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De novo transcriptome analysis reveals insights into different mechanisms of growth and immunity in a Chinese soft-shelled turtle hybrid and the parental varieties

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ABSTRACT

The Chinese soft-shelled turtle (*Pelodiscus sinensis*) is a highly important freshwater aquaculture species in China. The molecular mechanisms underlying changes in immunity and growth in hybrid vigor are not well understood. In the present study, the transcriptomes from significantly different *P. sinensis* strains (Qingxi black turtle, B and Japanese strain, J) and the resulting hybrid (Zajiao-1, F) were sequenced using an Illumina sequencing platform. Differentially expressed genes (DEGs) between Zajiao-1 and the Qingxi black turtle were enriched mainly in the HTLV-I infection and Hippo signaling pathways, while DEGs between the Zajiao-1 and Japanese strain were enriched mainly in tryptophan metabolism, caner-associated pathways, transcriptional dysregulation in cancer, amebiasis, Fcγ-mediated phagocytosis and the peroxisome pathway. Highly expressed genes involved in the regulation of disorders of the fatty acid biosynthesis, immune and cardiovascular systems in *P. sinensis* were found among the DEGs. Enrichment categories for gene ontology included cellular processes, metabolic pathways, and the actin cytoskeleton pathway. The reliability of the sequencing data was verified through quantitative real-time polymerase chain reaction analysis of 20 immunity or growth-related genes. These findings offer new insights into heterosis of growth traits and resistance to stresses and potential strategies for selective breeding.

1. Introduction

Chinese soft-shelled turtle (*Pelodiscus sinensis*) is a traditional food in China and other Asian countries (Haitao et al., 2008). This species has become one of the most important species in the Chinese aquaculture industry, with production of 355,000 t in 2014 and a total value of 250 million (He et al. 2015). However, deterioration of the breeding stock, the prevalence of severe infectious diseases and a lack of basic research on its biology have greatly affected the sustainability of the turtle farming industry. Genetics and genomics techniques have immense potential for enhancing aquaculture production through selective breeding programs. Therefore, the identification and characterization of the genes that regulate heterosis of growth and immunity traits in soft-shelled turtles is urgently required.

Immunologic defenses in vertebrates consist of the innate and adaptive immunologic subsystems (Clark et al. 2005). The innate immune response is a fast, effective, and evolutionarily ancient defense system, while the flexibility of the adaptive immune system provides stronger defense in future encounters with the same pathogen (Macagno et al. 2010). The immune signaling network comprises both intercellular communication and intracellular signaling pathways (Huang et al. 2008). Some important genes related to immune defense have been reported in vertebrates, including TLRs (toll-like receptors) in the Toll/IL-1 signal transduction pathway, and C1q (complement component 1, q subcomponent) in the innate and acquired immune systems (Lin et al. 2011, Zhang et al. 2014b). Defense-related genes such as *tMyD88*, *tHsc70*, *tGRP78* and *tHsp72*) have been identified in the Chinese soft-

Abbreviations: AGBL2, ATP/GTP binding protein-like 2; BMPR1A, Bone morphogenetic protein receptor, type 1A; CLEC3B, C-type lectin domain family 3; C1QTNF1, C1q and tumor necrosis factor related protein1; DDAH1, Dimethylarginine dimethylaminohydrolase 1; DEGs, Differentially expressed genes; DHA, Docosahexaenoic acid; ECSIT, Evolutionary conserved signaling intermediate in Toll pathway; EPA, Eicosapentaenoic acid; GABRG2, Gamma-aminobutyric acid A receptor; GDA, Guanine deaminase; GH, Growth hormone; GHITM, Growth-hormone inducible transmembrane protein; GHRH, Growth hormone releasing hormone; GRB2, Growth factor receptor-bound protein 2; HSPA14, Heat shock 70 kDa protein 14; IGF, Insulin-like growth factor; LAP, Linguales antimicrobial peptide; MAPK, Mitogen-activated protein kinase; MEF2C, Myocyte enhancer factor 2C; MHC, The major histocompatibility complex; MYD88, Myeloid differentiation factor 88; NOS1, Nitric oxide synthase 1; SMAD4, Mothers Against Decapentaplegic Drosophila Homolog of 4; SOX18, Sry related HMG box18; SST, Somatostatin; THRSP, Thyroid hormone responsive spot; TLR, Toll-like receptor; TRAF2, TNF Receptor-Associated Factor 2.

