The First De Novo Transcriptome Assembly From the Gonads of a Scleractinian Coral Euphyllia Ancora: Molecular Mechanisms Underlying Scleractinian Gametogenesis

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Abstract

Background: Sexual reproduction of scleractinians has captured the attention of researchers and the general public for decades. Although extensive ecological data has been acquired, underlying molecular and cellular mechanisms remain largely unknown. In this study, to better understand mechanisms underlying gametogenesis, we isolated ovaries and testes at different developmental phases from a gonochoric coral, *Euphyllia ancora*, and adopted a transcriptomic approach to reveal sex- and phase-specific gene expression profiles. In particular, we explored genes associated with oocyte development and maturation, spermiogenesis, sperm motility / capacitation, and fertilization.

Results: 1.6 billion raw reads were obtained from 24 gonadal samples. De novo assembly of trimmed reads, and elimination of contigs derived from symbiotic algae (Symbiodiniaceae) and other organisms yielded a reference *E. ancora* gonadal transcriptome of 35,802 contigs. Differential gene expression analysis of 4 developmental phases of ovaries and testes identified 2,023 and 678 differentially expressed genes in oogenesis and spermatogenesis, respectively. In premature/mature ovaries, 631 genes were specifically upregulated, with 538 in mature testes. Upregulated genes included those involved in gametogenesis, gamete maturation, sperm motility / capacitation, and fertilization in other metazoans, including humans. Meanwhile, a large number of genes without homology to sequences in the SWISS-PROT database were also observed among upregulated genes in premature / mature ovaries and mature testes.

Conclusions: Our findings show that scleractinian gametogenesis shares many molecular characteristics with that of other metazoans, but it also possesses unique characteristics developed during cnidarian and/or scleractinian evolution. To the best of our knowledge, this study is the first to create a gonadal transcriptome assembly from any scleractinian. This study and associated datasets provide a foundation for future studies regarding gametogenesis and differences between male and female colonies from molecular and cellular perspectives. Furthermore, our transcriptome assembly will be a useful reference for future development of sex-specific and/or stage-specific germ cell markers that can be used in coral aquaculture and ecological studies.

Background

Since the discovery of scleractinian mass spawning events in the Great Barrier Reef in the 1980s [1–3], sexual reproduction of scleractinians has captured the attention of researchers and the general public. Studies on various aspects of sexual reproduction, such as the timing of broadcast spawning or brooding, general cellular processes of gametogenesis, and sexuality (hermaphroditic or gonochoric), have been undertaken mainly from an ecological perspective in many scleractinian species in many locations during the past 3 decades [4–6]. Although large amounts of data are available from more than four hundred species to date [7], our current understanding of intrinsic mechanisms underlying key processes of sexual reproduction, such as sex determination/differentiation, gametogenesis, and ovulation/spawning, is quite limited.
Gametogenesis is a highly organized process whereby genetically diverse haploid gametes are created from diploid germ cells through meiosis with recombination. Generally, scleractinian germ cells are developed in endodermal mesenteries of polyps [4, 8, 9]. Sites of germ cell development are often observed as swellings in polyps during active gametogenesis, and are termed gonads [4]. Oogenesis begins with mitotic division of a small number of oogonia along the gonadal mesoglea, a thin layer composed of extracellular matrix. After oogonia differentiate into oocytes by entering a meiotic phase, oocytes increase in size and migrate into the mesoglea layer [4, 9–11]. There, oocytes further increase in size until maturation by accumulating yolk proteins, lipids, and other essential materials for embryonic development [12, 13]. Spermatogenesis begins with the active mitotic division of spermatogonia on the gonadal mesogleal layer. After spermatogonia form many small clusters comprising dozens of spermatogonia, they migrate into the mesoglea layer and form many spermatogenic compartments called spermarys. Further proliferation of spermatogonia, meiotic differentiation into spermatocytes, and spermiogenesis take place within each spermary [4, 14].

Studies on molecular and cellular aspects of scleractinian gametogenesis have just recently begun. Only several reports are available describing genes related to oogenesis including vitellogenesis [12–20] and to spermatogenesis [21, 22]. Currently, in order to cope with recent declines of coral reefs, reef restoration efforts via aquaculture are being initiated worldwide [23–25]. A comprehensive understanding of intrinsic mechanisms of gametogenesis will enable us to approach coral reef restoration from a new perspective. For instance, hormonal induction of gametogenesis and spawning under artificial rearing systems would allow more efficient propagation of target species [14]. Sex-and stage-specific molecular markers for germ cells would also enable us to monitor and to evaluate the developmental status of germ cells in corals cultured in captivity [21]. Moreover, because scleractinians belong to the phylum Cnidaria (e.g., corals, sea anemones, hydras, and jellyfish), which are regarded as evolutionarily basal in the animal kingdom, studies highlighting common mechanisms of sexual reproduction between scleractinians and advanced animals (e.g., vertebrates) would provide insights into the evolution of sexual reproduction in metazoans [14].

Transcriptome analysis using high-throughput sequencing has greatly enhanced identification of transcripts involved in sexual reproduction in various taxa [26–30]. This study performed gonadal transcriptome sequencing of a scleractinian coral, *Euphyllia ancora*, commonly known as the anchor or hammer coral (Fig. 1a-c). *E. ancora* was selected for the following reasons: (i) These corals are common in the Indo-Pacific region. (ii) They are gonochoric, and their annual gametogenic cycle in reefs along southern Taiwan has been studied histologically in both male and female colonies [8, 9]. For instance, a single oogenic or spermatogenic cycle in this region takes approximately a year in females and half a year in males. The annual spawning occurs within a week after full moon in April or May, or occasionally in June. Finally, (iii) They have large polyps (3–5 cm in diameter) that allow us to isolate ovaries and testes with relative ease [12]. This transcriptomic analysis of isolated gonads was undertaken in order to discover genes participating in gametogenesis.
The present study isolated ovaries and testes at different developmental phases from wild *E. ancora* colonies in order to reveal sex- and phase-specific gene expression profiles. In particular, we focused on premature and mature phases of gonads to identify candidate genes associated with oocyte development and maturation, spermiogenesis, sperm motility and capacitation, and fertilization, because of their importance for coral aquaculture (e.g., induction of sexual maturation) and ecological studies (e.g., monitoring germline development or predicting spawning time). The findings could highlight conserved molecular mechanisms of gametogenesis between scleractinians and other animals including humans.

