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Ovarian transcriptome analysis of *Mactra chinensis* provides insights into genes expressed during the intermediate and ripening stages

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ABSTRACT

Enhancing the production of aquatic animals is very important for fishery management and aquaculture applications. Ovaries have important functions in producing oocytes and hormones. The Chinese clam (*Mactra chinensis*) is a nutritious saltwater shellfish. Significant biochemical changes take place during the sexual maturation of *M. chinensis*; however, the genetic mechanisms of this process are unclear. Transcriptome sequencing can determine gene expression changes as development occurrs. In the present study, transcriptome sequencing was used to produce a comprehensive transcript dataset for the ovarian development of *M. chinensis*. The different ovarian developmental stages were determined using hematoxylin-eosin staining. There was identification of 54,172 unigenes at the intermediate stage and 63,081 at the ripening stage, and 80,141 all-unigenes were assembled to determine the molecular mechanism of ovarian development in *M. chinensis*. Quantitative real-time PCR for nine mRNAs confirmed the RNA-seq data. Functional annotation of the transcripts indicated there were important pathways in ovarian development, such as those involving the vitellogenin gene. Six pathways associated with ovarian development were identified: estrogen signaling pathway, GnRH signaling pathway, progesterone-mediated oocyte maturation, ovarian steroidogenesis, steroid hormone biosynthesis, and steroid biosynthesis. Significant upregulation of protein kinase alpha (PKA) and calmodulin (CAM) in four of the pathways indicates that PKA and CAM are active in *M. chinensis* ovarian development during maturation. Results of the present study provide the first comprehensive transcriptomic resource for *M. chinensis* ovaries, which will increase understanding of the molecular mechanisms underlying sexual maturation and promote molecular nutritional studies of *M. chinensis*.

1. Introduction

The Chinese clam (*Mactra chinensis*) is an edible shellfish with high nutritional value and tasty meat, found in the oceans around China, Korea, and Japan, and belonging to the Mollusca, Bivalvia, Veneridae, Mactridae. The shortage of *M. chinensis* germchit has been a key factor in restricting the scale of its artificial breeding in China. The lack the basic knowledge about its growth and maturation has limited artificial culture of *M. chinensis*. The ovaries are important tissues in reproduction with many physiological functions occurring in these tissues, including oocyte production, hormone secretion, and fertilization; therefore, it is important to

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explore the genetic mechanisms of the ovarian functions, with an emphasis during the time of development and sexual maturation. Thus, there was investigation of the mechanisms of *M. chinensis* growth and maturation in a natural habitat. An overarching aim was to enhance the understanding of how there can be effective and efficient artificial production of *M. chinensis*.

Next generation sequencing technologies provide opportunities to develop molecular resources for species of biological and economic interest that lack reference genome sequences (Riesgo et al., 2012). Massive parallel sequencing of RNA (RNA-seq) and *de novo* assembly approaches have been used to understand the transcriptomes of various species (Zhu et al., 2014; Guo et al., 2015; Kim et al., 2016). Zhang et al. (2016a,Zhang et al., 2016b identified the functional genes involved in the Cd2+ response of Chinese surf clams (*Mactra chinensis*) through transcriptome sequencing. Feng et al. (2009) performed central nervous system transcriptome analysis of *Lymnaea stagnalis*, and Clark et al. (2010) used the 454 pyrosequencing platform to sequence the mantle tissue of Antarctic scallops. In addition, Werner et al. (2013) reported that there were 14 genes that could be associated with shell mineralization using Illumina sequencing. Hou et al. sequenced and analyzed the transcriptome of *Patinopecten yessoensis* at different growth stages. Wang et al. (2013) investigated the transcriptome of *Chlamys farreri* and compared it with the transcriptome to that of *Patinopecten yessoensis*. There have been, however, few studies of bivalve gonad development-related molecular mechanisms and these previous studies have mainly focused on the pacific oyster (*Crassostrea gigas*) (Naimi et al., 2009), *Lymnaea stagnalis* (Feng et al., 2009), pearl oyster (*Pinctada matensii*) (Yu et al., 1998) and species in which the genes involved were confined to several more classic homologs of vertebrate development genes (Matsumoto et al., 2003; Fleury et al., 2008; Meistertzheim et al., 2009; Liu et al., 2012). Most of the research focused on *M. chinensis* breeding has been on fertilization (Chung, 2007), developmental histology, cytology (Chung et al., 1987; Li et al., 2011), and larval nutrition (Shen, 2005; Tyurin and Drozdov, 2005; Zhang et al., 2016a,b). The regulatory mechanism of development, however, has been reported only a few times (Reunov et al., 2014; Kim et al., 2016), and there has been no research about the genes related to ovarian development. Research on the *M. chinensis* transcriptome during ovarian development could, therefore, increase knowledge of shellfish gonadal development, and further elucidate the changes occurring development of germ cells. Furthermore, research that is focused on the molecular mechanisms of ovarian maturation would provide a scientific basis for the development of the *M. chinensis* breeding industry.

2. Materials and methods

2.1. Sample collection

All animals used in this study were 2-year-old clams obtained from the Yalv river estuary (39°80′N, 124°20′E), Dandong, Liaoning Province, China, in April 2016. The gonads were dissected immediately after collection. For each clam, gonad tissues were utilized for RNA extraction and fixed for histology. There were five biological replicates for each group. Each replicate consisted of one sample. For total RNA isolation and extraction, individual samples of gonad tissues were conserved in RNA preservation solution and stored at −80 °C. Five samples of RNA were mixed in equal amounts for RNA-sequencing (RNA-seq).

Sex and ovarian development stages were determined histologically using hematoxylin and eosin staining, and samples were classified into two different stages of ovarian development: Intermediate stage (developing gonads) and the mature stage (the clam is ready to spawn). The female gonads at the intermediate stage were termed “FI” and the gonads at the mature stage were termed “FR”.

