RESEARCH ARTICLE

Molecular Characterization and Gene Expression of the Channel Catfish Ferritin H Subunit After Bacterial Infection and Iron Treatment

JOURNAL OF EXPERIMENTAL ZOOLOGY
PART A
ECOLOGICAL GENETICS AND PHYSIOLOGY
A Journal of Integrative Biology

HONG LIU[1,2], TOMOKAZU TAKANO[1], ERIC PEATMAN[1], JASON ABERNATHY[1], SHAOLIN WANG[1], ZHENXIA SHA[1,3], HUSEYIN KUCUKTAS[1], DE-HAI XU[4], PHILLIP KLESIUS[4], AND ZHANJIANG LIU[1]*

[1]The 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, Alabama
[2]College of Fisheries, Huazhong Agricultural University, Wuhan, China
[3]Key Lab for Sustainable Utilization of Marine Fisheries Resources, Ministry of Agriculture, Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao, China
[4]Aquatic Animal Health Research Laboratory, Agricultural Research service, United States Department of Agriculture, Auburn, Alabama

ABSTRACT

Ferritins are the major iron storage protein in the cytoplasm of cells, responsible for regulating levels of intracellular iron. Ferritin genes are widely distributed in both prokaryotes and eukaryotes. In mammals, ferritin molecules are composed of heavy- (H) and light- (L) chain subunits; amphibian genomes contain three ferritin-type genes (H; middle, M; and L subunits); and teleost genomes to date contain H and M subunits. The objective of this study was to characterize the ferritin H gene in channel catfish (*Ictalurus punctatus*) to determine its genomic organization and copy numbers, to determine its patterns of tissue expression, and to establish if it is involved in defense responses of catfish after bacterial infection. The catfish ferritin H gene was completely sequenced and characterized, using both mRNA and genomic DNA. Catfish ferritin H gene has a full-length mRNA sequence of 999 bp, an open reading frame of 534 bp, and 4,704 bp genomic DNA sequence. Catfish ferritin H has a 5 exon and 4 intron genetic organization, containing a long 5′-untranslated region, which shares high similarity with mammalian and zebrafish genes. Based on phylogenetic analyses, the catfish ferritin H gene is highly conserved throughout evolution. Southern blot analysis suggested that the ferritin H gene has only one copy in the catfish genome. The catfish ferritin H gene was widely expressed in various healthy tissues. The catfish ferritin H gene was significantly up-regulated in the liver after intraperitoneal injection of iron dextran and coinjection of *Edwardsiella ictaluri* and iron dextran treatment, suggesting its role in iron metabolism and immunity. *J. Exp. Zool. 313A:359–368, 2010.* © 2010 Wiley-Liss, Inc.

J. Exp. Zool. 313A:359–368, 2010

How to cite this article: Liu H, Takano T, Peatman E, Abernathy J, Wang S, Sha Z, Kucuktas H, Xu D, Klesius P, Liu Z. 2010. Molecular characterization and gene expression of the channel catfish ferritin H subunit after bacterial infection and iron treatment. J. Exp. Zool. 313A:359–368.

Grant Sponsor: USDA ARS Aquatic Animal Health Laboratory; Grant number: 58-6420-5-030; Grant Sponsor: USDA NRI Animal Genome Basic Genome Reagents and Tools Program; Grant number: 2009-35205-05101; Grant Sponsor: National Natural Science Foundation of China; Grant number: 30871941.

Additional Supporting Information may be found in the online version of this article.

*Correspondence to: Zhanjiang Liu, The 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. E-mail: zliu@acesag.auburn.edu

Received 18 November 2009; Revised 9 February 2010; Accepted 23 February 2010

Published online 24 March 2010 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jez.606

