEG*, differentially expressed gene; SCRiP, small cysteine-rich protein; ROS, reactive oxygen species; OM, organic matrix.

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doi:10.1016/j.margen.2009.07.002

regulating metamorphosis in marine invertebrates. Using cDNA microarrays, Williams et al. (2009) studied the gene expression changes underlying settlement and metamorphosis in the abalone *Haliotis asinina* (which also experiences a pelagic to benthic transition upon settlement). Additionally, Grasso et al. (2008) compared gene expression profiles between four key stages during the development of the Complex/Long clade scleractinian coral *Acropora millepora*. Although several groups have described metamorphosis in scyphozoans (Nakanishi et al., 2008; Yuan et al., 2008), hydroids (Katsukura et al., 2003), and non-calcifying anthozoans (Hand and Uhlinger, 1992), Grasso et al.'s (2008) is to date the only study describing broad scale transcriptional changes during metamorphosis and settlement in cnidarians.

There is evidence demonstrating that the order Scleractinia is composed of two main clades (i.e. Robust/Short and Complex/Long) that diverged approximately 240–288 million years ago (Kerr, 2005; Romano and Palumbi, 1997; Medina et al., 2006). Marlow and Martindale (2007) reported different gastrulation mechanisms between scleractinian clades. While Robust clade corals experience both invagination and minor cell ingressions during this morphogenetic transition, Complex clade corals gastrulate via a modified flat blastula known as the "prawnchip". These observations reveal a great deal of complexity during gastrulation in reef-building corals that might reflect phylogenetic history, and suggest that variation in the mechanisms regulating metamorphic morphogenesis and larval calcification are likely to exist within the order. Thus, characterizing metamorphosis and settlement in the two main clades of reef-building corals represents a suitable system to compare the molecular mechanisms underlying early stages of calcification and metamorphic transitions across Scleractinia. This approach will allow for the identification of convergent and/or ancestral molecular pathways regulating morphogenetic changes not only in reef-building corals but also across Cnidaria. Furthermore, studies concerning the molecular basis of coral calcification in both scleractinian lineages are necessary because they are likely to provide valuable information for the management and conservation of coral reefs.

In this study, we examined changes in transcriptional profiles during the ontogeny of *Montastraea faveolata*, a Robust clade coral. A cDNA array containing 1310 genes was used to compare changes in gene expression among planulae, aposymbiotic juvenile polyps, and adult symbiotic polyps in order to understand the molecular mechanisms regulating metamorphosis and the initiation of skeletogenesis in this species.

2. Materials and methods

2.1. Collection of samples

Montastraea faveolata gamete bundles and adult fragments were collected on 3 September 2007 during the annual spawning event in Puerto Morelos, Quintana Roo, Mexico. Gamete bundles were collected and fertilized as described in Miller and Szmant (2006). After 1 h, excess sperm was rinsed away, and the eggs were examined for signs of cleavage. Embryos/larvae were maintained in 5 µm filtered seawater, with water changes several times per day. After a week, larvae reached the planula stage. At this point, a subset of planulae was collected and preserved in RNAlater (Ambion) for subsequent RNA extraction and microarray hybridizations. Remaining planulae were transferred to polycarbonate culture bins and allowed to metamorphose and settle. After 2 weeks post-fertilization, aposymbiotic, calcifying settled polyps were collected and preserved in RNAlater. Coral larvae/embryos were kept in filtered sea water at 28–29 °C throughout the entire experiment.

Three adult fragments were obtained using a hammer and chisel from one colony of *M. faveolata* at 2.7 m depth near Puerto Morelos (20°52′28.77″N and 86°51′04.53″W) on 31 July 2007. Fragments were transported back to the station and acclimated to a flow-through (~0.6 L/min) 50 L aquarium for 27 days. Mean temperature of the aquarium was 28.4 ± 0.9 °C (as measured by a HOBO Light/Temperature Data Logger — Onset Corp.). The fragments were frozen in liquid nitrogen prior to and during transport to Merced, CA. All samples were exported to the USA through a CITES permit (MX-HR-007-MEX).

2.2. RNA extraction and amplification

Total RNA from frozen coral tissues was isolated using Qiazol lysis reagent (Qiagen) following product specifications. Both adult and larval tissues were homogenized using a pre-chilled mortar and pestle. Frozen coral powder was transferred directly to Qiazol. Two chloroform extractions were performed, followed by isopropanol precipitation and two washes in 80% ethanol. RNA pellets were re-dissolved in nuclease-free water and cleaned further with RNeasy Mini columns (Qiagen). RNA quantity and integrity were assessed with a NanoDrop ND-1000 spectrophotometer and an Agilent 2100 Bioanalyzer, respectively. Due to the scarcity of planula and polyp tissue, all RNA samples were amplified using the MessageAmp II aRNA kit (Ambion) with 1 µg of total RNA as input. Bias introduced by RNA amplification is minimal (Feldman et al., 2002; Li et al., 2004).

2.3. Microarray construction and hybridization

Microarrays were constructed using PCR-amplified cDNAs chosen from EST libraries described in Schwarz et al. (2008). Microarray features include clones from five different stage-specific libraries (all designated by four-letter prefixes given by the Department of Energy Joint Genome Institute): 1) unfertilized egg — AOSB — 5.9% of all printed features; 2) early planulae — AOSC — 10.7% of all printed features; 3) late planulae aposymbiotic — CAON — 25.6% of all printed features; 4) late planulae symbiotic — CAOO — 11.7% of all printed features; and 5) adult colony — AOSF — 46.1% of all printed features. All features were printed in duplicate to control for hybridization efficiency. The 1310 genes printed on the microarray conservatively represent 12% of the total coral larval transcriptome (Meyer et al., 2009) and a lower percentage of the entire genome. The microarray is 28% annotated, and 91% of the annotated clones are estimated to be unique (i.e. the microarray is 9% redundant). All gene sequences (searchable by clone ID, blast hit, and GO annotation) are accessible via our EST database at http://sequoia.ucmerced.edu/SymBioSys/index.php. Annotations presented herein represent the top blastx hit to the SwissProt database with an *e*-value cut-off equal to 1×10^{-5}.

All hybridization protocols are previously described in DeSalvo et al. (2008). Briefly, prior to hybridization, microarrays were post-processed by: 1) UV crosslinking at 60 mJ; 2) a "shampoo" treatment (3× SSC, 0.2% SDS at 65 °C); 3) blocking with 5.5 g succinic anhydride dissolved in 335 mL 1-methyl-2-pyrrilidinone and 15 mL sodium borate; and 4) drying via centrifugation. Five µg of aRNA per sample were primed with 5 µg/µL random nonamer for 10 min at 70 °C. Reverse transcription (RT) lasted for 2 h at 50 °C using a master mix containing a 4:1 ratio of aminoallyl-dUTP to TTP. Following RT, single-stranded RNA was hydrolyzed by incubating the RT reactions in 10 µL 0.5 M EDTA and 10 µL 1 M NaOH for 15 min at 65 °C. After hydrolysis, RT reactions were cleaned using MinElute Reaction Purification columns (Qiagen). Cy3 and Cy5 dyes (GE Healthcare) were dissolved in 12 µL DMSO, and the coupling reactions lasted for 2 h at room temperature in the dark. Dye-coupled cDNAs were cleaned (MinElute columns), and appropriate Cy3 and Cy5 labeled cDNAs were mixed together in a hybridization buffer containing 0.25% SDS, 25 mM HEPES, and 3× SSC. The hybridization mixtures were boiled for 2 min at 99 °C then allowed to cool at room temperature for 5 min. The cooled hybridization mixtures were pipetted under a mSeries Lifterslip (Erie Scientific), and hybridization took place in Corning hybridization chambers overnight at 63 °C. Microarrays were washed

twice in 0.6× SSC and 0.01% SDS followed by a rinse in 0.06× SSC and dried via centrifugation. Slides were immediately scanned using an Axon 4000B scanner.