2.2. Library construction and illumina sequencing

An Agilent 2100 Bioanalyzer (Agilent RNA 6000 Nano Kit) was used to measure the total RNA concentration, the RNA integrity (RIN) value, 28S/18S, and size. The purity of the samples was assessed using a NanoDrop™ instrument. Total RNA (200 ng) was purified using oligo-dT beads, and then the poly (A)-containing mRNA was fragmented into small pieces using Fragment Buffer. First-strand cDNA was generated using First Strand Master Mix and Super Script II (Invitrogen) and reverse transcription (reaction condition: 25 °C for 10 min; 42 °C for 50 min; 70 °C for 15 min). The Second Strand Master Mix was subsequently added to synthesize the second-strand cDNA (16 °C for 1 h). The purified fragmented cDNAs were combined with End Repair Mix, and incubated at 30 °C for 30 min. The end-repaired DNA was purified with using Ampure XP Beads (AGENCOURT). The A-Tailing Mix was subsequently added, and the samples were incubated at 37 °C for 30 min. To adenylate the 3′ ends of the DNA, RNA Index Adapter and Ligation Mix were combined and ligated at 30 °C for 10 min. The end-repaired DNA was purified using Ampure XP Beads (AGENCOURT). Several replications of PCR amplification using a PCR Primer Cocktail and PCR Master Mix were performed to enrich the cDNA fragments. The PCR products were subsequently purified using Ampure XP Beads (AGENCOURT). The final library was quantified in two ways: by determining the average molecule length using the Agilent 2100 bioanalyzer instrument (Agilent DNA 1000 Reagents), and by quantifying the library using quantitative real-time PCR (qPCR) (TaqMan Probe). The qualified and quantified libraries were first amplified within the flow cell on the cBot instrument for cluster generation (HiSeq® 4000 PE Cluster Kit, Illumina). The clustered flowcell was subsequently loaded onto the HiSeq 4000 Sequencer for paired-end sequencing (HiSeq® 4000 SBS Kit, Illumina) with recommended read lengths of 100 bp.

2.3. De novo assembly and functional annotation

The raw reads were filtered by removing the following sequences: (1) Reads that contained adapters, (2) low quality reads with ambiguous sequence of which the phred score was less than 20, and (3) low quality reads that contained more than 5% ambiguous nucleotides. After filtering, the remaining reads were termed “Clean Reads” and stored in the FASTQ format (Cock et al., 2010).

After removing duplicate reads the Trinity software was used to perform *de novo* assembly with the remaining clean reads (Grabherr et al., 2011). Tgicl was then used to cluster the transcripts into Unigenes (Pertea et al., 2003). Trinity software (*de novo* assembly software), which combined three independent software modules: Inchworm, Chrysalis, and Butterfly, was applied sequentially to process the large volumes of reads. Trinity partitions the sequence data into many individual de Bruijn graphs, each representing the transcriptional complexity at a given gene or locus, and then processes each graph independently to extract full-length splicing isoforms and to discriminate transcripts derived from paralogous genes. The resulting sequences generated by Trinity are termed transcripts. The Tgicl analysis was subsequently repeated for each of the Unigenes of each sample to obtain a final Unigene set for downstream analyses. Gene family clustering was subsequently performed using Tgicl. The Unigenes were divided into two classes, one was clusters, with the prefix CL, followed by the cluster number. Each cluster comprised several Unigenes for which similarity was greater than 70%. The other classes were the singletons, which were given the prefix Unigene.

After assembly, all Unigenes were subjected to functional annotation using seven databases: the NCBI non-redundant (Nr) protein database, the NCBI nucleotide (Nt) database using BLASTn with E value $< 10^{-5}$; the Clusters of Orthologous Groups (COG) of in the protein database; the Kyoto Encyclopedia of Genes and Genomes (KEGG) with an E value $< 10^{-5}$; and the Swiss-Prot protein database. Using the Blast2 GO program (Conesa et al., 2005), the number of Unigenes associated with each gene ontology (GO) category was calculated for the categories of biological processes, cellular components, and molecular functions.

2.4. Quantitative real-time PCR for mRNA relative abundance

To validate the sequencing data, six mRNAs with different relative abundances between the two stages were selected. The mRNA relative abundances of the six selected Unigenes were quantified using specific primers (Table S1). The SYBR Green PCR Master Mix (TaKaRaTakara, Japan, RR820A) was used to perform qPCR on a Roche Light Cycler 480 System II (Roche, Mannheim, Germany). *Mactra chinensis* 18s RNA was used as the internal control for qPCR. All of the real-time reactions were performed in triplicate and the Livak and Schmittgen (2001) $2^{-\Delta\Delta Ct}$ method was used for the relative quantification of the qPCR results (Livak and Schmittgen, 2001). To examine the differences between the means, the SPSS 17.0 software was used (IBM Corp., Armonk, NY, USA) to conduct an analysis of variance (ANOVA), as well as Turkey's pairwise comparison tests. A P value < 0.05 was considered significant.

3. Results

3.1. Characteristics of ovarian development

To study gonadal differentiation, there was identification of two representative stages of ovarian development, FI and FR. The intermediate stage (FI) was from late April to early June, in which the oogonia gradually differentiated and most of the oocytes had a short stipitate attached to the follicle wall, with an average size of 41.4 μm, and a small amount of mature oocytes were observed (Fig. 1a). When there was complete maturation of the ovaries, the female gland was purple red, the oocytes stalk disappeared from the follicle wall, and the oocytes were irregular, with an average diameter of (48.0 × 36.8) μm (Fig. 1b).

3.2. Sequencing and de novo assembly

In the present study, the first transcriptome data obtained from next-generation sequencing of the female *M. chinensis* gonad are reported (Table 1). In total, 50.6 Mb of raw reads were generated in intermediate stage and 52.3 Mb in the mature stage. After filtering the raw data, 44.67 Mb of clean reads with a Q20 percentage of 99.0% were obtained for the intermediate stage, and 45.4 Mb of clean reads with a Q20 percentage of 98.7% were obtained for the mature stage.

De novo assembly of the reads using the Trinity software (Ketata et al., 2007) resulted in generation of 192,391 unique transcript fragments (unigenes) in FI and 218,895 unigenes in FR. More than one sample was sequenced; therefore, Tgicl was conducted again with each sample's Unigene set to obtain the Unigene set for downstream analyses, termed "All-Unigenes". The total number of All-Unigenes was 80,141 and the total length was 62,898,299 bp, with a mean length of 784 bp. The N50 length was 1374 bp. The assembly results indicated that the total number and mean length of the unigenes were similar to those reported as a result of a previous clam gonad transcriptome study (Teaniniuraitemoana et al., 2014), suggesting that the data from *M. chinensis* gonad tissues were effectively assembled. The sequence data were deposited in the NCBI Sequence Read Archive (SRA) database with the accession IDs SRX4715528 and SRX4715529.

3.3. Functional annotation

Based on BLASTx analysis, the unigenes were aligned with sequences deposited in the major databases (Nt, Nr, Swissprot, COG, GO, and KEGG). At an E cutoff value of 10^{-5}, unigenes had the following significant hits in these databases: 20,843 to Nr (26.0%); 16,252 to Nt (20.3%); 16,724 to Swissprot (20.9%); 15,616 to KEGG (19.5%); 7478 to COG (9.3%); 15,469 to Interpro (19.3%); and 3291 to GO (4.1%). Overall, 27,064 unigenes were annotated with at least one functional database.

