GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Gene expression profile analysis of testis and ovary of oriental river prawn, *Macrobrachium nipponense*, reveals candidate reproduction-related genes

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Genet. Mol. Res. 14 (1): 2041-2054 (2015)
Received February 21, 2014
Accepted June 13, 2014
Published March 20, 2015
DOI http://dx.doi.org/10.4238/2015.March.20.14

ABSTRACT. This study utilized high-throughput RNA sequencing technology to identify reproduction- and development-related genes of *Macrobrachium nipponense* by analyzing gene expression profiles of testis and ovary. More than 20 million 1 x 51-bp reads were obtained by Illumina sequencing, generating more than 7.7 and 11.7 million clean reads in the testis and ovary library, respectively. As a result, 10,018 unitags were supposed to be differentially expressed genes (DEGs) between ovary and testis. Compared to the ovary library, 4563 (45.5%) of these DEGs exhibited at least 6-fold upregulated expression, while 5455 (54.5%) DEGs exhibited at least 2-fold downregulated expression in the testis. The Gene Ontology (GO) enrichment analysis showed that 113 GO terms had potential molecular functions in reproduction. The Kyoto Encyclopedia of Genes and Genomes results revealed that the most important pathways may be relevant to reproduction and

included 7 pathways. Forty-two genes were identified as reproduction-, development-, and sex-related genes based on GO classification and sequence comparison with other publications, including male reproductive-related LIM protein, spermatogenesis-associated protein, gametocyte-specific factor 1, VASA-like protein, vitellogenin, sex-determining protein fem-1, and other potential candidates. These results will advance research in the field of molecular genetics in *M. nipponense* and offer a valuable resource for further research related to reproduction in crustaceans.

Key words: Reproduction-related genes; Differentially expressed genes; Testis; Ovary; *Macrobrachium nipponense*

INTRODUCTION

The oriental river prawn, *Macrobrachium nipponense* (Crustacea; Decapoda; Palaemonidae), is a commercial freshwater prawn species that is widely distributed in many Asian countries, including Japan, Korea, Vietnam, Myanmar, and China (Cai and Ng, 2002). Now, it has entered into a rapid development period and has become an important way to increase efficiency and income of agriculture with a total fishing production of 230,248 tons per year in China (Bureau of Fishery MoAPRC, 2011) and an annual production value of more than 100 million RMB. However, the oriental river prawn is the only species of freshwater crustaceans in large-scale production that can propagate themselves to overpopulation, especially in autumn (known as autumn-propagation). This causes a multi-generation reunion, increased breeding density, and risk of lack of oxygen. As a result, the autumn-propagation has become a problem in cultured stocks and restrained the health development of prawn aquaculture, which leads to low market value of the product because of low growth rate, poor survival, and short life span. Therefore, it is of great practical significance to study the regulatory mechanism of reproduction in oriental river prawn, which would help to delay early sexual maturity, regulate the reproduction rate, and improve the production benefit.

Various factors can influence the reproduction of crustaceans. Previous studies showed that some hormones produced by special glands, such as methyl farnesoate, gonad-inhibiting hormone (GIH), and mandibular organ-inhibiting hormone, can obviously stimulate or inhibit gonad development (Somka et al., 1993; Wainwright et al., 1996; Olmstead and Leblanc, 2002). Removing these special glands, such as the androgenic gland, can also be effective by promoting sex-reversed populations (Sagi et al., 1990). Some other exogenous factors, such as sex hormones (17β-estradiol and progesterone) and environmental factors (temperature, light, and feed conditions), were proven to affect gonad development in crustaceans (Sasser and Singhas, 1992; Pascual et al., 1998). These methods, rather than genetics, were not applied well in practical production. In recent studies, researchers expected to reveal the regulatory mechanism of gonadal development of crustaceans by constructing cDNA expression libraries and discovering reproduction- and gonad development-related genes (Krungkasem et al., 2002; Qiu et al., 2008a,b; Treerattrakool et al., 2008; Wu et al., 2009; Xie et al., 2010; Ma et al., 2012). For oriental river prawn, there were only two expressed sequence tag (EST) cDNA libraries (testis and ovary) with a total of 81,411 EST records in the National Center for Biotechnology Information (NCBI) database and 1 transcriptome (several tissues) reported;

several genes (*Ubc9*, gustavus, and kazal-type protease inhibitors) were reported to play an important role in gonad development in crustaceans (Wu et al., 2009; Qian et al., 2012; Qiao et al., 2012; Ma et al., 2012). However, tissue-specific gene expression profiles in gonad of *M. nipponense* are not currently available.

