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Channel catfish hemoglobin genes: Identification, phylogenetic and syntenic analysis, and specific induction in response to heat stress

Jianbin Feng [a,b,1], Shikai Liu [a,1], Xiuli Wang [a,c], Ruijia Wang [a], Jiaren Zhang [a], Yanliang Jiang [a], Chao Li [a], Ludmilla Kaltenboeck [a], Jiale Li [b], Zhanjiang Liu [a,*]

[a] Fish Molecular Genetics and Biotechnology Laboratory, Department of Fisheries and Allied Aquacultures and Program of Cell and Molecular Biosciences, Aquatic Genomics Unit, Auburn University, Auburn, AL 36849, USA
[b] Key Laboratory of Exploration and Utilization of Aquatic Genetic Resources, Shanghai Ocean University, Ministry of Education, Shanghai 201306, China
[c] College of Fisheries and Life Science, Dalian Ocean University, Dalian 116023, China

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ABSTRACT

Hemoglobins transport oxygen from gill to inner organs in fish, and this process is affected by temperature, one of the major environmental factors for fish. The hemoglobin gene clusters have been well studied in humans and several model fish species, but remain largely unknown in catfish. Here, eight α- and six β-hemoglobin genes were identified and characterized in channel catfish. Genomic synteny analysis showed that these hemoglobin genes were separated into two unlinked clusters, the MN cluster containing six α- and six β-hemoglobin genes, and the LA cluster consisting of two α-hemoglobin genes. Channel catfish hemoglobin genes were ubiquitously expressed in all the 10 tested tissues from healthy fish, but exhibited higher expression level in spleen, head kidney, and trunk kidney. In response to heat stress, hemoglobin genes, especially MN *Hbα4*, MN *Hbα5*, MN *Hbα6*, MN *Hbβ4*, MN *Hbβ5*, MN *Hbβ6*, LA *Hbα1*, and LA *Hbα2*, presumably the embryonic hemoglobin genes, were drastically up-regulated in the gill and head kidney of heat-tolerant fishes, but not in these tissues of the heat-intolerant fish, suggesting the importance of the embryonic hemoglobin genes in coping with the low oxygen conditions under heat stress.

1. Introduction

Fish are ectotherms, whose body temperature is virtually identical to environmental temperature. Therefore, any rise in the ambient water temperature due to natural variation or human activity would directly increase their body temperature. The elevated temperatures not only reduce the availability of dissolved oxygen in water, but also accelerate the metabolic processes, alter the respiration rate and enhance the oxygen consumption, eventually place a burden on the oxygen transport system of fish (Portner, 2001).

In teleost, hemoglobin, the best-known member of respiratory proteins, plays a critical role in reversibly binding oxygen and transporting oxygen from gill to peripheral tissues. The hemoglobin molecule is made up of two α- and two β-subunits, each having a prosthetic group called heme, which is bound to oxygen. The heme-oxygen bond is exothermic, so increasing temperature could weaken the bond and eventually decrease the hemoglobin's affinity for oxygen (Schmidt-Nielsen, 1997). However, there are exceptions, crucian carp (*Carassius carassius*) hemoglobin increases its oxygen affinity in response to increased temperature, especially at pH levels below 7.0 (Kamshilov and Kamshilova, 2007).

Like other vertebrates, fish has multiple hemoglobin genes including embryonic and adult α-hemoglobin (*Hbα*) and β-hemoglobin (*Hbβ*) chains. To date, the genomic organization of the fish *Hbα*–*Hbβ* clusters has been investigated in fugu (*Takifugu rubripes*) (Flint et al., 2001; Gillemans et al., 2003), medaka (*Oryzias latipes*) (Maruyama et al., 2004a,b; Hardison, 2008), platyfish (*Xiphophorus maculatus*) (Hardison, 2008; Patel et al., 2008), zebrafish (*Denio rario*) (Brownlie et al., 2003), three-spined stickleback (*Gasterosteus aculeatus*) (Wetten et al., 2010), tilapia (*Oreochromis niloticus*) (Opazo et al., 2013), Atlantic salmon (*Salmo salar*) (Quinn et al., 2010), and Atlantic cod (*Gadus morhua*) (Borza et al., 2009; Wetten et al., 2010). In these studies, the adult *Hbα* and *Hbβ* genes, along with the embryonic hemoglobin genes, were observed to be clustered adjacently in the genome. Furthermore, in most of these fish species, two hemoglobin gene clusters were found to be located on separate chromosomes or linkage groups, which support the hypothesis that the teleost lineage experienced whole genome duplication (WGD) events subsequent to the divergence from tetrapod (Taylor et al., 2001).