2.4. Microarray data analysis

The experiment followed a loop design in which all life stages (i.e. planula, polyp, and adult) were directly compared against each other. Hybridizations were performed in triplicate and included dye swapping between technical replicates ($n=6$ for each developmental stage for a total of 18 hybridizations). Pre-processing of microarray intensity data was performed using TIGR TM4 software (Saeed et al., 2003). Briefly, spot-finding was performed in SpotFinder 3.11 according to the *otsu* algorithm. We used default values in determining whether a spot was flagged "bad". Background-subtracted median intensities were extracted. Using MIDAS 2.19, the data were first lowess normalized, and the dye swap pairs were averaged and spots showing a dye bias were filtered out using default parameters. Secondly, the in-slide duplicates were averaged. Resulting intensities in both channels (for nine total microarrays since dye swaps were collapsed) were compiled into a spreadsheet. Genes were included in subsequent statistical analysis only if there were data in two out of three replicates for each developmental stage. Of 1310 possible genes, 1243 passed filtering criteria. Microarray data is deposited in Gene Expression Omnibus with the series accession number GSE15962.

The ratio between the fluorescence intensity of the two channels was used as input for BAGEL (Bayesian Analysis of Gene Expression Levels) (Townsend and Hartl, 2002). The BAGEL software uses Bayesian probability to infer a relative expression level of each gene (the life stage with the lowest expression was set to one and the expression of the other stages is relative to one). An estimated mean and 95% credible interval of the relative level of expression of each gene is computed for each life stage. We used the conservative gene-by-gene criterion of non-overlapping 95% credible intervals to regard a gene as differentially expressed. To assess over-representation of Gene Ontology (GO) terms in the lists of significant genes, we used default values in GOEAST (Zheng and Wang, 2008), however no correction was made for multiple testing. GOEAST identifies significantly enriched GO terms among a list of genes by calculating the hypergeometric probability that a given GO term is represented by more microarray features than would be expected by chance. These parameters were empirically chosen as they produced results that were best reflected by the data. We viewed GOEAST results with caution and only focused on over-represented categories that contained two or more genes.

2.5. Quantitative real-time PCR (qRT-PCR)

In order to prove that our microarray is effective at detecting differentially expressed genes, qRT-PCR was performed on 10 DEGs. HKGs were identified using previously described quantitative methods (Rodriguez-Lanetty et al., 2007). The three most stable HKGs were identified using geNORM software (Vandesompele et al., 2002). The pairwise variation of the normalization factor based on the three HKGs was 0.1 — less than the 0.15 cut-off suggested in Vandesompele et al. (2002). cDNAs were synthesized from 1 µg of aRNA and diluted to a final volume of 200 µL. qRT-PCR primers (Table S1) were designed using Primer Express 3.0 (Applied Biosystems), and test-PCRs confirmed specific amplification of the desired amplicons (70–100 bp). Two µL of cDNA (from all nine samples) were used in triplicate 12.5 µL qPCR reactions with 0.2 µM primers and Power SYBR Green PCR Master Mix (Applied Biosystems) for 40 cycles. Ten candidate Primers used for qRT–PCR can be found in Table S1. Using calculations outlined in Vandesompele et al. (2002), the qRT-PCR expression values for the 10 DEGs were normalized to the geometric mean of the three HKGs. Statistical significance in qRT-PCR data was assessed on the normalized expression levels for planula, polyp, and adult for each gene ($n=3$ for each developmental stage) using SigmaStat 3.11. If normality and equal variance tests were satisfied, then a one-way ANOVA and Tukey post-hoc testing was performed. If a normality test failed, then a Kruskal–Wallis one-way ANOVA on ranks was performed with Tukey post-hoc testing. In both cases, pairwise comparisons were also performed via paired *t*-tests.

3. Results

To identify differentially expressed genes (DEGs) encompassing coral metamorphosis and the early stages of calcification, we compared three developmental stages of *Montastraea faveolata* using cDNA microarrays. Bayesian analysis of gene expression identified 818 DEGs, or 66% of the 1243 assayed genes. Only 32% of DEGs had functional annotation. To organize the DEGs into groups displaying common expression, we created 12 possible patterns (Fig. 1) based on differences in expression between the three developmental stages (Microsoft Excel logic formulas used to group DEGs into the 12 patterns are in Table S2). Summary data for all patterns can be found in Table 1, and all genes mentioned below are also presented in Table 2 (for all differentially expressed genes see Table S2).

Pla Pol Adu	Pattern	# of genes	Expression differences
	1	21	Pla < Pol < Adu
	2	14	Pla > Pol > Adu
	3	58	Pla < Pol = Adu
	4	121	Pla > Pol = Adu
	5	84	Pla = Pol < Adu
	6	51	Pla = Pol > Adu
	7	88	Pla = Adu > Pol
	8	214	Pla = Adu < Pol
	9	23	Pla > Pol < Adu + Pla < Adu
	10	9	Pla < Pol > Adu + Pla > Adu
	11	93	Pla > Pol < Adu + Pla > Adu
	12	42	Pla < Pol > Adu + Pla < Adu

Fig. 1. The 12 different gene expression patterns used to analyze the differentially expressed genes. For each gene, BAGEL assigned the value of 1 to the life stage with the lowest expression, and calculated the expression of the other life stages relative to 1. The heat map was generated by averaging the BAGEL-estimated gene expression levels of all genes within each pattern. Genes were clustered into the 12 patterns by using the formulas indicated in the last column. Pla = planulae, pol = polyps, and adu = adults.

Table 1
Summary statistics of the 12 different gene expression patterns identified in this study (see Fig. 1 for a visual description of each pattern).

Pattern	# of DEGs	% of all DEGs	# of annot. DEGs	% annot.	Egg	Larval	Adult
1	21	2.6	2	9.5	0	0.10	0.90
2	14	1.7	5	35.7	0	0.50	0.50
3	58	7.1	6	10.3	0.03	0.14	0.83
4	121	14.8	49	40.5	0.01	0.60	0.40
5	84	10.3	17	20.2	0	0.11	0.89
6	51	6.2	10	19.6	0	0.73	0.27
7	88	10.8	36	40.9	0.03	0.34	0.63
8	214	26.2	55	25.7	0.20	0.66	0.14
9	23	2.8	13	56.5	0	0.13	0.87
10	9	1.1	1	11.1	0	0.44	0.56
11	93	11.4	56	60.2	0.01	0.42	0.57
12	42	5.1	9	21.4	0.29	0.36	0.36
	818		259				

A total of 818 differentially expressed genes were sorted into the 12 patterns, of which 259 genes (or 32%) contained functional annotation (the 1310 gene array is 43% annotated). The final three columns give the percentages of genes within a particular pattern that came from an egg cDNA library (AOSB), larval libraries (AOSC, CAON, and CAOO), and an adult library (AOSF).

Table 2
Genes of interest among the 818 differentially expressed genes identified by comparing mRNA transcript levels between three developmental stages of *M. faveolata*.