Iron is vital to nearly all organisms given its roles in many metabolic pathways: oxygen transport, electron transport, DNA synthesis, etc. However, excess free iron can be toxic with the potential to cause wide-ranging detrimental effects (Crichton et al., 2002). Thus, organisms have developed a tight regulatory system to balance uptake of iron, to prevent deficiency vs. potential toxicity, and to adapt efficient iron transport and storage mechanisms. This regulation is accomplished by the interaction of several genes (Aisen and Listowsky, '80; Anderson and Frazer, 2005; Graham et al., 2007). Ferritins, a group of widespread proteins found in prokaryotes and eukaryotes, play important roles in maintaining iron in a soluble, nontoxic, bioavailable form, and are capable of sequestering large amounts of iron. Ferritins, thus, carry out the dual function of iron detoxification and storage (Aisen and Listowsky, '80; Crichton and Charloteauxwauters, '87; Anderson and Frazer, 2005). Beyond iron metabolism, ferritin genes also have an important role in cellular defense against stress and inflammation (Torti et al., '88). Recently, a new role for ferritins has been suggested—that of a regulator of immune functions crucial in autoimmune diseases (Recalcati et al., 2008). Li et al. (2008) reported that a ferritin homolog in amphioxus plays a dual role in both immune response and iron metabolism. Ferritin proteins have a molecular weight of 450 kDa, and all eukaryotic ferritins consist of 24 protein subunits arranged to form a hollow shell with an 80 A° diameter cavity capable of storing up to 4,500 Fe (III) atoms as a biomineral (Aisen and Listowsky, '80; Harrison and Arosio, '96; Torti and Torti, 2002). In mammals, ferritin molecules are composed of heavy (*H*) and light (*L*) chain subunits, with molecular masses of 21 and 19 kDa, respectively. *H* chains are important for Fe (II) oxidation, whereas *L* chains assist in iron nucleation, mineralization, and long-term storage (Harrison and Arosio, '96). The *H* and *L* subunits coassemble in different ratios to form a protein shell of 24 subunits capable of acquiring iron atoms. In amphibian red blood cells, there are three types of ferritin subunits (Middle, M; *H*; and *L*) (Dickey et al., '87; Andersen et al.,'98).

Information on teleost ferritin genes is limited. From *Salmo salar*, H and M subunit cDNAs have been isolated and northern blot analysis revealed the presence of H chain mRNA in spleen, liver, and heart, and of M chain mRNA almost exclusively in the gonads (Andersen et al., '95). In rainbow trout, three cold-inducible H-chain isoforms (H1, H2, and H3) have been described and were found to be enhanced by cold acclimation (Yamashita et al., '96). Ferritin from the spleen of the antarctic teleost *Trematomus bernacchii* was found to be an M-type homopolymer (Mignogna et al., 2002). Recently, H-chain mRNA was observed in the spleen of ploughfish but not in ocellated icefish or emerald rockcod (Scudiero et al., 2007). Ferritins from the liver and spleen of the cold-adapted antarctic teleosts, *T. bernacchii* and *T. newnesi*, have been isolated and characterized and only *H*- and *M*-chains were found to be expressed (Giorgi et al., 2008). More recently, Neves et al. (2009) revealed a dual role for ferritin in sea bass, with functional involvement in both immune response and iron metabolism.

Channel catfish, *Ictalurus punctatus*, is not only the most abundant catfish species in North America, the most important aquaculture species in the United States (USDA, 2006), and an important game fish, ranking within the top three sports fish (Dunham, '84), but it also serves as a research model for comparative genomics and immunology (Liu et al., 2009). In this study, we aim to characterize the ferritin H gene in channel catfish, to determine its genomic organization and copy numbers, to determine its patterns of tissue expression, and to establish if it is involved in defense responses of catfish after bacterial infection.

MATERIALS AND METHODS

Identification and Sequencing of Channel Catfish Ferritin H Gene cDNAs

BLAST searches were used to identify channel catfish expressed sequence tags, encoding partial cDNAs for the catfish ferritin H gene (Ju et al., 2000; Cao et al., 2001; Karsi et al., 2002; Li et al., 2007; Wang et al., 2010). The cDNA clone AUF_Hdk_43_d24, which contained a complete cDNA of catfish ferritin H gene, was sequenced by using SP6 and T7 primers (Table 1). Sequencing reactions were performed on an ABI 3130 automated DNA sequencer using the BigDye terminator v3.1 ready reaction kit (Applied Biosystems, Foster City, CA) following manufacturer's protocols with modifications (Xu et al., 2006).

