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Transcriptome analysis of the *Larimichthys crocea* liver in response to *Cryptocaryon irritans*

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ABSTRACT

The large yellow croaker (*Larimichthys crocea*) is an economically important marine fish cultured in China and East Asian countries and is facing a serious threat from *Cryptocaryon irritans*, which is a protozoan ectoparasite that infects most reared marine fish species. To understand the molecular immune mechanisms underlying the response to *C. irritans*, we first performed a comparative gene transcription analysis using livers from *C. irritans*-immunized *L. croceas* and from a control group through RNA-Seq technology. After the removal of low-quality sequences and assembly, 51360 contigs were obtained, with an average length of 1066.93 bp. Further, a blast analysis indicates that 30747 contigs can be annotated based on homology with matches in the NT, NR, gene, and string databases. A gene ontology analysis was used to classify 21598 genes according to three major functional categories: molecular function, cellular component, and biological process. Moreover, 14470 genes were found in 303 KEGG pathways. We used RSEM and EdgeR to determine that 3841 genes were significantly differentially expressed (FDR < 0.001), including 2129 up-regulated genes and 1712 down-regulated genes. A significant enrichment analysis of these differentially expressed genes and isogenes revealed major immune-related pathways, including the toll-like receptor, complement and coagulation cascades, and chemokine signaling pathways. In addition, 28748 potential simple sequence repeats (SSRs) were detected from 12776 transcripts, and 62992 candidate single nucleotide polymorphisms (SNPs) were identified in the *L. croceas* liver transcriptome. This study characterized a gene expression pattern for normal and *C. irritans*-immunized *L. croceas* for the first time and not only sheds new light on the molecular mechanisms underlying the host-*C. irritans* interaction but also facilitates future studies on *L. croceas* gene expression and functional genomics.

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1. Introduction

The large yellow croaker (*Larimichthys crocea*) is a jewfish species, an economically important marine fish species in China, and composes the largest yield for a single species in Chinese marine net-cage farming [1]. In recent years, with rapid development of the *L. crocea* culture industry, parasites, bacteria, and viruses have caused increasingly severe outbreaks of infectious diseases [2–4]. The market has suffered significant economic loss due to these infectious diseases, particularly marine cryptocaryonosis (white spot disease). White spot disease is one of the most damaging salt water aquaculture diseases caused by *C. irritans*, which is a protozoan ectoparasite that infects most reared marine fish species [5,6]. However, thus far, information on the immune mechanisms underlying the *L. croceas* response to *C. irritans* is limited.

Transcriptome profiling analysis is a powerful method for genome studies and functional gene identification. Recent rapid developments in high-throughput deep-sequencing technologies have provided an overwhelming increase in transcriptome data [7,8]. RNA-Seq is a relatively new technology for transcriptomic studies across the entire genome of aquaculture species [9,10]. In recent years, several studies have reported the transcriptome profile of fish following exposure to pathogenic microorganisms. For

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instance, an Affymetrix microarray analysis was used for transcriptional profiling of the skin immune response to *Aeromonas hydrophila* infection of zebrafish (*Danio rerio*) [11]. Zhang et al. took advantage of a transcriptome analysis that revealed changes to multiple genes involved in immunity in *Cynoglossus semilaevis* during a *Vibrio anguillarum* infection [12]. Additional examples of transcriptome profiling analysis include *Epinephelus coioides* [13], the blunt snout bream (*Megalobrama amblycephala*) [14], *Concholepas* (*Gastropoda*, *Muricidae*) [15], grass carp (*Ctenopharyngodon idella*) [16], and *Takifugu rubripes* [17]. Several studies have reported transcriptomic and expression profiles for *L. crocea* in response to immune stimuli, pathogenic infection or environmental stress [18–21]. However, to our knowledge, no information is available on the gene expression profile for the entire transcriptome in response to *C. irritans*.

In the past decades, studies on resistance to *C. irritans* have mainly concentrated on immune-related genes, such as *Nrdp1* [22] and *MHC IIB* [23]; antimicrobial peptides (AMPs) [24,25]; proteomic analyses [26]; physical control strategies [27]; and chemical control strategies [28]. However, a series of knotty problems, such as fragmented studies, undefined infection and response mechanisms, hinder development of effective strategies to prevent the disease.

To systematically and comprehensively comprehend the molecular immune mechanisms underlying the response to *C. irritans*, we used Illumina/Solexa sequencing technology for a transcriptome profiling analysis of *L. croceas* infected with *C. irritans*. We determined gene expression at the transcriptome level, identified immune-related genes and pathways, and detected many simple sequence repeats (SSRs) and single nucleotide polymorphisms (SNPs). These data facilitate further investigation into the molecular mechanism underlying the host-*C. irritans* interaction.