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shelled turtle; however, the molecular mechanisms involved in innate immune responses of this species remain to be elucidated. Myeloid differentiation factor 88 (MyD88) is the key adaptor protein in the transduction of signals that trigger downstream cascades involved in innate immunity (Li et al. 2011).

Heterosis, also known as hybrid vigor, is improved or increased biological function in hybrid offspring compared to that of the homozygous parents (Darwin 1876, Shull 1908). Superior phenotypic performance can be observed in diverse traits, such as physiological vigor and resistance to disease. Although, the genetic mechanisms are not clearly understood, heterosis in animals and plants has been widely utilized in activities such as aquaculture production. Heterosis is a highly complex biological phenomenon controlled largely by allelic interactions among parental genomes and epigenetic regulation leading to altered programming of multiple genes that enhance growth vigor, stress tolerance and fitness (Chen 2013). Furthermore, heterosis involves molecular alterations in different levels of genetic, epigenetic, proteomic and metabolomic regulatory networks (Fu et al. 2015; Kaeppler 2012; Feng et al. 2010).

RNA sequencing has been performed in aquatic animals including *Salmo salar*, *Labeo rohita*, *Litopenaeus vannamei*, *Ostrea edulis*, and *Patellavulgate* (Micallef et al. 2012; Robinson et al. 2012; Zeng et al. 2013; Martín-Gómez et al., 2013; Werner et al. 2013). In the Western painted turtle (*Chrysemys picta bellii*), RNA-seq analysis has been used to determine mRNA expression during anoxia exposure. Highly differentially expressed genes (*APOLD1*, *FOS*, *JUNB*, *ATF3*, *PTGS2*, *BTG1/2*, and *EGR1*) were found to be involved in the control of cellular proliferation, cancers, and tumor suppression (Shaffer et al. 2013). RNA-seq analysis of the *P. sinensis* yielded >47 million sequencing reads and 73,954 unigenes (Wang et al. 2013a). Although some physiological, morphological, and phylogenomic analyses have been conducted (Wang et al. 2013a, b; Zhang et al. 2014a, b; Crawford et al. 2015), the genetic variation associated with different varieties of heterosis is still poorly understood in turtles. RNA-seq revealed that hybridization can cause accumulation of transcript levels equal to that of the mid-parent (additivity), the high or low parent (high or low parental dominance), greater than the high parent (over-dominance), or less than the low parent (under-dominance) in pufferfish (Gao et al. 2013).

Large-scale RNA sequencing array analyses in turtles have identified numerous candidate genes involved in embryogenesis, fatty acid biosynthesis and collagen formation (Wang et al. 2013a, b; Kaplinsky et al. 2013). Furthermore, high-throughput sequencing of the transcriptomes of the nutritional quality traits in soft-shelled turtles has been reported (Wang et al. 2013a, b). Recently, the genomes of the green sea turtle (*Chelonia mydas*), the Chinese soft-shelled turtle (*Pelodiscus sinensis*), and the Western painted turtle (*Chrysemys picta bellii*) have been reported (Shaffer et al. 2013; Wang et al. 2013a, b), providing the basis for investigation of their important traits. However, in-depth analysis of heterosis and the immune system has become very important in Chinese turtle aquaculture. In the present study, the whole transcriptomes of three Chinese soft-shelled turtle varieties: the Japanese strain (J), the Qingxi black mutant (B) and their F1 offspring Zajiao-1 (F) were sequenced to analyze the functional differences in the transcriptomes of hybrids and the parental strains, and to provide the basis for investigating the mechanisms of heterosis and immunity in the turtle.