Recent research has shown that high-throughput RNA-sequencing (RNA-Seq) technology is a powerful, cost-efficient tool for gene expression profiling analysis and provides a more sensitive tool than microarray methods (Marioni et al., 2008). In this study, we investigated the gene expression profiles of testis and ovary of oriental river prawn. We aimed 1) to develop a gene expression profile database of the testis and ovary of oriental river prawn to identify candidate genes that are involved in reproduction and differ in expression between males and females and 2) to gain a global view of the molecular mechanism (s) whereby gene expression may influence reproduction and gonad development in oriental river prawn.

MATERIAL AND METHODS

Prawn and tissue preparations

Healthy adult male and female *M. nipponense* weighing 2.10-4.85 g were captured from Tai Lake in Wuxi City, Jiangsu Province, China (120°13'44"E, 31°28'22"N). Male and female prawns were transferred to two 300-L tanks (20 prawns in each tank) and maintained in aerated freshwater at room temperature (20°C) for 72 h before sample collection. No prawns died during the period. After the period, 6 prawns from each tank were randomly selected and anesthetized under MS222 anesthesia to minimize suffering. The testes and ovaries from 6 individuals were collected and frozen in liquid nitrogen immediately until used for RNA extraction.

RNA extraction and Illumina sequencing

The differentially expressed gene (DEG) libraries (testis and ovary) were constructed as transcriptome libraries and an ovary library was constructed as a control library. Six male and 6 female prawns were dissected to obtain samples of testis and ovary. Total RNA was extracted using TRIzol® Reagent (Invitrogen, USA) according to the manufacturer protocol and treated with DNaseI. RNA integrity was confirmed by the 2100 Bioanalyzer (Agilent Technologies, Germany). The samples were prepared using the Illumina kit following the manufacturer recommendations. Two biological replicates were sequenced and analyzed. Two-strand cDNA libraries were prepared, and samples were sequenced on an Illumina HiSeq 2000 with a 51-bp single-end read length (GATC Biotech, Germany).

Sequencing and functional analyses

Clean reads were obtained by filtering the adaptor sequences and removing low-quality sequences (containing ambiguous bases). Then, the clean reads were mapped to the reference genome and genes of *M. nipponense* available at http://www.ncbi.nlm.nih.gov/sra/?term=SRA051767 and nucleotide basic local alignment search tool (BLASTn), allowing no more than 2 nucleotide mismatches. Only the tags with a perfect match or 1 mismatch were further considered and annotated based on the reference genes.

A statistical analysis of the frequency of each read in the different cDNA libraries was performed to compare gene expression in different tissues. The gene expression level was calculated using the reads per kb per million reads (RPKM) (Mortazavi et al., 2008) method. Both libraries of clean reads were normalized to the RPKM value to obtain the normalized gene expression level. A strict algorithm (Audic and Claverie, 1997) was performed to identify DEGs. The relative-change threshold in comparisons was performed using the absolute value of log2 (Testis-RPKM/Ovary-RPKM). The threshold with a false discovery rate (FDR) ≤ 0.001 and the absolute value of logFC ratio ≥ 2 were used to judge the significance of gene expression differences.

Gene Ontology (GO) and pathway annotation and enrichment analyses were based on the NCBI Gene Ontology database (http://www.geneontology.org/) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (http://www.genome.jp/kegg/), respectively.

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

To verify the DEGs, qRT-PCR was used to investigate expression levels. The qRT-PCR analysis was carried out using SYBR Green qRT-PCR analysis in a CFX96TM Real-Time System (Bio-Rad, USA). The protocol was performed as follows: a 96-well plate with a 20 μL reaction volume containing 10 μL 2X SYBR Green Premix Ex Taq (TaKaRa, Japan), 0.4 μL 2 μM of each primer, 2 μL template, and 7.2 μL PCR-grade water. The thermal profile for SYBR Green qRT-PCR was 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Diethylpyrocarbonate water was used in place of template as a negative control. A relative standard curve was constructed using 10-fold serially diluted cDNA. Each sample was run in triplicate along with the internal control gene, β-actin. To ensure that only 1 PCR product was amplified and detected, dissociation curve analysis of amplification products was performed at the end of each PCR. The relative copy number of mRNA was calculated according to the $2^{-\Delta\Delta Ct}$ comparative cycle threshold method (Livak and Schmittgen, 2001).

RESULTS

Sequence read processing and mapping

The Illumina HiSeq 2000 platform was used to perform high-throughput sequence analysis on the testis and ovary libraries to investigate the DEGs in gonad. More than 12.5 million single reads of 51 bp were obtained from testis, and over 92.6% of the total reads were identified as clean reads before mapping them to the reference database. Over 7.7 million clean reads (66.40%) in the testis library were successfully mapped to the reference database. In the ovary library, 15.2 million reads were generated, and more than 93.1% of them were clean. Among the clean reads, 83.07% were mapped to the database perfectly (Table 1).

Table 1. Major characteristics of the testis and ovary libraries.