**Results**

**Histological analysis of *E. ancora* gonads collected at different times**

Ovaries and testes were isolated from wild colonies at different times during a period of 9 months in 2016–2017 (Fig. 1d). Progress of gametogenesis was histologically confirmed as the spawning season approached (April–June, 2017). Gametogenesis is generally synchronized among polyps in a colony. Histological analysis of isolated ovaries showed that oocytes grew steadily during the 9-month investigation, and that ovaries isolated at 4 sampling dates generally displayed different oocyte developmental stages: October 2016 (oocytes with cytoplasmic polarization, < 125 µm in diameter), December 2016 (oocytes with accumulation of yolk and other components, 126–200 µm in diameter), February 2017 (oocytes with accumulation of yolk and other components, 201–275 µm in diameter), and April 2017 (oocytes with 'U'-like germinal vesicles or GVBD, > 276 µm in diameter) (Fig. 1d, Table 1). Notably, in the April 2017 samples, most oocyte nuclei had translocated to the peripheral membrane (Fig. 1e-h), and some oocyte nuclei had disappeared (Additional File 1), indicating that germinal vesicle breakdown (GVBD) had occurred in those oocytes. These ovarian samples were then classified into 4 phases, early, middle, late, and premature/mature, and were used for RNA-seq (Fig. 1e-h, Table 1).

Similarly, testes isolated at the following 4 sampling dates in 2017 possessed germ cells in different developmental stages: February (spermatogonia), March (spermatogonia and primary spermatocytes), April (secondary spermatocytes and spermatids), and June (mature sperm) (Fig. 1d, Table 1) (Fig. 1i-l). In the June samples, although a small number of spermataries with both round spermatids and mature sperm were observed in some testes, cytological observation confirmed the presence of morphologically mature sperm (Additional File 1). Testis samples were then classified into 4 phases, early, middle, late, and mature, and were subjected to RNA-seq (Fig. 1i-l, Table 1).
De novo transcriptome assembly of E. ancora gonads, identification of coral contigs, and functional annotation

1.6 billion raw reads comprising approximately 240 Gb of clean transcriptomic sequencing data were obtained by Illumina paired-end sequencing from the selected 12 testes (3 colonies, 4 time points) and 12 ovary (3 colonies, 4 time points) samples. Clean reads were deposited in the Sequence Read Archive (SRA) of DDBJ under accession number PRJDB9831 (Additional File 2). De novo assembly of all clean reads produced 169,272 initial contigs with an average size of 2,321 bp and an N50 of 4,610 bp. Maximum contig length reached 52,720 bp (Table 2).

Since the initial transcriptome assembly contained contigs from E. ancora gonads, symbiotic algae (Symbiodiniaceae), and other organisms (e.g., bacteria), we first bioinformatically identified possible E. ancora contigs prior to detailed analyses (Fig. 2a). All assembled contigs were aligned to available genome databases of 4 scleractinian species (Acropora digitifera, Pocillopora damicornis, Stylophora pistillata, and Orbicella faveolata) and transcriptomic databases of 6 Symbiodiniaceae (Symbiodinium sp. A1, Symbiodinium sp. A2, Symbiodinium sp. B2, Symbiodinium muscatinei, Uncultured clade C Symbiodinium sp. and Uncultured clade D Symbiodinium sp.) (For more details, see Additional File 3), and contigs unambiguously matched to coral genomic databases (72,238 contigs) and to Symbiodiniaceae transcriptomic databases (31,353 contigs) were separated (Fig. 2a). Contigs matching
both databases (43,332 contigs) were further aligned to the combined databases of coral genomes and Symbiodiniaceae transcriptomes, and were separated into coral contigs (23,742 contigs) and symbiotic algal contigs (19,590 contigs) based on the top hit results of BLASTN (e-vale cut off of le^−3). Eventually, 95,980 contigs were assigned as *E. ancora*, and 50,943 to Symbiodiniaceae (Fig. 2a). *E. ancora* contigs had a GC peak at 41.5%, while symbiotic Symbiodiniaceae peaked at 50.6% (Fig. 2b). These GC contents correspond well to previous genomic studies of corals and *Symbiodinium minutum* [31–33].

In order to remove sequence heterogeneity originating from different individuals or different haplotypes in the same individual, translated sequences of the extracted 95,980 *E. ancora* contigs were further clustered using CDHIT with 95% amino acid sequence identity. Finally, 35,802 contigs totaling approximately 125 Mbp (N50, 5,019 bp) were used as the reference *E. ancora* gonadal transcriptome with bench-marking universal single-copy orthologs (BUSCO) of more than 90% (Table 2), which covers all *E. ancora* candidate genes involved in gametogenesis. BLAST homology searches (BLASTP, le^−5) revealed that 21,569 of 35,802 (60.2%) contigs had significant similarities to sequences in the SWISS-PROT database (Fig. 3a). Moreover, 23,686 of 35,802 (66.2%) contigs matched conserved protein domains in the Pfam database (Fig. 3b).

The reference *E. ancora* gonadal transcriptome contained reproduction-related genes identified in our previous studies using degenerate PCR or cDNA libraries (Additional File 4). Furthermore, evolutionarily conserved genes associated with germline development (*Gcl, Mago, Boule, and Pum1*) were identified. Genes involved in meiotic processes, such as invasion and pairing of the homologous strand (*Msh4, Msh5, Mlh1*), formation of a synaptonemal complex (*Sycp1, Sycp3*), and maintenance of chromosome structure integrity (Rad21) were also identified (Additional File 5).

**Differential gene expression analysis among different developmental stages of ovaries and testes**

Hierarchical cluster analysis of 24 selected samples (12 testes and 12 ovaries) samples determined that 2 samples (Oct-female-1 and Feb-male-1) were different from all others (Additional File 6). These were assigned as outliers, possibly resulting from accidental collection of allospecific samples adjacent to the labeled colonies. To minimize data variation, the foregoing 2 samples were removed, and the remaining 22 samples were used for downstream analysis. Differential gene expression analysis of 4 developmental phases of ovaries and testes identified 2,023 and 678 differentially expressed genes during oogenesis and spermatogenesis, respectively, and 67 differentially expressed genes in the both ovary and testis during gametogenesis (q-value < 0.05, Fig. 4a). There were 1165, 89, 138, and 631 upregulated genes in the early, middle, late, and premature/mature phases of ovary compared with the other 3 phases, respectively (Fig. 4b). In the testis, there were 6, 19, 115, and 538 upregulated genes in the early, middle, late, and mature phases compared with the other 3 phases, respectively (Fig. 4c).