CloneID	SwissProt annotation	*E*-val	Plan	Pol	Adu
Pattern 1					
AOSF1022	Vitellogenin-2	1.E-25	1	2.55	7.90
AOSF1277	Proprotein convertase subtilisin/kexin type 6	1.E-12	1	1.90	3.00
Pattern 2					
AOSF1016	Pre-mRNA branch site protein p14	8.E-52	4.28	2.07	1
AOSF523	Small G protein signaling modulator 3 homolog	1.E-33	1.51	1.20	1
CAON1411	Tubulin polymerization-promoting protein family member 2	1.E-10	4.98	2.62	1
CAON1415	Elongation factor 1-alpha	5.E-14	2.95	2.01	1
CAON1906	Lethal(2) giant larvae protein homolog 2	3.E-57	1.49	1.35	1
Pattern 3					
AOSB745	Pre-mRNA-splicing factor SPF27	9.E-73	1	1.93	1.51
AOSC633	FK506-binding protein 3	1.E-53	1	1.24	1.29
AOSC931	FUN14 domain-containing protein 1	3.E-13	1	1.54	1.44
AOSF1176	Galaxin	4.E-12	1	3.31	2.40
AOSF493	Putative cation exchanger C3A12.06c	3.E-08	1	1.57	1.39
AOSF976	Neurogenic locus notch protein homolog	6.E-08	1	1.83	1.42
Pattern 4					
AOSC617	Heat shock protein HSP 90-alpha	1.E-52	4.25	1.26	1
AOSF1096	Glutathione transferase omega-1	1.E-42	3.14	1	1.21
AOSF1308	15 kDa selenoprotein	3.E-36	2.09	1	1.35
AOSF1385	Aldose reductase	1.E-37	3.08	1	1.05
CAON1226	LWamide neuropeptide	6.E-10	1.96	1	1.02
CAON1605	Heat shock factor protein 1	1.E-31	3.36	1.26	1
CAOO1119	Glutathione transferase omega-1	1.E-42	2.61	1	1.16
Pattern 5					
AOSF1131	GFP-like fluorescent chromoprotein cFP484	1.E-66	1	1.04	5.85
AOSF1172	Cathepsin B	2.E-49	1.14	1	2.54
AOSF1190	SCRiP7	–	1.28	1	8.64
AOSF1199	SCRiP6	–	1.18	1	2.57
AOSF1214	SCRiP4	–	1.47	1	2.67
AOSF387	Tachylectin-2	3.E-40	1.06	1	1.86
AOSF631	Cytochrome b	6.E-53	1	1.21	25.00
AOSF642	Major yolk protein	5.E-12	1.28	1	2.32
AOSF907	Four-domain proteases inhibitor	1.E-16	1.36	1	3.49
CAON1101	Soma ferritin	5.E-35	1.34	1	1.95
Pattern 6					
AOSC663	Vesicle transport protein USE1	2.E-23	1.88	1.84	1
CAON637	Epididymal secretory protein E1	9.E-29	1.49	1.67	1
CAOO636	Epididymal secretory protein E1	9.E-29	1.44	1.41	1
Pattern 7					
AOSF1140	SCRiP5	–	4.53	1	3.84
AOSF1347	Ubiquitin	1.E-35	2.19	1	2.16
AOSF1373	Thioredoxin	2.E-25	1.66	1	2.14
AOSF997	Peroxidasin-like protein	1.E-24	4.98	1	5.99
CAON1056	Calumenin	3.E-19	2.99	1	2.35
Pattern 8					
AOSB874	Sorting nexin-7	1.E-07	1.08	1.73	1
AOSB847	Afadin	1.E-31	1.37	2.39	1
AOSB976	Calcium homeostasis endoplasmic reticulum protein	1.E-24	1	3.59	1.22
AOSC1122	Cysteinyl-tRNA synthetase, cytoplasmic	9.E-57	1	1.53	1.11
AOSC1134	Syntaxin-16	1.E-15	1	2.37	1.17
CAON1815	Ras-related protein Rab-10	8.E-57	1.02	1.55	1
CAON980	Zinc metalloproteinase nas-13	3.E-06	1.19	1.83	1
Pattern 9					
AOSF1109	SCRiP1	–	2.52	1	29.24
AOSF1192	SCRiP2	–	2.34	1	7.77
AOSF1361	Adenosine receptor A1	2.E-07	1.20	1	2.20
AOSF1521	Universal stress protein A-like protein	2.E-06	3.01	1	3.83
AOSF810	SCRiP8	–	2.06	1	3.29
AOSF882	CCAAT/enhancer-binding protein gamma	3.E-15	1.73	1	2.29
Pattern 10					
AOSF914	CDGSH iron–sulfur domain-containing protein 2 homolog	4.E-27	1.41	2.39	1
Pattern 11					
AOSC448	Calreticulin	1.E-127	1.56	1	2.39
AOSF1434	Piwi-like protein 1	2.E-65	3.31	1	1.75
Pattern 11					
AOSF1447	Glutathione S-transferase Mu 5	5.E-30	3.92	1	2.36
AOSF513	SCRiP3a	–	2.23	1	1.66
AOSF573	Calmodulin	2.E-79	2.78	1	1.70
AOSF761	Proapoptotic caspase adapter protein	3.E-07	2.82	1	1.60
AOSF836	Peptidyl-prolyl cis-trans isomerase FKBP1A	1.E-27	4.20	1	2.09
CAON1055	DNA mismatch repair protein Msh2	3.E-21	2.56	1	1.92
CAON969	Soma ferritin	3.E-66	3.83	1	1.37
Pattern 12					
AOSB1028	Ubiquitin-conjugating enzyme E2 S	2.E-54	1	5.18	1.50
AOSB472	Ubiquitin carboxyl-terminal hydrolase 5	5.E-28	1	16.14	2.20
AOSB788	Coatomer subunit delta	1.E-114	1	2.21	1.54
AOSC1006	Rab3 GTPase-activating protein catalytic subunit	8.E-17	1	3.23	1.92
CAON468	Flotillin-1	1.E-102	1	1.48	1.21
CAON500	Ubiquitin carboxyl-terminal hydrolase 24	1.E-24	1	2.92	1.21

Plan = planula, pol = polyp, and adu = adult. *E*-value and annotation are for the best blastx hit to the SwissProt database.

3.1. Patterns 1, 5, and 9: highest expression in adults

Patterns 1, 5, and 9 together encompass 128 genes, or 16% of all DEGs. Over 87% of genes within each pattern are from the adult cDNA library (AOSF). Pattern 1 includes genes that increase in expression incrementally during development from planula to polyp to adult. Twenty-one genes satisfied these criteria, 10% of which are annotated. The two annotated genes in pattern 1 are *vitellogenin*-2 (AOSF1022 — a yolk protein) and proprotein convertase subtilisin/kexin type 6 (AOSF1277 — a protein with endopeptidase activity). Pattern 5 includes genes that are not different between planulae and polyps but upregulated in adults. This pattern includes 84 genes, 20% of which are annotated. Notable annotated genes in pattern 5 include another yolk protein (AOSF642), a GFP-like protein (AOSF1131), ferritin (CAON1101 — iron homeostasis), cathepsin B (AOSF1172 — protein degradation), *SCRiP7* (AOSF1190), *SCRiP6* (AOSF1199), *SCRiP4* (AOSF1214), and cytochrome *b* (AOSF631 — respiration — 25-fold upregulated compared to the other stages). Pattern 9 includes genes that are upregulated in adults and planulae relative to polyps, and upregulated in adults compared to planulae. Twenty-three genes fulfilled these criteria, and 57% are annotated. Over-represented GO categories in pattern 9 include: regulation of transcription (GO:0045449); response to stress (GO:0006950); and regulation of metabolic process (GO:0019222) (see Table 3 for GOEAST results mentioned in text and Table S3 for all GOEAST results).

Table 3
Statistically over-represented gene ontology (GO) categories present in certain gene expression patterns mentioned in the text.

