Transcriptome Analysis of the Scleractinian Coral *Stylophora pistillata*

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Abstract

The principal architects of coral reefs are the scleractinian corals; these species are divided in two major clades referred to as "robust" and "complex" corals. Although the molecular diversity of the "complex" clade has received considerable attention, with several expressed sequence tag (EST) libraries and a complete genome sequence having been constructed, the "robust" corals have received far less attention, despite the fact that robust corals have been prominent focal points for ecological and physiological studies. Filling this gap affords important opportunities to extend these studies and to improve our understanding of the differences between the two major clades. Here, we present an EST library from *Stylophora pistillata* (Esper 1797) and systematically analyze the assembled transcripts compared to putative homologs from the complete proteomes of six well-characterized metazoans: *Nematostella vectensis*, *Hydra magnipapillata*, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Strongylocentrotus purpuratus*, *Ciona intestinalis* and *Homo sapiens*. Furthermore, comparative analyses of the *Stylophora pistillata* ESTs were performed against several Cnidaria from the Scleractinia, Actiniaria and Hydrozoa, as well as against other stony corals separately. Functional characterization of *S. pistillata* transcripts into KOG/COG categories and further description of Wnt and bone morphogenetic protein (BMP) signaling pathways showed that the assembled EST library provides sufficient data and coverage. These features of this new library suggest considerable opportunities for extending our understanding of the molecular and physiological behavior of "robust" corals.

Introduction

Corals and sea anemones include some of the morphologically simplest metazoan animals. As members of the phylum Cnidaria, a sister group of the Bilateria, they have proven to be particularly significant for deriving information about the gene content of the common metazoan ancestor, the Ureumetazoan [1]. Cnidarians are characterized as having a single body axis with only two germ layers and two to three cell lineages, which gives rise to a handful of different cell types [2]. This profile suggests an underlying genetic simplicity and that cnidarian body plans are likely to be specified and patterned by a subset of the genes known among 'higher' animals. However, several recent studies [3–5] have challenged this idea. Analyses of the *Hydra magnipapillata*, *Nematostella vectensis* and *Acropora digitifera* genomes have revealed that cnidarians possess homologs of many of the developmental genes that control the axial specification of the Bilaterian body plan [3,6–8]. Two examples are the Wnt and the BMP pathways.

The Wnt proteins are one of six families of signaling molecules that are responsible for the majority of developmental cell-cell interactions. They play important roles during vertebrate and invertebrate development and have the ability to activate different intracellular signaling pathways. In spite of the diverse roles of the Wnt's across the Bilateria, Kusserow et al. [4] have recognized certain conserved expression patterns in *Nematostella* by identifying a near-complete Wnt complement. They identified 12 of the 13 known vertebrate and invertebrate members of the Wnt subfamilies. This number is greater than that found in *Hydra*, which have most likely lost two of the ancestral Wnt subfamilies [9]. In contrast, the Ecdysozoa are known to have lost half of the ancestral Wnt diversity. The available data on the Wnt family reveal that there was greater diversity in the common ancestor of higher animals compared to extant species, which indicates gene loss over the course of animal evolution [10]. Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β (TGF-β) superfamily of proteins, which includes TGF-βs, activins, and

inhibins [11], and act as differentiation factors. BMP protein family members have been identified in a wide range of vertebrates (see [12] for review) as well as invertebrates such as nematode worms [13], flies [14,15], sea urchins [16], mollusks [17] and cnidarians [18–20]. Although the TGF-β superfamily exists in all animals, it appears that BMP2/4 homologues [20] and BMP receptors [21] arose after the Porifera (sponge)/(cnidarian,+ bilateral metazoa) split because BMP2/4 homologues are not found in sponges. Among Cnidaria, the BMP2/4 ortholog (bmp2/ 4-Am) is differentially expressed in the larval stage in *Acropora millepora* and is assumed to play a role in tissue differentiation and axis determination [19,22].

Among scleractinians, several phylogenetic studies have shown that the Scleractinia are divided into two primary lineages: the "complex" and "robust" clades [23–27]. Acroporidae, which includes *Acropora*, belongs to the "complex" clade. As shown by Shinzato et al. [7], there is a divergence between the "complex" and "robust" clades, necessitating studying corals from both clades. Four EST libraries from "complex" species [3,28–31] and two EST libraries of "robust" species [30–32] have been created to date. In this study, we performed EST sequencing of *Stylophora pistillata*, which is abundant in most coral reefs of the Indo-Pacific region (Fig. 1). This species has become a classic cnidarian model organism for studying symbiosis, physiology, cell biology, the calcification process and photosynthesis [33–44].

In the present study, 521,460 reads were generated from the coral holobiont, and were assembled as 15,052 contigs. We searched for putative coral protein homologs within a comprehensive non-redundant proteome database that included the complete genomes of six metazoan organisms: the cnidarians *Nematostella vectensis* (starlet sea anemone) and *Hydra magnipapillata*, the nematode *Caenorhabditis elegans* (worm), the arthropod *Drosophila melanogaster* (fruit fly), the echinoderm *Strongylocentrotus purpuratus* (purple sea urchin), the urochordate *Ciona intestinalis* (sea squirt) and the vertebrate *Homo sapiens* (human). Furthermore, comparative EST analyses were performed within the Cnidaria (Scleractinia, Actiniaria and Hydrozoa) and stony corals. Additionally, comparisons with the human proteome allowed us to delineate clusters of orthologous groups (COGs) and to focus on two developmental pathways (the Wnt and BMP pathways). This study provides a strong platform for further research on the molecular aspects of physiological and behavioral processes in corals.

