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Transcriptome analysis revealed positive selection of immune-related genes in tilapia

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A B S T R A C T

High-throughput sequencing of transcriptome promises a new approach for detecting evolutionary divergence among species. Up to now, the information about evolution of immune genes in cultured fish, especially in tilapias which would aid to understand the molecular basis of immune phenotypic differentiation is still lack. Thus, in the present study, we used high-throughput sequencing to obtain large amount of gene sequences in blue tilapia and characterized the diversity of orthologs among Nile tilapia, blue tilapia and zebrafish. A total of 52,424,506 raw reads, representing 31,404 unigenes were obtained from blue tilapia cDNA library of mixed tissues, including brain, pituitary, gill, heart, liver, spleen, kidney, intestine, muscle, testis and ovary. Based on Ks value, we calculated that the divergence time between Nile tilapia and blue tilapia is 2.93 million years ago. And the tilapias are both apart from zebrafish in 197 million years ago. Furthermore, the positive selected genes were identified by calculating of Ka/Ks ratio. Several immune-related genes were identified as positively selected genes, such as Notch2 and nfatc3b. Considering that these genes play crucial role in immune regulating function, the immune system genes met a great variation under environment selection in tilapias which suggests fast evolution in immune system of cultured tilapias.

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1. Introduction

Tilapia fishes are one of the most species-rich teleosts which have great commercial importance around the world including 85 countries due to the substantial improvement in growth performance [1]. Different species of tilapia have been cultured. The most popular cultivated varieties include Nile tilapia (*Oreochromis niloticus*), blue tilapia (*Oreochromis aureus*) and Mozambique tilapia (*O. mossambicus*) [1,2]. Moreover, interspecies hybrids tilapias were also used in aquaculture, such as Nile tilapia × blue tilapia [3] and Nile tilapia × Mozambique tilapia [4]. These abundant germplasm resources provide a rich genetic base for breeding in aquaculture industry. Meanwhile, these different species which are close relatives provide a suitable model for investigating speciation and variation during hybridization. Up to date, the most studies on evolution of tilapias are focus on speciation resulted from geographical isolation in east Africa [5,6]. These results give large information about natural selection to these species. However, in artificial culture pools, the evidence of variation and germplasm resource changes are still largely absent.

The evolution of immune system has been studied extensively in the past. Several studies showed that in mammalians, MHC genes are dynamic and natural selected in the adaptive immune system to confer protection against pathogens [7,8]. By using pyrosequencing technology, Savage and colleagues demonstrated that high-speed evolution of lysozyme genes in frogs [9]. In teleosts, the evolution of immune genes is more complicated because most fishes are polyploidy species. Polyploidization was confirmed in many fishes, including Salmonidae [10], Catostomidae [11] and Cyprinidae [12]. Moreover, zebrafish (*Danio rerio*) [13] and *tetraodon nigroviridis* [14] are presumed as polyploidy by comparative genomic analysis. The polyploid genome may lead to gene lost or duplicate. For instance, in Atlantic cod (*Gadus morhua*), the whole genome sequence indicated that the fish has evolved

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compensatory mechanisms and absent of MHCII, CD4 and invariant chain [15]. On contrary, another study demonstrated that multiple paralogous of TLR22 were found which indicated gene duplicate in Atlantic cod [16]. The lost and/or duplicated immune genes suggested that unique immune systems and mechanism in teleosts. In addition, immune genes in teleosts showed a high-speed evolution. Chen and colleagues gave evidence that TLR9 gene was positive selection in teleosts [17]. Positive selection suggested the excess of high-frequency variants results in changes of coding protein which may lead to new gene structure and function [18].

High-throughput sequencing of transcriptome provides a new approach for identifying genes with positive selection. Rapid evolution of biosynthesis, metabolic processes and development related genes were found between two close relatives in crater lake Apoyo [19]. Transcriptome data showed that several genes in yellow-cheek carp (*Elopichthys bambusa*) were adaptive genes by comparison with the publicly available databases, including RAS-related protein [20]. A transcriptomic scan study of *Sebastes caurinus* and *S. rastrelliger* also showed the genes with positive selection belonged to GO categories of immune function, metabolism, longevity, and reproductive behavior [21]. Thus, immune genes were usually found as positive selected genes in teleosts. This is due to the evolutionary selection of fish immune systems. However, large-scale investigation about the evolutionary immune genes in teleosts is far from completed until now.

Blue tilapia is close relative to Nile tilapia, which is a popular cultured species in aquaculture. Meanwhile, considering the annotated genome data of Nile tilapia, it is convenient to carry out comparative genomic study in the two species. In the present study, the goal is to unveil the evolutionary changes between blue tilapia and Nile tilapia. Further, by comparative genomic analysis, the positive selected genes were identified. To our knowledge, this is the first transcriptome report in blue tilapia.