Name	Testis	Ovary
Total nucleotides (nt)	640,341,006	775,342,290
Total reads	12,555,706	15,202,790
Clean reads	11,631,094	14,167,160
% Total clean reads	92.64%	93.19%
Mapped reads	7,723,096	11,768,079
% Total reads mapped	66.40%	83.07%

To discover the genes displaying a significant difference in expression in testis and ovary, the gene expression levels were calculated using the RPKM method (Mortazavi et al., 2008). A total of 81,397 entities were detected between the testis and ovary libraries. Using FDR ≤ 0.001 and the absolute value of the logFC ratio ≥ 2, we identified 10,018 significant DEGs that included 19,750 upregulated and 18,669 downregulated genes (Table 2). Compared to the ovary library, 4563 (45.5%) of these DEGs exhibited at least 6-fold upregulated expression, while 5455 (54.5%) DEGs exhibited at least 2-fold downregulated expression in the testis. The DEGs are shown in Figure 1.

Table 2. Differentially expressed genes.

	Total	Upregulated	Downregulated	Significant*
Testis *vs* Ovary	81,397	19,750	18,669	10,018

*Differentially expressed genes (DEGs).

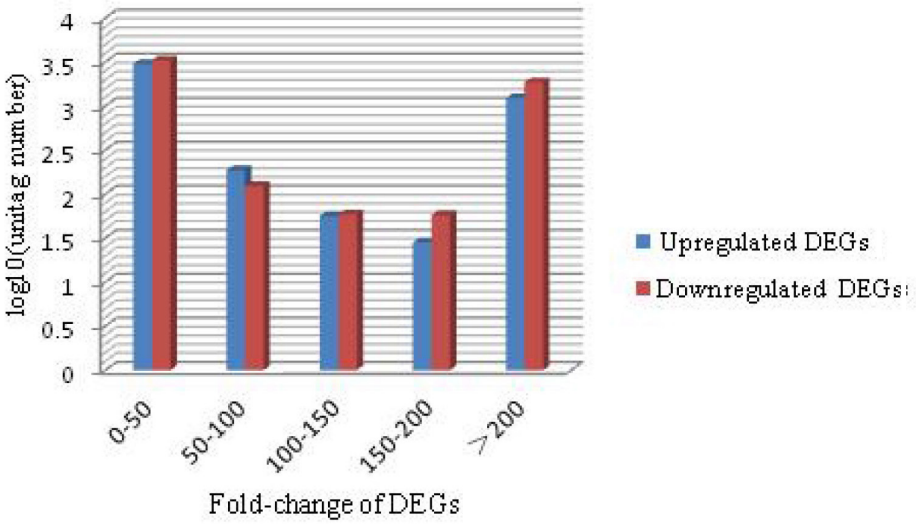

Figure 1. Differentially expressed genes (DEGs). The x-axis represents the fold-change of expression. The y-axis represents the number of DEGs (log 10).

GO function analysis

In GO analysis, 51,404 unique tags from *M. nipponense* were clustered based on matches with sequences with known function (Figure 2). These were divided into 3 functional categories: cellular component (16,618), molecular function (10,645), and biological process (24,141). Most of the corresponding biological process genes were involved in cellular processes (4663, 19.3%). Most of the cellular component genes encoded proteins associated with cells and cell parts (3697, 22.2%), and most of the molecular function genes were associated with binding (4609, 43.3%). The GO enrichment analysis showed that 113 GO terms had potential molecular functions in reproduction (Figure 3). The top 3 GO functions were estab-

lishment of localization (P = 3.08E-10), small molecule metabolic process (P = 3.43E-10), and transport (P = 3.59E-10).

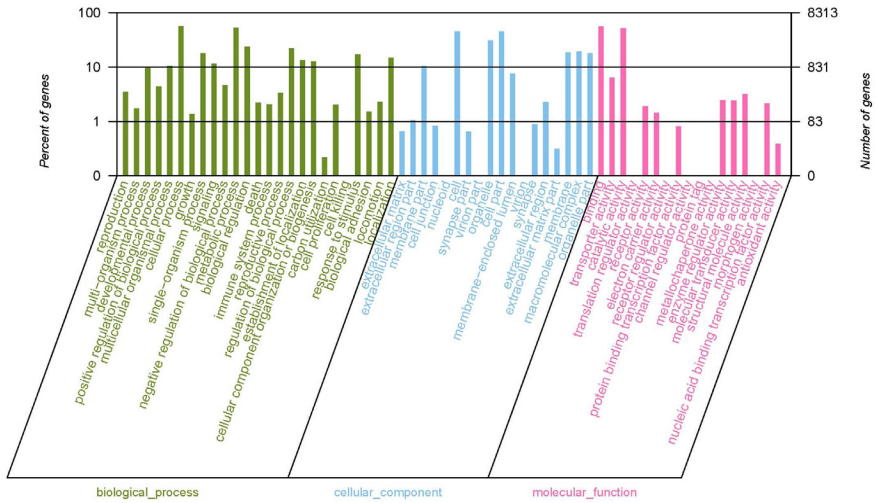

Figure 2. Distribution of gene ontology (GO) classifications.

