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Suppression subtractive hybridization PCR isolation of cDNAs from a Caribbean soft coral

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Abstract Transcriptomic studies of marine organisms are still in their infancy. A partial, subtracted expressed sequence tag (EST) library of the Caribbean octocoral Erythropodium caribaeorum and the sea fan Gorgonia ventalina has been analyzed in order to find novel genes or differences in gene expression related to potential secondary metabolite production or symbioses. This approach entails enrichment for potential non-"housekeeping" genes using the suppression subtractive hybridization (SSH) polymerase chain reaction (PCR) method. More than 500 expressed sequence tags (ESTs) were generated after cloning SSH products, which yielded at least 53 orthologous groups of proteins (COGs) and Pfam clusters, including transcription factors (Drosophila Big Brother), catalases, reverse transcriptases, ferritins and various "hypothetical" protein sequences. A total of 591 EST sequences were deposited into GenBank [dbEST: FL512138 - FL512331, GH611838, and HO061755-HO062154]. The results represent proof of concept for enrichment of unique transcripts over housekeeping genes, such as actin or ribosomal genes, which comprised approximately 17% of the total dataset. Due to the gene and sequence diversity of some ESTs, such sequences can find utility as molecular markers in current and future studies of this species and other soft coral biogeography, chemical ecology, phylogenetics, and evolution.

Keywords: cDNA, *Erythropodium caribaeorum*, EST, *Gorgonia ventalina*, gorgonian, sea fan

INTRODUCTION

Knowing the complete nucleotide genome sequence of any given organism does not guarantee complete elucidation of its molecular biology. For a more accurate profile of ongoing cellular processes, attention must turn to the mRNA transcripts and resulting proteins and enzymes (the phenotype) encoded by the nuclear genome. For this reason, the fields of genomics, transcriptomics, and proteomics all help advance our understanding of basic cellular metabolism (DeLong et al. 2006; Koonin and Wolf, 2006), and this paper focuses on the second tier of mRNA analyses.

Genomics and other "omics" analyses of marine organisms have only recently begun. Many marine invertebrates spark interest for their ecology and residence in unique and threatened habitats (coral reefs), or because they produce bioactive and potentially therapeutic natural products (Newman and Cragg, 2004). For example, the bryozoan *Bugula neretina*, in conjunction with a microbial endosymbiont, produces the macrocyclic lactone Bryostatin-1, which has been shown to have

immunomodulatory and anti-tumor capabilities through its modulation of protein kinase C activity (Lopanik et al. 2006).

Natural product biosynthesis in scleractinian corals appears relatively less pronounced, perhaps because these organisms already have other protective mechanisms, such as nematocysts, a hard skeleton, and mucus production. However, octocorals (soft corals) can yield a diverse array of terpenoids, such as the anti-inflammatory and analgesic agents, the pseudopterosins, derived from the octocoral *Pseudopterogorgia elisabethae* (Look et al. 1986). Another octocoral, the common encrusting gorgonian, *Erythropodium caribaeorum* (EC) fastens to rocky substrate on Floridian and Caribbean coral reefs. This coral is known for the production of eleutherobin, a potent terpenoid chemo-therapeutic agent with high activity against breast, renal, and lung cancer, and a mechanism of action similar to Taxol™. In addition to the anti-cancer activities, diterpene biosynthetic intermediates of EC have been shown to also have antimitotic properties (Britton et al. 2001). The production of these secondary metabolites within the octocoral has indicated defensive properties that deter predation by reef fish (Fenical and Pawlik, 1991). This study stemmed from a primary goal to characterize key biosynthetic enzymes (*e.g.* diterpene synthases), central to the production of eleutherobin by EC or its symbionts.