BAC Library Screening and Genomic Sequencing of Catfish Ferritin H Gene

Using the CHORI-212 BAC library (Wang et al., 2007), screening was conducted to determine the positive BAC clones of channel catfish ferritin H gene as described earlier (Takano et al., 2008; Liu et al., 2010). Briefly, cDNA probes were used for the screening of ferritin. The plasmid AUF_Hdk_43_d24 harboring ferritin H cDNA was used as the template for the amplification of the cDNA insert using PCR with SP6 and T7 primers (Table 1). A random primed DNA labeling kit (Roche Diagnostics, Indianapolis, IN) was used for the labeling of the cDNA fragment with ^{32}P-deoxycytidine triphosphate (PerkinElmer, Boston, MA). Sephadex G50 spin columns (Boehringer, Manheim, Germany) were used to remove unincorporated nucleotides. Probes were denatured at 95°C for 10 min and added into hybridization tubes that had been under prehybridization for 2 hr. High-density BAC filters containing the whole CHORI-212 BAC library, purchased from Children's Hospital of the Oakland Research Institute (CHORI, Oakland, CA), were hybridized at 63°C for 16 hr in 30 mL hybridization solution. The filters were washed twice at 60°C with washing buffer (2 × SSC, 0.1% SDS), and then exposed to X-ray film (Fujifilm, Tokyo, Japan) at −80°C for 20 hr. Positive clones were identified according to the clone distribution pattern

Table 1. PCR primers used for this study.

Primer name	Primer sequence (5′–3′)
SP6 sequencing primer	ATTTAGGTGACACTATAG
T7 sequencing primer	TAATACGACTCACTATAGGG
Forward PCR primer for screening ferritin H from BAC library	AAAGTCCAGAACCAGAGAGGA
Forward PCR primer for screening ferritin H from BAC library	ACCCAGTCAGAAAGCTCCTTA
Forward PCR primer for Southern blot probes	ACAGTGCTTGAACGGCAACC
Reverse PCR primer for Southern blot probes	CACTAATTGGACCACCATCTGG
Forward primer for RT-PCR and qRT-PCR	AAAGTCCAGAACCAGAGAGGA
Reverse primer for RT-PCR qRT-PCR	ACCCAGTCAGAAAGCTCCTTA
18S rRNA forward primer	GAGAAACGGCTACCACATCC
18S rRNA reverse primer	GATACGCTCATTCCGATTACAG

from CHORI. Positive clones were picked and cultured in 2XYT medium. After overnight culture, BAC DNA was isolated using R.E.A.L. Prep 96 BAC DNA isolation kit (Qiagen, Valencia, CA) as described earlier (Xu et al., 2006, 2007). BAC clone 7_K7, containing the catfish ferritin H gene, was sequenced using the primer-walking method and sequencing was conducted as described above.

Southern Blot Analysis

Southern blot analysis was conducted to determine the genomic copy numbers of channel catfish ferritin H gene as described earlier (Takano et al., 2008; Liu et al., 2010). Briefly, 10 μg of genomic DNA from three individual adult channel catfish each was digested with endonuclease *Eco*RI, *Hin*d III, or *Pst* I (New England Biolabs, Beverly, MA) and was electrophoresed on a 0.7% agarose gel. The gels were submerged in 0.25 N HCl for 10 min, denaturation buffer and neutralization buffer for 30 min, respectively. The DNA was transferred to an Immobilon nylon membrane (Millipore, Bedford, MA) by capillary transfer with 20 × SSC buffer for 18 hr. The DNA was fixed to the membrane using a UV cross-linker (Stratagene, La Jolla, CA). The filters were hybridized with probes amplified with ferritin H specific primers (Table 1), as described above.

Phylogenetic Analysis

A phylogenetic tree was constructed using the Molecular Evolutionary Genetics Analysis (MEGA 4.0) software (Tamura et al., 2007). The ferritin protein sequences, from various species retrieved from GenBank, were aligned by ClustalW and then a neighbor-joining tree was created. Data were analyzed using Poisson distance correction and gaps were removed by complete deletion. The topological stability of the trees was evaluated by 10,000 bootstrap replications.