**Upregulated genes of premature/mature ovaries**
The 631 genes specifically upregulated in premature/mature ovaries were further analyzed. 446 of those genes (71%) matched the human SWISS-PROT database (Fig. 4b). Analysis of enriched functional terms found that 18 GO terms were enriched in premature/mature ovaries (P < 0.05 and enrichment > 4-fold; Additional File 7): 16 biological processes (BP) and 2 molecular functions (MF). Of the enriched BP terms, terms related to neuronal activity such as positive regulation of synaptic transmission, GABAergic (GO: 0050806), calcium ion-regulated exocytosis of neurotransmitter (GO: 0048791), neurotransmitter transport (GO:0006836) and neuronal action potential (GO: 0019228) were highly enriched. Among enriched MF terms, extracellular ligand-gated ion channel activity (GO: 0005230) was most enriched (Additional file 7).

In premature/mature ovaries, genes upregulated > 5-fold more than in the other three phases (log2, FDR < 0.05), 9 genes, including those encoding GFP-like fluorescent chromoprotein, neurogenic locus notch homolog protein 3, carbonic anhydrase 2, octopamine receptor beta-1R, beta-1,4-galactosyltransferase galt-1 were identified. One of the 9 genes could not be annotated (Table 3).

Evolutionarily conserved genes associated with oocyte development (vitellogenin-A2, low-density lipoprotein receptor-related proteins), formation of chromosome structure (histone H2B), and oocyte maturation (serine/threonine-protein kinase mos, mitogen-activated protein kinase 1) were identified (Table 4). Additionally, several sequences similar to components of skeletal organic matrix proteins of scleractinians (mucin-like protein, MAM and LDL-receptor class A domain-containing protein 2, cephalotoxin-like protein, uncharacterized skeletal organic matrix protein 5, polycystic kidney disease protein 1-like, and hemicentin) were also identified (Table 4, Fig. 5).

| Table 4. Upregulated genes of interest in premature/mature ovaries showing similarities to oocyte development/maturation-related genes in other animals |
|---------------------------------|----------------|
| Category                        | Annotation                                                                                             |
| Oocyte development              | Vitellogenin-A2                                                                                         |
|                                 | Uncharacterized skeletal organic matrix protein 5                                                      |
|                                 | Neurogenic locus notch homolog protein 1                                                                |
|                                 | Low-density lipoprotein receptor-related protein 2                                                     |
|                                 | Low-density lipoprotein receptor-related protein 4                                                     |
| Chromosome structure            | Histone H2B                                                                                             |
| Oocyte maturation               | Serine/threonine-protein kinase mos                                                                     |
|                                 | Mitogen-activated protein kinase 1                                                                     |
| Neurotransmitter receptors      | Octopamine receptor beta-1R                                                                            |
|                                 | Octopamine receptor beta-2R                                                                            |
|                                 | Dopamine receptor                                                                                      |
| Skeletogenesis                  | Mucin-like protein                                                                                     |
|                                 | MAM and LDL-receptor class A domain-containing protein 2                                               |
|                                 | Cephalotoxin-like protein                                                                              |
|                                 | Uncharacterized skeletal organic matrix protein 5                                                      |
|                                 | Poly cystic kidney disease protein 1-like                                                               |
|                                 | Hemicentin                                                                                             |
| Assembly ID                     | Reference                                                                                              |
| CL4556.Contig5_All              | (E. acora vitellogenin) [12]                                                                           |
| Unigene22577_All                | (E. acora egg protein) [12]                                                                            |
| Unigene262293_All               | (E. acora egg protein) [12]                                                                            |
| Unigene39647_All                | [36, 37]                                                                                                |
| Unigene20580 All                | [36, 37]                                                                                                |
| Unigene6692_All                 | [132]                                                                                                  |
| Unigene58091_All                | [41]                                                                                                    |
| CL13032.Contig3_All             | [41]                                                                                                    |
| CL4990.Contig1_All              | [133-135]                                                                                                |
| Unigene7363_All                 | [133-135]                                                                                                |
| Unigene35882_All                | [54]                                                                                                    |
| CL7569.Contig3_All              | [38]                                                                                                    |
| CL3169.Contig2_All              | [38]                                                                                                    |
| Unigene21179_All                | [38]                                                                                                    |
| Unigene22577_All                | [38]                                                                                                    |
| CL5263.Contig3_All              | [39]                                                                                                    |
| CL1601.Contig3_All              | [40]                                                                                                    |
Upregulated genes of mature testes

There were 538 specifically upregulated genes in mature testes. Of those, 305 (57%) matched human genes in the SWISS-PROT database (Fig. 4c). GO functional enrichment analysis found that 32 GO terms were enriched (P < 0.05 and > 4-fold enrichment; Additional File 7): 21 biological processes (BP), 8 cellular components (CC), and 3 molecular functions (MF). Of the enriched BP terms, response to corticosteroid (GO: 0031960), sequestering of TGF beta in extracellular matrix (GO:0035583), and regulation of cellular response to growth factor stimulus (GO: 0090287) were highly enriched. The term spermatid development (GO: 0007286) was also identified, and further queries of genes representative of the term identified genes encoding testis-specific serine/threonine-protein kinases, outer dense fiber protein 2, alstrom syndrome protein 1, radial spoke head 1 homolog, and dynein regulatory complex protein 9. Of the enriched CC terms, microfibril (GO: 0001527) was among the most enriched. Of the 3 MF terms identified, the term extracellular matrix structural constituent (GO: 0030021) was most enriched (Additional file 8).