The approach to find potential biosynthetic pathway genes related to eleutherobin or other secondary metabolism was based on enrichment. Genomic studies suggest that highly expressed housekeeping or “core” genes probably evolve more slowly, while rare (but active) transcripts exhibit more divergent sequences (Green et al. 1993). This is reasonable, if it is assumed that the cell has to “test” novel sequences before committing a large amount of metabolic energy into its coding and translation of a potentially useless protein (Krylov et al. 2003). Thus, this study aims to bypass most housekeeping transcripts by enriching for novel sequences via the utilization of suppression subtractive hybridization polymerase chain reaction (PCR) (Diatchenko et al. 1999). This method has been previously applied to find symbiosis-specific genes (Rodriguez-Lanetty et al. 2006). Other studies have applied transcriptomic analyses to soft corals, but only the more recent high throughput studies have yielded expressed sequence tags (EST) sequence data (Overbeek et al. 2005; Hoover et al. 2007; Hoover et al. 2008; Schwarz et al. 2008). The present study also represents an exercise to apply mRNA enrichment methods to a non-model marine coral (rather than *Montastraea* or *Acropora*). *Gorgonia ventalina* was used as the suppression subtractive hybridization (SSH) reference strain (driver) since it has different and fewer secondary metabolites than the target *Erythropodium caribaeorum*. Similar to M. Matz's and other initiatives to allow more open access to DNA sequences (http://www.bio.utexas.edu/research/matz_lab/matzlab/Data.html) it is expected that the release of these data can spur further experiments and hypothesis testing in coral species.

MATERIALS AND METHODS

Sample and RNA extraction

EC was collected by SCUBA at Dania Beach, Fort Lauderdale, Florida, USA at a depth of 10 m. *Gorgonia ventalina* (GV) samples were obtained from live cultured specimens at Harbor Branch Oceanographic Institution. All samples were immediately frozen in liquid nitrogen and stored at -80ºC or placed in RNALater upon collection and stored at -20ºC.

Total RNA from EC and from GV was extracted using the RNeasy Plant Mini kit (Qiagen, Valencia, CA, USA) or RNAwiz reagents (Ambion, Austin, TX, USA) according to manufacturers’ instructions. Microbial RNA was not separated from eukaryotic mRNA during this process. DNA digestion was performed with Amplification Grade DNAse I (Invitrogen, Carlsbad, CA, USA). RNA quantity was assessed by spectrophotometry (A260/A280), while RNA quality was assessed by gel electrophoresis.

SSH PCR

cDNA synthesis was performed with approximately 3 µg of total RNA. Enrichment via EC *vs*. GV for suppression subtractive hybridization PCR (EC x GV SSH PCR) to obtain novel non-housekeeping transcripts was accomplished with a combination of SuperSmart PCR cDNA Synthesis (oligo dT primers) and PCR-Select™ cDNA Subtraction Kits (Clontech, Mountain View, CA, USA). The pCRII

Topo TA plasmid vector (Invitrogen) was then used to clone enriched cDNAs after their PCR amplification with supplied adapters and primers.

Sequencing

Sequences were generated by Symbio Corporation (Menlo Park, CA) on ABI 3730xl DNA sequence analyzers. The resulting chromatograms (.scf extension) were base called via Phred (Ewing et al. 1998) which generated quality scores for each nucleotide. Phred quality scores are on a logarithmic scale, with a score of 20 (Q20) indicating a 99% probability that the base was called correctly.

Table 1. Representative *E. caribaeorum* EST and unigenes after BLASTX searches.