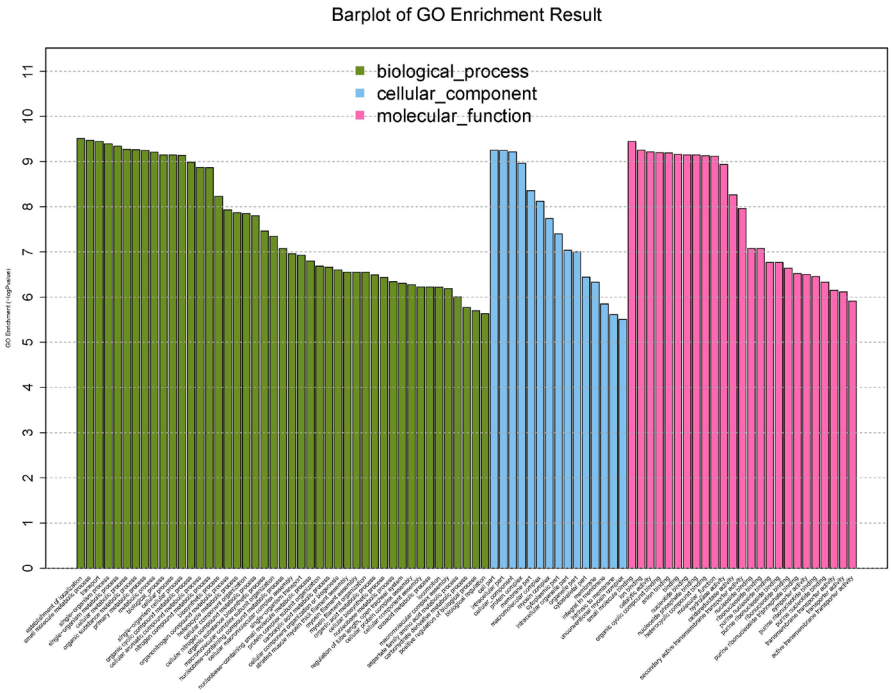

Figure 3. Gene ontology (GO) enrichment classification of differentially expressed genes (DEGs) from ovary and testis in *Macrobrachium nipponense*.

KEGG pathway analysis

In order to determine the functional annotation of the large lists of DEGs, significantly enriched metabolic pathways and signal transduction pathways were identified. A total of 251 predicted KEGG metabolic pathways were associated with 2729 DEGs. Many of the pathways, including progesterone-mediated oocyte maturation, oocyte meiosis, MAPK signaling pathway, and ubiquitin-mediated proteolysis, might be related to reproduction and gonad development. Table 3 lists the top 25 pathways with the highest tag numbers. Pathways with $P < 0.05$ were significantly enriched. Our results revealed that the most important pathways that may be relevant to spermatogenesis or reproduction included 7 pathways (Figure 4). They included metabolism of xenobiotics by cytochrome P450 (P = 1.14E-04); drug metabolism by cytochrome P450 (P = 2.61E-04); glycine, serine, and threonine metabolism (P = 7.52E-04); retinol metabolism (P = 1.38E-03); viral myocarditis (P = 5.62E-03); naphthalene degradation (P = 0.024); and tight junction (P = 0.048). These predicted pathways are likely to be useful in future investigations focusing on their functions in *M. nipponense*.

Table 3. Top 25 pathways with the highest tag numbers.

No.	Pathway terms	No. of tags	Pathway ID
1	Lysosome	52	ko04142
2	Phagosome	50	ko04145
3	Tight junction	45	ko04530
4	Protein processing in endoplasmic reticulum	41	ko04141
5	Alzheimer's disease	41	ko05010
6	Focal adhesion	37	ko04510
7	Insulin signaling pathway	36	ko04910
8	Endocytosis	36	ko04144
9	Pathways in cancer	36	ko05200
10	Huntington's disease	35	ko05016
11	Viral myocarditis	34	ko05416
12	Regulation of actin cytoskeleton	34	ko04810
13	Oxidative phosphorylation	34	ko00190
14	HTLV-I infection	34	ko05166
15	Purine metabolism	33	ko00230
16	Spliceosome	33	ko03040
17	Pathogenic *Escherichia coli* infection	32	ko05130
18	Influenza A	30	ko05164
19	*Vibrio cholerae* infection	29	ko05110
20	RNA transport	29	ko03013
21	Oocyte meiosis	28	ko04114
22	Amino sugar and nucleotide sugar metabolism	25	ko00520
23	Parkinson's disease	25	ko05012
24	Ubiquitin-mediated proteolysis	25	ko04120
25	Glycine, serine, and threonine metabolism	24	ko00260

ID represents the pathway ID in the Kyoto Encyclopedia of Genes and Genomes (KEGG). The pathway term represents the pathway name.