2. Materials and methods

2.1. Fish and sampling

All the blue tilapias were provided by Guangxi Academy of Fishery Sciences. Four adult males and four adult females (1-year old) were collected. After anaesthetized with 2-phenoxyethanol (Sigma, St. Louis, MO, USA), the fish were executed and the tissues including brain, pituitary, gill, heart, liver, spleen, kidney, intestine, muscle, testis and ovary were excised. Immediately, the tissues were frozen by liquid nitrogen and stored at −80 °C.

2.2. RNA extraction and high-throughput sequencing

Total RNAs were extracted according to protocol of Trizol reagent (Invitrogen, Carlsbad, CA, USA). After detected by Agilent 2100 bioanalyzer, the total RNAs of tissues were mixed with equal amount and the mixed 1 μg total RNA was used for sequencing in Illumina Genome Analyzer (Illumina, San Diego, CA, USA). Sequence reads with 100 bp pool was generated. All sequencing reads were deposited in the Short Read Archive (SRA) database (http://www.ncbi.nlm.nih.gov/sra/), which are retrievable under the accession number SRS676061. The reads were obtained and then filtering out adapter and low-quality reads with Q-value ≤ 20. The genome data of Nile tilapia were downloaded from Ensembl genome data (http://www.ensembl.org/Oreochromis_niloticus/Info/Index). Using TopHat v2.0.6 and Cufflinks v2.1.1 package [22,23], the clean reads were mapped to the genome of Nile tilapia. The parameters of TopHat and Cufflinks were performed using default parameters as previous report [22,23]. The r parameter of TopHat was −r 200 and the output file was saved as GTF format.

2.3. Bioinformatic analyses and identification of positively selected genes

By local BLAST against the Nile tilapia cDNA database (e-values $< 10^{-10}$), the sequenced genes were annotated, and then WeGo software was used for analysis of the Gene Ontology (GO) annotations (http://wego.genomics.org.cn/cgi-bin/wego/index.pl). For identifying orthologous ESTs, reciprocal BLASTN searches were used to identify orthologous ESTs in the blue tilapia, Nile tilapia and zebrafish. Zebrafish was used as outgroup in evolutionary analysis. The sequences were considered as orthologous if the best reciprocal BLAST e-value was $<10^{-40}$ [24]. The pairwise Ka/Ks ratios of the number of nonsynonymous substitutions (Ka) to the number of synonymous substitutions (Ks) in orthologs were obtained using a maximum-likelihood method (YN00) implemented in the PAML package version 4.2 [25]. Orthologous ESTs with Ka/Ks ratios >1 were selected for GO annotation analysis to identify the function of the genes. The divergence time was estimated using overall substitution rate for the orthologous EST pairs among Nile tilapia, blue tilapia and zebrafish reciprocally. The rate (r) of 3.51×10^{-9} for coding region analysis as Li described [26] was used to calculate the divergence time between species. The divergence time was calculated from the mean of genetic distance (Ks) between sequences divided by the mutation rate (2r).

3. Results

3.1. High-throughput sequencing and read mapping

After sequencing of the mixture cDNA library from adult blue tilapia, a total of 4.16 Gb data were obtained. In total, the cDNA library represented 52,424,506 reads. After cleaning the raw reads, the reads were mapped into genome of Nile tilapia. The 85.37% reads were matched to genomic locations. The remaining 14.63% reads were not matched. By annotated with the genome, 31,404 unique genes were identified in blue tilapia, providing abundant ESTs sequences for analysis the gene function and comparative genomic study of tilapia.

3.2. Functional annotation

The sequenced genes of blue tilapia were assigned into Gene Ontology (GO) terms to annotate the gene function. The 13,942 (44.40%) unigenes were annotated with an inferred GO terms based on the proteins of tilapia in the Ensembl database. The unigenes were assigned into three major divisions according to the GO database. The first major division of GO terms is “biological process” which indicates the “biological objective to which the gene or gene product contributes” (The Gene Ontology Consortium 2000). The unigenes were identified into 23 categories of “biological process”. The most abundant categories were: (1) “cellular processes” to which 5858 unigenes were dedicated; and (2) “metabolic process” to which 4644 unigenes were dedicated. The second major division of GO terms, “molecular function”, indicated the biochemical activity of the genes (The Gene Ontology Consortium 2000). The unigenes were assigned into 14 categories of “molecular function”. The most abundant categories were binding (7921 genes) and catalytic activity (4066 genes). The third division is “cellular component” which refer to sub-cellular location of the gene product (The Gene Ontology Consortium 2000). The unigenes were found in 11 categories of “cellular component”. The genes were mainly assigned into cell part terms (4634 genes) (Fig. 1).

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