Methods

Coral Sampling and Growth Conditions

Adult colonies of *Stylophora pistillata* were collected either from the field or from corals maintained in tanks for at least 20 years within the aquarium system of the Centre Scientifique de Monaco. The corals collected from the field (the Israeli Nature and National Parks Protection Authority approved the collection of corals in this study, permit No. 2011/38353) came from the Gulf of Aqaba in the Red Sea and were transferred after collection to tanks at the Marine Station of Eilat, Israel. The tanks were supplied continuously with seawater from the Red Sea. After an acclimation period of two weeks, colonies of *S. pistillata* were separated into different tanks that exposed the colonies to different environmental conditions, as described below. The cultured corals were maintained in a 300-liter aquarium supplied with seawater from the Mediterranean Sea (exchange rate 2% h^{-1}) under controlled conditions, as follows: semi-open circuit, temperature of $26.0 \pm 0.2°C$, salinity of 38.2‰, and light intensity of 175 μmol photons m^{-2} s^{-1} (using fluorescent tubes from Custom Sea Life®) under a 12:12 h photoperiod. The corals were fed three times

weekly with a mixture of *Artemia salina* nauplii, frozen adults of *Artemia salina*, and frozen krill.

Colonies from field and cultured sources were grown for two weeks under different conditions (Fig. 2) in order to maximize the expression of the greatest variety of genes. These conditions include different temperatures (18°C, 26°C and 32°C), different light/dark cycles, different pH levels (7 to 8.2), and either fed or not fed. Different field conditions were also included, with colonies being exposed to depths ranging from 5 to 50 meters. After exposure to different treatments, each colony was divided into fragments and snap-frozen in liquid nitrogen.

RNA Extraction and cDNA Library Construction

Total RNA was isolated from each of the fragments from the different treatments described above using TRIzol (Invitrogen) according to manufacturer's instructions. The quality of all of the RNA was checked using a Bioanalyzer (Agilent) RIN ≥9.5, and pools with the same amount of RNA were then created. The cDNA library was constructed using a Clontech SMARTer PCR cDNA synthesis kit and amplified using the Advantage 2 PCR kit according to the manufacturer's instructions. Subsequently, 2 μg of the amplified cDNA was normalized using the Trimmer kit (Evrogen) following the manufacturer's instructions and purified using the Qiaquick PCR purification kit (Qiagen). The normalized and non-normalized cDNA was sent to Roche for further analysis. Sequencing was performed using a 454 GS-Flx instrument according to the manufacturers' instructions (Roche). In order to obtain the abundant and rare transcripts, the library placed on the 454 plate was divided into two, half containing normalized cDNA, and the other half with non-normalized cDNA. The normalized and non-normalized cDNAs were sheared by sonication to produce short random fragments (300–400 bp) appropriate for 454 sequencing, and oligonucleotide adaptors were then ligated to the fragmented sequences. The 454 GS-Flx running plate was divided for internal comparison of normalized and non-normalized libraries (data not shown).

Sequence, Data Assembly and Analysis

The sequences were submitted to Newbler assembler version 2.6 for de novo assembly of 454-sequenced EST libraries using the default parameters. The assembled sequences were first automatically annotated with the SwissProt databases and then with several species-specific databases using the BLAST program. The species utilized in this analysis are as follows: the sea anemones *Nematostella vectensis*, *Aiptasia pallida*, *Metridium senile*, and *Anemonia viridis*; the stony corals *Acropora millepora*, *Acropora palmata*, *Acropora digitifera*, *Montastraea faveolata*, and *Porites astreoides*; the hydrozoans *Clytia hemisphaerica* and *Hydra vulgaris*; and the protist *Symbiodinium* sp. The best matches obtained using the following blast parameters: e-value ≤1e-10, best-hit-overhang = 0.1, best-hit-score-edge = 0.1; in order to avoid random short hits. Pathway analysis was later performed by running a pairwise sequence search compared with the KEGG-curated set of human proteins [45].

Phylogenetic Analyses

The alignments of all amino acid sequences were performed with the Multalin server [46] and phylogenetic relationships were investigated using Bayesian techniques as implemented in the computer program MrBayes v3.1.2 [47], starting from a random tree, generating 3,500,000 generations with sampling every 1000 generations, and with four chains in order to obtain the final (consensus) tree and to determine the posterior probabilities at the different nodes.
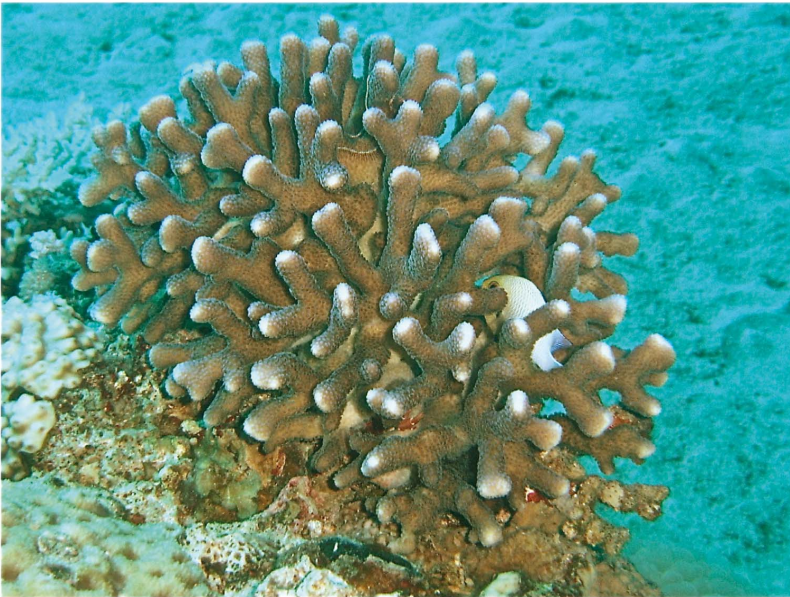
Figure 1. *Stylophora pistillata* (Esper, 1797) is a member of the "robust" lineage of stony corals. (A) A colony of *S. pistillata* growing in the Gulf of Eilat, Israel. (B) A colony in the aquarium at the Centre Scientifique de Monaco. (C) Magnification of a polyp in which the symbiotic dinoflagellates (*Symbiodinium* sp.) can be observed. (Credits-A: D. Zoccola; B &C: E. Tambutté/CSM).
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Results