Bacterial Challenge and Iron Dextran Treatment

All experimental procedures involving fish were approved by the Institutional Animal Care and Use Committee of Auburn University. Channel catfish of the Marion strain were artificially spawned at the hatchery of the Auburn University Fish Genetics Research Unit. At 1 week posthatch, they were transferred to troughs or aquaria at the Auburn University Fish Pathology wet lab. The use of recirculating systems and well water sources ensured that the catfish fingerlings remained naïve to *Edwardsiella ictaluri* during grow-out. Bacterial challenge and iron dextran treatment experiments were conducted as described earlier (Liu et al., 2010) using channel catfish, *I. punctatus* (average weight 6.1 g and average length 9.5 cm). The fish were kept at 27°C in a flow-through system, utilizing heated well water for a week before experiment and then experiment was conducted in the same system. Nine hundred and sixty fish were divided between four groups: (1) control group (phosphate-buffered saline, PBS injected); (2) *E. ictaluri* challenged group; (3) iron dextran treated group; (4) iron dextran treated and *E. ictaluri* infected group. The bacterial culture from a single colony of *E. ictaluri* cultured in BHI broth media at 28°C overnight was diluted with PBS, and then 1×10^5 cfu of bacteria in 100 μL PBS were injected intraperitoneally into the channel catfish. For the iron dextran group, 100 μL of the iron dextran solution (Sigma, St. Louis, MO) diluted in sterile PBS to a final concentration of 12.5 mg/mL was injected intraperitoneally. The fish in the combination group were injected with the same amount of bacteria and iron dextran. Injections were carried out under light anesthesia utilizing tricaine methanesulfonate (MS 222) at 100 mg/L.

Tissue Sampling and RNA Extraction

Fish used in this study were euthanized by prolonged MS 222 exposure at 300 mg/L before dissection. From the healthy, untreated group, 11 tissues including brain, gill, heart, head kidney, trunk kidney, intestine, liver, muscle, skin, spleen, and stomach were collected to determine tissue distribution of expression using real time quantitative RT-PCR (qRT-PCR) from a pool of 15 fish. The tissues were immediately frozen in liquid nitrogen, homogenized under liquid nitrogen using a mortar and

pestle, and stored at −80°C until RNA extraction. From the PBS injected, *E. ictaluri* challenged and/or iron-treated fish, liver tissues were collected from 45 fish at each timepoint (4 hr, 24 hr, 3d and 7d after treatment) and tissues from 15 fish were pooled together (3 pools per timepoint/treatment) for qRT-PCR analysis.

Total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen) and the concentration of total RNA was quantified using an Ultrospec 1100 pro (Amersham Biosciences Fairfield, CT). The RNA samples were subsequently used for determination of gene expression.

Quantitative Real-Time RT-PCR Analysis

Quantitative real-time RT-PCR was carried out using a Fast Start RNA Master SYBR Green I Reagents Kit (Roche Applied Science, Indianapolis, IN) on a LightCycler 1.0 (Roche Applied Science), following manufacturer's instructions with modification (Peatman et al., 2007, 2008). To assess the relative expression of ferritin H in various catfish tissues, the spleen RNA sample was arbitrarily chosen as the calibrator (1×) and the relative abundance of ferritin H was calculated for the remaining 10 tissues by ratio with the calibrator. The fold induction of the channel catfish ferritin H gene after different treatments was quantified in the liver. Total RNA of 100 ng was used in one reaction. The 18s rRNA gene was used as an internal control for normalization of expression levels. The PCR primers that were used in this analysis are listed in Table 1. The triplicate fluorescence measured by crossing point (Ct) values, were compared and converted to fold differences by the relative quantification method using the Relative Expression Software Tool 384 v. 1 (REST) (Pfaffl et al., 2002). Expression differences between control and treatment groups were assessed for statistical significance using a pairwise fixed reallocation randomization test within the REST software. PCR specificity was assessed by melting curve analysis.

RESULTS

Identification and Sequencing of Channel Catfish Ferritin H cDNA

The cDNA clone AUF_Hdk_43_d24 containing the full-length cDNA of ferritin H of channel catfish was directly sequenced. The full-length cDNA of channel catfish ferritin H is 999 bp containing an ORF of 531 bp encoding 177 amino acids with a non-coding first exon. The channel catfish ferritin H cDNA sequence has been deposited in GenBank with the accession number of GU122920.

Analysis of the deduced amino acid sequences by multiple sequence alignments indicated that the ferritin H genes are highly conserved throughout evolution (Fig. 1). The catfish ferritin H showed 80% identity with those protein sequences of mammals and 87–91% with zebrafish and other fish (Table 2).