Among significantly upregulated genes in mature testes (log$_2$ > 8- fold change compared to the other 3 phases, FDR < 0.05), we identified 28 genes, including those encoding creatine kinase, S-type mitochondrial, creatine kinase flagellar, omega-6 fatty acid desaturase, glutamate receptor ionotropic, kainate 2, testis-specific serine/threonine-protein kinase 4, and fibrillin-2 (Table 3). Ten of the 28 genes could not be annotated.
Evolutionarily conserved genes involved in spermiogenesis and fertilization process were further explored among upregulated genes in mature testes (Table 5). We identified a number of important genes encoding proteins associated with spermiogenesis (spermatogenesis-associated protein 6, cilia- and flagella-associated protein 69), sperm motility and/or capacitation (dynein regulatory complex subunit 7, sodium/hydrogen exchanger 10, creatine kinase, flagellar, adenylate cyclase type 10, and cation channel sperm-associated protein 3), and fertilization process (hapless 2/generative cell specific 1 and receptor guanylate cyclase). We also identified a gene encoding steroid 17α-hydroxylase/17,20-lyase (Cyp17a), a key enzyme in sex steroid and cortisol production (Fig. 5, Table 5).

Discussion

First scleractinian gonadal transcriptome assembly

Since scleractinian gametogenesis occurs exclusively in gonads, isolated gonads (but not whole polyps) are useful to explore genes associated with gametogenesis. However, gonad isolation is technically difficult in many scleractinians due to small polyp sizes. Gonad isolation not only requires an understanding of polypl anatomy, but also technical skill. The present study applied previously established techniques for gonad isolation from *E. ancora* polyps [12] to the current transcriptomic study. Bioinformatics methods to eliminate contigs from symbiotic algae or other contaminants were also employed [31]. The established transcriptome assembly allowed us to identify sex- and gonadal, phase-specific, upregulated genes, as well as evolutionarily conserved genes associated with germ cell...
development. The resulting dataset will provide a foundation for future research investigating molecular and cellular mechanisms of gametogenesis in scleractinians.

**Characteristics of premature/mature ovaries as assessed by anatomical and histological analyses**

The observed growth of oocytes and the loss of germinal vesicles in oocytes of premature/mature ovaries suggest that oocytes were still actively accumulating essential materials (e.g., yolk and other components) for survival and development of embryos until just before maturation. Also, the oocyte maturation process, including germinal vesicle breakdown (GVBD) and resumption of meiosis occurred in some oocytes.

**Upregulated genes in premature/mature ovaries**

Yolk formation and accumulation is one of the most important aspects of oogenesis for oviparous animals. In scleractinian eggs, several major yolk proteins, including vitellogenin (Vg), a female-specific phosphoglycolipoprotein, and large amounts of lipids (e.g., wax esters, fatty acids, phosphatidylethanolamines, and phosphatidylcholines) have been identified to date [12, 13, 19, 20, 34, 35]. The present study found that transcripts encoding 3 major yolk proteins were upregulated (Vg, Egg protein, and Euphy, Fig. 5, Table 4), in agreement with histological observations, indicating that oocytes were actively accumulating yolk materials. Those yolk proteins are produced by ovarian somatic cells adjacent to oocytes [12, 13]. However, little is known about the uptake mechanisms of yolk proteins by oocytes. Although receptor-mediated endocytosis has been hypothesized, related receptor molecules have not been identified yet [12]. The present study also identified transcripts encoding two types of low-density lipoprotein receptor related proteins (Lrps) as upregulated genes in premature/mature ovaries (Fig. 5, Table 4). In some teleosts, a member of Lrps, Lrp13, serves as one of the Vg receptors expressed on oocyte membranes [36, 37]. Thus, the identified Lrps may be involved in uptake mechanisms of yolk materials in scleractinians, and are promising candidate receptors for Vg and/or other lipoproteins in future studies.

In addition to the major yolk materials, eggs of scleractinians are assumed to accumulate materials essential for larval development. Among the upregulated genes in premature/mature ovaries, we identified several sequences similar to components of skeletal organic matrix proteins found in *A. digitifera* [38], *A. millepora* [39], and *S. pistillata* [40]. Since no skeleton formation occurs in ovaries, it is likely that these gene products (mRNA and proteins) are stored in oocytes during oogenesis to be used for skeleton formation during larval development.

The occurrence of GVBD in some oocytes of ovaries collected in April 2017 was an unexpected finding, because mature gametes were not observed in testes collected at the same time. It is possible that timing of oocyte maturation was split among oocytes and/or ovaries over April and May (or June) for unknown reasons. Nevertheless, this study successfully identified two sequences similar to serine/threonine-protein kinase mos (Mos) gene and mitogen-activated protein kinase 1 (Mapk1) gene, which contribute to
signaling pathways of oocyte maturation in a variety of animals, including cnidarians [41] (Fig. 5). Upregulation of these two genes in premature/mature ovaries implies that they may also function in oocyte maturation in scleractinians. Previous studies regarding oocyte maturation in scleractinians were limited to histological observations and focused on the presence and timing of GVBD [42, 43]. To the best of our knowledge, this is the first study to identify these candidate molecules in oocyte maturation of scleractinians.

In a variety of animals, hormones (i.e., steroids, growth factors, peptides, and other substances) are involved in these processes [44–51]. In scleractinians, transcriptome studies suggest that melanopsin-like homolog and/or neuropeptides [52] and Rhodopsin-like receptors [53] are involved in the signaling pathway for spawning in Acropora species. Enriched BP terms in E. ancora premature/mature ovaries imply that neuronal activity is significantly higher than during other phases. Upregulation of transcripts similar to the genes encoding monoamine receptors (e.g., octopamine receptors, dopamine receptors, adrenergic receptors, and serotonin receptors, Fig. 5, Table 4) also support this assumption. Recent studies show that some neurotransmitters (dopamine and serotonin) are also involved in regulation of scleractinian spawning. Treatment of Acropora tenuis with dopamine during the final phase of gametogenesis resulted in inhibition of spawning [54]. By contrast, treatments with serotonin and its precursor, L-5-hydroxytryptophan (5-HTP) induced spawning of Acropora cervicornis [55]. Taking all these lines of evidence into account, the identified monoamine receptors may also be essential during the premature/mature phase of E. ancora oogenesis. It will be of interest to investigate whether treatment of female E. ancora with these neurotransmitters induces/inhibits oocyte maturation and spawning.