EC clone	GenBank Best Hit (Acc. No.)	Top BLASTX Hit Description	Gen-Bank No. (*)	Score	E Value
353 C	XP_002786203	RAS small GTpases RIC1/ypt1, putative [*Perkinsus marinus* ATCC 50983]	FL512138	168	4.00E-47
353 P	XP_002433402	UDP-galactose transporter, putative [*Ixodes scapularis*]	GH611838	114	1.00E-23
355 M	ABK29471	CHK1 checkpoint-like protein [*Helicoverpa armigera*]	FL512144	88.6	2.00E-16
356 BV	ABA28990	DNA J-like protein 2 [*Symbiodinium* sp. C3]	FL512146	125	1.00E-27
356 CP	NP_001174722	Os06g0286310 [*Oryza sativa Japonica Group*]	FL512148	91.7	3.00E-17
356 U	XP_002112452	Expressed hypothetical protein [*Trichoplax adhaerens*]	FL512151	136	9.00E-31
382 AP	XP_001624566	Core-binding factor subunit beta [*Nematostella vectensis*] and Big brother CG7959-PA [*Drosophila melanogaster*]	FL512174	199	2E-49
382 AY	XP_001624566	Core-binding factor subunit beta [*Nematostella vectensis*] and Big brother CG7959-PA [*Drosophila melanogaster*]	FL512176	193	1.00E-47
382 DI	XP_002742298	PREDICTED: ferritin-like protein-like [*Saccoglossus kowalevskii]*	FL512182	149	9.00E-37
382 DV	XP_002742298	PREDICTED: ferritin-like protein-like [*Saccoglossus kowalevskii*]	FL512192	172	5.00E-42
382 FD	ZP_06579711	Putative reverse transcriptase [*Zingiber officinale*]	FL512203	94.7	2.00E-17
386 C	AAF91388 XP_001676261	SocE [*Myxococcus xanthus*]	FL512217	99.4	2.00E-19
418 BB	XP_002644180	Hypothetical protein CBG17156 [*Caenorhabditis briggsae*]	FL512261	51.6	1.00E-04
418 BC	ZP_03289628	Hypothetical protein CLONEX_01831 [*Clostridium nexile DSM 1787*]	FL512262	142	1.00E-31
386 B	ZP_03104354	SocE [*Bacillus cereus W*]	FL512216	80.9	5.00E-14
386 AB	NP_608540	CG2839 CG2839-PA [*Drosophila melanogaster*]	FL512212	37.4	1.00E-04
386 K	XP_001633321	Predicted protein [*Nematostella vectensis*]	FL512221	71.2	4.00E-11
422 E-1	XP_001633321	Predicted protein [*Nematostella vectensis*]	FL512303	68.2	2.00E-09
419 CJ	XP_002165056	PREDICTED: similar to acidic ribosomal protein A1 [*Hydra magnipapillata*]	FL512277	101	7.00E-20
381 AY	XP_002128607	PREDICTED: similar to actin [*Ciona intestinalis*]	FL512160	182	1.00E-44

(*) Newly assigned GenBank EST Accession # for the corresponding EC clone shown in first column.

Bioinformatics data analyses

The sequences were mapped, 6-frame translated, to the NR protein database, using the BLASTX option in NCBI BLAST (http://blast.ncbi.nlm.nih.gov/). Matches with E-value $<10^{-2}$ were further analyzed using in-house scripts that select the most specific gene annotations from among all the high-quality alignments to proteins in the database. This procedure is necessary in order to avoid a common annotation error caused by situations where the top BLAST hit has a non-specific label (*e.g.* hypothetical protein) yet other high-quality matches exist that have been assigned a specific functional label.

Putative housekeeping genes were identified through keyword searches using a manually curated list of keywords associated with highly expressed microbial (Karlin and Mrazek, 2000) and eukaryotic genes (Eisenberg and Levanon, 2003). Furthermore, each gene was assigned to one of the three groups (Eukaryotes, Prokaryotes or Viruses) based on the taxonomic assignment of the nearest BLAST hit when such an assignment was available in the database.

Sequence Submission. Novel ECEST sequences were deposited into GenBank (dbEST: FL512138-FL512331, GH611838, and HO061755-HO062154).