Phylogenetic Analysis of Channel Catfish Ferritin H Gene

Phylogenetic analysis was conducted, based on amino acid sequences to analyze the catfish ferritin H genein the larger context of vertebrate ferritin genes. As shown in Figure 2, the

```
CATFISH    -------21 UUCUCCGCUUCAACAGUGCUUGAACGGCAACCU-----------------    217
ZEBRAFISH  -------6  UUACCUGCUUCAACAGUGCUUGAACGGCAACCG-----------------    214
HUMAN      -------58 UUUCCUGCUUCAACAGUGCUUGGACGGAACCCG-----------------    235
MOUSE      -------29 UUUCCUGCUUCAACAGUGCUUGAACGGAACCCG-----------------    167
                     **  * ***************** **** * **

CATFISH    ----MSSQVRQNFHQDCEAAINRQINLELYASYVYLSMSYYFDRDDQALHNFAKFFRKQS     56
ZEBRAFISH  ----MSSQVRQNFEEACEAAVNRQINMELYASYVYLSMSYYFDRDDQALHNFAKFFRHQS     56
HUMAN      MTTASTSQVRQNYHQDSEAAINRQINLELYASYVYLSMSYYFDRDDVALKNFAKYFLHQS     60
MOUSE      MTTASPSQVRQNYHQDAEAAINRQINLELYASYVYLSMSCYFDRDDVALKNFAKYFLHQS     60
           .******:.: .***:*****:************ ****** **:****:* :**

CATFISH    HEEREHAEKLMKVQNQRGGRIFLQDIKKPERDEWGSGMEALECALQLEKNVNQSLLDLHK    116
ZEBRAFISH  HEEREHAEKLMKFQNQRGGRIFLQDVKKPEKDEWGSGVEALECALQLEKSVNHSLLELHK    116
HUMAN      HEEREHAEKLMKLQNQRGGRIFLQDIKKPDCDDWESGLNAMECALHLEKNVNQSLLELHK    120
MOUSE      HEEREHAEKLMKLQNQRGGRIFLQDIKKPDRDDWESGLNAMECALHLEKSVNQSLLELHK    120
           ************.************:***: *:* **::*:****:***.**:***:***

CATFISH    VATDHNDPHMCDFIEAHYLDEQVKSIKELSDWVTNLRRMGAPQNGMAEYLFDKHTLGSES--S 177
ZEBRAFISH  LASQHNDPHMCDFIETHYLDEQVKSIKELGDHVTNLRRMGAPQNGMAEYMFDKLTLGKES--S 177
HUMAN      LATDKNDPHLCDFIETHYLNEQVKAIKELGDHVTNLRKMGAPESGLAEYLFDKHTLGDSDNES 183
MOUSE      LATDKNDPHLCDFIETYYLSEQVKSIKELGDHVTNLRKMGAPEAGMAEYLFDKHTLGHGD-ES 182
           :*:::****:*****::**.****:****.* *****:****: *:***:*** ***   .  *
```

Figure 1. Comparison of ferritin H IRE in the 5′ UTRs and amino acid sequences from catfish and other species. Sequences used are from channel catfish (*Ictalurus punctatus*), human (*Homo sapiens*, P02794), mouse (*Mus musculus*, P09528), and zebrafish (*Danio rerio*, AAG37837). Grey boxes indicate residues involved in iron binding.

Table 2. Pairwise similarities of selected ferritin H proteins by ClustalW.

Catfish	Sablefish	Atlantic salmon	Rainbow trout	Zebrafish	Human	Rat	Mouse	
	91	90	90	87	80	80	80	Catfish
		92	92	88	79	80	80	Sablefish
			97	88	77	77	77	Atlantic salmon
				87	77	78	78	Rainbow trout
					76	77	77	Zebrafish
						94	92	Human
							97	Rat
								Mouse

teleost and the mammalian ferritin H orthologies are supported by very strong bootstrap values. The vertebrate ferritin genes formed three distinct subclades: (1) zebrafish ferritin-like, catfish ferritin M, and other fish ferritin M genes; (2) mammalian and teleost ferritin H genes; and (3) mammalian ferritin L genes.