GO ID	Ont	Term	*E*-val	Clone ID	SwissProt annotation
Pattern 4					
GO:0006414	BP	Translational elongation	1.E-133	AOSC1120	Elongation factor 1-alpha
			5.E-65	CAOO2069	Elongation factor 1-alpha
GO:0032956	BP	Regulation of actin cytoskeleton org.	6.E-10	AOSF1089	ADP-ribosylation factor 6
			3.E-12	AOSF1307	Severin
GO:0031497	BP	Chromatin assembly	8.E-63	AOSF1233	Histone H3.3
			5.E-50	AOSF622	Histone H2A.V (fragment)
Pattern 7					
GO:0005840	CC	Ribosome		–	20 Ribosomal proteins
			2.E-14	CAON1748	Ubiquitin-like protein FUBI
GO:0010941	BP	Regulation of cell death	6.E-20	AOSB1072	Protein BTG1
			9.E-82	CAON1026	40S ribosomal protein S3a
Pattern 8					
GO:0009888	BP	Tissue development	5.E-10	AOSB1074	Putative golgin subfamily A member 6-like protein 4
			1.E-09	AOSB574	Trichohyalin
			2.E-31	AOSB668	Lethal(2) giant larvae protein
			3.E-15	AOSC865	Peptide methionine sulfoxide reductase
GO:0007155	BP	Cell adhesion	8.E-08	AOSB564	Integral membrane protein DGCR2/IDD
			1.E-31	AOSB847	Afadin
			3.E-06	AOSC876	Tyrosine-protein phosphatase Lar
GO:0005694	CC	Chromosome	6.E-53	AOSB1138	Chromodomain-helicase-DNA-binding protein 1
			1.E-18	AOSB632	Histone H1.0-A
			2.E-07	AOSB875	Stress response protein NST1
			6.E-37	CAON1314	DNA topoisomerase I mitochondrial
GO:0009792	BP	Embryonic development ending in…	4.E-23	AOSB596	Nuclear hormone receptor family member nhr-6
			2.E-31	AOSB668	Lethal(2) giant larvae protein
			3.E-06	AOSC876	Tyrosine-protein phosphatase Lar
			1.E-08	AOSF1368	Titin
Pattern 9					
GO:0019222	BP	Regulation of metabolic process	3.E-22	AOSF533	Transcription factor HES-1
			3.E-15	AOSF882	CCAAT/enhancer-binding protein gamma
			4.E-13	CAOO2203	Thymosin beta-4
GO:0045449	BP	Regulation of transcription	3.E-22	AOSF533	Transcription factor HES-1
			3.E-15	AOSF882	CCAAT/enhancer-binding protein gamma
GO:0006950	BP	Response to stress	2.E-06	AOSF1521	Universal stress protein A-like protein
			3.E-15	AOSF882	CCAAT/enhancer-binding protein gamma
Pattern 11					
GO:0006886	BP	Intracellular protein transport	1.E-127	AOSC448	Calreticulin
			8.E-17	AOSF1278	Protein transport protein Sec61 subunit gamma
			2.E-14	CAOO1095	Protein transport protein Sec61 subunit beta
			1.E-48	CAOO526	Protein transport protein Sec61 subunit alpha
GO:0005739	CC	Mitochondrion	2.E-19	AOSC391	ATP synthase lipid-binding protein mitochondrial
			2.E-31	CAON1087	ATP synthase subunit b mitochondrial
			5.E-18	CAON1556	ATP synthase lipid-binding protein mitochondrial
			4.E-81	CAON943	NADH dehydrogenase [ubiq] iron–sulfur protein 7 mito.
			6.E-14	CAOO440	Cytochrome b-c1 complex subunit 6 mitochondrial
			2.E-19	AOSC391	ATP synthase lipid-binding protein mitochondrial
GO:0016021	CC	Integral to membrane	8.E-17	AOSF1278	Protein transport protein Sec61 subunit gamma
			4.E-06	AOSF1397	*N*-acetylglucosaminyl-phosphatidylinositol de-*N*-acetylase
			2.E-12	AOSF621	DBH-like monooxygenase protein 2 homolog
			5.E-18	CAON1556	ATP synthase lipid-binding protein mitochondrial
			2.E-14	CAOO1095	Protein transport protein Sec61 subunit beta
			1.E-48	CAOO526	Protein transport protein Sec61 subunit alpha
GO:0051082	MF	Unfolded protein binding	1.E-127	AOSC448	Calreticulin
			3.E-98	AOSF1451	Heat shock protein HSP 90-alpha
GO:0031497	BP	Chromatin assembly	3.E-63	AOSF1219	Histone H3.3
			2.E-71	CAON910	SWI/SNF-rel'd matrix-assoc actin-dep reg of chromatin A5
GO:0006979	BP	Response to oxidative stress	5.E-41	AOSF550	Catalase
			5.E-49	AOSF666	RING-box protein 2
GO:0015630	CC	Microtubule cytoskeleton	1.E-44	AOSF1400	Dynein light chain 2 cytoplasmic
			3.E-54	AOSF634	Dynein light chain Tctex-type 1
			5.E-32	AOSF651	Dynein light chain roadblock-type 2
			4.E-56	CAOO2335	Dynein heavy chain domain-containing protein 2
GO:0003735	MF	Structural constituent of ribosome		–	14 Ribosomal proteins
			1.E-57	CAON1092	Probable ribosome biogenesis protein RLP24

Over-representation was assessed using GOEAST, an on-line tool that identifies statistically significant enrichment of GO terms within a list of genes. All *p*-values are less than 0.01. Column "Ont" refers to the three main ontologies within GO: BP = biological process; CC = cellular component; and MF = molecular function. *E*-value and annotation are for the best blastx hit to the SwissProt database. Missing patterns did not contain notable GO category over-representation.

3.2. Patterns 8, 10, and 12: highest expression in polyps

Patterns 8, 10, and 12 hold a total of 265 genes, or 32% of all DEGs. Pattern 8 genes are not significantly different between planulae and adults but upregulated in polyps relative to the two other stages. This pattern has 214 genes, the most of any gene expression pattern. 66% of the genes are from larval cDNA libraries (AOSC, CAOO, and CAON), 20% from egg (AOSB), and 14% from adult. Fifty-five pattern 8 genes (26%) are annotated. According to the analysis of GO category representation, the following notable categories were statistically over-represented: cell adhesion (GO:0007155); tissue development (GO:0009888); embryonic development ending in birth or egg hatching (GO:0009792); and chromosome (GO:0005694). Pattern 8 also included numerous genes involved in vesicle transport (e.g. sorting nexin-7 — AOSB874, syntaxin-16 — AOSC1134, and Rab10 — CAON1815). Pattern 10 contained only nine genes, which are upregulated in polyps relative to adults and planulae but upregulated in planulae relative to adults. 56% of genes are from the adult cDNA library, and the remaining 44% are larval. One of these nine genes was annotated: CDGSH iron–sulfur domain-containing protein 2 homolog (AOSF914 — an ER membrane protein). Genes in pattern 12 were upregulated in polyps relative to adults and planulae but upregulated in adults relative to planulae. This pattern contains 42 genes of which 21% are annotated. 35.7% of pattern 12 genes are from larval and adult libraries, and the remaining 28.6% are from the egg library. Annotated genes include two ubiquitin carboxyl-terminal hydrolases (AOSB742 and CAON500), ubiquitin-conjugating enzyme E2S (AOSB1028 — protein ubiquitination), coatomer subunit delta (AOSB788 — intracellular protein transport), Rab3 GTPase-activating protein catalytic subunit (AOSC1006 — exocytosis), and flotillin-1 (CAON468 — vesicle formation).