EST Library Construction and Assembly

The normalized and non-normalized cDNA libraries constructed from *S. pistillata* holobiont RNA starting materials were based on an RNA pool collected from different environmental conditions to maximize the diversity of rarely expressed genes. Normalization of the library decreased the amounts of abundant transcripts and maximized the chances of finding new genes. We divided the 454 plate to run normalized and non-normalized cDNA libraries to obtain both abundant and rare transcripts. The two datasets were merged before assembly to generate a database of 523,533 sequenced reads. Assembly of these reads produced in 15,052 contigs with a mean length of 1,078 bp and N50 1,256 bp. These results are available from the NCBI and from (http://data.centrescientifique.mc/). Using BLAST searches against SwissProt database we were able to annotate 51% of the obtained sequences.

Comparative Analysis

S. pistillata transcript similarity searches were first performed against proteome libraries using blastX (see methods for specific parameters) (Table 1). At this level, 10,050 out of the 15,052 contigs (~67%) exhibited a positive match to proteins from one or more species. As expected, most of the *S. pistillata* transcripts matched sequences from the coral *A. digitifera* [7] and *N. vectensis*. The human proteome ranked third in terms of the number of hits, followed by the third diploblast *Hydra magnipapillata*. The two protostomes (the fruit fly and nematode) presented the lowest numbers of hits and were ranked last. The general tendency of the homolog presence across lineages is even more apparent if we group *S. pistillata* homologs across taxonomic groups, as shown in Fig 3. Most of the coral transcripts (5,381) were found in all three groups followed by transcripts found specifically in at least one of the species belonging to the diploblast group, (2,977). Moreover, a large set of diploblast homologs was found among the deuterostome representatives (1,579), supporting the findings of previous work in cnidarians [3,4,6,10] that has shown an unexpectedly high homology of diploblasts with deuterostomes in comparison to protostomes [48].

To study the differences between our EST library and those of the Cnidaria in general, we compared it to the available cnidarian EST libraries (see methods). Cross matches of *Stylophora* transcript homologs across Cnidaria (Table 2 and Fig. 4) showed 13,216 hits with stony corals, 8,787 with anemones and 6,246 with hydrozoans. Out of these, 5,988 were common to all three families.

Functional Characterization

After the evolutionary comparisons of homology between related and non-related lineages were complete, we successfully classified 7,764 *S. pistillata* transcripts using the STRING database v.6.3 [49] and a stringent assignment process. The graphical distribution of functional classes is illustrated in Figure 5. When comparing functional data between *S. pistillata* transcripts and the
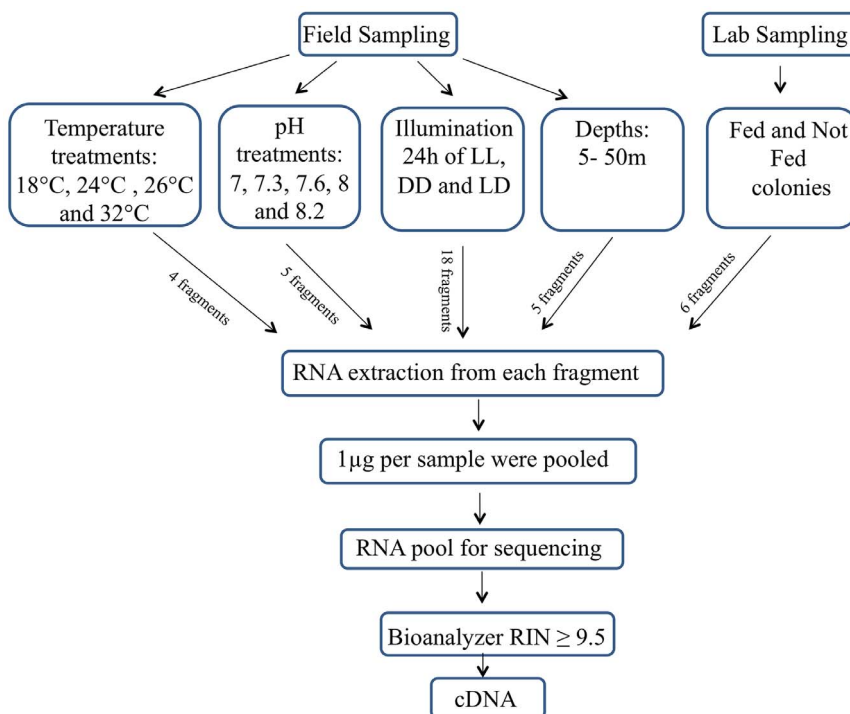

