Gene 677 (2018) 10–16

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Research paper

Transcriptome analysis for identification of candidate genes related to sex determination and growth in *Charybdis japonica*

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ARTICLE INFO

Keywords:
Charybdis japonica
De novo assembly
Transcriptome
Sex differentiation
Growth

ABSTRACT

Charybdis japonica is an important cultured crab in China and it exhibits sex differences in their growth. Growth is an important economic trait that is controlled by many genes. In order to discover the growth-related regulatory mechanisms, whole-body transcriptomic sequencing and comparative analyses in different genders of *C. japonica* were conducted based on Illumina RNA-seq technology. In the present study, we obtained 62,879,204 and 60,226,334 clean reads in female and male libraries, respectively. 25,000,000 clean reads of every library were randomly selected and compared with Nt database to examine the possible contamination. Results showed that all clean reads were distributed among *C. japonica* or other species that were closely relative to this species, indicating no-pollution. *De novo* assembly was performed and a total of 32,543 and 44,174 unigenes were produced in female and male of *C. japonica*, respectively. Among all the unigenes, 12,591 and 14,455 unigenes of female and male crabs were annotated based on protein databases. Moreover, a total of 33,926 unigenes were found to contain ORFs and 52,839 SSRs were detected. The contrast between male and female *C. japonica* identifying 1939 unigenes were significantly differentially expressed. In addition, we specifically discussed some gene functions and pathways potentially associated with sex determination and growth. This is the first systematic report of whole transcriptome in *C. japonica*. The transcriptome information provides a basic resource for further studies on understanding the molecular basis of biological processes in *C. japonica* and other crustaceans.

1. Introduction

Sex determination is an evolutionary process that regulates the differentiation of gender characteristics (Chen et al., 2017; Liu et al., 2015). This process is controlled by multi-factors and is important in many respects for animals, such as morphological, physiological and behavioral levels (Teaniniuraitemoana et al., 2016; Trabzuni et al., 2013; Mackey et al., 2016). Therefore, elucidating the sex determination is critical in understanding the phenotypic, behavioral, and cellular differences between genders (Cribbin et al., 2017).

The Asian paddle crab, *Charybdis japonica* belongs to the family Portunidae and is the prevalent species in the South East Asia (Micheletti et al., 2007; Gao et al., 2010). *C. japonica* typically inhabits estuaries with firm sand or muddy fine sand bottoms. They can survive in low oxygen levels, wide range of salinity and temperature (Gust and Inglis, 2006; Liu et al., 2008). Female crabs usually spawn when sea temperature reaches between 20 °C and 28 °C and lay an average of 85,000 eggs per brood (Yu et al., 2005). The pelagic larval stage of *C. japonica* is approximately four weeks, during which time they may be dispersed at large distances by tides and currents. The culture of *C. japonica* has been promoted in the past years due to significant economic value. However, genetic heterozygosity reduction and

Abbreviations: C. japonica, *Charybdis japonica*; SSRs, simple sequence repeats; ORFs, open reading frames; E1, ubiquitin activating enzyme; E2, ubiquitin conjugating enzyme; E3, ubiquitin ligase; *JHE*, juvenile hormone esterase; *STAT*, signal transducer and activator of transcription; *SPARC*, Secreted protein acidic and rich in cysteine; *TRAX*, translin-associated factor X; *RERG*, Ras-related and estrogen-regulated growth inhibitor-like protein; *FABP7v*, Fatty acid-binding protein 7; *HR4*, Hormone receptor 4; *MEGF6*, Multiple epidermal growth factor-like domains protein 6, partial; *IGF2BP*, Insulin-like growth factor 2 mRNA-binding protein 1 isoform X4; *CCNG2*, Cyclin G2; *GPAM*, Glycerol-3-phosphate acyltransferase 1, mitochondrial-like; *MEGF8*, Hypothetical protein DAPPUDRAFT_194252; *MAP2K1*, mitogen-activated protein kinase kinase; *SOCS2*, Cytokine signaling 2; *IRS1*, Insulin receptor substrate 1-B-like; *JAK2*, Janus kinase; *GRB2*, Growth factor receptor-bound protein; *RAP1A*, Ras-related and estrogen-regulated growth inhibitor-like; *ZMAT3*, Zinc finger matrin-type protein 3; MAPK, mitogen-activated protein kinase; *NDKB*, Nucleoside diphosphate kinase B; *CALM*, Calmodulin; *CATA*, Catalase; *TNNCB*, Troponin C, isoform 2B; *CUL2*, E3 ubiquitin ligase, Cullin 2 component; APC5, Anaphase-promoting complex subunit 5; *UBE2O*, E2 ubiquitin-conjugating enzyme

