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Adaptive response of pearl oyster *Pinctada fucata martensii* to low water temperature stress

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ABSTRACT

The pearl oyster *Pinctada fucata martensii* is a warm-water shellfish that is sensitive to cold environments. To investigate its potential adaptation to low-temperature stress, the selected line (SL) and based population (BP) were sampled to undergo transcriptome sequence. Results of transcriptome analysis showed 572 significant differentially expressed genes. The typical *HSP70* and *HSP40* exhibited the polar expression model in the two groups. Meanwhile, the related genes that involved in energy release mediated by oxidative phosphorylation and the biosynthesis of unsaturated fatty acid were increased in the SL. The apparent enrichment of different expressed genes in amino acid metabolism indicated that the small molecule system with amino acids was one of the main regulator for low-temperature stress. The different expressions of immune-related and lysosome protein encoding genes also reflected the variation of immunity in the two groups and indicated that it could affect the adaptation ability in different temperature. In addition, the similar trends of different expression of typical genes between two groups were obtained by using RNA-seq and qRT-PCR. These results suggested that multi-system adjustments are involved in the processes of low water temperature stress in pearl oyster, providing insights into the response systems of shellfish to acclimatise with ambient environment change.

1. Introduction

Temperature change is a major environmental stress for poikilothermic animals. Temperature stress determines habitat selection and spatial distribution and also affects survival, growth, reproduction and productivity of poikilothermic animals [1]. Under cold conditions, decreased cardiac frequency and intensity caused by cold stress could slow the hunting frequency of gill cilia and reduce their filtering and feeding rates [2,3]. The change in temperature also influences the activity of metabolism-related enzymes in the metabolic process to support their growth and survival [4,5].

To deal with the cold stress, antifreeze proteins, small molecule system, heat shock proteins and other systems were evocated in poikilotherms. Typically, organisms recruit different types of heat shock proteins (HSPs) to deal with low- or high-temperature stress [6]. In teleost and arthropods, antifreeze proteins help the organisms to adapt and survive in subzero temperature environments by preventing water from freezing [7]. However, until now no conserved antifreeze protein was identified in molluscs. The variety of unsaturated fatty acids, no matter in prokaryotes, animals or plants, are as important components in biological membranes and can change the fluidity of membranes to protect the cell from damage brought about by cold stress [8]. Moreover, the small molecular system including amino acid and glucide plays critical roles in the low-temperature stress in insects [9]. What's more, the oxidative stress proteins [10], which are important components of the immune system [11,12] and lysosomal protein degradation pathways, are increased during cold acclimation in mussels and gilthead sea breams [13,14]. Recently, a series of studies was conducted to explore the mechanism of temperature stress through exposing experiments in bivalves [13,15,16]; however, their response to cold stress was not determined.

Pearl oyster *Pinctada fucata martensii*, the main seawater pearl-produced shellfish in China, is a warm-water shellfish that has a weak endurance capacity to low temperature. This temperature sensibility limited its culture areas, thereby affecting the development of the pearl industry. Discovering of cold adaptation potential of pearl oyster could contribute to purposefully select the cryophylactic variety and help insight into the adaptability system and population genetic diversity for the north migration accompanied with overwintering events under global warming condition in future. In this study, we constructed a

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selected line (SL) with high survival rate in low water temperature and performed RNA-seq analysis between based population (BP) and SL. We aimed to determine the long-term adaptability, instead of excitability, under sustainably low ambient temperatures.

2. Materials and methods

2.1. Development of the selected line

The pearl oysters were sampled from the first generation line selected for resistance to low water temperature. The details for development of the line was as follows. In September 2013, a total of 1200 pearl oysters were sampled a base population farmed at Leizhou of Zhanjiang, Guangdong. They were transported to Nan'ao Island of Shantou, Guangdong, where water temperature is 2–3 °C lower than that at Leizhou of Zhanjiang, Guangdong. The pearl oyster overwintered at Nan'ao Island of Shantou, and the survived individuals were used to produce the first generation line in April 2014. The procedures for larval, juvenile and adult rearing were detailed by Deng et al. [17]. In December 2015, 30 individuals were each sampled from the selected line (SL) and the base population (BP). The individual was dissected and adductor tissue were stored in RNAlater in a −80 °C refrigerator.

2.2. Total RNA extraction and cDNA synthesis

Trizol (Invitrogen) was used to extract the total RNA. SMARTer™ PCR cDNA Synthesis Kit (Clontech) was then used for reverse transcription. The integrity of RNA and cDNA was examined by 1% agarose EB gel electrophoresis. The concentrations of total RNA and cDNA were measured using NanoDrop ND-1000 microvolume UV/VIS spectrophotometer.