Of particular interest is the upregulation of three genes encoding neurogenic locus notch homolog proteins in premature/mature ovaries (Additional File 9). The Notch signaling pathway is conserved across animal taxa, and regulates cell-cell interactions and cell fate determination [56]. One of the identified genes, neurogenic locus notch homolog protein 1, encodes Euphy, a novel major yolk protein in E. ancora oocytes identified in our previous study [13]. The remaining 2 genes have not been previously reported. Although both sequences possess EGF-like domain repeats typifying notch homolog proteins, they are structurally distinct from Notch1 identified in vertebrates (e.g., human Notch1). These may be novel genes that emerged after gene duplication, domain shuffling, and rapid molecular evolution in cnidarian/scleractinian lineages [38, 39]. Interestingly, one of them, neurogenic locus notch homolog protein 3, was highly and significantly upregulated, and contains a zona pellucida (ZP) protein and transmembrane domains (Fig. 5, Additional File 9). The ZP is the extracellular matrix (ECM) surrounding mammalian oocytes, composed of four glycoproteins (ZP1-ZP4). ZP functions during oogenesis, fertilization, and preimplantation development in mammals [57]. In jellyfish, a ZP domain-containing protein called mesoglein, which resembles mammalian ZP, was identified in the contact plate of oocytes [58]. Although scleractinian oocytes have neither a protective coat nor a membrane surrounding them, this finding implies that the identified ZP domain-containing protein probably participates in oogenesis and subsequent fertilization processes.
GFP is one of the natural pigments of corals [59–62]. Although the exact functions of GFP remain obscure, proposed functions include photoprotection from high UVA/blue irradiation, photosynthetic enhancement, phototaxis of zooxanthellae [63–66], and antioxidant activity [67, 68]. We previously showed that *E. ancora* oocytes express an endogenous RFP with H$_2$O$_2$ degradation activity from early to mature stage of oocytes, and suggested a possible role of RFP in protecting oocytes from oxidative stress during oogenesis [15]. Our finding implies that not only RFP, but also GFP may serve in oogenesis, particularly during the premature/mature phase (Fig. 5).

**Characteristics of mature testes as assessed by histological and cytological analyses**

Spermiogenesis is a process by which haploid spermatids undergo a complex series of morphological changes, and eventually become elongated functional sperm. The presence of spermarys having both round spermatids and mature sperm in testes collected in June 2017 suggested that spermiogenesis was occurring in the testes at the time of collection, and that genes involved in regulation of spermiogenesis were being expressed in testes.

**Upregulated genes in mature testes**

Morphological changes of male germ cells during spermiogenesis include flagellum formation, nuclear DNA condensation, and elimination of organelles and cytoplasm. Scleractinian spermiogenesis is generally morphologically similar to that of vertebrates, except that male germ cells possess long flagella from early to late stages of development [21]. Nevertheless, scleractinian male germ cells possess typical flagellar axonemes, characterized by a “9 + 2” arrangement of microtubules [21, 69]. In this study, further queries of genes associated with spermatid development (GO term:0007286), together with literature-based gene identification, allowed us to identify various candidate genes encoding proteins of flagellar components. The presence of a conserved molecular toolkit for spermiogenesis suggests that scleractinians and vertebrates share similar characteristics at both morphological and molecular levels.

Sperm motility is important for most scleractinians, which fertilize externally in seawater. Sperm of acroporid corals are completely immotile in seawater, but become motile when sperm come close to eggs [70]. The presence of chemoattractants and the involvement of intracellular pH elevation and Ca$^{2+}$-dependent signal transduction in sperm motility have also been experimentally demonstrated [70, 71]. Molecules regulating flagellar motility still remain largely unexplored in scleractinians. This study identified a number of important genes encoding proteins involved in sperm motility and/or capacitation in mammals and sea urchins, such as cation channel sperm-associated protein 3 (CatSper3), sodium/hydrogen exchanger (sNHE), and adenylate cyclase type 10 (sAC) (Fig. 5, Table 5). These findings, therefore, support the hypothesis of Romero and Nishigaki, who suggested that CatSper3, sNHE, and sAC form prototypical machinery for sperm flagellar beating in metazoans [72]. This study further identified the gene encoding creatine kinase, flagellar, which was first identified from the flagellum of sea urchin sperm, participating in energy transport from the sperm head to the flagellum during sperm motility [73]. Genes associated with sperm motility and/or capacitation in scleractinians suggest that
these features were most likely present in the common ancestor prior to divergence of the cnidarian and bilaterian lineages.

Sex steroids are critical for sex differentiation, gametogenesis, and gamete maturation in vertebrates \([74–78]\). Sex steroids (e.g., estrogen, testosterone, and progesterone) have been demonstrated in several scleractinians, including \textit{E. ancora}\ [79–82]. Additionally, the correlation between sex steroids levels and gametogenic cycles has led to the hypothesis that sex steroids may be involved in regulation of scleractinian reproduction [80, 82]. Steroid biosynthesis is catalyzed by activities of various steroidalogenic enzymes. Although steroid biosynthesis activities are known from extracts of some scleractinian tissues [80–85], only one gene encoding a steroidalogenic enzyme, 17β-hydroxysteroid dehydrogenase type 14 (17β-hsd 14), has been identified and characterized so far [86]. In the present study, a gene encoding steroid 17α-hydroxylase/17,20-lyase (Cyp17a) (Fig. 5, Table 5), a key enzyme in production of sex steroids and cortisol [87], was upregulated in mature testes. Although further analysis is required to clarify the activity, this implies that steroid biosynthesis may be occurring in mature testes, and the produced sex steroids/cortisol could be associated with maturation of male germ cells in scleractinians.