Figure 2. Flow chart of the experimental procedure. RNA was extracted from adult colonies of *Stylophora pistillata* from field and laboratory maintained colonies. The coral colonies were held under the presented treatments. LL – constant illumination; LD –12:12 h light/dark; DD – constant dark.
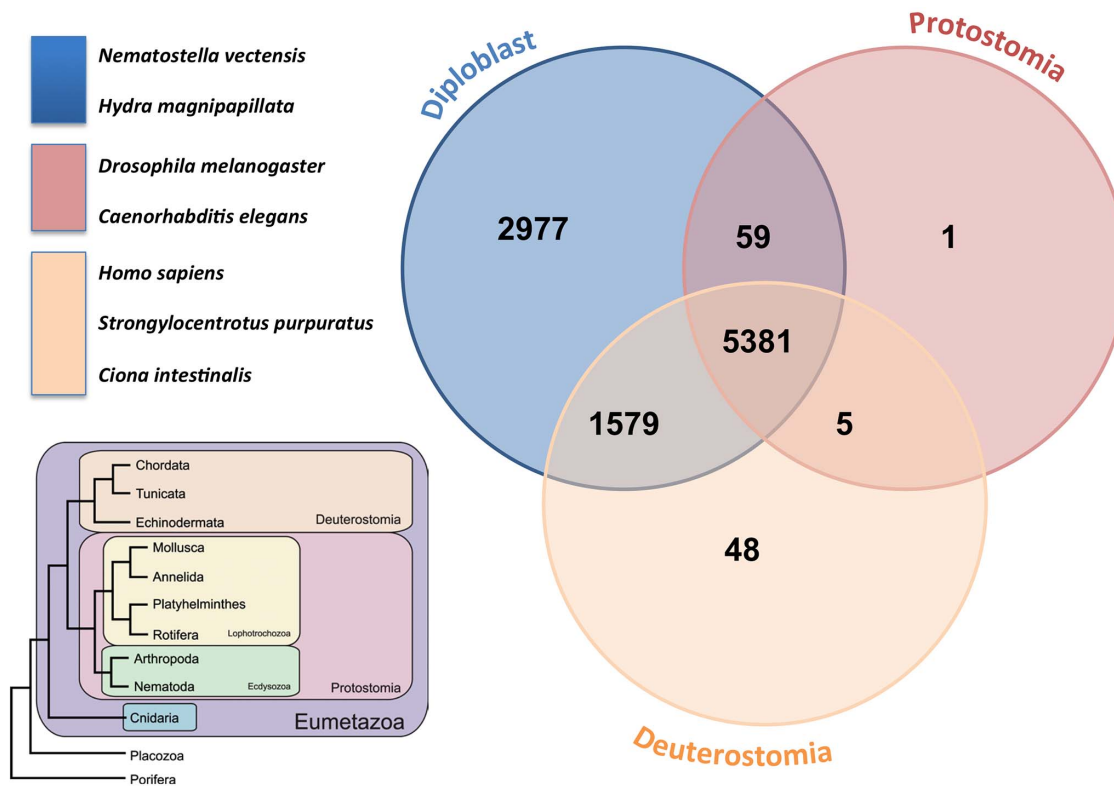doi:10.1371/journal.pone.0088615.g002

Figure 3. Venn diagram of transcript homologues from the stony coral *Stylophora pistillata* across taxonomic groups, including diploblasts (A. digitifera, *H. magnipapillata, N. vectensis,***); protostomes (***D. Melanogaster* and *C. elegans***); and deuterostomes (***S. Purpuratus, C. intestinalis* and *H. sapiens***).**
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human proteome, we observed the presence of equivalent functional elements. These elements are sufficient to reconstitute key processes in signaling and cell adhesion, which we chose to further explore by focusing on Wnt and BMP pathways (Fig. 6). The wingless (Wnt) and bone morphogenetic protein (BMP) pathways are essentially independent signaling mechanisms, although they often regulate similar biological processes. Using DAVID bioinformatics resources [50], we revealed the presence of equivalent functional elements in the Wnt and TGF-β signaling pathways (Fig. 6).

Discussion

EST Library Construction and Assembly

The transcriptome reported in this study comprises 15,052 sequences, which is less than the estimated number of genes in the cnidarian genomes described to date (32,338, 27,273 and 23,668 for *Hydra magnipapillata*, *Nematostella vectensis*, and *Acropora digitifera*, respectively). The raw reads were also assembled by MIRA 3.02 software [54] which produced a significantly higher number of contigs (48,075), however, a comparison between the two assemblies demonstrated that Newbler assembly was much less fragmented (data not shown), and hence was used for downstream

Table 1. The number of contigs with at least one valid hit obtained using blastX to search the *S. pistillata* EST library against various proteome libraries.