3.3. Patterns 2, 4, and 11: highest expression in planulae

There are 228 total genes within patterns 2, 4, and 11, or 28% of all DEGs. While pattern 2 only holds 14 genes, pattern 4 has 121 genes, and pattern 11 has 93 genes. The 14 genes in pattern 2 (5 of which are annotated) show the opposite expression scheme as pattern 1 — they decrease step-wise during development from planula to polyp to adult. Pattern 2 genes were 50% larval and 50% adult. The annotated genes include: pre-mRNA branch site protein p14 (AOSF1016 — RNA splicing), small G protein signaling modulator 3 homolog (AOSF523 — signal transduction), a tubulin polymerization-promoting protein family member (CAON1411), elongation factor 1α (EF1α — CAON1415 — translation), and lethal (2) giant larvae protein homolog 2 (CAON1906 — establishment of cell polarity). Genes in pattern 4 show distinct upregulation in planulae relative to both polyps and adults. This pattern is the second-most represented pattern with 121 genes, 41% of which are annotated. 60% of the genes are from larval libraries, and 40% are from the adult library. Over-represented GO categories include: translational elongation (GO:0006414); regulation of actin cytoskeleton organization (GO:0032956); and chromatin assembly (GO:0031497). Other striking trends in pattern 4 include the differential expression of: ten ribosomal proteins, an LWamide neuropeptide (CAON1226), and numerous stress-related proteins (e.g. heat shock factor protein — CAON1605, hsp90 — AOSC617, glutathione transferase omega-1 — AOSF1096 and CAOO1119, a 15 kDa selenoprotein — AOSF1308, and aldose reductase — AOSF1385).

Pattern 11 is the third-most populated pattern with 93 genes at a striking annotation rate of 60%. Like pattern 4, these genes are upregulated in planulae relative to both polyps and adults; however, their expression in adults is greater than in polyps. 57% of genes in pattern 11 are from the adult library, and 42% are from larval libraries. Many of the same processes differentially expressed in pattern 4 are present in pattern 11. This is illustrated by the following over-represented GO categories: structural constituent of ribosome (GO:0003735); response to oxidative stress (GO:0006979); chromatin assembly (GO:0031497); microtubule cytoskeleton (GO:0015630); and unfolded protein binding (GO:0051082). Trends unique to pattern 11 include an over-representation of mitochondrial genes (GO:0005739) and intracellular protein transport proteins (GO:0006886).

3.4. Pattern 3: lowest expression in planulae

This pattern contains 58 genes (7% of DEGs) downregulated in planulae relative to the other two stages. Only 10% of these genes are annotated and 83% of them are from the adult library. The six annotated genes in pattern 3 include: pre-mRNA-splicing factor SPF27 (AOSB745), FK506-binding protein 3 (AOSC633), FUN14 domain-containing protein 1(AOSC931), galaxin (AOSF1176), a putative cation exchanger C3A12.06c (AOSF493), and a neurogenic locus Notch protein homolog (AOSF976).

3.5. Pattern 6: lowest expression in adult

Pattern 6 contains 51 genes (6% of DEGs), which are downregulated in adults relative to planulae and polyps, and consistently, 73% of the genes are from larval cDNA libraries. Fifty-one genes are in pattern 6 and 20% are annotated, including vesicle transport protein USE1 (AOSC663 — required for secretion) and epididymal secretory protein E1 (CAON637 and CAOO636 — lipid transport).

3.6. Pattern 7: lowest expression in polyp

There are 88 genes (11% of DEGs) within pattern 7, which are downregulated in polyps relative to planulae and adults. 63% of genes are adult and 34% are larval. 41% of pattern 7 genes are annotated, and based on their identities, differentially expressed processes in pattern 7 are similar to patterns 4 and 11. Over-represented GO categories include: ribosome (GO:0005840) and regulation of cell death (GO:0010941). Furthermore, three stress-related proteins (thioredoxin — AOSF1373, ubiquitin — AOSF1347, and peroxidasin — AOSF997), SCRiP5 (AOSF1140), and a Ca^{2+} signaling molecule (calumenin — CAON1056) are in pattern 7.

3.7. RT-qPCR validation of microarray gene expression

The ten DEGs validated with qRT-PCR came from five of the 12 patterns: one from pattern 3; two from pattern 5; one from pattern 7; three from pattern 9; and three from pattern 11. All DEGs validated with qRT-PCR were significantly differentially expressed in at least one pairwise comparison between the three developmental stages ($p<0.05$ for one-way ANOVA between normalized qRT-PCR expression values of planula, polyp, and adult). A comparison between the microarray and qRT-PCR results is summarized in Table 4. Similar to the data output from BAGEL, we set the lowest expression class to 1.0 and calculated the expression of the other two classes relative to the lowest. Nine DEGs showed consistency in which class had the lowest expression. AOSC587 was the only DEG that gave inconsistent results in that planulae had the lowest expression according to the microarray, which was opposed to polyps in the qRT-PCR results.

Comparing the expression estimates for the nine validated genes, it is evident that the microarray and qRT-PCR results are very consistent in measuring relative differences between the three developmental stages. Based on the results of statistical analyses, four genes (*AOSF722*, *SCRiP7*, *peroxidasin*, and *AOSF1123*) showed complete validation of their pattern assignment. The remaining five genes, however, lacked statistical significance in at least one pairwise comparison. In some cases clear differences in gene expression were evident, but high variance among the most upregulated stage reduced statistical power (e.g. *SCRiP8* and *SCRiP2* expression in adults). Similar to another study utilizing the same *M. faveolata* cDNA microarray platform (DeSalvo et al., 2008), many qRT-PCR estimates of gene expression were magnified compared to the microarray. For example, six genes showed qRT-PCR inflation

Table 4
Differentially expressed genes validated with RT-qPCR.

Unique ID	Gene name	Pattern	Microarray				RT-qPCR			
			Pla	Pol	Ad	N/S	Pla	Pol	Ad	N/S
AOSF722	Non-annotated	3	1	1.87	2.11	3	1	2.53	3.29	3
AOSC587	RNA helicase *DDX19B*	5	1	1.15	1.40	1	4.51	1	5.43	2, 1
AOSF1190	*SCRiP7*	5	1.28	1	8.64	1	1.10	1	378.08	1
AOSF997	Peroxidasin-like protein	7	4.98	1	5.99	2	104.04	1	189.32	2
AOSF810	*SCRiP8*	9	2.06	1	3.29		13.11	1	22.19	2, 3
AOSF882	*C/EBPγ*	9	1.73	1	2.29		3.83	1	4.77	2
AOSF1192	*SCRiP2*	9	2.34	1	7.77		10.86	1	1763.32	2
AOSF1123	EF-hand protein	11	7.63	1	3.44		20.61	1	10.56	
AOSF1095	GLI-pathogen.-related 1	11	3.27	1	1.75		11.77	1	8.08	2
CAOO440	Cytochrome *b-c1* subunit 6	11	2.88	1	1.82		3.12	1	1.88	3

Pla = planula, pol = polyp, and ad = adult. Pairwise comparisons not statistically significant are listed in the columns labeled "N/S": 1 = planula–polyp, 2 = adult–planula, and 3 = adult–polyp.

greater than 2.5 times the microarray estimates in at least one of the developmental stages. Three genes (*SCRiP2*, *SCRiP7*, and *peroxidasin*) had qRT-PCR inflation by a factor greater than 20, while the remaining three genes (*SCRiP8*, *AOSF1123*, and *AOSF1095*) had qRT-PCR estimates that were 2.7–6.7 times greater than the microarray estimates. While our qRT-PCR expression estimates are in some places very different than the microarray estimates, overall, we see directionally consistent differences in gene expression among the three developmental stages using both quantitative platforms.