Molecules involved in fertilization remain largely unknown in scleractinians. We found that a gene similar to Hapless 2/Generative Cell Specific 1 (Hap2/Gcs1) was upregulated in mature testes (Fig. 5, Table 5). Hap2/Gcs1 was first identified as a male gamete-specific transmembrane protein in lilies [88]. The coding gene is found in genomes of most major eukaryotic taxa (e.g., protozoa, plants, and animals) except fungi [89, 90]. Functional analysis with the mutant/gene targeting system showed that Hap2/Gcs1 are essential for gamete fusion in \textit{Arabidopsis} [89], the protozoan parasite, \textit{Plasmodium} [90], and the green alga, \textit{Chlamydomonas} [91]. Expression of Hap2/Gcs1 was also confirmed in male germ cells of some cnidarians, such as \textit{Hydra} [92] and the starlet sea anemone, \textit{Nematostella vectensis} [93], and involvement in fertilization has been demonstrated in sea anemones [93]. Upregulation of Hap2/Gcs1 in \textit{E. ancora} mature testis raises the possibility that Hap2/Gcs1 participates in scleractinian sperm-egg fusion. Most recently, we reported that a receptor guanylate cyclase A (rGC-a) (also known as atrial natriuretic peptide receptor 1 in mammals) is expressed in \textit{E. ancora} sperm flagella [22] (Fig. 5, Table 5). rGCs are expressed on sperm and serve as receptors for egg-derived sperm-activating and sperm-attracting factors in some echinoderms and mammals [94–97]. Taken together, evolutionarily conserved proteins underlie fertilization mechanisms of scleractinians.

**Other major findings and potential applications**

Genes encoding Histone H2B and Histone H2A were upregulated in premature/mature ovaries and mature testes, respectively (Fig. 5, Table 4, 5). Histones are the major protein components of chromatins in eukaryote cell nuclei. Five histone protein families exist: the core histone families (H2A, H2B, H3, and H4) and the linker histone family (H1) [98]. Core histones are components of the nucleosome core, whereas linker histones are present in adjacent nucleosomes, where they bind to nucleosomal core particles, and stabilize both nucleosome structure and higher-order chromatin architecture [98, 99]. Various isoforms of each family have been identified as histone variants, and their importance in diverse cellular processes (e.g., transcriptional control, chromosome segregation, DNA repair and recombination,
and germline specific translational regulation) have been revealed [100, 101]. In scleractinians, although nucleotide sequences of the histone gene cluster containing H3, H4, H2A, and H2B have been identified in *Acropora formosa* [102], differences in gene expression levels between ovaries and testes have not been reported so far. This study revealed for the first time the existence of histone variants showing sexually dimorphic expression in scleractinians. In the cnidarian model organism, *Hydractinia echinata*, 19 genes encoding histones were identified, and some of them, such as histone H2A.X and five H2B variants, are specifically expressed in female and male germ cells, respectively [103]. Our findings imply that the identified histone may control gene expression in female and male germ cells during scleractinian gametogenesis.

Studies of a variety of animals have revealed that a set of specialized and highly conserved genes govern germline specification, development, meiosis, and/or maintenance in metazoans [104, 105] (Additional File 5). In the gonadal transcriptome, and for the first time in scleractinians, we could identify many genes associated with germline specification and meiotic processes (Additional File 5). Although further spatiotemporal expression analyses and functional assays are required to clarify their functions, their expression in gonads implies that these genes participate in scleractinian germline development and meiosis.

The identified *E. ancora* gonadal transcriptome assembly includes a large number of genes without homology to sequences in the SWISS-PROT database. These findings suggest that although scleractinian gametogenesis shares many common molecular characteristics with gametogenesis in other metazoans, it also possesses characteristics that developed in evolutionarily unique ways. Further characterization and functional studies of these unannotated genes will clarify unique features in scleractinian gametogenesis, and this will eventually lead to comprehensive understanding of scleractinian gametogenesis.

The knowledge obtained in the present study will be useful for ecological studies and coral aquaculture. For instance, since scleractinian corals have no secondary sexual characteristics, histological analysis has traditionally been used to investigate sex of polyps or colonies, as well as to determine the status of germ cell development. However, histological analysis of scleractinians is time consuming. It generally requires decalcification steps, and the whole histological process sometimes takes 1–2 weeks. Identification of molecular markers for determining colony sex and germ cell development status offers a useful alternative process. Colony sex and germ-cell type could be determined faster using PCR with markers, than by histological means. Sex- and gonad phase-specific genes identified in this study would be candidates.

**Conclusions**

A scleractinian gonadal transcriptome was created for the first time. Analysis of upregulated genes in premature/mature gonads allowed us to identify many genes probably involved in oocyte development, oocyte maturation, spermiogenesis, sperm motility/capacitation, and fertilization processes (Fig. 5). The
gonadal transcriptome efficiently identified a large number of sex-biased or sex-specific genes and shed light on possible molecular mechanisms of scleractinian gametogenesis, which appear to be coordinated by both conserved and novel genes. This study and its generated datasets thus provide a foundation for future studies regarding gametogenesis and differences between sexes from molecular and cellular perspectives. Furthermore, our transcriptome assembly will be a useful reference for future development of sex-specific and/or stage-specific markers for germ cells for use in coral aquaculture and ecological studies.

Methods

Sample collection

*E. ancora* specimens were collected by scuba divers at Nanwan Bay, Kenting National Park, in southern Taiwan (21°57'N, 120°46'E). Approximately 10 colonies were labeled, and gonads (> 20 gonads) of labeled colonies were microscopically isolated at different times during a 9-month period from October 2016 (non-spawning period) to June 2017 (spawning period) (Fig. 1d). Collection was approved by the administration office of Kenting National Park (issue number: 1010006545). For RNA-seq, collected samples were snap frozen in liquid nitrogen, and stored at -80 °C until use. Isolated gonads were also fixed with 20% Zinc-Formal-Fixx (Thermo Fisher Scientific, Pittsburgh, PA, USA) for histological analysis. Experiments were performed in accordance with principles and procedures approved by the Institutional Animal Care and Use Committee, National Taiwan Ocean University, Taiwan.

Histological analysis for sample selection

Histological analyses were performed to determine developmental phases of gonads, and to select samples for RNA-seq. Isolated gonads (> 10 gonads/colony/time point) were analyzed according to the methodology in our previous studies [8, 9]. Developmental stages of germ cells were determined according to previous criteria [8, 9] with some modifications (see Table 1).

