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Research paper

Transcriptome profiling of the eyestalk of precocious juvenile Chinese mitten crab reveals putative neuropeptides and differentially expressed genes

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ABSTRACT

Chinese mitten crabs that reach maturity 1 year earlier than normal crabs are known as precocious juvenile crabs. The molecular mechanisms underlying the precocity of the Chinese mitten crab are poorly understood. To identify the genes that may be involved in the control of precocity in Chinese mitten crab, we measured the expression profile of eyestalk genes in precocious and normally developed juvenile crabs using high-throughput sequencing on an Illumina HiSeq 2500 platform. We obtained 56,446,284 raw reads from the precocious crabs and 58,029,476 raw reads from the normally developed juvenile crabs. Reads from the two libraries were combined into a single data set. *De novo* assembly of the combined read set yielded 78,777 unigenes with an average length of 1563 bp. A total of 41,405 unigenes with predicted ORFs were selected for functional annotation. Among these genes, we identified three neuropeptide genes belonging to the crustacean hyperglycemic hormone family and two neuropeptide genes encoding the chromatophorotropic hormones. Transcriptome comparison between the two libraries revealed 42 genes that exhibited significant differential expression, of which 29 genes were up-regulated and 13 genes were down-regulated in the precocious crabs. To confirm the sequencing data, six differentially expressed genes with functional annotations were selected and validated by qRT-PCR. In conclusion, we obtained the comprehensive transcriptome of the eyestalk tissues of precocious juvenile crabs. The sequencing results may provide new insights into the biomolecular basis of precocity in the Chinese mitten crab.

1. Introduction

The Chinese mitten crab *Eriocheir sinensis*, a member of the Grapsidae family of decapod crustaceans, is an indigenous and economically important species in China. It has also been introduced to Europe and North America where it is considered an invasive species (Herborg et al., 2007). In recent years, the aquaculture of Chinese mitten crab has expanded rapidly, and is now an intensive, large-scale industry that provides high value crabs for wealthy consumers. In 2012, the annual production of the Chinese mitten crab was 650,000 tonnes, which was worth approximately 6.6 billion dollars (USD) (Fishery Bureau of Ministry of Agriculture PRC, 2013). Although significant advances have been made in the Chinese mitten crab aquaculture industry during the past two decades, there remains a number of constraints in the intensive aquaculture of the species. Among these, precocity is one of the primary problems hindering the development of the crab aquaculture industry in recent years (X. Li et al., 2011). Under normal growth conditions, the lifespan of the Chinese mitten crab is about 24 months. However, some crabs may reach maturity one year earlier than normal crabs in natural or cultured conditions, and these crabs are known as precocious crabs. The precocious rate of the Chinese mitten crab in the natural population is about 5–10%, but the rate can be up to 20–30% or higher under intensive aquaculture conditions. Precocious crabs usually weight between 15 and 50 g, which is much smaller than the normal size of the adult crabs (usually 100 ~ 200 g/crab), and consequently they have much lower commercial value (X. Li et al., 2011; X.W. Li et al., 2011). The majority of precocious crabs do not undergo a regular molting cycles. Therefore, inadvertent selection of precocious crabs for cultivation in the second year results in mass mortality (60–90%) before June. Because of this low growth rate and poor survival, precocious crabs typically result in significant economic losses to crab farmers.

The mechanisms controlling precociousness in crabs are poorly understood. It has been widely believed that over-nutrition and high cumulative temperature are the main reasons for precociousness in Chinese mitten crab reared under aquaculture conditions. Additionally,

Abbreviations: ORF, open reading frame; XO, X-organ; SG, sinus gland; GIH, gonad-inhibiting hormone; MIH, molt-inhibiting hormone; CHH, crustacean hyperglycemic hormone; MOIH, mandibular organ-inhibiting hormone; PDH, pigment-dispersing hormone; PCH, pigment-concentrating hormone; βGBP, beta-1,3-glucan-binding protein.

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genetic characteristics are also thought to be related to the precocity of the Chinese mitten crab (X. Li et al., 2011). However, the exact molecular mechanisms underlying the precocity of the Chinese mitten crab remain to be elucidated. In recent years, research focusing on the endocrine control of molting or gonadal maturation in crustaceans has attracted increased attention, with emphasis placed on the roles of the cephalothorax and eyestalk neuropeptides and hormones (Chang and Mykles, 2011; Sartaj et al., 2011; Brady et al., 2012). Eyestalk ablation, which involves the surgical removal of one or both eyestalks, has been widely used to induce gonadal development and maturation in many crustaceans (Murugesan et al., 2008; Nagaraju, 2011). In a recent study, a cDNA library from the pooled tissues of the eyestalk, Y-organ, and hepatopancreas of juvenile Chinese mitten crab was sequenced using an Illumina HiSeq 2000 platform. Eyestalk ablation resulted in differential gene expression in the hepatopancreas, further proof of the important role of the eyestalk in various physiological processes in the Chinese mitten crab (Sun et al., 2014). The crustacean eyestalk consists primarily of a cluster of peptidergic neurons located in the medulla terminalis X-organ (XO) and their clustered axon endings that constitute the sinus gland (SG) (Sartaj et al., 2011; Jung et al., 2012). It has been commonly accepted that the functions of the XO–SG complex in crustaceans is equivalent to the functions of the hypothalamus pituitary system in vertebrates (Jung et al., 2012). The XO–SG complex releases a number of peptide hormones into the hemolymph, and these hormones are involved in a range of physiological activities such as growth and postembryonic development in crustaceans (Devaraj and Natarajan, 2006; Allayie et al., 2011; Jung et al., 2012; Toullec et al., 2013).