4. Discussion

4.1. Differential gene expression patterns were identified between larval, juvenile and adult stages during the life cycle of M. faveolata

Our gene expression microarray analysis identified stage-specific gene expression profiles for each of the three developmental time points used in this study. The 818 differentially expressed genes (DEGs) were sorted into twelve possible patterns relating the three different developmental stages (Fig. 1). Interestingly, patterns 4 and 8, which are specific to planulae and polyps, respectively, contain the greatest number of DEGs. Pattern 5, which is specific to adults, holds 84 genes and is one of the most populated patterns. These results suggest that planula, primary polyp, and adult are transcriptionally distinct stages in which precise gene networks are likely to control and regulate the contrasting physiological and developmental conditions. Consistently, Schwarz et al. (2008) found a strikingly low number of unigenes that overlap between the five stage-specific cDNA libraries used to construct the microarray used in the present study. However, our results also show that many genes are similarly expressed in different life stages (e.g. patterns 3, 6, and 7), and that genes isolated from stage-specific cDNA libraries are also expressed in other life stages. For example, 40% of DEGs within pattern 4 (planula-specific expression) were from the adult cDNA library, 11% of DEGs within pattern 5 (adult-specific expression) were from larval cDNA libraries, and 20% of DEGs within pattern 8 (polyp-specific expression) were from the egg cDNA library.

Similar to gastrulation, metamorphosis is a morphogenetic process by which tissues reorganize and cell populations differentiate into specific phenotypes (Magie and Martindale, 2008). Thus, it is reasonable to expect distinct transcriptional changes during the planula to polyp transition, since this is the time that tissue rearrangement is occurring. The greater number of DEGs that were differentially expressed in larval and juvenile stages compared to adults is likely to reflect the developmental changes experienced by *M. faveolata* larvae.

Changes in gene expression patterns have been reported for *Ciona intestinalis* over its life cycle (Azumi et al., 2007), and five temporal gene expression profiles were reported during metamorphosis and settlement of the abalone *Haliotis asinine* (Williams et al., 2009). Likewise, Grasso et al. (2008) identified stage-specific transcription profiles during development of the Complex clade coral *Acropora millepora*. Azumi et al. (2007) reported that 87.5% of all assayed genes were up or downregulated during development of *C. intestinalis*. This result is consistent with our finding that 66% of all assayed genes were differentially expressed, as large numbers of DEGs are to be expected when comparing such disparate life stages. In contrast, Grasso et al. (2008) found that only 21% of all assayed genes were differentially expressed during development of *A. millepora*. This discrepancy is likely the result of the conditions under which the microarrays were constructed. While our microarray contains 52% egg/larval and 48% adult sequences, the microarray in Grasso et al. (2008) contains only genes from larval cDNA libraries. It is not surprising then that their least-populated synexpression cluster (CV) contained genes solely upregulated in adults. If adult cDNAs had been printed on their microarray, it is likely that many more adult-specific transcripts would have been identified thus increasing the total number of DEGs.

4.1.1. Planula-specific processes

Genes highest expressed in planulae (patterns 2, 4, and 11) include an over-representation of the translational machinery (EF1α and ribosomal proteins), and proteins involved in the oxidative stress response, cytoskeleton, chromatin assembly, and mitochondria (Fig. 2). LWamide, a well-known neuropeptide molecule able to regulate settlement and metamorphosis in hydrozoans (Katsukura et al., 2003, 2004), is 2-fold upregulated in *M. faveolata* planulae. There is evidence of RFamide-producing neurons in the aboral end of *Acropora millepora* larvae (Hayward et al., 2001), and it is known that planula migration and metamorphosis in the hydroid *Hydractinia echinata* is regulated by an antagonistic mechanism involving LWamide and RFamide (Katsukura et al., 2003, 2004). The presence of RFamide-positive neurons in planulae of *A. millepora* (Hayward et al., 2001) and the upregulation of a LWamide precursor in the planulae of *M. faveolata* suggest that metamorphosis in scleractinian corals may resemble that described for hydrozoan cnidarians (Katsukura et al., 2003, 2004). However, functional data are needed to show that metamorphosis in scleractinian corals is regulated by the neuropeptides LWamide and RFamide.

Over-representation of the GO categories: mitochondrial part (GO:0044429), oxidative phosphorylation (GO:0006119), and response to oxidative stress (GO:0006979) among pattern 11 genes (Table S3) points to the possibility that reactive oxygen species (ROS) may be involved in the regulation of metamorphosis during development in *M. faveolata*. Incomplete oxygen reduction during mitochondrial electron transport leads to the formation of ROS (Kang and Hamasaki, 2003; Raha and Robinson, 2000). It is estimated that 0.2 to 2% of the oxygen consumed by a cell is transformed to ROS by electron leakage during oxidative phosphorylation (Chance et al., 1979; Hansford et al., 1997). Although uncontrolled ROS production can cause oxidative damage to cellular structures (Raha and Robinson, 2000), ROS molecules are also

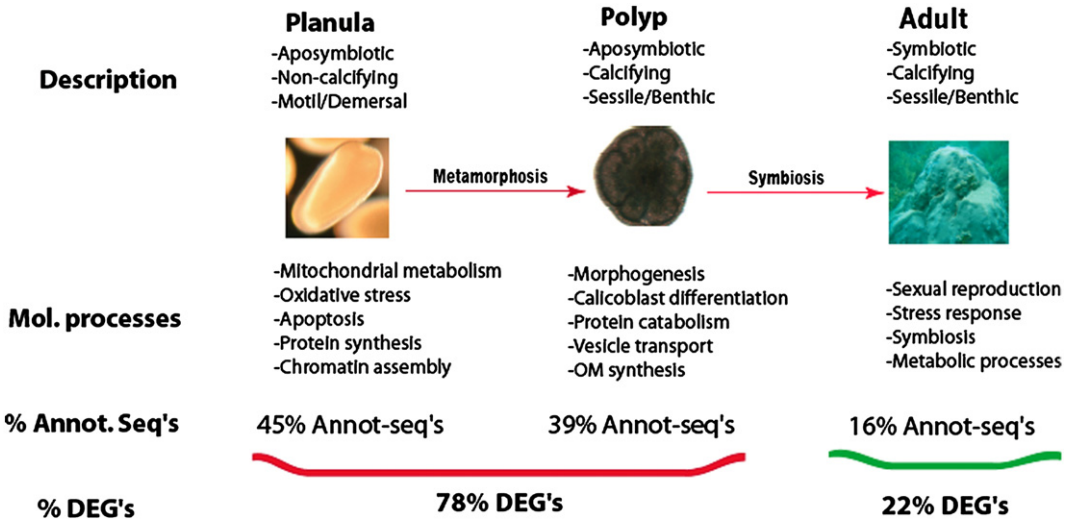

Fig. 2. After settlement and metamorphosis, coral planulae experience an aboral ectodermal modification that will originate the calicoblastic ectoderm. Calicoblastic cells produce an organic matrix that induces skeleton deposition in juvenile polyps. Settled primary polyps establish a symbiotic relationship with unicellular organisms from the genus *Symbiodinium*. This symbiotic interaction allows higher calcification rates and provides the coral host with nutrients. Stage-specific molecular processes were identified in our experiment. The higher percentage of annotated sequences in larval stages suggests conserved mechanisms regulating metamorphosis across metazoans with biphasic life cycles as well as a great number of uncharacterised putative specific *cnidarian/scleractinian* genes. While the 78% of DEGs in larval stages are likely to reflect the genetic networks underlying settlement and metamorphosis, the 22% of adult DEGs might be involved in the regulation of symbiosis and responses to environmental stress.

able to regulate specific signaling pathways (Brookes et al., 2002; Finkel, 2000) including those regulating apoptosis (Salas-Vidal et al., 1998). A link between apoptosis and morphogenesis has already been described in the cnidarian *H. echinata*, where morphogenesis of both apical and basal adult structures correlate with apoptotic recycling of larval tissues (Seipp et al., 2001). Furthermore, ROS play a direct role as primary and secondary messengers during embryonic development in mammals by regulating cellular pathways involved in cell proliferation, differentiation, and apoptosis (reviewed in (Dennery, 2007)). Consistently, there is an over-representation of molecules involved in apoptosis (Table S3) within the group of genes upregulated in planulae and adults relative to polyps (pattern 7). Apoptotic mechanisms initiated in planulae are likely responsible for a gradual remodeling of non-functional larval structures. These observations might also suggest that ROS and apoptosis may be involved in the differentiation of adult structures in planula larvae. For example, mesenterial filaments and the calicoblastic epithelium — structures present in adults — begin forming in coral planulae (Vandermeulen, 1974; Babcock and Heyward, 1986; Szmant-Froelich et al., 1980). Commonalities between planulae and adults may explain the transcriptomic similarities observed in the two stages (see Section 4.2).