A total of 3291 All-unigenes of *M. chinensis* were subjected to GO analysis using the Blast2GO software. These transcripts were assigned with GO terms from the three major GO categories of biological process, cellular component, and molecular function (Fig. 2). In the biological process category, cellular and metabolic processes were the most abundant GO terms. For the cellular component, the most abundant categories were cell and cell part. For molecular function, the GO terms of binding and catalytic

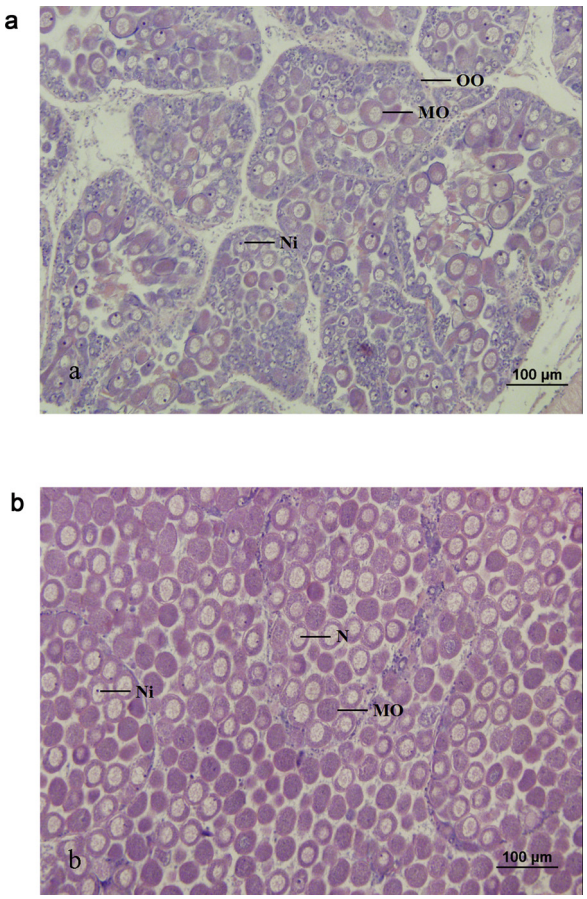

Fig. 1. Histological characteristics of two critical periods of ovarian development in *M. chinensis*; Histological sections (thickness, 6 μm) of the gonads of *M. chinensis* stained with hematoxylin and eosin; Samples at different developmental stages were selected to study the molecular mechanism of ovarian development using RNA-sequencing; a, intermediate stage female ovarian development (FI); b, mature stage female ovary (FR); Ni: nucleolus, OO: oocyte, MO: mature oocyte.

Table 1
Quality metrics of Unigenes.

Sample	Total Number	Total Length	Mean Length	N50	N70	N90	GC(%)
FI	54172	39035004	720	1152	624	290	32.89
FR	63081	46477863	736	1220	640	288	32.67
All-Unigene	80141	62898299	784	1374	716	296	32.71

activity were highly represented.

Among 15,616 All-unigenes with KEGG annotation, 27.7% were classified into human diseases pathways, with most of these being involved in infectious diseases (viral and bacterial). About 19.2% of the All-unigenes were classified in the metabolism category, such as global and overview maps, amino acid metabolism, and carbohydrate metabolism. Organismal systems were represented by 18.9% of the KEGG annotated All-unigenes, with most of these involved in the endocrine system, immune system, and digestive system. There were 12.5% and 12.1% of the All-unigenes classified into the cellular processes and genetic information processing categories, respectively. Additionally, 9.6% of the All-unigenes were involved in environmental information processes, including signal transduction, signaling molecules and interaction, and membrane transport.

3.4. DEGs between different intermediate and ripe stage gonads

The differentially expressed genes (DEGs) were examined based on the results of the relative amounts of gene expression in the individual samples. The reads per kb per million reads (RPKM) method was used to calculate the relative amount of gene expression. The upregulated genes were those where expression in FR relative to FI was greater and the downregulated genes were those where expression in FR relative to FI was less in the ovaries. In total, 10,324 genes were differentially expressed, including 5933 upregulated