Structural Analysis of the Channel Catfish Ferritin H Gene

Screening of a channel catfish BAC library (Wang et al., 2007) led to the identification of two BAC clones containing the catfish ferritin H gene. The two BAC clones containing catfish ferritin H were 56_K11 and 7_K7. The clone 7_K7 was used for sequencing of ferritin H gene. A genomic sequence of 4,703 bp of the channel catfish ferritin H gene was obtained by primer walking sequencing, and the nucleotide sequence has been deposited in GenBank with the accession number of **GU122919**.

Alignment of the genomic DNA with the cDNA sequences revealed the presence of 5 exons and 4 introns in the catfish ferritin H gene (Fig. 3). Notably, there is a non-coding first exon in the catfish ferritin H gene, similar to the ferritin H1 gene organization in zebrafish. With the exception of this first non-coding exon, the overall gene structures are highly conserved in vertebrates throughout evolution (Fig. 3).

Determination of Genomic Copies for Ferritin H Gene in Channel Catfish

Genomic Southern blot analysis was conducted to determine the copy number of the ferritin H gene in the channel catfish genome. A single copy of ferritin H was found to exist in the channel catfish genome. As shown in Figure 4, a single band was observed with restriction enzyme digestion using *Eco*RI, *Hin*d III, and *Pst* I, suggesting the presence of only a single copy gene of ferritin H. We determined the location of the two BAC clones positive for the ferritin H gene on the physical map of the fish genome and found that only one clone (7_K7) was physically mapped, falling into contig 1,067 (Xu et al., 2007).

Expression of the Channel Catfish Ferritin H Gene in Various Tissues

qRT-PCR was used to determine relative tissue expression patterns of the catfish ferritin H gene. As shown in Figure 5, the channel catfish ferritin H gene is expressed in all 11 tissues tested, including brain, gill, intestine, head kidney, trunk kidney, heart, liver, muscle, skin, spleen, and stomach. The highest expression of ferritin H was detected to be in the liver, followed by trunk kidney, spleen, head kidney, heart, intestine, gill, muscle, stomach, brain, and skin.

Ferritin H Expression After Bacterial Infection and Administration of Iron Dextran

In groups of fish injected with *E. ictaluri* alone, catfish ferritin H gene expression was not significantly different from the controls. In groups injected with iron dextran alone, the expression of channel catfish ferritin H was upregulated about 1.9 fold 4 hr after injection. In groups of fish injected with both *E. ictaluri* and iron dextran, catfish ferritin H was up-regulated 2.1 fold at 4 hr and 2.4 fold 7d after injection (Fig. 6).

DISCUSSION

In this study, the channel catfish ferritin H gene was identified and its structure and expression characterized. The catfish ferritin H gene was highly expressed in various healthy tissues and was significantly up-regulated in the liver after intraperitoneal injection of iron dextran and coinjection of *E. ictaluri* and iron dextran treatment, suggesting its role in both iron metabolism and immunity.

The ferritin H proteins are highly conserved, likely owing to the critical role of ferritin H in the maintenance of iron homeostasis. In mammals, ferritin H genes have 3 introns and 4 exons, with the intron–exon boundaries occurring at similar locations (Harrison et al., '91). The catfish and zebrafish ferritin H1, though sharing much of the gene organization pattern with mammals, harbor a non-coding first exon. The sizes of coding exons are close to each other in these species. In contrast, the introns are quite different in size for these species. In iron metabolism related genes, mRNA regulation depends on a family of non-coding sequences called iron responsive elements, which were found in the 5′ untranslated regions (UTRs) or 3′ UTRs to control gene expression in response to changes in the iron level (Thomson et al., '99; Piccinelli and Samuelsson, 2007). In ferritin