RNA extraction and RNA-seq library construction

In total, 12 testes and 12 ovaries (3 colonies x 4 time points) were selected based on the results of histological analyses. Total RNA of the 24 samples was extracted using TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer’s protocol. DNase I treated-RNA samples were sent to Beijing Genomics Institute (BGI, Shenzhen). RNAs were qualified using a Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA) with RNA 6,000 labchip kit (Agilent Technologies) and all samples were confirmed as high-quality RNA (RIN > 8). 24 RNA-seq libraries were constructed using TruSeq mRNA Library Prep Kits v2 (Illumina, San Diego, CA, USA), and sequenced with 150-bp paired-ends (150PE) on an Illumina HiSeq X Ten. Illumina adaptors, low-quality sequences (Phred value Q < 20), and reads with a high proportion of N (> 5%) were removed. Cleaned sequencing data were used for subsequent analyses.

De novo assembly and annotation of the *E. ancora* transcriptome
The transcriptome assembly of the *E. ancora* “holobiont”, the host and symbiotic organisms, was created by BGI as follows. Clean reads of 24 individual samples were assembled *de novo* using Trinity v2.0.6 software [106] (parameter settings: \(-\text{min\_contig\_length} \ 150\ – \text{CPU}\ 8\ – \text{min\_kmer\_cov}\ 3\ – \text{min\_glue}\ 3\ – \text{bfly\_opts} \ ‘V\ 5\ – \text{edge\_thr}\ =\ 0.1’\)) and assembled sequences were clustered using Tgicl v2.0.6 software [107] (parameter settings: \(-l\ 40\ -c\ 10\ -v\ 25\ -O\ ‘-repeat\_stringency\ 0.95\ -minmatch\ 35\ -minscore\ 35’\)). Since gonadal samples contained substantial numbers of symbiotic algal cells, we bioinformatically separated sequences originating from *E. ancora*, algal symbionts (Symbiodiniaceae), or microbes as follows. All assembled sequences were aligned to available genomic databases of 4 scleractinian corals and 6 Symbiodiniaceae transcriptomic databases using BLASTN $e^{-3}$. These databases included *A. digitifera* [31, 108], *P. damicornis* [109, 110], *S. pistillata* [111], and *O. faveolata* [112], *Symbiodinium* sp. A1 [113], *Symbiodinium* sp. A2 [114], *Symbiodinium* sp. B2 [114], *S. muscatinei* [115], Uncultured clade C *Symbiodinium* sp. [116] and Uncultured clade D *Symbiodinium* sp. [116] (For more detailed information on the databases, see Additional File 3). Contigs aligned exclusively to the coral genome database were annotated as “*E. ancora* contigs”, while those that aligned only to the Symbiodiniaceae transcriptome databases were annotated as “Symbiodiniaceae contigs”. To separate contigs aligned to both the coral genome and Symbiodiniaceae transcriptomic databases, contigs were re-aligned (BLASTN $e^{-3}$) using a combined database of coral genomes and Symbiodiniaceae transcriptomes. Based on the top hit results of BLASTN (corals or Symbiodiniaceae), contigs were annotated as “*E. ancora* contigs” or “Symbiodiniaceae contigs” (Fig. 2). All databases used in the present study were downloaded on 3/18/2019. Nucleotide sequences were again clustered using CDHIT [117] with 97% identity for removing sequences possibly originating from different individuals or haplotypes in a single individual. Finally, contigs were translated into amino acid sequences using the longorf script [118] and clustered using CDHIT with 95% identity. Completeness using the assembled sequences was assessed using BUSCO (bench-marking universal single-copy orthologs) version 3 [119, 120] in transcriptome mode. Reference *E. ancora* gonadal transcriptome contigs were annotated as follows: 1) BLAST homology searches against public protein databases: SWISS-PROT database (e-value cutoff of $e^{-5}$) (Consortium 2011) (3/18/2019), 2) Identification of conserved protein domains with the Pfam database (e-value cutoff of $e^{-3}$) [121, 122].

**Identification of reproduction-related genes in ovaries and testes**

Genes important in metazoan reproduction were searched in the reference *E. ancora* gonadal transcriptome, based on the literature. Two strategies were adopted. 1) Full-length cDNA sequences of genes in vertebrates and invertebrates were retrieved from Genbank (NCBI), and local BLAST searches were conducted (BLASTP, cut-off e-value of $< 1e^{-5}$) against translated sequences from the reference *E. ancora* gonadal transcriptome. 2) Gene names or keyword searches for target categories were performed in SWISS-PROT annotation results.

**Differential gene expression analysis**
First, possible outlier RNA-seq samples were examined by mapping raw reads to assembled sequences with Bowtie2 v2.2.6 software [123] (parameter setting: -q –phred33 –sensitive –dpad 0 –gbar 99999999 –mp 1,1 –np 1 –score-min L,0,-0.1 -l 1 -X 1000 –no- mixed –no-discordant -p 1 -k 200) and the mapping coverage of contigs was determined with RSEM v1.2.12 software [123] under default settings. The hclust package in R was used to perform a hierarchical cluster analysis of RNA-seq samples [124]. The above analyses were performed by BGI. Illumina adaptors and low-quality sequences (quality score > Q20, reads length > 25 bp) were removed from raw RNA sequences of the remaining samples using CUTADAPT v1.16 [125]. Using SALMON v0.13.1 [126], clean reads were mapped to the reference *E. ancora* transcriptome contigs, and numbers of reads mapped were counted and then converted to Counts Per Million (CPM) with edgeR v3.24.3 [127, 128]. CPM values were used to identify genes that were differentially expressed in each phase of ovaries and testes, respectively (ANOVA with q-value < 0.05). For heatmap generation, CPM values were scaled to row Z-scores for each of the genes that were highly expressed in each phase of gonads.

**Gene enrichment analysis**

UniProt IDs were assigned for each of the reference *E. ancora* gonadal transcriptome contigs based on best matches against the human SWISS-PROT database with BLASTP (e-value cutoff of $10^{-5}$) [129]. Gene enrichment analysis of Gene Ontology (GO) was performed with the assigned UniProt ID using DAVID Bioinformatics Resources 6.8 (> 4-fold enrichment and P < 0.05) [130, 131]. UniPort IDs of the reference *E. ancora* gonadal transcriptome were used as a background for the DAVID analysis.