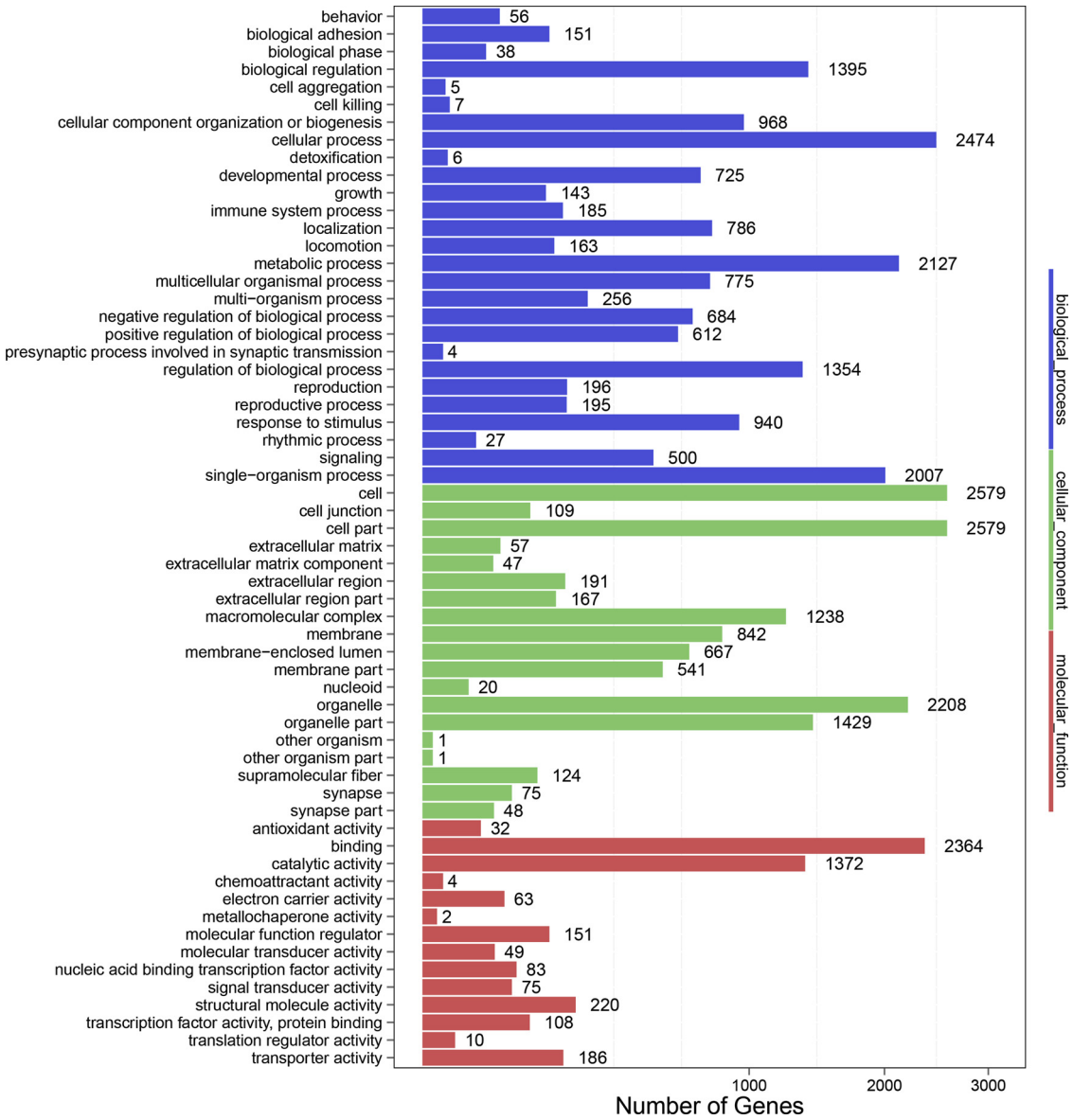

Fig. 2. Functional distribution of gene ontology (GO) annotation; X axis represents the number of Unigenes; Y axis represents the Gene Ontology functional category.

and 4391 downregulated genes in FR compared with FI. In Fig. 3, there is depiction of a MA plot of the DEGs; the x-axis represents value A (log2 transformed mean expression level) and Y axis represents value M (log2 fold change). Among the DEGs, there were more upregulated genes than downregulated genes.

The results of the GO enrichment analysis indicated that there were 811 DEGs, including 340 biological processes, 292 cellular components and 179 molecular functions, accounting for 41.9%, 36.0%, and 22.1% of the total, respectively. With the GO enrichment analysis, there was a majority of the DEGs that were reproduction-related as assigned to the processes of oogenesis, apoptosis, protein ubiquitination, gamete generation, chromosome organization, asymmetric division of female reproductive stem cells, sperm development, mitotic nuclear division, male meiosis, negative regulation of cyclin-dependent protein serine/threonine kinase, regulation of protein stability, germline stem cell maintenance, neuronal remodeling, and microtubule-associated complex.

To identify the biological pathways activated during the development of the ovaries, the DEGs of the two stages were mapped to the reference pathways recorded in the KEGG database. There were 5426 DEGs that mapped to known KEGG pathways. In Fig. 4, there is a depiction of the 20 most significantly enriched pathways. The results indicate that the DEGs were mainly involved in the energy metabolism, notch signaling, NOD-like receptor signaling, NF-kappa B signaling, neuroactive ligand-receptor interaction, Fanconi anemiapathway, drug metabolism-cytochrome P450, DNA replication, and GnRH signaling pathways. Most of these pathways are involved in ovarian development.

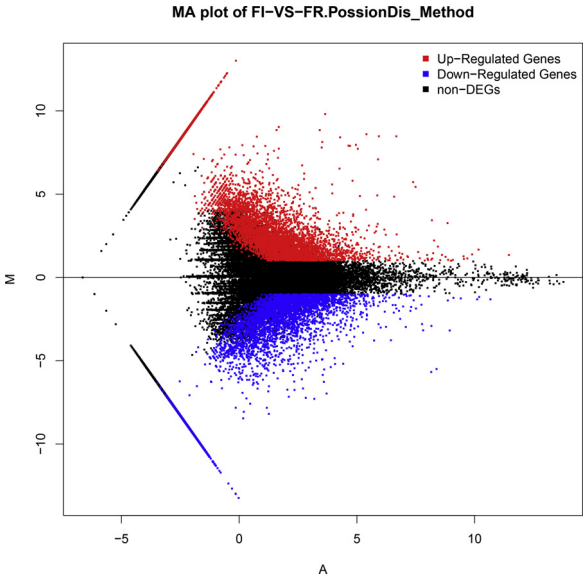

Fig. 3. MA plot of DEGs; X axis represents value A (log2 transformed mean amount of expression); Y axis represents value M (log2 transformed fold change); Red points represent up regulated DEG Blue points represent down regulated DEG; Black points represent non-DEGs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.5. Abundance of mRNA at different developmental stages

The qPCR results for nine mRNAs indicated there were similar changes in relative abundances as compared with those detected using RNA-seq (Fig. 5). These selected unigenes were related to gonadal development and reproduction-related pathways. The relative abundances of the nine mRNAs at the development stage were different from those in the mature stage. In Fig. 5, there is a depiction of the relative mRNA abundances of the CL7814.Conting2, Unigene12808, Unigene12216, CL5995.Contig3, CL4408.Contig1, CL1705.Contig3, Unigene35444 and Unigene33814 that were greater than those in the development stage ($P < 0.05$). The relative abundance of Unigene169 mRNA was less than that at the developmental stage ($P < 0.05$). The nine mRNAs encoded vg, 17β-HSD, 17β-estradiol, cytosolic phospholipase A2, PKA, carboxylesterase, calmodulin, braf and BUB1, respectively. The results validated the molecular resources and sequencing data of the present study.

4. Discussion

Although ovarian development is an important physiological process for *M. chinensis*, the underlying molecular mechanisms have not been evaluated. In the present study, 54,172 unigenes at the intermediate stage and 63,081 unigenes at the mature stage were identified, and 80,141 all-unigenes were assembled to enhance the molecular mechanism information concerning ovarian development in *M. chinensis*. The aim of this transcriptome sequencing project was to identify the genes involved in ovarian development.

4.1. Morphological and gene expression of the two ovarian developmental stages

Ovarian development is a complex process involving regulation of numerous genes. More genes were expressed in FR than in FI, possibly because during the FR stage there are a greater number of follicles with a greater function than during FI (Peters, 1969). It is speculated, therefore, that the transcriptional activity of FR would be greater in FR than FI. During the FI stage there is the initial stage of primary oocyte differentiation and growth, thus, a series of genes associated with growth and metabolism, such as *vasa*, *piwil*, *ND1*, and *Dax1*, were upregulated. The *Vasa* and *piwil* genes are marker genes of germ cells (Xu et al., 2014; Li et al., 2012). The *ND1* protein is an important molecule in energy metabolism, and its relatively greater expression may be required to generate a large amount of energy for oogonia differentiation. The *Dax1* protein, for which there was relatively greater gene expression in the FI than FR developmental stages is important in regulation of steroid hormone synthesis. It is speculated that oogonia undergo steroid-mediated differentiation. Oocytes are mature at the FR stage and genes associated with oocyte maturation, meiosis, and DNA modification were all upregulated, such as the *Sycp2* and *dnmt* genes. The *Sycp2* protein is important in the meiotic process involving the recombination and proper segregation of homologous chromosomes (Page and Hawley, 2004; Costa and Cooke, 2007). During oogenesis, DNA methylation changes are dynamic with the *Dnmt* protein having an important function in DNA methylation. Thus, the gene expression data obtained by using the two libraries provided information for the future study of ovarian development.