Channel catfish (*Ictalurus punctatus*), a commercially important freshwater fish in North America, is a temperate species. It has the

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[*] Corresponding author. Tel.: +1 334 844 8727; fax: +1 334 844 9208.
E-mail address: liuzhan@auburn.edu (Z. Liu).

[1] These authors contributed equally to this work.

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ability to cope with seasonal temperature changes from near freezing temperature during winter in the north to over 36 °C in summer in the south. Channel catfish exhibits a greater adaptability, geographic range, and a larger amount of genetic variation than other catfish species such as blue catfish (*I. furcatus*), black bullhead (*Ameiurus melas*), and white catfish (*A. catus*) in North America (Taylor et al., 1984). In previous studies, a variable electrophoretic pattern of hemoglobins were observed in various catfish species including channel catfish (Taylor et al., 1984), but the genomic organization of the catfish α- and β-hemoglobin clusters and their respective encoding genes remained unknown.

Changes in gene expression under heat stress have been extensively studied in many teleost fish. Many genes were up-regulated in response to heat stress, including heat shock protein genes (Hori et al., 2010; Quinn et al., 2011b; Dalvi et al., 2012), ribosomal protein genes (Aursnes et al., 2011), and hemoglobin genes (Imsland et al., 1997; Methling et al., 2010; Quinn et al., 2011a). To date, several channel catfish hemoglobin genes have been identified (Skow, 1971; Taylor et al., 1984; Yeh et al., 2006; Chen et al., 2010), but systematic analysis of the whole hemoglobin repertoire was still unavailable. A recent RNA-seq analysis of global gene expression profiling in catfish following heat stress suggested that various short read assemblies representing hemoglobin genes were noted, but gene identities of such short reads could not be fully addressed (Liu et al., 2013). In the present study, we identified and characterized the channel catfish hemoglobin clusters containing a total of eight *Hb*α genes and six *Hb*β genes. Here we report the identification, phylogenetic and syntenic analysis, and expression profiling of the hemoglobin genes in response to heat stress challenge.

2. Materials and methods

2.1. Identification and sequence analysis of hemoglobin genes

To identify the hemoglobin genes, RNA-seq and whole genome sequence databases of channel catfish were searched using available zebrafish and tilapia hemoglobins as queries. The RNA-seq database was generated from the transcriptome assembly of expressed short reads of a doubled haploid channel catfish (Liu et al., 2012). The quality of the transcriptome assembly obtained from RNA-seq database was confirmed by comparison with the draft catfish whole genome sequences (unpublished), which also originated from sequencing a doubled haploid channel catfish (Waldbieser et al., 2010). The transcripts were translated using FGENESH (Salamov and Solovyev, 2000) and ORF Finder (Sayers et al., 2012). The translated proteins were verified by protein BLAST (BLASTP) (Altschul et al., 1997) against NCBI non-redundant protein database. Functional domains were identified using SMART v 7 (Letunic et al., 2012).

2.2. Assessments of genomic synteny

To identify conserved syntenic regions, the genes in the upstream and downstream of hemoglobin gene clusters were annotated. Initial orthologous predictions were derived from Ensembl Compara Database (Flicek et al., 2013) and were visualized using the program Genomicus v 72.01 (Muffato et al., 2010). The genes lying upstream and downstream of the annotated hemoglobin genes were identified using FGENESH, and their identities determined by BLASTP searches against the non-redundant protein database.