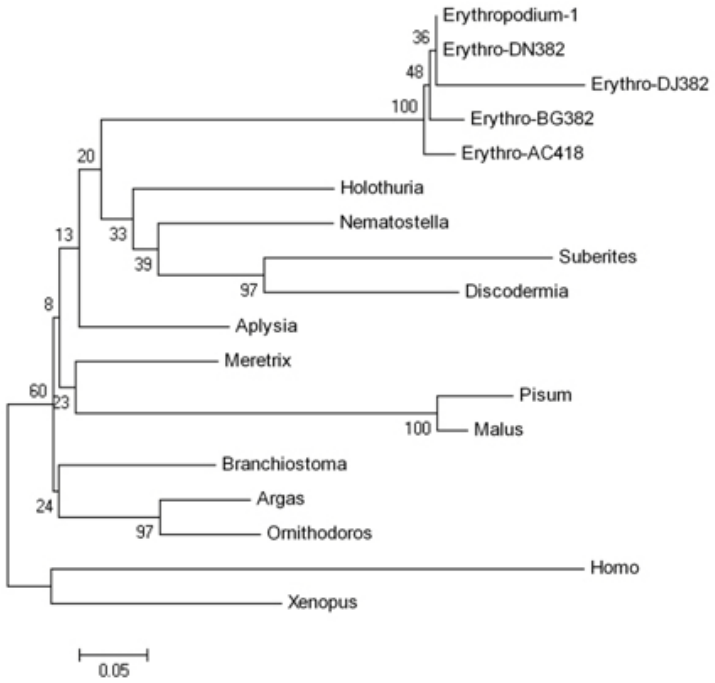

Fig. 1. Evolutionary relationships of ferritin of 18 taxa inferred from amino acid sequences using the Minimum Evolution (ME) method (Rzhetsky and Nei, 1992). The bootstrap consensus tree inferred from 100 replicates is taken to represent the evolutionary history of the taxa analyzed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths equal to the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm (Nei and Kumar, 2000) at a search level of 3. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 107 positions in the final dataset. Phylogenetic analyses were conducted with MEGA4 (Tamura et al. 2007). *Erythropodium* accession numbers are listed in the text, while accession numbers for reference sequences are as follows: *Nematostella vectensis* - XP_001632011; *Aplysia californica* - ABF21074.1; *Suberites ficusgi* - CAG25529.1; *Dermatophagoides pteronyssinus* AAG02250; *Argas monolakensis* - ABI52633; *Holothuria glaberrima* - ABS29643; *Ornithodoros parkeri* - ABR23389; *Meretrix meretrix* - CAB72315; *Dermacentor andersoni* - AAG02250.1; *Pisum sativum* - CAA51786.1; *Xenopus laevis* - AAB20316.1; *Branchiostomagi belcheri* - AAQ21039.1; *Malus xiaojinensis* - AAK83702.1|AF315505_1; *Homo sapiens* - CCDS33070.1.

RESULTS AND DISCUSSION

More than 591 EST sequences were generated in this study, with 238 ESTs [dbEST: FL512138 - FL512331, GH611838, and HO061755-HO062154] having significant matches against proteins in the NR database. Table 1 shows a sample representation of the top sequence identities obtained after BLASTX searches, showing relatively high hit scores and significant E values. As expected, a considerable fraction of EST hits (37 sequences) showed similarities to the sequenced anthozoan *Nematostella vectensis* genome (Sullivan et al. 2006). A large fraction of these sequences (25) had matches to unknown *N. vectensis* proteins (*e.g*. 358A, 382 EL) while the others matched a predicted core-binding factor (*e.g.* 382AF, 382AP).

Among potential primary (core) metabolic genes, only 15 actin sequences were identified in this limited dataset with identity matches to diverse taxa. Only one ribosomal related sequence was identified. Comprehensive profiles of different gene categories are shown in Table 2.

The majority of the sequences found (73.1%) appear to be of eukaryotic origin. This result was expected due to the use of oligo dT primers during first strand cDNA synthesis that typically tend to minimize the presence of prokaryotic and bacterial sequences, although the presence of these is still expected. Also, the EC dataset showed a high proportion of hypothetical sequences, consistent with similar analyses of larger EST datasets derived from 454 sequencing (Meyer et al. 2009). At least 60 “unknown” or “hypothetical” sequence designations comprised a large proportion of database matches in this dataset (19% of the eukaryotic and 46% of prokaryotic sequences, respectively), while several other gene categories also appeared in relatively high proportions, such as 22 total ferritin sequences (9.3%), 16 *Myxococcus* and *Bacillus* SocE proteins (6.7%), and 7plant reversetranscriptase sequences (2.9%).

Specific EC cDNAs, such as several transcription factors (with lower scoring thresholds), similar to those previously characterized in cnidarians (Seipel et al. 2004) also appear to have been detected. Within this category, ten clones such as 382AF and 382AP matched to a predicted core-binding factor in *Nematostella vectensis*, and a protein homologous to the *Drosophila* protein Big Brother, which has transcriptional regulatory functions (Kaminker et al. 2001). Another clone (386 R) showed similarity to a galaxin protein found in coral exoskeletons and used in coral systematics (Wirshing and Baker, 2008).