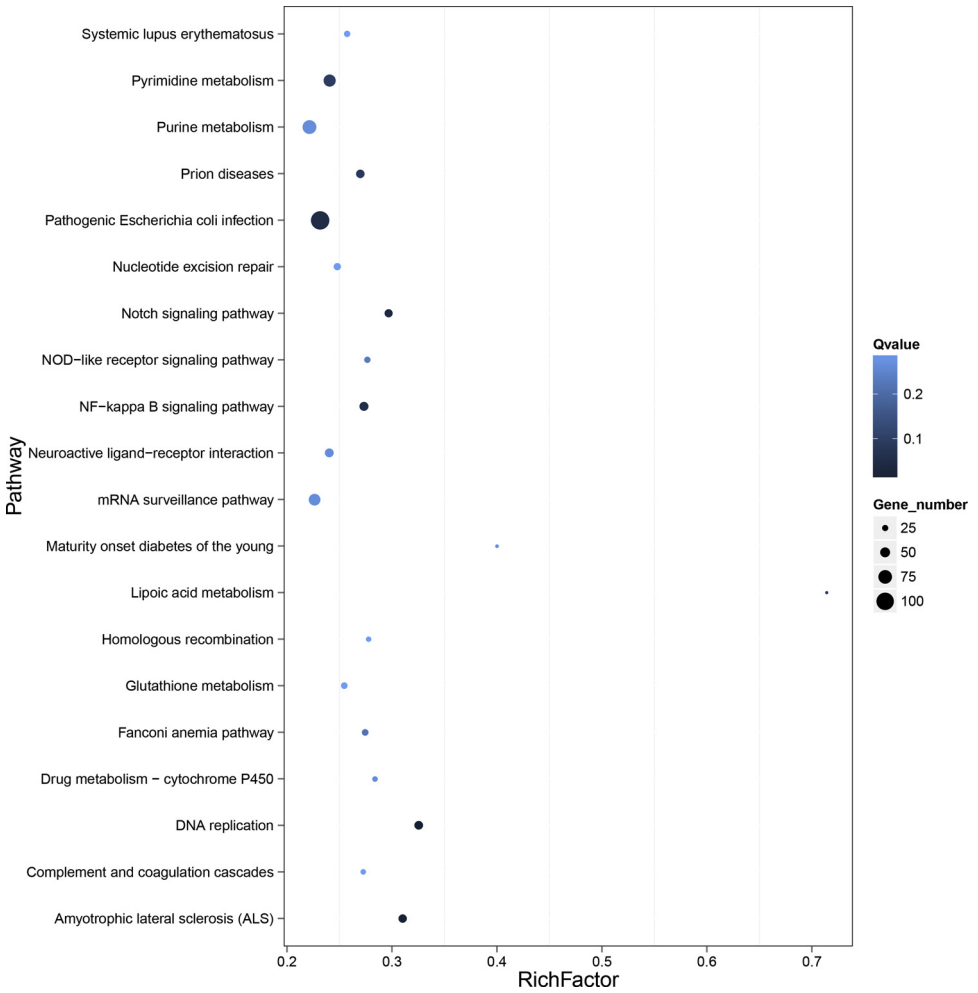

Fig. 4. Top 20 pathways with the least Q values of differentially expressed genes (DEGs); X axis represents enrichment factor; Y axis represents pathway name; Coloring indicates qvalue (high: white, low: blue), the lesser qvalues indicates more significant enrichment; Pointsize indicate DEG number (more: big, less: small). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4.2. Genes and related processes in ovarian development of M. chinensis

In the present study, genes involved in six pathways associated with ovarian development were identified, including the estrogen-signaling pathway, GnRH-signaling pathway, progesterone-mediated oocyte maturation, ovarian steroidogenesis, steroid hormone biosynthesis, and steroid biosynthesis, respectively. There were six important genes for *M. chinensis* ovarian steroidogenesis (map04913), including those encoding for β-hydroxysteroid dehydrogenase, hydroxysteroid (17-beta) dehydrogenase 2, luteinizing hormone beta polypeptide, steroid 17-alpha-hydroxylase, cytochrome P450, and estrone sulfotransferase genes (Table 2). Androstenedione can be converted to estrone as a result of CYP19A1 activities and there is a subsequent catalyzing as a result of 17β-HSD actions in conversion of this precursor into estradiol. Additionally, testosterone is converted to 17β-estradiol as a result of activation of CYP19A1. The CYP17 and 17β-HSD enzymes are, therefore, important for sex steroid hormone synthesis and metabolism. Sequencing results in the present study allowed for identification of some fragments of the vitellogenin genes. Besides vitellogenin, eight transcripts of for the vitelline membrane outer layer 1 protein were identified as being present in the ovaries.

4.3. KEGG classifications of DEGs in different ovarian developmental stages

In the present study, genes related to ovarian development were identified among the annotated genes. Genes associated with ovarian steroidogenesis, steroid hormone biosynthesis, steroid biosynthesis, estrogen-signaling pathway, GnRH-signaling pathway, and progesterone-mediated oocyte maturation were detected. The important DEGs from the seven pathways were identified and many candidate genes related to ovarian development were also identified, which might be important background information in future functional gene research studies. The pathways included steroid biosynthesis, steroid hormone biosynthesis, and ovarian