Proteome	Species	Number of proteins	Number of contigs
DIPLOBLASTS	*Hydra magnipapillata* (JGI)	32338	6084
	Nematostella vectensis (JGI)	27273	8228
	Acropora digitifera (Shinzato et al,2011)	23677	8868
DEUTEROSTOMES	*Homo sapiens* (Ensembl v.70)	104785	6377
	Ciona intestinalis (Ensembl)	17289	5578
	S. purpuratus (NCBI)	44303	5288
PROTOSTOMES	*Drosophila melanogaster* (Ensembl v.70)	26950	5123
	Caenorhabditis elegans (Ensembl v.70)	31234	4493

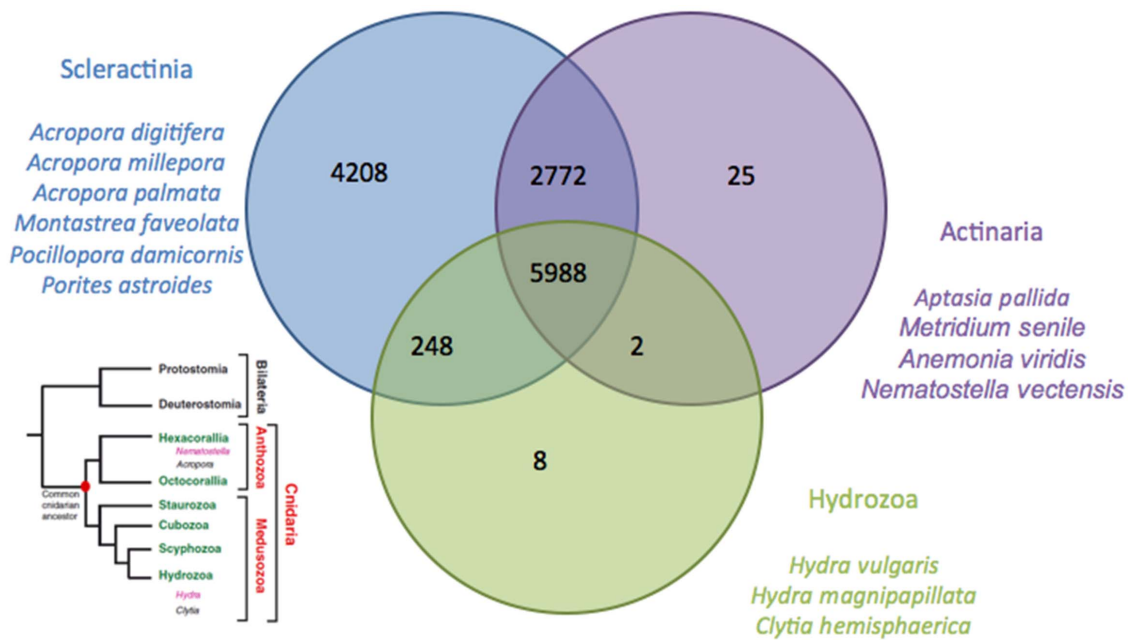doi:10.1371/journal.pone.0088615.t001

Figure 4. Venn diagram of transcript homologues from *Stylophora pistillata* across cnidarians (Scleractinia, Actiniaria, and Hydrozoa). The positions of the different groups are indicated in the left tree, obtained from Technau and Steele [63].
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analysis. We assume that the low number of contigs assembled is due to limited coverage, and that Newbler software produced an uncompleted albeit un-fragmented EST library.

Comparative Analysis

The outcome of the cross matches from the Venn diagram shown in Figure 3 illustrates that the highest number of unique hits exists between *S. pistillata* and the stony corals, as expected. Actiniaria are also hexacorallia but are solitary animals and do not produce a skeleton. The number of unique cross matches between

these two groups is lower, suggesting that biomineralization involves an important number of specific genes, as suggested by Shinzato et al. [7]. Finally, the lowest numbers of cross matches are obtained among the hydrozoans, which are more divergent (see the phylogenic tree in Fig. 4 obtained from Technau and Steele 2011), solitary and freshwater animals, which presumably accounts for part of the greater difference in the transcriptome.

It should be noted here that the cDNA library was constructed from holobiont tissue; hence, both zooxanthellae and host sequences are present in our library. Most coral libraries are

Table 2. The number of contigs with at least one valid hit obtained from a blastN search using the EST library from *S. pistillata* as a query against various cnidarian EST libraries.

Groups	Species	Number of ESTs	Number of contigs
Stony corals (Cnidaria; Anthozoa; Hexacorallia; Scleractinia)	*Acropora digitifera* (Shinzato et al,2011)	36,780	9,822
	Acropora millepora (Moya et al 2012)	52,963	10,560
	Acropora palmate (NCBI)	43151	6,626
	Montastraea faveolata (NCBI)	33,206	6,000
	Pocillopora damicornis	72,890	11,661
	Porites astreoides (matzLAB)	92,142	8,755
xxSea anemones (Cnidaria; Anthozoa; Hexacorallia; Actiniaria)	*Aiptasia pallida* (NCBI)	19,404	3,193
	Anemonia viridis (NCBI)	49,667	5,419
	Metridium senile (NCBI)	29,471	4,259
	Nematostella vectensis (JGI)	164,163	7,842
Hydrozoans (Cnidaria; Hydrozoa; Hydroida)	*Clytia hemisphaerica* (NCBI)	85,991	4,066
	Hydra vulgaris (NCBI)	179,642	4,025
	Hydra magnipapillata (JGI)	146,429	5,244