2. Materials and methods

2.1. Sample collection

All Chinese soft-shelled turtles used in the study were cultivated in the national turtle breeding center (Deqing, Huzhou, China). Protocols for the generation of crosses and rearing have been described previously (Zhang et al. 2011). Two Chinese soft-shelled turtle varieties (*P. sinensis*): Japanese strain (female, J) and Qingxi black turtle (male, B) were both from inbred lines produced through several generations of sibling mating; the hybrid turtles (Zajiao-1, F) were the F1 offspring of these two lines (Fig. 1A). The soft-shelled turtle Japanese strain is characterized by rapid growth, large size, tolerance to environmental parameters, good table quality, and significant breeding efficiency (60–110 eggs/year), with the growth performance of 25% higher than ordinary turtle (Zhang et al. 2012). Qingxi black turtle has a unique black belly, while its growth rate is normal (He et al. 2015). The yield and growth ratio of hybrid turtles are 15.6% and 14.8% higher than Japanese strain, respectively (He et al. 2016).

2.2. RNA extraction

The livers were immediately sampled, stored in RNA Later (Ambion) at 4 °C, and then frozen in liquid nitrogen for storage at −80 °C. Total RNA was extracted from liver tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The samples were then treated with RNase-free DNase I (New England Biolabs, Ipswich, MA, USA) to remove any contaminating genomic DNA. The Qubit RNA Assay Kit was used to measure RNA concentration with the Qubit 2.0 Flurometer (Life Technologies, CA, USA). The RNA Nano 6000 Assay Kit was used to evaluate RNA integrity with the Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

2.3. Library construction

The Illumina mRNA-Seq Sample Prep Kit (Illumina, San Diego, CA USA) was used to prepare the three sequencing libraries with 10 μg of RNA. Total RNA from the liver of a single turtle was pooled in equal proportions to generate one sample. The fragmentation buffer in the kit was added directly to heterogeneous nuclear RNA (hnRNA) to produce short fragments (200–700 bp) for use as the templates for first-strand cDNA synthesis using random hexamers. Second-strand cDNA was synthesized using the kit according to the manufacturer's instructions and was purified using a QIAquick PCR Extraction Kit (Qiagen, Valencia, CA USA) before being eluted in elution buffer (EB). Suitable fragments of approximately 200 bp were selected as templates for amplification in a MyCycler PCR instrument (Bio-Rad, Hercules, CA USA).

The ribosomal RNA was removed using the RiboMinus Eukaryote Kit for RNA-Seq (Invitrogen) following the manufacturer's instructions. The library was sequenced using the Illumina Genome Analyzer IIx by Zhihao Life Science Co., Ltd. (Hangzhou, China).

2.4. Read alignment

For each sequenced library, the read counts were adjusted using the edge R program package through one scaling normalized factor. The raw reads were filtered before data analysis by removing reads consisting of adaptors only, those with >10% unknown bases, and those in which more than half of the bases gave a quality score of <5.0. The clean reads were aligned to the reference genome of Chinese soft-shelled turtle (PelSin_1.0, ftp://ftp.ensembl.org/pub/release-78/fasta/pelodiscus_sinensis/dna/) using Tophat v1.4.1 and Bowtie v0.12.7 (Trapnell et al. 2009; Langmead et al. 2009). Some homemade Perlscript was used to extract the gene loci coordinates from the gene set file of *P. sinensis* downloaded from the Ensembl database. Mismatches of no more than two bases were allowed in the alignment. The DEG seq program package was used to compare expression level between samples. The reads per kb per million reads (RPKM) method was used to calculate unique gene expression levels (Mortazavi et al. 2008). Significantly differentially expressed genes (DEGs) were identified by Benjamini and Hochberg corrected *P*-value of <0.05 (Benjamini et al. 1995, 2001). Cufflinks v1.3.0 was used to calculate transcript abundances and identify DGEs based on the mapping results (Roberts et al. 2011). All groups were compared with each other in a