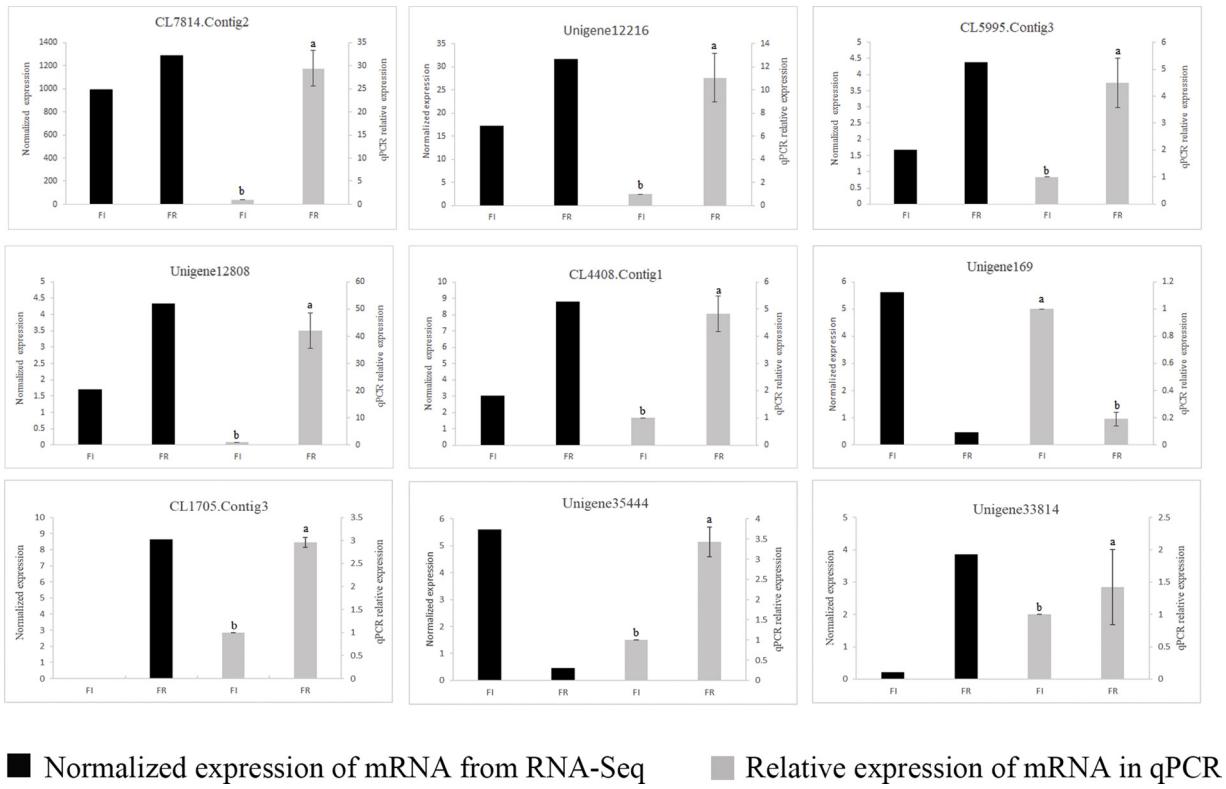

Fig. 5. Comparison of the relative abundances of nine mRNAs using RNA-sequencing and quantitative real-time polymerase chain reaction (qPCR).

Table 2
Candidate genes involved into ovarian development of *M. chinensis*.

Name	Query_id	Identity	Align_length	E_value	Score	Subject_annotation
3β-HSD	Unigene22953_All	39.78	93	7.00E-07	54.3	3 beta-hydroxysteroid dehydrogenase type 3
	Unigene44423_All	36.59	123	7.00E-08	57.8	3 beta-hydroxysteroid dehydrogenase/Delta 5 > 4-isomerase-like
17β-HSD	Unigene4909_All	31.2	234	2.00E-15	85.5	hydroxysteroid (17-beta) dehydrogenase 2
	Unigene4910_All	30.8	250	1.00E-17	93.6	hydroxysteroid (17-beta) dehydrogenase 2
	Unigene19949_All	41.51	53	9.00E-07	53.9	dihydrodiol dehydrogenase 3-like
LH	Unigene474_All	42.47	73	3.00E-09	65.5	luteinizing hormone beta polypeptide
CYP17	Unigene11337_All	31.43	509	9.00E-60	234	steroid 17-alpha-hydroxylase/17,20 lyase-like
	Unigene20807_All	45.78	83	7.00E-15	80.9	cytochrome P450 2U1
	Unigene22796_All	51.47	68	5.00E-11	68.2	steroid 17-alpha-hydroxylase/17,20 lyase
	Unigene51260_All	42.03	69	2.00E-07	55.8	steroid 17-alpha-hydroxylase/17,20 lyase
	CL1385.Contig1_All	40.95	337	4.00E-67	257	steroid 17-alpha-hydroxylase/17,20 lyase-like
	CL6230.Contig2_All	50.71	140	1.00E-31	137	steroid 17-alpha-hydroxylase/17,20 lyase-like
	Unigene19441_All	42.31	78	9.00E-13	73.9	steroid 17-alpha-hydroxylase/17,20 lyase-like
CYP1A1	Unigene22391_All	44	75	3.00E-13	75.5	Cyp1a1
	Unigene22796_All	42.03	69	1.00E-10	67	cyp17a1
	Unigene26063_All	41.43	70	5.00E-08	58.2	cytochrome P450 1A1
	Unigene27525_All	46.59	88	4.00E-15	81.6	cyp1a1
	Unigene34874_All	45.88	85	2.00E-18	92.8	CYP1A1
	Unigene3854_All	25.73	171	3.00E-07	56.6	cytochrome P450 1A1-like
CYP1B1	Unigene3854_All	27.22	180	3.00E-07	56.6	cyp1b1
Estrogen	Unigene12390_All	41.07	280	2.00E-53	212	sulfotransferase family 1E, estrogen-preferring, member 1
vitellogenin	CL7814.Contig2_All	22.8	1610	3.30E-90	340.502	Vitellogenin-6
	CL7814.Contig2_All	23.96	1006	4.07E-56	227.254	vitellogenin
	Unigene2388_All	47.83	92	2.47E-07	62.7734	vitellogenin
	CL8756.Contig6_All	36.02	211	4.15E-23	115.546	Vitelline membrane outer layer protein 1-like protein, partial
	Unigene757_All	76.24	101	3.53E-32	141.739	vitelline envelope zona pellucida domain 14, partial
	Unigene3284_All	43.38	136	8.02E-24	114.005	Vitelline membrane outer layer protein 1
	Unigene10971_All	45.96	161	4.24E-31	139.428	Vitelline membrane outer layer protein 1
	Unigene17149_All	44.44	189	2.32E-35	155.221	Vitelline membrane outer layer protein 1

Table 3
Selected steroid biosynthesis genes identified in the transcriptome.