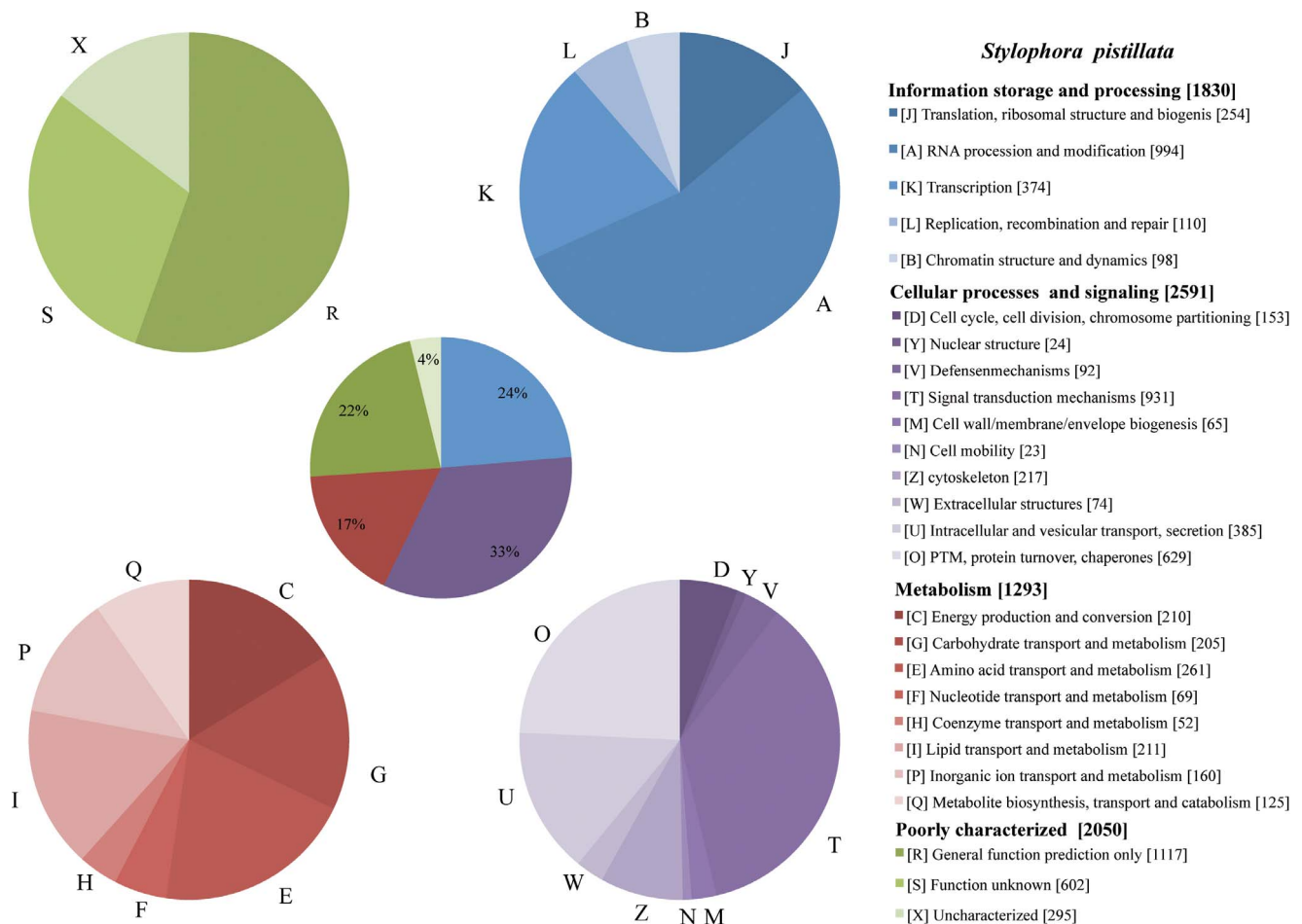doi:10.1371/journal.pone.0088615.t002

Figure 5. Functional characterization of *S. pistillata* transcripts. A total of 8,667 transcripts were classified into KOG/COG categories, giving rise to a total of 9,769 class assignments (some COGs belong to more than one class). The distribution among the functional classes is given in the central pie chart, with each super category slice broken down into separate pie charts in the corners (poorly characterized and uncharacterized functional categories [R and S] are combined with the uncharacterized category [X] in a separate pie chart at the top left corner). The overall class distribution follows those of other metazoan genomes, with the most abundant functions being signal transduction, protein turnover, translation and transcription.

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constructed from larvae, which do not contain symbionts, with *P. damicornis* and *P. astreoides* being exceptions. However, a blastX comparison with *Symbiodinium* EST libraries [51,52] downloaded from NCBI shows that few sequences mapped exclusively to the *Symbiodinium* genome and not to the coral *A. digitifera* (375 out of 15,052, i.e., ∼ 2.5% of sequences) illustrating that most of the contigs belong to the coral itself.

Among *Stylophora* ESTs, there are sequences coding for a specific organic matrix protein that provides the skeleton shape of the coral. The only protein to have been fully characterized from the calcifying matrix of scleractinian corals is galaxin, which was originally identified from the coral *Galaxea fascicularis* [53]. Based on similarity searches performed in an EST library from *Acropora millepora* [3,54], Reyes-Bermudez et al. [55] reported the sequences of three genes encoding galaxin-related molecules and their expression patterns during settlement and metamorphosis. A fourth galaxin protein, galaxin 2, has been characterized by Shinzato et al. [7], based on the sequencing of *Acropora digitifera*. blastX and blastn searches of the galaxin sequences against the *S. pistillata* EST library identified two ESTs with strong similarity. A phylogenetic tree generated by MrBayes program classified these ESTs to galaxin 2 and galaxin-like 1 (Figure 7). No homolog for

Amgalaxin-like 2 was found, probably since its expression is restricted to post-settlement polyps and is not observed in adults [56]. The presence of an Amgalaxin-like 1 homolog in an adult expression library is surprising, as Reyes-Bermudez et al. [55] showed that its expression remains restricted to the settlement and metamorphosis phases. Though, a species-specificity in galaxin-like diversity cannot be excluded. The availability of new coral genomes, from both robust and complex clades, is then a key feature in understanding the role and the impact of matrix proteins in coral calcification.