A number of polypeptide neurohormones that are produced by neurosecretory cells of the eyestalks, including crustacean hyperglycemic hormone (CHH) family peptides and chromatophorotropic hormones, have been identified and characterized in a range of crustaceans (Harzsch et al., 2009; Chung et al., 2010; Webster et al., 2012). However, at this point, little is known about the gene regulation and expression profiles of the eyestalk of the Chinese mitten crab, and particularly in precocious juvenile crabs. Sequencing the eyestalk transcriptome of precocious juvenile crabs may lead to discovery of functional genes that are involved in the precocity of the Chinese mitten crab.

In this study, we prepared two cDNA libraries from total RNA extracted from eyestalk tissues of precocious and normal juvenile crabs. We used the Illumina HiSeq 2500 massively parallel sequencing method to sequence the two cDNA libraries. Five putative neuropeptide genes were identified and characterized *via* comparisons to previously reported neuropeptides in other crustaceans. A number of differentially expressed genes between the two libraries were screened and validated, which may help to elucidate the potential roles of the eyestalk in the precocity of the Chinese mitten crab. Overall, this study not only provided us a comprehensive transcriptome of the eyestalk of the Chinese mitten crab, but also helped us to identify a number of differentially expressed genes between the precocious and normal juvenile crabs. Our results can inform our understanding of the molecular mechanisms involved in the development of precocious Chinese mitten crab.

2. Materials and methods

2.1. Preparation of tissue samples

Precocious and normal juvenile crabs were obtained from the *National Research Center for Chinese Mitten Crab Breeding*, China. Approximately 15 kg of crab megalopae (crab larva) were cultured in a pond under standard culture conditions in April 2013. After 12 months of culture, precocious and normal juvenile crabs were randomly sampled in April 2014. Precocious and normal juvenile crabs were determined according to the descriptions in X. Li et al. (2011). Twenty precocious juvenile crabs (precocious group) and 20 normally developed juvenile crabs (normal group) were used for the study. All samples were anesthetized on ice and dissected to collect the eyestalks. The eyestalks from each crab were collected and immediately submerged in RNAlater solution (Ambion, USA). All crabs used in the present study were in the intermolt period, and there were an equal number of male and female crabs in each group.

2.2. RNA extraction, cDNA synthesis, and sequencing

The eyestalks that were submerged in the RNAlater solution were dissected to remove the exoskeleton. To obtain the complete transcriptome of the eyestalk, the whole eyestalk tissue was used for total RNA extraction. Total RNA was exacted from the eyestalk tissue of each crab using TRIzol Reagent (Invitrogen, Shanghai, China) and then purified using an RNA purification reagent (Invitrogen) according to the manufacturer's instructions. Equal quantities of RNA from every crab within each group were then pooled together for the transcriptome analysis. RNA integrity and quantity were determined with an Agilent 2100 Bioanalyzer (Agilent, Shanghai, China) before cDNA synthesis. Oligo (dT) linked beads were used to isolate the mRNA from the total RNA (Illumina; San Diego, CA, USA). The isolated mRNA strands were broken into small strands with Fragmentation Buffer (Illumina). The fragmented mRNAs were used as templates to construct the cDNA libraries using a TruSeq RNA Sample Prep Kit following the manufacturer's instructions (Illumina). The two cDNA libraries, derived from the precocious and the normal crabs, were sequenced on an Illumina HiSeq 2500 system at Shanghai Majorbio Bio-pharm Biotechnology Co., Ltd.

2.3. Sequence assembly and annotation of the transcriptome

Adaptor sequences, empty reads, and low quality sequences were removed from the raw reads using the SeqPrep program (https://github.com/jstjohn/SeqPrep). Reads from the two libraries were combined into a single data set. The combined read set was assembled by Trinity to generate a reference *de novo* transcriptome assembly (http://trinityrnaseq.github.io/; version: trinityrnaseq-r2013-02-25). Three independent software modules (Inchworm, Chrysalis, and Butterfly) of the Trinity were used for the data analysis with the default parameters. If a gene had more than one transcript, the longest one was selected to represent the assembled component to eliminate redundancy (Haas et al., 2013). Functional annotations for the unigenes were carried out using BLAST (Version 2.2.25) against the Nr (www.ncbi.nlm.nih.gov/protein), String (http://string-db.org/), and KEGG (http://www.genome.jp/kegg/genes.html) database with an E-value of less than 1E-05. Additionally, GO terms (http://www.geneontology.org) were extracted from the best hits obtained from the BLAST against the Nr database using Blast2GO (http://www.blast2go.com/b2ghome) (Conesa et al., 2005). The open reading frames (ORFs) of the polypeptide neurohormone genes were predicted using the ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/). The putative amino acid sequences were analyzed for the presence of signal peptides using SignalP 4.1 server (www.cbs.dtu.dk/services/SignalP/) (Emanuelsson et al., 2007). Domain predictions were performed using the SMART server (http://smart.embl-heidelberg.de/) (Schultz et al., 1998), and were further confirmed in the Conserved Domain Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The phylogenetic trees of the neuropeptides were constructed using the neighbor-joining method with MEGA 6.0 (Tamura et al., 2013).

2.4. Identification and validation of differentially expressed genes

The RSEM program was used to calculate the number of mapping reads to every assembled unigene and estimate the gene expression levels according to a maximum likelihood estimate with FPKM-values (Fragments Per Kilobase of gene model per Million fragments mapped) (Li and Dewey, 2011). To compare expression levels of different