Sequence ID	Accession No.	Gene Name	FI	FR	Regulation (FR/FI)
Steroid biosynthesis					
CL5995.Contig3_All	hro:HELRODRAFT_194195	17beta-estradiol	1.67	4.38	Up
Unigene12808_All	crg:105346644	carboxylesterase 5A-like	1.7	4.34	Up
CL6230.Contig3_All	crg:105334514	steroid 17-alpha-hydroxylase	4.05	0.18	Down
CL231.Contig3_All	crg:105346644	carboxylesterase 5A-like	2.54	0.34	Down
Unigene1994_All	aml:100463734	steroid 5 alpha-reductase 3	1.8	8.46	Up
CL5995.Contig3_All	hro:HELRODRAFT_194195	17beta-estradiol	1.67	4.38	Up
CL6230.Contig3_All	crg:105334514	steroid 17-alpha-hydroxylase	4.05	0.18	Down
Ovarian steroidogenesis					
CL4408.Contig1_All	crg:105336635	cAMP-dependent protein kinase catalytic subunit-like	3.03	8.79	Up
CL5995.Contig3_All	hro:HELRODRAFT_194195	17beta-estradiol 17-dehydrogenase	1.67	4.38	Up
Unigene6613_All	crg:105337022	growth differentiation factor	1.49	3.85	Up
CL2600.Contig1_All	tgu:100228552	myosin XVIIIB	1.84	0.01	Down
Unigene14450_All	crg:105344258	prostaglandin-endoperoxide synthase 2	9.65	0.22	Down
CL6230.Contig3_All	crg:105334514	steroid 17-alpha-hydroxylase	4.05	0.18	Down
Unigene14452_All	crg:105344258	prostaglandin G/H synthase 2-like	7.14	0.33	Down
Unigene169_All	crg:105340783	cytosolic phospholipase A2-like	5.61	0.45	Down
Unigene15213_All	lcm:102350971	cytochrome P450 2K1-like	2.22	0.24	Down
CL6462.Contig1_All	crg:105324861	cytochrome P450 2J6-like	3.54	0.84	Down
CL3062.Contig2_All	crg:105340783	cytosolic phospholipase A2-like	20.17	6.39	Down
CL2600.Contig2_All	lcm:102359035	low-density lipoprotein receptor 1-like	3.31	1.07	Down
Unigene170_All	crg:105340783	cytosolic phospholipase A2-like	10.86	3.78	Down

steroidogenesis (Table 3). The steroid hormone, 17β-estradiol, has an important function in reproduction of shellfish and was identified in these three pathways.

There is much ongoing research focused on modulation of estradiol concentrations in gonads or other tissues during the reproductive cycle of shellfish (Lavado et al., 2006; Ketata et al., 2007; Mouneyrac et al., 2008; Devier et al., 2010; Yan et al., 2010; Ni et al., 2013). There were 30 DEGs identified as being associated with the estrogen-signaling pathway, most of which were calmodulin and heat shock protein related genes, including 20 upregulated genes (e.g, calmodulin; Table 4). There were 26 DGEs related to the

Table 4
Selected estrogen signaling pathway genes identified in the transcriptome.

Sequence ID	Accession No.	Gene Name	FI	FR	Regulation (FR/FI)
CL1705.Contig3_All	spu:575365	calmodulin	0.01	8.63	Up
Unigene33698_All	mmu:70405	calmodulin-like 3	0.01	1.48	Up
Unigene36974_All	crg:105320743	transcription factor Sp3-like	0.09	4.01	Up
CL2679.Contig14_All	ngi:103737133	calmodulin-like 5	0.4	16	Up
CL2634.Contig3_All	phu:Phum_PHUM373530	calmodulin-A	0.23	5.25	Up
Unigene42541_All	xma:102219929	RING finger protein 38-like	0.62	8.16	Up
Unigene10525_All	tru:101075813	heat shock cognate 71 kDa protein-like	2.34	13.26	Up
CL6023.Contig2_All	xtr:100037899	heat shock 70 kDa protein	2.4	11.21	Up
CL7378.Contig2_All	crg:105334510	heat shock protein 68-like	5.55	25.56	Up
CL4019.Contig3_All	crg:105322085	calmodulin-like	15.71	66.29	Up
CL2152.Contig1_All	der:Dere_GG23119	mitogen-activated protein kinase 1/3	0.96	3.93	Up
CL4420.Contig1_All	phi:102107817	Na + /K + transporting	14.35	54.02	Up
Unigene31446_All	crg:105348304	heat shock protein 70 B2-like	5.36	19.12	Up
CL2162.Contig4_All	crg:105340223	heat shock protein 90 kDa beta	4.82	12.83	Up
CL7378.Contig3_All	crg:105348304	heat shock protein 70 B2-like	6.95	18.03	Up
Unigene12305_All	spu:575365	calmodulin	7.19	17.87	Up
CL1705.Contig2_All	smm:Smp_026560.2	calmodulin	21.7	50.1	Up
CL4707.Contig1_All	crg:105342292	calmodulin-like	3.68	7.97	Up
Unigene672_All	crg:105334510	heat shock protein 68-like	3.74	8.04	Up
CL2546.Contig2_All	nvi:100679877	V-type proton ATPase subunit C	10.49	0.01	Down
CL1428.Contig1_All	crg:105326506	cyclic AMP-responsive element-binding protein 3-like protein 2	7.42	0.01	Down
CL1428.Contig2_All	crg:105326506	cyclic AMP-responsive element-binding protein 3-like protein 2	7.34	0.01	Down
CL344.Contig1_All	tru:101079118	heat shock 70 kDa protein-like	6.74	0.67	Down
CL2863.Contig3_All	nvi:100679877	V-type proton ATPase subunit C	10.87	1.55	Down
CL8510.Contig1_All	phi:102107817	Na + /K + transporting, alpha 2 polypeptide	5.22	1.05	Down
Unigene17575_All	nvi:100679877	V-type proton ATPase subunit C	4.8	1.05	Down
CL6023.Contig1_All	xtr:100037899	heat shock 70 kDa protein	23.33	5.15	Down
CL2162.Contig1_All	crg:105340223	heat shock protein 90 kDa beta	6.72	2.68	Down
CL7964.Contig1_All	crg:105330118	epidermal growth factor receptor	24.14	11	Down
Unigene31499_All	phi:102107817	Na + /K + transporting, alpha 2 polypeptide	7.02	3.44	Down

Table 5
Selected GnRH signaling pathway genes identified in the transcriptome.