A small portion (12% or 22 sequences) of the 172 of the eukaryotic ESTs also grouped into iron-sulfur assemblages that included ferritin-like proteins. As stated above, ferritin represents a ubiquitous iron storage protein (found in animals, plants, bacteria, and archaea) with key functions of iron detoxification and sequestration (Harrison and Arosio, 1996). Ferritin has also been implicated in redox stress responses (Rocha and Smith, 2004; Theil, 2007).

Table 2. ***Erythropodium caribaeorum*** **ESTs.**

Category	ESTs Number	% of Total
Total	591	100
Eukaryotes	174	29.44
Prokaryotes	62	10.49
Viruses	1	0.17
Unknown	1	0.17
No hit	353	59.73
Euk hypothetical	32	18.39
Euk housekeeping	30	17.24
Euk other	112	64.37
Prok hypothetical	28	45.16
Prok housekeeping	0	0.00
Prok other	34	54.84

Alignments and cursory phylogenetic analysis of diverse ferritin amino acid sequences were performed to place the present EC ferritin sequences within a taxonomic framework (Figure 1). The alignment revealed the presence of conserved tyrosine sequences and extends the finding of Schwarz et al. (2008) that indicated several conserved motifs, likely relevant to ferritin function, which could be used for future primer or probe design. The minimum evolution trees showed that EC sequences allied with each other formed a weak clade with other marine invertebrates, but not close with the cnidarian *Nematostella*. The ambiguity of positioning may be due to homoplasious residues in ferritin sequences, suggesting potential drawbacks as a deep phylogenetic marker despite the protein's likely ancient origin. Although paralogous EC ferritin sequences are possible, the number of potential coral-derived ferritins derived from this subtraction may highlight the importance of this protein for the coral or its microbial community.

As expected, bacterial and protozoan sequences were also detected in the library, albeit in fewer numbers (62 sequences - 26%), and could be associated with either transient or resident symbionts associated with the corals (Lopez et al. 2010). For example, sequences included a SocE gene, involved in protein degradation and amino acid scavenging in *Myxococcus*, a *Streptomyces* two-component system sensor kinase, and diverse *Clostridium, Arthrobacter sp.* and *Plasmodium chabaudi* hypothetical sequences.

Although the inclusion of specific metabolic genes or organismal source of these products has not yet been validated by quantitative PCR (qPCR), the current expression SSH analyses places the EC-derived sequences in an interesting context. For example, these genes may be active in distantly related species, or may represent expression of rare genes. It would also be interesting to test for the expression of novel transcription factor sequences, which may be correlated with secondary metabolite biosyntheses.

It was unfortunate that the originally targeted eleutherobin or related diterpene synthase genes were undetected in this study. However to date, relatively few diterpene genes have been characterized or isolated, with none from marine organisms. Moreover, very few biosynthetic pathway genes appeared to be detected with the exception of a putative RTX toxin (ZP_01442131). Nonetheless, this does not preclude the possibility that biosynthetic gene sequences occur among the 353 EC EST sequences that show no hits with current databases. For example, proteins similar to those in the moenomycin-producer *Streptomyces ghanaensis* genome appear to be present in the *Erythropodium* dataset.

One could also begin to monitor gene expression variation across different coral taxa that would help characterize an unknown sequence's importance and function**.** For example EC can reside in much different reef substrates than GV and thus may produce more bioactive natural products as a consequence. Other explanations for a particular cDNA profile may stem from the presence of specific microbial associates or environmental cues. Overall, this study shows the utility and benefits of enriching and subtracting cDNAs to remove abundant core genes, identify novel genes, metabolic pathways, or variation in gene expression related to particular ecologies (Matus et al. 2008). The 591 soft coral ESTs (with 353 without hits) newly isolated for this study represent a valuable library since the majority of the published genetic sequence data belongs to hard corals but not much work has been devoted to soft corals.

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