Candidate reproduction-related genes and qRT-PCR validation

All 10,018 DEGs were subjected to BLASTn using the NCBI nr/nt database, and 3061 were assigned to known sequences in the databases. In other word, a total of 6957 (69.4%) DEGs were novel. According to our results and published reports, many DEGs involved in reproduction in *M. nipponense*, including male reproductive-related LIM protein, sperm gelatinase, cathepsin D, gametocyte-specific factor 1, VASA-like protein, vitellogenin, and other

potential candidates, were identified. Table 4 lists 42 DEGs that are related to reproduction, development, and sex selection from the expression profile. qRT-PCR analysis was used to validate the Solexa expression profiles. Six genes (spermatogenesis-associated protein 13, kunitz-type protease inhibitor 1, arginine kinase 1, vitellogenin receptor, male reproduction-related protein Mar-Mrr, and extra sex combs) were randomly selected for qRT-PCR validation (Figure 5). qRT-PCR analysis of independent samples of testis and ovary tissues confirmed the results obtained by sequencing. The primers used in qRT-PCR validation are listed in Table 5.

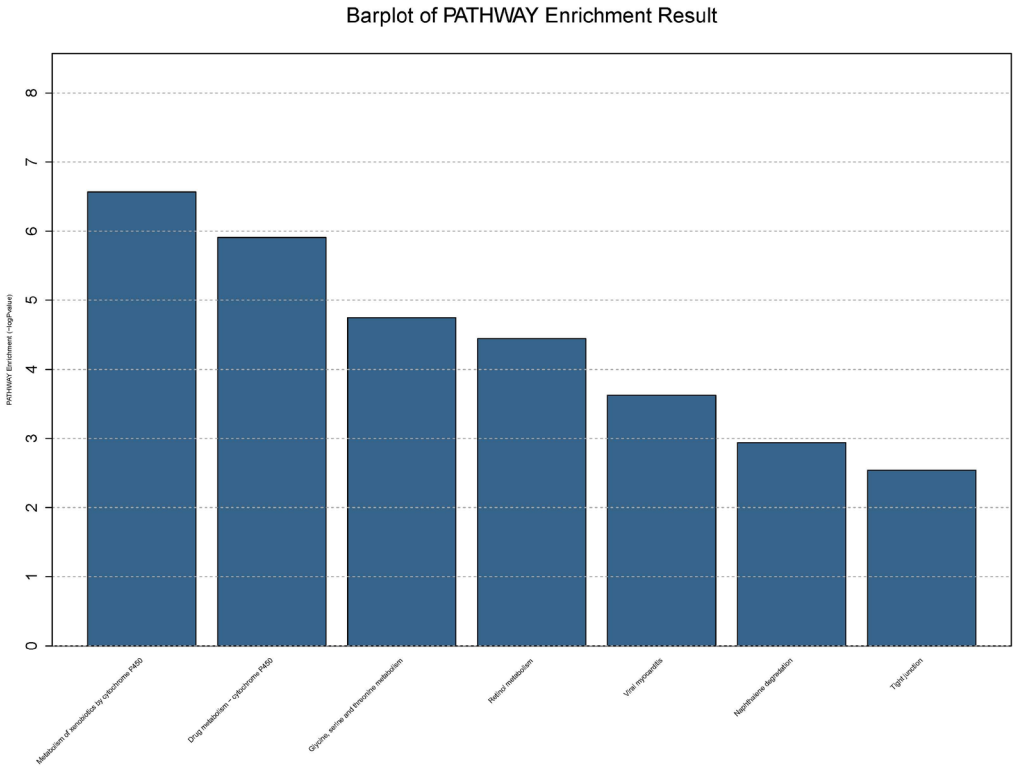

Figure 4. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of differentially expressed genes (DEGs) from ovary and testis of *Macrobrachium nipponense*.

DISCUSSION

Reproduction and development were very complicated processes with many unclear genetic regulatory mechanisms. The animal tests and ovary are multifunctional organs that play a key role in reproduction, sexual maturity, and secretion of hormones to regulate growth and development (Chu et al., 2006). Recent studies showed that constructing cDNA libraries and transcriptome profiles provide a rapid method for gene discovery to fill in the gaps in the genome database of *M. nipponense* (Wu et al., 2009; Qiao et al., 2012; Ma et al., 2012). In the expression profiling of testis and ovary of *M. nipponense* described in this study, the tag-based RNA-Seq approach proved successful for expression profiling, and over 27 million Illumina

reads were generated with more than 10,000 significant DEGs being identified. Compared to the EST libraries of *M. nipponense* (Wu et al., 2009; Qiao et al., 2012), which generated 3256 (ovary) and 5202 (testis) ESTs, this approach was more effective and productive with lower cost. Comparing the 2 libraries, ovary DEGs with upregulated expression (54.5%) were more abundant than testis DEGs with upregulated expression. Of these DEGs, only a small number were annotated in the NCBI database, and novel genes accounted for 69.4% of the DEGs. These DEGs can provide a large candidate database to mine novel genes that play key roles in reproduction and gonad development in *M. nipponense* and other crustaceans.