Sequence ID	Accession No.	Gene Name	FI	FR	Regulation (FR/FI)
CL1705.Contig3_All	spu:575365	calmodulin	0.01	8.63	Up
Unigene33698_All	mmu:70405	calmodulin-like 3	0.01	1.48	Up
CL966.Contig1_All	crg:105328474	rho-related GTP-binding protein RhoQ-like	0.05	2.56	Up
CL2679.Contig14_All	ngi:103737133	calmodulin-like 5	0.4	16	Up
CL966.Contig2_All	crg:105328474	rho-related GTP-binding protein RhoQ-like	0.05	1.47	Up
CL2634.Contig3_All	phu:Phum_PHUM373530	calmodulin-A	0.23	5.25	Up
Unigene3836_All	crg:105337849	cell division control protein 42-like	0.35	5.52	Up
Unigene42541_All	xma:102219929	RING finger protein 38-like	0.62	8.16	Up
CL4019.Contig3_All	crg:105322085	calmodulin-like	15.71	66.29	Up
CL2152.Contig1_All	der:Dere_GG23119	mitogen-activated protein kinase 1/3	0.96	3.93	Up
CL4420.Contig1_All	phi:102107817	ATPase, Na + /K + transporting, alpha 2 polypeptide	14.35	54.02	Up
CL4408.Contig1_All	crg:105336635	cAMP-dependent protein kinase catalytic subunit-like	3.03	8.79	Up
Unigene12305_All	spu:575365	calmodulin	7.19	17.87	Up
CL1258.Contig2_All	umr:103681847	toll-like receptor adaptor molecule 1	3.22	7.58	Up
CL1705.Contig2_All	smm:Smp_026560.2	calmodulin	21.7	50.1	Up
CL4707.Contig1_All	crg:105342292	calmodulin-like	3.68	7.97	Up
CL2546.Contig2_All	nvi:100679877	V-type proton ATPase subunit C	10.49	0.01	Down
Unigene25674_All	crg:105328474	rho-related GTP-binding protein RhoQ-like	2.77	0.01	Down
Unigene169_All	crg:105340783	cytosolic phospholipase A2-like	5.16	0.45	Down
CL2863.Contig3_All	nvi:100679877	V-type proton ATPase subunit C	10.87	1.55	Down
CL8510.Contig1_All	phi:102107817	ATPase, Na + /K + transporting, alpha 2 polypeptide	5.22	1.05	Down
Unigene17575_All	nvi:100679877	V-type proton ATPase subunit C	4.8	1.05	Down
CL3062.Contig2_All	crg:105340783	cytosolic phospholipase A2-like	20.17	6.39	Down
Unigene170_All	crg:105340783	cytosolic phospholipase A2-like	10.86	3.78	Down
CL7964.Contig1_All	crg:105330118	epidermal growth factor receptor	24.14	11	Down
Unigene31499_All	phi:102107817	ATP1A2	7.02	3.44	Down

GnRH-signaling pathway in *M. chinensis* ovaries, including calmodulin (CAM), ATPase, cytoplasmic and phospholipase A2, and these were comprised of 16 up-regulated genes, and 10 down-regulated genes (Table 5). In the GnRH-signaling pathway, the predominant upregulated gene was CAM. A total of 21 DGEs were associated with progesterone-mediated oocyte maturation, which mainly comprised those encoding for serine/threonine-protein kinase mos-like, protein kinase C substrate 80K-H, and the cell division cycle protein 16 homolog. In addition to the two downregulated genes, the remaining 18 genes in the pathway were up-regulated genes (Table 6). In the present study, the gene encoding for PKA in these pathways was upregulated, while in the estrogen-signaling and GnRH-signaling pathways, CAM was upregulated. The PKA enzyme, also termed cyclic-AMP dependent protein kinase A, and CAM are associated with cell signal transduction. These proteins function in the transmission of the extracellular signal to the cells or nucleus via a cell nucleus information route, modulating biological effects, such as influencing cell growth, development, differentiation, and proliferation. The significant upregulation of PKA and CAM in the four pathways, therefore, may indicate that PKA and CAM function in *M. chinensis* ovarian development during maturation.

Table 6
Selected Progesterone-mediated oocyte maturation genes identified in the transcriptome.

Sequence ID	Accession No.	Gene Name	FI	FR	Regulation (FR/FI)
Unigene35222_All	crg:105324657	serine/threonine-protein kinase mos-like	0.14	73.4	Up
Unigene320_All	crg:105317174	G2/mitotic-specific cyclin-B-like	0.78	58.98	Up
CL6917.Contig1_All	crg:105348733	G2/mitotic-specific cyclin-A-like	10.5	321.18	Up
Unigene35444_All	xtr:779570	braf, B-Raf, b-raf1, braf1, raf, rafb1	0.46	13.95	Up
Unigene33814_All	gga:421226	BUB1; BUB1 mitotic checkpoint serine/threonine kinase	0.21	3.86	Up
Unigene3487_All	crg:105317174	G2/mitotic-specific cyclin-B-like	10.24	171.4	Up
Unigene53604_All	cge:100753910	Prkcsh; protein kinase C substrate 80K-H	0.72	0.77	None
Unigene4765_All	crg:105341494	serine/threonine-protein kinase PLK1-like	0.29	3.06	Up
CL6928.Contig1_All	cmk:103181515	M-phase inducer phosphatase 1-like	0.95	9.45	Up
Unigene6402_All	crg:105329118	mitotic spindle assembly checkpoint protein MAD1-like	11.96	80.25	Up
CL2152.Contig1_All	der:Dere_GG23119	GG23119 gene product from transcript GG23119-RA	0.96	3.93	Up
Unigene6299_All	crg:105347974	G2/mitotic-specific cyclin-B3-like	1.62	5.94	Up
CL4408.Contig1_All	crg:105336635	cAMP-dependent protein kinase catalytic subunit-like	3.03	8.79	Up
CL4500.Contig1_All	crg:105333859	anaphase-promoting complex subunit 7-like	3.04	7.78	Up
Unigene4105_All	dpo:Dpse_GA14145	GA14145 gene product from transcript GA14145-RA	90.81	219.71	Up
Unigene17606_All	crg:105329592	G2/mitotic-specific cyclin-B-like	27.04	60.51	Up
Unigene10937_All	crg:105331959	anaphase-promoting complex subunit cdc26-like	3.56	7.92	Up
Unigene11696_All	crg:105321354	anaphase-promoting complex subunit 1-like	2.41	5.35	Up
CL246.Contig4_All	crg:105317224	cell division cycle protein 16 homolog	7.62	1.66	Down
Unigene5731_All	xtr:549513	cdc23; cell division cycle 23	7.15	2.64	Down

5. Conclusions

This is the first report of transcriptome data obtained from next-generation sequencing of the female *Mactra chinensis* gonads. There was generation of about 13.51 Gb of bases using Illumina Hiseq sequencing. Assembly of all the samples resulted in identification of 80,141 Unigenes, which were annotated using seven functional databases (NR, NT, Swissprot, COG, KEGG, GO, and Interpro). The mRNA analysis resulted in identification of 5933 upregulated and 4391 downregulated DEGs between the FR and FI samples, indicating the involvement of the proteins encoded by these genes in gonadal development. Results from the present study could be important in conducting subsequent studies focused on functional gene research. The qPCR results for six mRNAs indicated mRNA abundance changes were similar to the data obtained using RNA-seq procedures, which confirmed the validity of the sequencing data conducted in the present study. This study has theoretical and practical significance for use in the development of the *M. chinensis* cultivation and breeding industry, as well as in protection of the natural seedling resources.

Conflict of interest

The authors declared that they have no conflicts of interest to this work. We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.anireprosci.2019.05.007.

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