2.3. Sequence alignment and phylogenetic analysis

The deduced amino acid sequences of *Hb*α and *Hb*β genes were separately aligned using MUSCLE v 3.8 (Edgar, 2004) and the L-INS-i, G-INS-i, and E-INS-i of MAFFT v 6.8 (Katoh et al., 2005). The best-scoring multiple alignment was selected by MUMSA for phylogenetic tree construction (Lassmann and Sonnhammer, 2005, 2006). The phylogenetic tree of the *Hb*α and *Hb*β genes was constructed by using both maximum likelihood (ML) and Bayesian methods. The best-fitting models were selected using the Akaike Information Criterion with correction for small sample size (AICc) in Treefinder software (v March 2011) (Jobb et al., 2004). Simulations of 1000 bootstraps were performed to provide statistical support of the phylogenetic tree. Bayesian phylogenies were conducted in MrBayes v 3.2.1 (Ronquist et al., 2012), running four simultaneous chains for 10,000,000 generation runs of four chains, sampling every 1000 generations, under a mixed model with gamma distribution of rate and invariant site categories of amino acid substitution and using default priors. Support for the nodes and parameter estimates were derived from a majority rule consensus of the last 2500 trees. MrBayes analyses were run by using the CIPRES Portal (Miller et al., 2009).

2.4. Heat stress challenge and tissue sampling

All experimental protocols concerning the use of catfish were approved by the Institutional Animal Care and Use Committee (IACUC) at Auburn University. The heat stress experiments were conducted at the hatchery of the Auburn University Fish Genetics Research Unit. The fish and heat treatment were previously reported (Liu et al., 2013). Briefly, tanks were set up with a constant flow system with fresh pond water at 24 ± 0.5 °C and ambient oxygen level (8.9–9.2 ppm). A total of 300 fingerling catfish (Average length: 13.20 ± 1.04 cm, average weight: 13.26 ± 2.69 g) were transferred to an experimental tank (Length: 3.2 m, width: 50 cm and depth: 1 m) and left to acclimate for 72 h at ambient temperature. After acclimation and before the heat stress treatment, 45 fish were removed to another tank serving as a control group with the water temperature kept at 24 °C. The water temperature in the treatment tank was increased by 4 °C h^{-1} until it reached 32 °C, and then the water temperature was increased at 1 °C h^{-1} until it reached 36 °C. Dissolved oxygen was allowed to fluctuate naturally and decreased from approximately 8.5 ppm to a minimum of 6.8 ppm during the trial.

When the water temperature reached 36 °C, the temperature was held constant and the fishes were closely monitored for signs of stress. The first 45 (intolerant group) and the last 45 (tolerant group) individuals showing loss of balance were quickly removed from the tank for sampling, similarly as described in Arctic charr (Quinn et al., 2011a). Fish were euthanized by MS-222 exposure at a concentration of 300 ppm. The fish were weighed and their body lengths were measured.

Ten tissues including brain, gill, heart, liver, head kidney, trunk kidney, spleen, intestine, muscle, and skin were dissected from the 45 fish of intolerant and of tolerant group, respectively. For each group, the same tissues of 15 individuals were pooled together (3 pools of 15 fish each) and immediately immersed in RNA*later* Solution (Invitrogen, Carlsbad, CA, USA). Similarly, the 10 tissues from 45 fish (3 pools of 15 fish each) of the control group were collected. All samples were stored at 4 °C overnight to allow RNA*later* Solution to thoroughly penetrate the tissues, removed supernatant, and then moved to −80 °C until RNA extraction.

2.5. Quantitative real-time RT-PCR analysis

Total RNA was extracted using the TRIzol Reagent (Qiagen, Germantown, MD, USA) following manufacturer's instructions, and then quantified using UV-spectrophotometer. First strand cDNA synthesized using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA) was used for determination of gene expression by quantitative real-time RT-PCR (qRT-PCR). The qRT-PCR was performed using EvaGreen Supermix (Bio-Rad) on the CFX Real Time PCR Detection System (Bio-Rad) following manufacturer's protocol. The primers used in qRT-PCR were designed using primer 3 plus (Untergasser et al., 2012) and listed in Table 1. The 18S rRNA gene was used as an internal control for