2.3. Transcriptome sequencing

The adductors from SL and BP were used as samples for transcriptome sequencing. In each population, the mix RNA samples with 5 individuals were used to perform the transcriptome sequencing. The whole genome of *P. f. martensii* obtained in the earlier assembly was taken as the reference for transcriptome sequencing analysis, which was completed by BGI Genomics Co., Ltd [18]. The total RNA of samples were extracted, and the DNA was digested using DNase I. Subsequently, magnetic beads with Oligo (dT) were applied for the enrichment of eukaryotic mRNA. The reagents were added into the Thermomixer under appropriate temperature to cut the mRNA into short fragments, which were used as template to synthesise single-strand cDNA. Afterwards, the two-strand synthesis reaction system was prepared to synthesise the two-strand cDNA. In addition, the kit was used for the purification, recycle and repair of the cohesive end. Base 'A' was added to the 3′ end of the cDNA and connected to the adapter. The next step was to select the size of the fragment, and then PCR amplification was performed. Illumina HiSeq TM 2000 sequencer was used for sequencing after the constructed library passed the quality inspection using Agilent 2100 Bioanalyzer and ABI StepOnePlus Real-Time PCR System.

2.4. Assembly and function annotations of the newly built transcriptome

To ensure the reliability of subsequent data analysis, the raw reads were processed to remove inferior sequences, sequencing adapters and redundant sequences (unknown nucleotides > 5%). De novo assembly was carried out after the acquisition of clean reads. TGICL software was applied to de-redundancy and splicing so as to obtain the longest possible non-redundant unigene set. Further statistics and quality-control analysis were conducted on the unigene set. The unigene set obtained by the assembly using De novo that had passed the quality inspection Map to the completed genus *P. f. martensii* genome [18]. Gene Ontology (GO) [19] and Kyoto Encyclopedia of Genes and Genomes (KEGG) [20] significance enrichment analyses and analyses on clustering were conducted using the differentially expressed genes between the selected samples.

2.5. Verification of transcriptome data by qRT-PCR

In this experiment, ten gene sequences of *P. f. martensii* (i.e. *heat shock 40 kDa protein*, *heat shock 70 kDa protein*, *Δ^6-desaturase*, *cathepsin C-1*, *cathepsin F*, *cathepsin L-2*, *lysosomal-associated membrane protein*, *carnosine synthase*, *phosphoenolpyruvate carboxykinase*, *toll-like receptor 2-1*) were comparatively analysed with the obtained genome, a highly conserved region was selected for the primer design of qRT-PCR. The primer design was completed using Primer Premier 5 software. The primer sequence is shown in Table S1. The reaction system consisted of 0.4 μL cDNA, 0.4 μL upstream and downstream primers and 5 μL SYBR master mix, with a total volume of 10 μL. The reaction conditions were as follows: 95 °C for 2 min, 1 cycle; 95 °C for 15 s, 60 °C for 1 min, 40 cycles. Singularity of PCR products was confirmed by observing the melting curve after each reaction. Standard curve of each gene was established with template concentration as the gradient and *β-actin* as the internal reference to verify the linearity of gene expression. Gene expression multiples were calculated using delta CT method ($2^{\wedge(C_T\ \beta\text{-actin} - C_T\ \text{Target gene})}$).

2.6. Data analysis

In this study, the expression levels of target genes detected by qRT-PCR in the two groups (SL and BP) were compared by T-test. The significance level in the analyses was considered at $P < 0.05$.

3. Results

3.1. Assembly assessment

Illumina platform was used for sequencing. The transcriptome of SL obtained 45,404,820 clean reads after filtering original reads, accounting for 95.57% of the original reads. On the other hand, the transcriptome of BP obtained 45,630,530 clean reads after filtering original reads, accounting for 96.05% of the original reads. Quality analysis showed that SL and BP had 43.38% and 43.54% GC (%) content of clean reads, Q20 of 97.8% and 97.68% and Q30 of 94.44% and 94.19%, respectively. Clean reads had good randomness in reference gene, and the clean read number from map to genome was 66.3% for SL and 66.57% for BP (Table S2). The data above indicated good sequencing quality.

3.2. Screening of differentially expressed genes and functional enrichment of SL and BP

A total of 572 differentially expressed genes ($|\log 2$ Ratio (BP/SL)$| > 1$ FDR ≤ 0.001) were obtained by comparative transcriptomics analysis of transcriptome data of SL and BP (Fig. 1), with 282 and 290 highly expressed genes belonging to SL and BP, respectively. Among 572 differentially expressed genes, 551 annotations were obtained by functional annotation. The explanatory database included non-redundant (Nr), KEGG and GO. Among them, 551 (96.33%) were injected into the Nr database, 405 (70.80%) were annotated into the database of KEGG, and 101 (17.66%) of the genes were uploaded to the GO database. KEGG enrichment analysis showed that the significant differentially expressed genes were involved in 193 signal pathways, in which 13 were significantly enriched (P-value < 0.05) (Fig. S1). GO functional enrichment results showed that the most significant differentially expressed genes were annotated into the biological process, followed by cellular component and finally molecular function (Fig. S2). In GO enrichment, only the synapse part (P-value = 0.01493) of cellular component had P-value < 0.05.