Upregulation in planulae of calmodulin (AOSF573 — pattern 11), calumenin (CAON1056 — pattern 7), and calreticulin (AOSC448 — pattern 11) suggest that, similar to mammalian bone homeostasis (Koga et al., 2004), calcium signaling might regulate calicoblast differentiation. There are several lines of evidence supporting this hypothesis. Calmodulin is a highly conserved calcium sensor known to regulate cytoskeleton organization, cellular metabolism, cell differentiation, proliferation, apoptosis, and metamorphosis in eukaryotes (Benaim and Villalobo, 2002; Cyert, 2001; Vetter and Leclerc, 2003). Calumenin regulates the bone protein osteocalcin and the transforming growth factor BMP-2 (Wajih et al., 2006, 2004), which induces skeletogenesis across Eumetazoa (Zoccola et al., 2008). Calreticulin belongs to the group of intracellular integrin ligands (Vallar et al., 1999) and constituents of integrin-based adhesion complexes which induces osteoblast differentiation in mammals (Feng et al., 2000; Tran et al., 2002).

4.1.2. Polyp-specific processes

While the planula stage is marked by large changes in protein synthesis, chromatin architecture and mitochondrial metabolism likely involved in the planula-to-polyp transition, genes highest expressed in polyps (patterns 8, 10, and 12) also bear signatures of the morphogenetic transitions occurring during this life stage. Strikingly, pattern 8, which includes genes only upregulated in polyps, is the most populated pattern, and is over-represented by genes involved in development (GO:0009888), cell adhesion (GO:0007155), morphogenesis (GO:0048729), and chromosome structure (GO:0005694) (Table S3). Furthermore, there are many genes differentially expressed in polyps likely involved in calcification (Fig. 2).

The calicoblastic epithelium secretes a macromolecular network of proteins, lipids and polysaccharides known as the organic matrix (OM), which controls $CaCO_3$ nucleation (Clode and Marshall, 2003; Fukuda et al., 2003; Watanabe et al., 2003). Only calicoblastic cells in direct contact with the skeleton appear to be responsible for the synthesis and secretion of OM components (Puverel et al., 2005). Upregulation in polyp (pattern 8 and 12) of numerous genes involved in vesicle transport (Table 2), suggest that OM synthesis is actively happening. Other pattern 8 genes that may be involved in OM synthesis and calcification include zinc metalloproteinase nas-13 (CAON980 — an astacin-like metalloproteinase) and cysteinyl-tRNA synthetase (AOSC1122). Nas-13 belongs to a family of metalloproteinases involved in bone growth and morphogenesis (Xiong et al., 2006), and cysteinyl-tRNA synthetase regulates cysteine (cys) metabolism and protein synthesis (Hauenstein and Perona, 2008). Cys residues are common in many types of extracellular matrix proteins (Bhattacharya et al., 2002; Pearsall et al., 2002) since disulfide bonds act to create a structural framework. Furthermore, the upregulation of a neurogenic locus notch protein homolog (AOSF976 — pattern 3) in polyps and adults suggests that notch signaling may be involved in the development of calcifying cell types resembling that of mammals (Sciaudone et al., 2003). However, the assignment of AOSF976 to a protein involved in notch signaling is not strong (i.e. *e*-value = $6e^{-8}$). Similar to the neurogenic locus notch protein homolog, two other genes in pattern 3 (upregulated equally in polyp and adult relative to planula) seem to be involved in calcification. Galaxin (AOSF1176) is considered to be an important OM structural protein in the calcifying matrix of the scleractinian coral *Galaxea fascicularis* (Fukuda et al., 2003), and cation exchanger C3A12.06c (AOSF493) is involved in calcium transport.

4.1.3. Adult-specific processes

Pattern 3 genes mentioned above highlight how our approach can pinpoint gene expression patterns likely to be involved in a process of interest, e.g. calcification and the symbiotic lifestyle of adult corals.

While patterns 8 and 12 (in which polyps have the highest expression) bear many genes likely involved in calcification, it is interesting that patterns 1 and 5 (in which adults have highest gene expression) do not appear to contain calcification genes. These patterns, however, are also among the least annotated (10 and 17% respectively). Additionally, pattern 3 is also 10% annotated, thus highlighting the possibility that these patterns contain many coral-specific genes involved in skeleton deposition.

The 22% of genes differentially expressed in adults (pattern 1, 5, and 9) are likely involved in adult processes, such as sexual reproduction, bleaching, and symbiosis (Fig. 2). Azumi et al. (2007) suggested that adult gene expression responds to physiological and biological stimulations such as exposure to pollutant chemicals, physiological stress, and bacterial and viral infections. Reef-building corals are able to associate with a diverse range of marine organisms such as *Symbiodinium* spp., protists, archaea, bacteria, as well as viral communities, thus establishing the biological entity known as the coral holobiont (Harel et al., 2008; Rowher et al., 2002; Marhaver et al., 2008; Siboni et al., 2008). Indeed, perusal of genes within patterns 1, 5, and 9 supports these assumptions. Pattern 1 contains vitellogenin-2 (AOSF1022), an egg yolk protein involved in egg production across Metazoa (Byrne et al., 1989). Pattern 5 includes another yolk protein (AOSF642) and 33 genes previously identified in a thermal stress and bleaching study of adult *M. faveolata* (DeSalvo et al., 2008). Only four of these overlapping genes are annotated: *tachyletin-2* (AOSF387), four-domain protease inhibitor (AOSF907), a GFP-like protein (AOSF1131), and *SCRiP7* (AOSF1190). Likewise, 78% of the genes in pattern 9 were differentially expressed during thermal stress and bleaching, including: *SCRiP1* (AOSF1109), *SCRiP2* (AOSF1192), *SCRiP8* (AOSF810), adenosine receptor A1 (AOSF1361), universal stress protein A-like (AOSF1521), neurofascin (CAOO655), and CCAAT/enhancer-binding protein gamma (AOSF882). Thus, patterns 5 and 9 definitely include genes involved in environmental stress and symbiosis.

4.2. The planula and adult transcriptomes are more similar to each other than to the polyp transcriptome

Although planula larvae and adult corals display distinct physiological and morphological characteristics as well as different environmental niches, their gene expression profiles were more similar to each other than to that of the polyp. There are three lines of evidence that support this notion: 1) a distant matrix which explicitly shows that planula and adult are the most similar of the three stages based on the expression of all assayed genes (Fig. 3); 2) the number of genes that fall within each expression pattern; and 3) the cDNA library representation of genes within each pattern.