Table 4. Selected genes of interest for reproduction, development, and sex in the *Macrobrachium nipponense* expression profile.

No.	Description	Up or Down[1]	Fold (T/O)*	Accession No. of Hit	E value
1	Arginine kinase 1	↑	23.6	gi|307715368|	7.56E-05
2	Cathepsin D	↑	28.1	gi|146217392|	7.35E-04
3	Chitinase 1 precursor	↑	34.0	gi|195969366|	8.34E-03
4	Crustin	↑	80.8	gi|146432699|	1.31E-10
5	Cytochrome P450 V20	↑	76.4	gi|374923109|	1.10E-06
6	Cytochrome c oxidase subunit I	↑	10.3	gi|322422337|	3.15E-25
7	Kazal-type serine proteinase inhibitor	↑	28.4	gi|391234179|	8.49E-07
8	Male reproduction-related LIM protein	↑	14.3	gi|146432689|	3.73E-04
9	Male reproduction-related protein A	↑	89.0	gi|146432625|	3.87E-11
10	Male reproduction-related protein B	↑	77.64	gi|146432657|	2.42E-10
11	Male reproduction-related protein Mar-Mrr	↑	1027.3	gi|146432671|	5.52E-11
12	Sperm gelatinase	↑	294.3	gi|391234181|	7.12E-16
13	Spermatogenesis-associated protein 13	↑	6.85	gi|307179210|	5.15E-03
14	Sox14 protein	↑	7.83	gi|3913352|	0.018
15	Serine proteinase inhibitor 8	↑	165.1	gi|288188858|	1.92E-11
16	JHE-like carboxylesterase 1	↑	13.9	gi|326579691|	9.71E-03
17	Juvenile hormone esterase	↑	9.0	gi|157109005|	0.010
18	Peritrophin	↑	42.25	gi|19879412|	4.53E-07
19	Heat shock protein 90	↑	13.8	gi|301299151|	1.24E-04
20	Cyclin-L1	↑	19.9	gi|307189308|	1.34E-05
21	Cathepsin L	↓	0.286	gi|330434686|	3.47E-03
22	Extra sex combs	↓	0.384	gi|2352418|	1.59E-03
23	Gametocyte-specific factor 1	↓	3.37E-03	gi|225710058|	1.21E-07
24	PREDICTED: ubiquitin-conjugating enzyme E2 S-like	↓	0.128	gi|383850836	1.18E-05
25	VASA-like protein	↓	0.367	gi|349806885|	8.01E-03
26	Vitellogenin	↓	7.18E-03	gi|16151644|	9.31E-03
27	Vitellogenin receptor	↓	7.98E-03	gi|301070472|	1.59E-16
28	Kunitz-type protease inhibitor 1	↓	5.51E-03	gi|405978887|	3.89E-15
29	PREDICTED: gonadotropin-releasing hormone receptor-like	↓	0.013	gi|380019458|	5.57E-11
30	PREDICTED: oocyte zinc finger protein XlCOF7.1-like	↓	0.237	gi|301629236|	1.86E-04
31	PREDICTED: oocyte zinc finger protein XlCOF6-like	↓	0.340	gi|326666714|	8.74E-04
32	Cdc42 homolog	↓	0.024	gi|290562982|	1.47E-11
33	Trypsin-like serine proteinase 2	↓	0.312	gi|229258304|	1.21E-04
34	Cathepsin C	↓	0.192	gi|195971120|	1.03E-03
35	Broad-complex protein isoform 4	↓	0.114	gi|358442122|	2.91E-06
36[#]	Protein fem-1-like protein	-	-	gi|307214932|	8.31E-11
37[#]	Fem-1 homolog B-like protein	-	-	gi|375493328|	1.83E-04
38[#]	Sex-determining protein fem-1	-	-	gi|170058345|	5.62E-06
39[#]	Fushi tarazu factor 1	-	-	gi|5306097|	0.089
40[#]	Estrogen-related receptor			gi|283825466|	0.073
41[#]	Ecdysteroid receptor isoform 1			gi|226316403|	0.074
42[#]	Sex-determining region Y protein			gi|326430125|	0.052

[1]An upward arrow indicates upregulation in testis; a downward arrow indicates downregulation in testis (upregulation in ovary). [#]Gene had no significant differential expression between testis and ovary. *Fold (T/O) means the expression ratio of DEGs between testis and ovary.