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https://doi.org/10.1016/j.gene.2018.07.044
Received 23 March 2018; Received in revised form 7 July 2018; Accepted 13 July 2018
Available online 20 July 2018
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precocious puberty have brought about multiple negative effects on *C. japonica* aquaculture industry, which ultimately lead to economic losses (Zhang and Qiu, 2010). Additionally, several biology and economic traits are related to genders in *C. japonica*s (Gao et al., 2014). For example, male *C. japonica* usually have a higher growth rate than that of females, while female crabs show higher economic values (Lee and Hsu, 2003; Kolpakov and Kolpakov, 2011). Growth rate is a primary biology and economic trait. It has an impact on productivity and profitability of aquaculture industries (Ma et al., 2016). Jung et al. (2013) considered that the growth rate in crustaceans could be influence by many candidate genes. However, the internal regulation mechanisms of these genes controlling the growth difference between genders of *C. japonica* are still unknown. Therefore, screening and identifying growth-related genes and their functions are an initial step towards to understanding phenotypic differences in *C. japonica*.

Despite next-generation sequencing provided important gene sequences for crustaceans, it was difficult to directly assemble the genome information due to high heterozygosity and numerous repetitive sequences were existed in crustacean genome (Colbourne et al., 2011). Therefore, the available genomic resources were inadequate to offer an extensive detection on growth-related regulatory mechanisms in crustacean. Transcriptome represented almost all effective genes expressed in particular cells or organs. When the genomic sequences were not available, transcriptome sequencing could provide a comprehensive understanding of regulatory mechanisms involved in specific biological processes based on the structures and functions of differential gene (Wolf, 2013; Ekblom and Galindo, 2011; Tirosh et al., 2006). In addition, transcriptome sequencing allowed simultaneous analyses of all of the processes, including metabolism, protein homeostasis and other regulatory cellular processes (Wen et al., 2016; Yang et al., 2015; Schroder et al., 2012). Recently, transcriptome sequencing were applied to identify the growth-related regulatory mechanisms of crustaceans and many special genes were detected in other crabs, for example, juvenile hormone esterase (*JHE*), signal transducer and activator of transcription (*STAT*) in *Eriocheir sinensis* (Xu et al., 2017); Secreted protein acidic and rich in cysteine (*SPARC*), translin-associated factor X (*TRAX*) and others in *Portunus trituberculatus* (Lv et al., 2014). However, little information was known about regulation and expression profiles of growth-related genes of *C. japonica* due to limited amount of genomic data.

In the present study, the whole transcriptomes of pooled multiple tissues from mature males and females of *C. japonica* were sequenced based on Illumina sequencing platform. This was the first transcriptomic study of *C. japonica* and it provided significant portion information to enrich the genetic resources. The comparative transcriptome analyses were used to generate expression profiles and discover differential expression genes between two genders. Subsequently, we determined the candidate genes and regulatory mechanisms associated with sex determination and growth. In addition, abundant markers potentially useful population studies including simple sequence repeats (SSRs) were also reported. Our study aimed to provide valuable transcriptomic resource for understanding regulatory mechanisms associated with sex determination, growth and other biological processes of *C. japonica*. Finally, this information will improve aquaculture breeding programs for *C. japonica* and other crustaceans.