Functional Characterization

Concerning the functional classification, half the transcripts show similarity to sequences in the KEGG database (51%). The distribution of functional classes is consistent with those in both the human and fruit fly complete proteomes [56], with the most abundant categories being RNA processing and modification (A) signal transduction (T), protein turnover (O), and translation (J), indicating that the coverage of the EST library presented here was adequate (Figure 5). We decide to focus on the Wnt and BMP signal transduction pathways because of their importance in coral
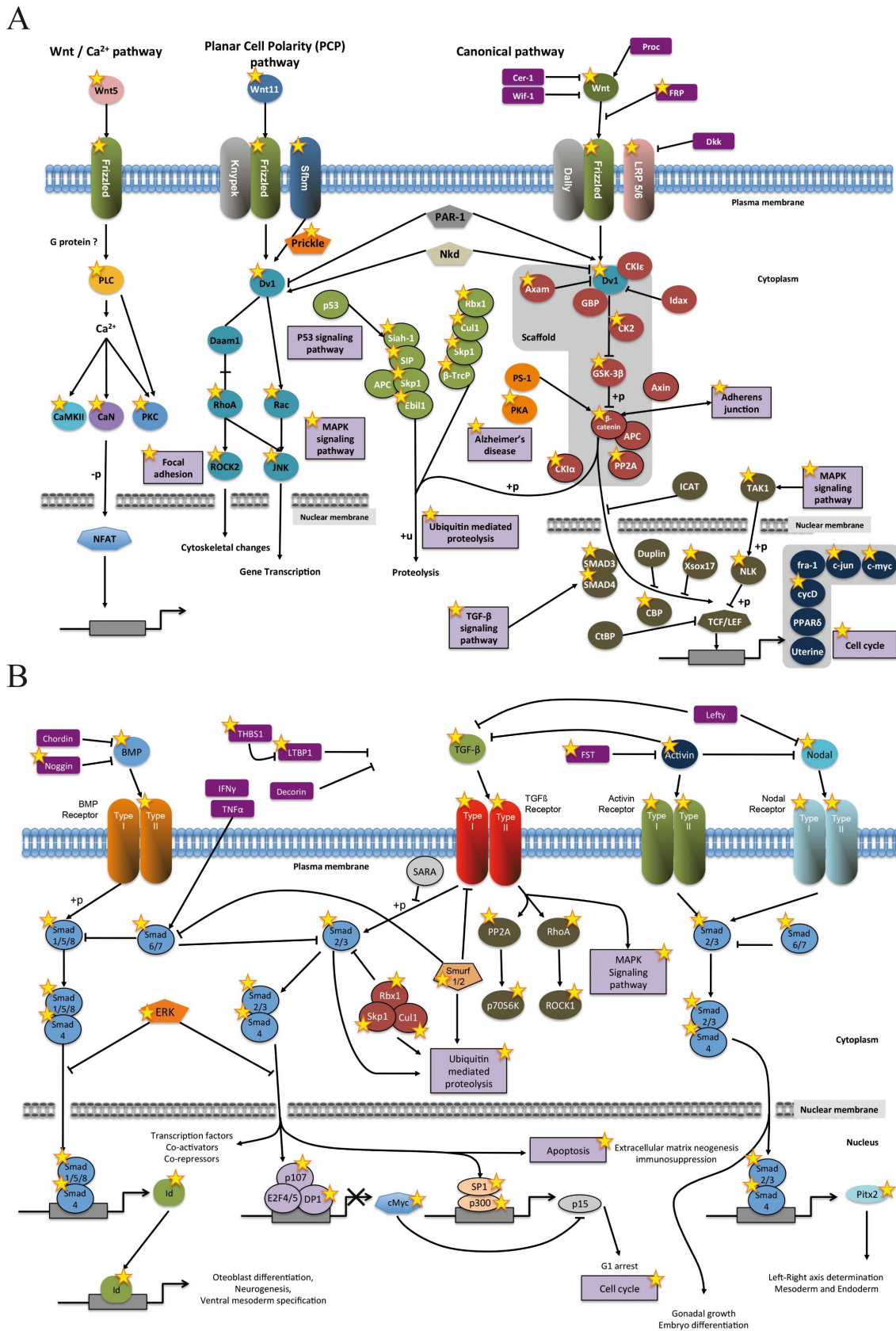
Figure 6. Human Wnt (A) and BMP (B) signaling pathways from KEGG pathways [45]. Sequences found in the *Stylophora pistillata* EST library based on sequence similarity with equivalent human homologues are labeled with a yellow star.
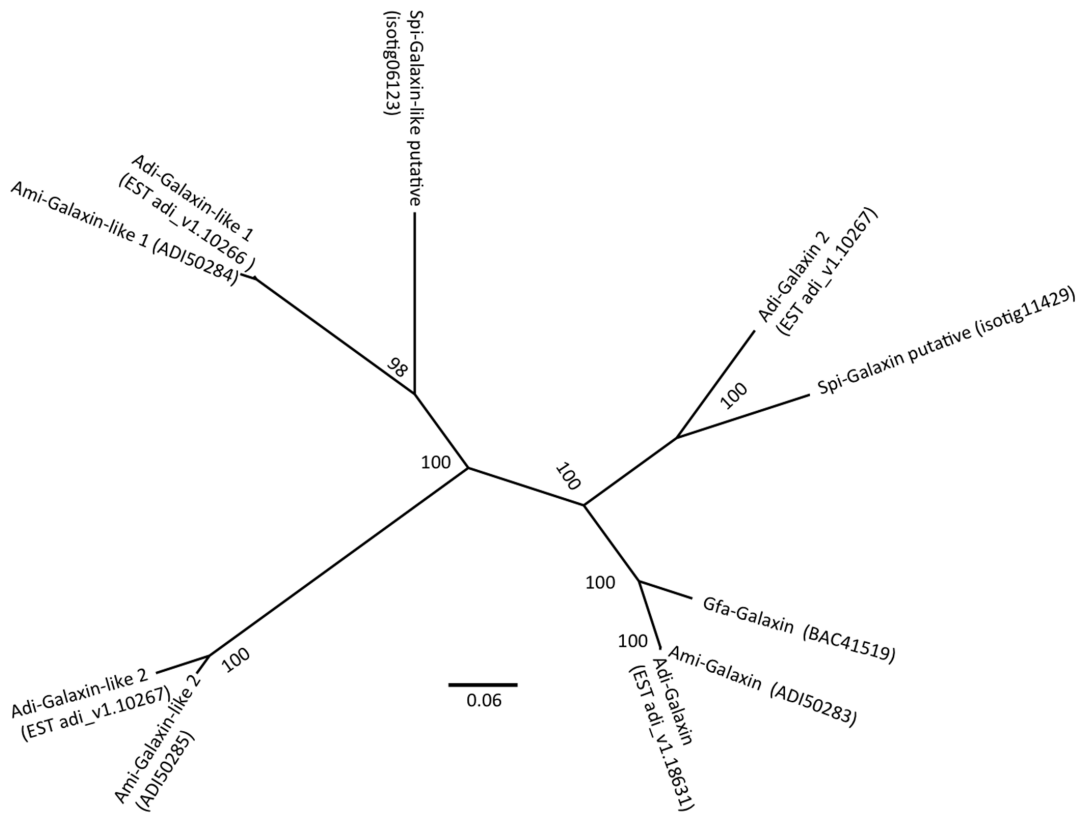doi:10.1371/journal.pone.0088615.g006