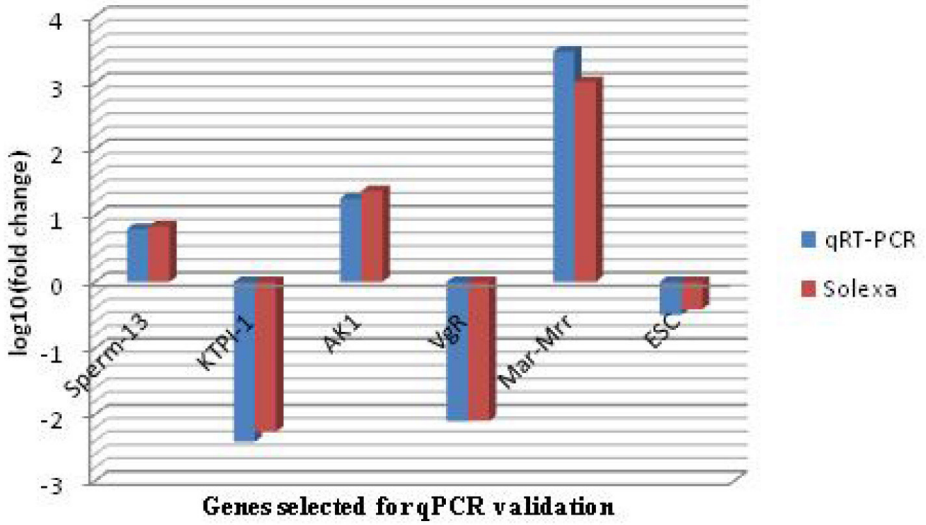

Figure 5. Six genes randomly selected for quantitative real-time polymerase chain reaction (qRT-PCR) validation. The x-axis indicates the name of genes selected for qRT-PCR validation. The y-axis represents the fold-change of expression between testis and ovary (log 10). qRT-PCR indicates the data from the qRT-PCR validation; Solexa indicates the data from the expression profile. Sperm-13 = spermatogenesis-associated protein 13; KTPI-1 = kunitz-type protease inhibitor 1; AK1 = arginine kinase 1; VgR = vitellogenin receptor; Mar-Mrr = male reproduction-related protein Mar-Mrr; ESC = extra sex comb.

Table 5. Primers used in quantitative real-time polymerase chain reaction (qRT-PCR) validation.

Name	Primer sequence (5'-3')
Sperm-13	F: TCTCTTTGGATGGCTTCCTT
	R: CGTTCATTGACAAGAAGTGC
KTPI-1	F: ATTGGTGCTCCCTGAGTTTGTAA
	R: GCTTGCAATCGTCATCATAGCTT
AK1	F: AAGATCAAGGGCAACATCAACGCC
	R: AGGCGCTCTTCATGAGGTTCTCAA
VgR	F: AGAGAAATGGGCGAGTACAAGTT
	R: CTCGATAGTCACCAAGGAGCTTT
Mar-Mrr	F: ATTCATCCTGGTCTCTGAAGCTG
	R: CCCATCCTAGGGCAAATGTATGA
ESC	F: TGGCTACCTTTGGTGGAGTAGAG
	R: TGCAGGTGCCCTCAATGTC

GO analyses classified the unitags from 2 libraries into various terms based on the annotation in the NCBI nr protein database. The terms were similar to those from the reference transcriptomic data of this and other species, indicating that genes encoding these functions may be conserved and easy to annotate in the database. It also provides a comprehensive and structured vocabulary for describing genes in the gonads of *M. nipponense*. Enrichment analyses were performed for the significant DEGs. The enrichment analysis results support the biological significance of expression profiles. The KEGG enrichment pathway results revealed that significant DEGs were strongly associated with metabolism of xenobiotics by cytochrome P450, and drug metabolism by cytochrome P450. Cytochrome P450 genes are important for the biosynthesis of sex steroids, which have crucial roles in reproduction, including sex differ-

entiation, gametogenesis, and gamete maturation, in vertebrate gonads and some invertebrates (Callard et al., 2001; Mizuta and Kubokawa, 2007). Actually, many gene tags were related to cytochrome P450 in the expression profiles, which indicated that these genes might have important functions in the reproduction process and gonad development of *M. nipponense*. The predicted metabolic pathways, together with the GO analysis, are useful for further investigations of gene function.

In this research, 10,018 DEGs involved in reproduction in *M. nipponense* and other potential candidates were identified. Of these, 69.4% were new without any annotation and require further investigation. The other DEGs with annotations were divided into 2 groups, those that were upregulated in testis and those that were upregulated in ovary. Without considering the conserved housekeeping genes, many important reproduction-related genes were discovered.