2. Materials and methods

2.1. *Larimichthys crocea* experiment

L. croceas specimens (weight: 85.5 ± 15.1 g, length: 19.2 ± 1.3 cm) were purchased from Ningde Marineland (Fujian, China) and acclimatized for two weeks prior to the experiment. The protozoan was previously isolated from a croaker suffering from a deadly *C. irritans* infection and was identified by observing the morphology and through molecular biology methods. Next, larvae concentration was calculated using an inverted microscope. A *C. irritans* challenge was performed as previously described [25]. In brief, 60 healthy *L. croceas* were immersed in a suspension containing the fish pathogen *C. irritans* at 26665 theronts per fish in 500 L of treated seawater for 4 h, subsequently transferred into a new cement pool containing treated seawater, and observed daily for infections. On the fourth day post-infection (trophont came off from the fish), the fish were transferred to a new cement pool to avoid reinfection with *C. irritans*. The liver tissue was collected separately from five unchallenged fish and five 3-days post-infection (dpi) fish. The fresh tissues were placed in RNAfixer (TaKaRa) immediately after collection and then stored at −20 °C for preservation before use.

2.2. Library construction and sequencing

Using RNAiso Plus (TaKaRa), the total RNA from five *L. croceas* per group was extracted in accordance with the manufacturer's instructions and then treated with RNase-free DNase I (TaKaRa) to remove DNA contaminants. Subsequently, the purified RNA quality and quantity were determined using a Nanodrop spectrophotometer (LabTech, USA), and only RNA samples with an A260/A280 from 1.8 to 2.0 and an A260/A230 from 2.0 to 2.5 were used for the subsequent analyses. The RNA pool was obtained from each group following the verification of the RNA integrity using an Agilent 2100 Bioanalyzer (RIN ≥8) (Agilent Technologies, USA). Subsequently, poly (A) mRNA was isolated from the total RNA using poly (dT) oligo-attached magnetic beads, and the cDNA libraries were prepared using the TruSeq RNA Sample Preparation Kit (Illumina) following the TruSeq protocol. The cDNA libraries were sequenced using the Illumina HiSeq2000 sequencing platform with 2 × 101 bp paired-end (PE) reads.

2.3. Transcriptome data analysis

The raw reads from the Illumina Solexa sequencing experiment were first preprocessed by eliminating adaptor sequences and low quality reads. The clean reads were assembled and stitched using the Trinity program (http://trinityrnaseq.sourceforge.net/) into contigs [29]. Subsequently, Trinity (http://trinityrnaseq.sourceforge.net/analysis/extract_proteins_from_trinity_transcripts.html) was used to analyze the open reading frame (ORF). The assembled transcripts were divided into two categories (contigs with ORFs or without ORFs) based on these ORF predictions. The isogenes were annotated using local BLAST programs with the NCBI non-redundant (nr) protein database as well as nt, gene, and string databases. The Blast2GO program was used to obtain GO annotation based on BLASTx hits with the NCBI Nr database [30]. The number of isogenes associated with each GO term was calculated using the categories molecular function, cellular component, and biological process [7]. The Blastx/blastp 2.2.24+ tools were used to analyze the KEGG pathways, and KO (KEGG Orthology) annotations were obtained based on the BLASTx hits with the KEGG database.

2.4. Differentially expressed genes and enrichment analysis

The clean sequencing reads from each of the two libraries (Liver and Liver-Ci) were mapped back to the transcriptome assembly using the software Bowtie2 with the default parameters [31]. Differentially expressed sequences between the two libraries were sifted using RSEM (http://deweylab.biostat.wisc.edu/rsem/) [32], and EdgeR (http://www.bioconductor.org/packages/elease/bioc/tml/edgeR.html) [33] was used to analyze differential expression based on a threshold false discovery rate (FDR) <0.001. Heatmap and cluster analysis were performed using R package. Subsequently, a significant enrichment analysis of the differentially expressed genes and isogenes was performed to analyze the immune-related genes through hypergeometric distribution testing using the software Goatools (https://github.com/tanghaibao/goatools) and KOBAS (http://kobas.cbi.pku.edu.cn/home.do).

2.5. Experimental validation using RT-qPCR

The differentially expressed genes were validated using RT-qPCR amplification to confirm the RNA-seq results. Quantitative real-time PCR was performed using an ABI Quantstudio 6 Flex system with SYBR® Premix Ex Taq™ (TaKaRa, Japan) in accordance with the manufacturers' instructions. Primer sequences were carefully designed based on each identified gene sequence from the transcriptome library using the Primer Premier 6 software (Premier Biosoft, USA) (Table S4). PCR amplification experiments were performed in triplicate under the following conditions: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 55 °C for 30 s, and 72 °C for 30 s. The results were normalized using β-actin for each sample and

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