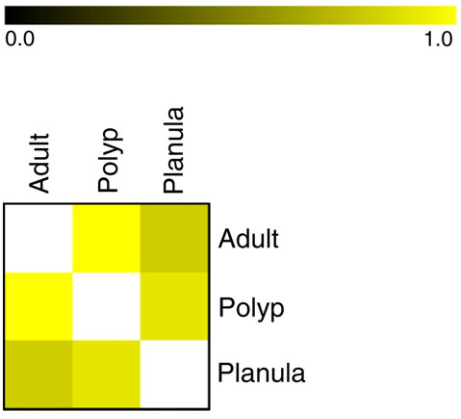

Fig. 3. A gene distance matrix illustrating that the adult and planula transcriptomes are more closely related to each other than to the polyp transcriptome. Black = identical gene expression and yellow = highly different gene expression in a comparison of two life stages. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The distance matrix (Fig. 3) shows the degree of distance between the three life stages based on the expression of all assayed genes. We see that the adult and polyp are the most distantly related transcriptomes, followed by the planula–polyp comparison. The adult–planula comparison is the least distant; thus, the adult and planula transcriptomes are the most closely related. With respect to the second point, patterns 7 and 8 (adult and planula are similar) contain 37% of all DEGs. In contrast, patterns 5 and 6 (polyp and planula are similar) contain only 17% of all DEGs, and patterns 3 and 4 (adult and polyp are similar) contain 22% of genes. Thus, adults and planulae share more DEGs than either share with polyps. With respect to the third point above, if we look at the cDNA library assignments of genes within each pattern, then we see a similar result (last three columns of Table 1). The mean percent of transcripts from the adult cDNA library (AOSF) is 89% for patterns in which adults were the highest (1, 5, and 9); 35% for polyps (8, 10, and 12); and 49% for planulae (2, 4, and 11). This result suggests that planulae use more genes isolated from an adult cDNA library than polyps. Also evident from this type of analysis is the percentage of genes from the egg library (AOSB) used by polyps: 16%, which is in stark opposition to 0% in adults and 1% in planulae. Twenty percent of genes in pattern 8 are from the egg library, and interestingly these egg-stage genes are 62% annotated; the remainder of genes in pattern 8 are 17% annotated. Similarly, 29% of genes in pattern 12 are from the egg library, and they are 50% annotated while the rest of the genes in pattern 12 are 10% annotated. These findings suggest that the egg-stage genes highest expressed in polyps are evolutionarily conserved genes. For example, the AOSF genes in pattern 8 are involved in development, cell adhesion, and chromosomal interactions (Table 3).

Our results are similar to the development of *Drosophila melanogaster* — many genes are expressed in two waves with embryonic expression being repeated in pupae and larval expression being repeated in adults (Arbeitman et al., 2002). Interestingly, we see egg-stage genes being expressed later in polyps, and planula-stage genes being expressed later in adults. While more research is necessary to fully understand co-expressed gene networks in contrasting life stages, it is possible that some aspects of the genetic programming used in planulae, for example, to generate polyps during metamorphosis are also used in adults to generate polyps during asexual reproduction. It is also important to note that our microarray represents a small portion of the coral genome and lacks cDNAs from a polyp-stage- specific library, and thus future studies will be needed to definitely prove the results discussed above.

4.3. Coral-specific genes: taxon-restricted or lack of genomic data?

By comparing the subset of genes that were differentially expressed in each developmental stage, we observed clear differences in the level of annotation. Genes that were differentially expressed in planula (patterns 2, 3, 4, and 11 — 116 annotated DEGs) represent 45% of all annotated sequences; whereas, polyp-specific patterns (7, 8, 10, and 12 — 101 annotated DEGs) and adult-specific patterns (1, 5, 6, and 9 — 42 annotated DEGs) account for 39% and 16% of all annotated genes, respectively (Fig. 2). The higher number of annotated sequences in larval stages might reflect the common ancestry of the mechanisms regulating metamorphosis across Bilateria. It has been proposed that the basic mechanisms regulating metamorphosis have been conserved and that similar groups of genes control morphological transitions across metazoans with biphasic life cycles (Heyland and Moroz, 2006). This is consistent with the idea that conserved signaling pathways regulate the expression of taxon-specific genes during *Haliotis asinina* metamorphosis (Williams et al., 2009), and that molecules regulating coral metamorphosis and early calcification are also present in other organisms (Grasso et al., 2008). These observations suggest that although conserved signaling pathways are

likely to regulate basic cellular processes during metamorphosis such as apoptosis and protein synthesis, they also control the expression of taxon-specific molecules responsible for taxon-specific variations.

The low annotation of transcripts differentially expressed in adults suggests the presence of a high number of uncharacterized cnidarian- or scleractinian-specific genes likely involved in the regulation of cnidarian/scleractinian-specific functions, such as symbiosis and calcification. Among these genes are members of a recently identified scleractinian-specific, cysteine-rich, secreted protein family (SCRiPs) (Sunagawa et al., 2009). We found eight SCRiPs differentially expressed in the present analysis, six of which were highest expressed in adults (three SCRiPs each in both patterns 5 and 9). While the functions of the SCRiPs are currently unknown, this is an illustrative example of how many coral-specific genes may be predominantly active in adult stages. Consistently, Azumi et al. (2007) reported that 29% of all *C. intestinalis* DEGs had no similarity to known proteins. Relatively high proportions of non-annotated genes were differentially expressed in adult clusters, which suggest that these genes are ascidian-specific genes involved in morphogenesis and the function of adult organs. Grasso et al. (2008) found that 15% of DEGs were specific to *Acropora millepora*, but in contrast to our results, most *Acropora*-specific genes were identified in planulae and polyps. The percent of *Acropora*-specific genes (i.e. non-annotated) in CII (planula-specific, 24%) and CIV (polyp-specific, 26%) were the highest among all clusters; thus, these two clusters still had over 70% of the genes annotated. Again, these differences are likely connected to the scarcity of adult material printed on the microarray used in Grasso et al. (2008), which ultimately supports the notion that adult corals utilize many taxon-restricted genes. We believe the high number of non-annotated genes in adult corals reflects adaptations unique to this life stage in which the coral holobiont takes form. However, as suggested by Williams et al. (2009) and Grasso et al. (2008), it is also possible that the number of taxon-restricted genes reflects the lack of genomic data for a vast range of non-model organisms and that some of these molecules have a wider distribution.

4.4. Conclusions and future outlook

This study represents the first microarray analysis focused on the development of a Robust/Short clade coral. While our microarray represents a small proportion of the coral genome, we nevertheless identified key molecular and cellular components underlying metamorphosis and the onset of calcification in *M. faveolata*, which include genes involved in: protein synthesis, chromatin assembly, and mitochondrial metabolism in planulae; morphogenesis, protein catabolism, and organic matrix synthesis in polyps; and sexual reproduction, stress response, and symbiosis in adults (Fig. 2). Furthermore, the gene expression patterns identified in this study are a valuable resource for those searching for genes involved in processes which occur exclusively in a particular life stage or in a combination of life stages, such as lineage-specific cell differentiation, initiation of skeleton deposition, and establishment of symbiosis.

Transcriptomic studies in scleractinian corals are necessary to generate hypotheses and ultimately accelerate discovery in many areas of coral biology as they represent a powerful tool to study gene expression profiles during both experimental treatments and key developmental time points. We kindly encourage researchers to test hypotheses borne out of this study. At the present time, the functions and physiological interactions of gene products identified in coral microarray studies are restricted to conceptual models based on analogous pathways in model organisms, thus we cannot overstate the need for cellular assays such as coral cell lines, microinjection methodologies, and RNA interference assays to validate these models. In addition, these techniques will also help to elucidate the cellular localization and physiological function of cnidarian/scleractinian-specific genes (Mohrlen et al., 2006), as well as the ancestral function of genes homologous between cnidarians and vertebrates (Kortschak et al., 2003).

Acknowledgements

The authors would like to thank members of the Medina Laboratory at UC Merced, and members of the photobiology group in Puerto Morelos, Mexico for aid in many aspects of this study. We are especially grateful to the numerous members of the spawning team in Mexico. The comments of two anonymous reviewers greatly improved this manuscript. This study was supported through a UC MEXUS-CONACYT Grant to MM and RI-P, NSF awards to MM (BE-GEN 0313708 and IOS 0644438), and start-up funds from UC Merced to MM.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.margen.2009.07.002.

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