Vitellogenin, which is a good indicator of female ovarian activity, has been proposed to be synthesized in the ovary and hepatopancreas in decapod crustaceans (Tsukimura, 2001). It was reported to be expressed in both the hepatopancreas and ovaries in many prawns, such as *Penaeus vannamei*, *P. semisulcatus*, and *P. monodon*, but it is only expressed in the female hepatopancreas in *M. rosenbergii* (Avarre et al., 2003; Tiu et al., 2006). It was also not identified in the ovary cDNA library (Wu et al., 2009). In this study, vitellogenin and vitellogenin receptor were found to be expressed at a moderate level in ovary of *M. nipponense*. Whether vitellogenin mRNAs exit the mature ovary, hepatopancreas, or both is not clear and requires further studies.

Furthermore, many other reproduction-related genes were also discovered in the profile. Cathepsin L has activities that have been associated with yolk processing during vitellogenesis in rainbow trout (Kwon et al., 2001). Cathepsin C was found to be involved in the final stages of oocyte maturation in the kuruma prawn *Marsupenaeus japonicus* (Qiu et al., 2008a). These cathepsins also presented significantly upregulated expression in ovary in this paper, which is consistent with the previous reports. Cathepsin D, which was found to have the greatest tissue-specific immunoexpression in the testis in rat male reproductive organs (Burdan et al., 2006), displayed significantly upregulated expression in testis in this study. Male reproductive-related proteins such as kazal-type serine proteinase inhibitor, sperm gelatinase, male reproductive-related LIM protein, male reproductive-related protein Mar-Mrr, and male reproductive-related protein A and B showed strong expression in testis. These proteins were proven to be male-specific proteins in *M. rosenbergii* (Dai et al., 2009). The functions of these proteins in *M. nipponense* remain to be determined. Gonadotropin-releasing hormone, which has been demonstrated by immunocytochemistry in *M. rosenbergii* and *P. monodon* to be located in the pre-vitellogenic and early vitellogenic oocytes and mature ovaries, was also found to display high expression in ovary of *M. nipponense*, suggesting a specific role in ovarian maturation in this prawn (Ngernsoungnern et al., 2008a,b). The extra sex comb gene, which had significantly higher expression in ovary than in testis in this paper, plays important roles in determining segment identity (Struhl, 1981). Broad-complex isoform 4 was identified in the profile and showed significant high expression in ovary. It contributes to ovarian development in *P. monodon* (Buaklin et al., 2013). Many other reproduction- or development-related factors, such as gametocyte-specific factor 1, vitelline membrane outer layer 1-like protein, and spermatogenesis-associated protein 13, displayed relative upregulated expression in ovary and testis. Characterization of these genes with significant differences in expression levels in oriental river prawn may lead to great advances in our understanding of the molecular mecha-

nism of reproduction.

In this study, we also identified putative genes related to reproduction and sex determination with no significant difference in expression levels in *M. nipponense*. Recent studies indicate that ecdysteroids, which are also considered as molting hormones, play a major role in regulating vitellogenesis, ovarian maturation, and protein synthesis in decapods (Brown et al., 2009). A high titer of estrogens was found during vitellogenic stages, suggesting a possible role in the stimulation of vitellogenesis (Fairs et al., 1990). We found estrogen-related receptor, ecdysteroid receptor, and ecdysteroid regulated-like protein homologs in our expression profile. Besides, we identified other putative genes related to sex determination, such as Fushi tarazu factor 1, sex-determining protein fem-1, and sex-determining region Y protein. Fushi tarazu factor 1, which was originally found as a regulator of the *Drosophila* homeobox segmentation gene *FTZ*, is an essential factor in sex differentiation (Lavorgna et al., 1991). The sex-determining protein complex of FEM-1/FEM-2/FEM-3 promotes male development by inhibiting the activity of the terminal sex-determination factor TRA-1A, a zinc-finger transcription factor that promotes hermaphrodite development by repressing the expression of genes required for sperm production and somatic male development (Mapes et al., 2010). Sex-determining region Y protein acts dominantly to trigger differentiation of testes from undifferentiated gonads (Berta et al., 1990; Koopman et al., 1991). However, these important genes showed no significant difference in expression levels in this study. The distribution and function of these genes in *M. nipponense* require further study.

In conclusion, our study constructed a database from an RNA-Seq project to identify genes with significant differences in expression level between the mature testis and ovary of *M. nipponense*. One of the most important aspects of this study is the association of sequences and related expression information with reproduction functions. These annotations will advance research in the field of molecular genetics in *M. nipponense* and offer a valuable resource for further research related to reproduction in crustaceans.

ACKNOWLEDGMENTS

Research supported by the Jiangsu Provincial Natural Science Foundation for Young Scholars of China (Grant #BK2012091), the National Natural Science Foundation of China (Grant #31272654), the National Science & Technology Supporting Program of the 12th Five-year Plan of China (Grant #2012BAD26B04), the Science & Technology Supporting Program of Jiangsu Province (Grant #BE2012334), and the Basic R&D Fund for National Research Institutions of China (#2013JBFM15).

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