2. Methods

2.1. Samples collection, RNA extraction and Illumina sequencing

Healthy individuals of *C. japonica* were obtained from an aquatic products market in Zhoushan (China) on December 28, 2016. Female and male individuals were acclimated in tanks for 4 days with fully aerated seawater and we did not feed them to eliminate the food contamination before dissection. Then, tissues (gill, muscle, heart and intestinal) from different genders (each 5 females and males) were rapidly sampled, snap-frozen in liquid nitrogen and stored at −80 °C prior to the RNA extraction. The same tissue samples from each gender were mixed equally, respectively. Then, the RNA of each tissue sample was isolated using the mirVana™ miRNA Isolation Kit (AM1561) following the manufacturer's protocol. RNA integrity was evaluated using the Agilent 2100 Bioanalyzer and the sample with RNA Integrity Number (RIN) ≥ 7 was subjected to the subsequent analysis. Then the RNAs from tissues of each gender were pooled in equal amounts and mRNA was extracted from the total RNA using magnetic beads with Oligo (dT) probes. Fragmentation buffer was applied to lyse the mRNA into fragments with a suitable size and the fragmented mRNA was used to construct a cDNA library using TruSeq Stranded mRNA LTSample Prep Kit. Then the library was sequenced on the Illumina HiSeq™ 2500 platform and 150 bp paired-end reads were generated.

2.2. Pollution test, transcriptome de novo assembly and functional annotation

All raw reads in FASTQ format quality control relied on NGS QC ToolKit software and clean reads were obtained by removing raw reads containing adapter, ploy-N (N ratio > 10%) and low quality reads (quality scores ≤ 5). In addition, 25,000,000 clean reads were randomly selected and compared with Nt database to examine the possible contamination. Subsequently, all the remaining high-quality clean reads were *de novo* assembled using Trinity software package (version: trinityrnaseq_r20131110). Then, the redundant transcripts were removed using TGICL software package and further spliced them to the longest unigenes. All unigenes of two genders were applied to analyze the gene ontology and orthologous classifications based on NR, Swiss-Prot, KOG, KEGG and GO database using the Blastx alignment (E-value < 0.00001).

2.3. Predict the unigene structure

We further analyzed the structure of all unigenes using TransDecoder software, MISA and Primmer3 software in the present study. Simple sequence repeats (SSRs) were identified and the minimum repeat number was 10 for Mono-nucleotide (1 bp), 6 for Di-nucleotide (2 bp), 5 for Tri-nucleotide (3 bp), Quad-nucleotide (4 bp), Penta-nucleotide (5 bp) and Hexa-nucleotide (6 bp), respectively.

2.4. Differentially expression analyses

In order to analyze gene expressed variation of different genders, we mapped all unigenes to the multifasta file using BWA-mem (Li and Durbin, 2009). Then the expression level of overall unigenes was normalized to determine FPKM (Fragments per kilobase of exon model per million mapped fragments) using RSEM and Bowtie2 with default settings (Li and Dewey, 2011; Langmead and Salzberg, 2012). Differentially expressed genes of different genders were identified using edgeR package (Robinson et al., 2010). Subsequently, $P \leq 0.05$ and the absolute value of $\log_2$Ratio ≥ 2 were used as the filtering thresholds to determine the significance of the differentially expressed genes. Then the differentially expressed genes of different genders were applied to identify functional categories using Blast2GO program and the threshold parameter of significant difference was $P \leq 0.05$. In addition, the biochemical metabolic pathways and functions of gene products were predicted based on KEGG pathway annotation using KAAS (http://www.genome.jp/kaas-bin/kaas_main), and we set the $Q < 0.05$ as the threshold parameter to determine the abundance of pathways.

2.5. Quantitative real-time PCR validation

Quantitative real-time PCR (qRT-PCR) was applied to validate the transcriptomic data. Within each of the categories for up- and down-

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