Figure 7. Galaxin phylogenetic tree. Galaxin and Galaxin-like sequences from different species: Spi-*Stylophora pistillata*, Adi-*Acropora digitifera*, Ami-*Acropora millepora*, Gfa- *Galaxea fascicularis* were used for phylogenetic analysis. The tree was constructed using Mrbayes program [46].
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development [4]. These two pathways do not share any major components and can function independently of each other, which is accomplished by means of different ligands, different receptors and different cytoplasmic and nuclear signal transducers. Most of the proteins found in humans are also present in corals. However, some of them are missing in the EST data obtained in this study due to 1) the technical methodology applied (for example, the chordin gene in the BMP pathway, absent from our EST library, is present in other cnidarian species [57,58] and was found in a new library constructed using genome sequencing (in prep.) and/or 2) the absence of these genes in Cnidaria in cases where the genes appeared after the coelomata split, as observed for the secreted cytokines IFN gamma [personal data].

Wnt and BMP play roles in vertebrate biomineralization in addition to their roles in development. Studies have shown that both BMP and Wnt pathways are involved in osteogenesis [59,60]. Furthermore, in adult coral, BMP expression is restricted to the skeletogenic ectoderm [42], and it has been suggested that BMP might be an organic matrix protein [61]. Although biomineralization and the nature of the calcifying organic matrix have been extensively studied in other taxa, such as mollusks [62], there have been relatively few corresponding studies of these elements in stony corals. We show here that some signaling pathways are conserved in *S. pistillata* providing new tools to understand the mechanisms of biomineralization. For instance, the study of BMP receptors and their location in polyps would help us to better understand the role of BMPs in adult corals.

Conclusions

Databases of expressed sequence tags have become a widely used tool in fundamental biological research, primarily due to the considerable resources now available for searching novel gene sequences. Our study of the ubiquitous robust coral *Stylophora pistillata* provides an opportunity to explore a series of questions concerning comparative and functional genomics and the evolutionary relationships among the stony corals and invertebrates in general. In conducting this study, we have been able to investigate the ancestry of several important metazoan genes. The basal phylogenetic position of the anthozoans and the surprising extent to which key genes appear to have been conserved between Cnidaria and chordates implies that further research in corals is likely to provide unique perspectives on common molecular principles of animal development. Most of our knowledge on the physiology of coral calcification comes from studies in *Stylophora pistillata*, the present EST library will constitute a valuable tool for determining the molecular mechanisms underlying coral growth and development and the molecular targets of environmental disturbances such as global warming and ocean acidification [61].

Acknowledgments

Author Contributions

Conceived and designed the experiments: SKL DZ OL. Performed the experiments: SKL DZ AB OL. Analyzed the data: SKL DZ MK ED MDS. Contributed reagents/materials/analysis tools: OHG ST DA OL. Wrote the paper: SKL DZ AB MK ST AB OHG DA OL.

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