**Abbreviations**

GVBD: Germinal vesicle breakdown

SRA: Sequence read archive

DDBJ: DNA Data Bank of Japan

bp: base pair

GC content: Guanine-Cytosine content

Pfam: Protein family

Gcl: germ cell-less

Mago: mago nashi

Boule: boule-like

Pum1: Pumilio homolog 1
Dmc1: DNA meiotic recombinase 1
Muts4: MutS protein homolog 4
Muts5: MutS protein homolog 5
Mlh1: DNA mismatch repair protein Mlh1
Symp1: Synaptonemal complex protein 1
Symp2: Synaptonemal complex protein 2
Symp3: Synaptonemal complex protein 3
Rad21: Double-strand-break repair protein rad21
FDR: False discovery rate
GO: Gene ontology
BP: Biological processes
MF: Molecular functions
CC: cellular components
SOMPs: Skeletal organic matrix proteins
Lrps: Low-density lipoprotein receptor related proteins
Lrp13: Low-density lipoprotein receptor-related-protein 13
Vg: Vitellogenin
Mos: Serine/threonine-protein kinase mos
Mapk1: Mitogen-activated protein kinase 1
5-HTP: 5-Hydroxytryptophan
Notch1: Neurogenic locus notch homolog protein 1
ZP: Zona pellucida
ECM: Extracellular matrix
GFP: Green fluorescent protein
UVA: Ultraviolet A

RFP: Red fluorescent protein

CatSper3: Cation channel sperm-associated protein 3

sNHE: Sodium/hydrogen exchanger

sAC: Adenylate cyclase type 10

17beta-hsd 14: 17beta-hydroxysteroid dehydrogenase type 14

Cyp17a: 17α-hydroxylase/17,20-lyase

Hap2/Gcs1: Hapless 2/Generative Cell Specific 1

rGC: receptor guanylate cyclase

PCR: Polymerase chain reaction

BGI: Beijing Genomics Institute

RIN: RNA integrity number

PE: Paired-end

BUSCO: Bench-marking universal single-copy orthologs

CPM: Count per million

ANOVA: Analysis of variance

DAVID: Database for annotation, visualization and integrated discovery

**Declarations**

**Availability of data and materials**

The datasets created and/or analyzed in this study are available in the Sequence Read Archive (SRA) of DDBJ under accession number PRJDB9831.

**Consent for publication**

Not applicable.
Competing interests

The authors declare that they have no competing interests.

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Author’s Contributions

SS conceived the idea and designed the experiments. YLC and SS performed the sampling and experiments. CS, YY, YLC, and SS analyzed the data. YLC, SS, CFC, and CS wrote the manuscript.

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**Figures**
Figure 1

Euphyllia ancora and its germ cells histologically observed in isolated gonads at different sampling times. (a) External appearance of an E. ancora colony. (b) External appearance of tentacles of an E. ancora colony. Anchor-like tentacles and the flabello-meandroid skeleton typify E. ancora. (c) A top view of an E. ancora skeleton after removal of polyp tissue. (d) Periods of oogenesis (pink arrow) and spermatogenesis (blue arrow) and predicated spawning timing (*). Letters (e-l) on the arrows correspond with to figures e-l below, and indicate the timing (month) of sampling for ovaries and testes. (e-h) The external appearance of isolated ovaries in October and December 2016 and February and April 2017. (e'-h') Histological observation of the isolated ovaries. (e, e') The early phase of ovaries. (f, f') The middle phase of an ovary. (g, g') The late phase of an ovary. (h, h') The premature/mature phase of an ovary. (i-l) The external appearance of isolated testes in February, March, April, and June 2017. (i'-l') Histological observation of isolated testes. (i, i') The early phase of a testis with spermatogonia. (j, j') The middle phase of testis having spermatogonia and primary spermatocytes. (k, k') The late phase of a testis with secondary spermatocytes and spermatids. (l, l') The mature phase of a testis with mature sperm. Sections were stained with hematoxylin and eosin. Scale bars= 1 cm (c); 500 µm (e-l); 50 µm (e'-h'); 10 µm (i'-l').
Figure 2

Identification of E. ancora contigs from the transcriptome assembly of an E. ancora holobiont. (a) A flow chart for identification of E. ancora contigs from the transcriptome assembly (all contigs) that contains contigs from the host coral, symbiotic (algae), and other organisms (bacteria). (b) Distribution of GC percentages of the assembled contigs. Red line: all contigs, green line: the E. ancora contigs, blue line: extracted Symbiodiniaceae contigs. (c) Proportions of contigs from E. ancora, Symbiodiniaceae, and other symbiotic organisms (others contigs) in the initial whole holobiont transcriptome assembly. Only extracted E. ancora contigs (56.7%) were used for further analysis.
Figure 3

Contig numbers in the reference E. ancora gonadal transcriptome that matched SWISS-PROT and Pfam databases. (a) Results of BLAST homology searches against the SWISS-PROT database (e-value cutoff of le-5). Note that 21,569 out of 35,802 contigs (60.2%) had significant similarities with database sequences. (b) Identification of protein domains using the Pfam database (e-value cutoff of le-3) for contigs from the reference E. ancora gonadal transcriptome. Note that 23,686 out of 35,802 contigs (66.2%) had conserved protein domains.
Figure 4

Differentially expressed genes in oogenesis and spermatogenesis of E. ancora. (a) 2,023 and 678 genes were differentially expressed during oogenesis and spermatogenesis, respectively, and 67 of those genes were differentially expressed in both oogenesis and spermatogenesis (q-value<0.05, ANOVA). (b) Relative gene expression levels of differentially expressed genes (2023 genes) at different phases of ovaries. CPM values were scaled to row Z-scores for each of the genes. In premature/mature ovary, 631 genes were expressed at higher levels than in the other 3 phases. Among the 631 genes, 446 genes (71 %) matched the SWISS-PROT human database, as shown in the pie chart. (c) Relative gene expression levels of differentially expressed genes (678 genes) at different phase of testes. CPM values were scaled to row Z-scores for each of the genes. In mature testis, 538 genes were expressed more highly than in the other 3 phases. Among the 538 genes, 305 (57 %) matched the SWISS-PROT human database, as shown in the pie chart. In the heatmaps, each row represents a differentially expressed gene and the columns represent time points. The color bar on the left indicates expression levels.
Figure 5

Genes possibly involved in oocyte development and maturation, sperm motility/capacitation, and fertilization in E. ancora. Genes indicated in red have been reported in our previous studies.

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