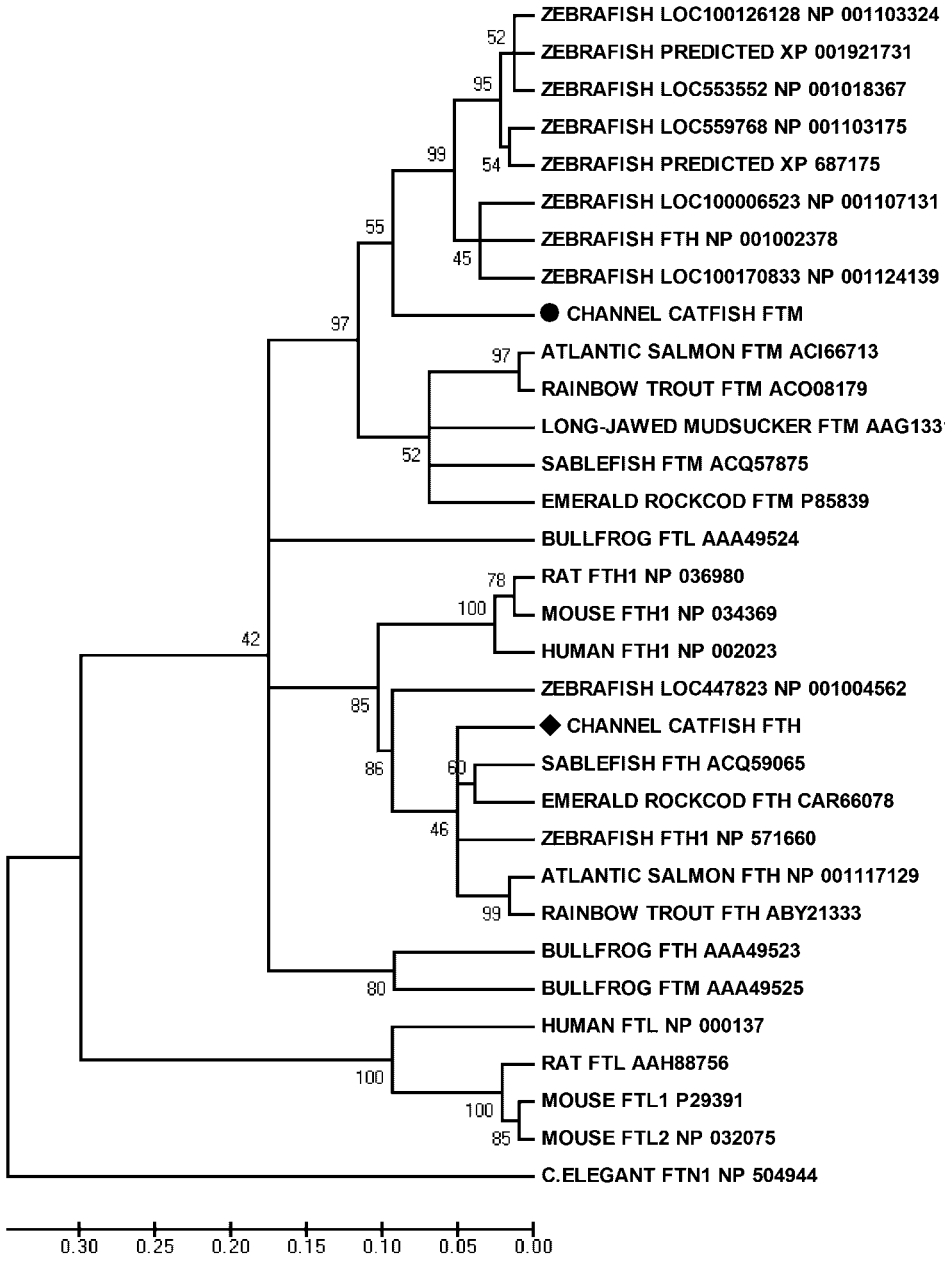

Figure 2. Phylogenetic analysis of ferritin gene from catfish and other species followed by the GenBank accession numbers. The phylogenetic tree was constructed based on ClustalW generated multiple sequence alignment of amino acid sequences using the neighbor-joining method which ignores gaps within the MEGA 4.0 package. The topological stability of the neighbor joining trees was evaluated by 10,000 bootstrapping replications, and the bootstrapping percentage values are indicated by numbers at the nodes.

mRNAs, a single IRE is located in the 5′-UTR, which is highly conserved and plays an important role in the control of iron metabolism in animal cells. As expected, in the catfish ferritin H mRNA described here, we identified an IRE located in the 5′-UTR with a CAGUGN apical loop sequence and a conserved C residue five bases upstream.

Phylogenetic analysis suggested that teleost fish likely have ferritin H and M subunits while mammalian species have ferritin H and L subunits. The ferritin H genes from all vertebrate species, including catfish, formed a clade while the mammalian ferritin L genes formed another distinct clade. Catfish ferritin M (Genbank ID: GU122921), together with zebrafish ferritin-like genes, and

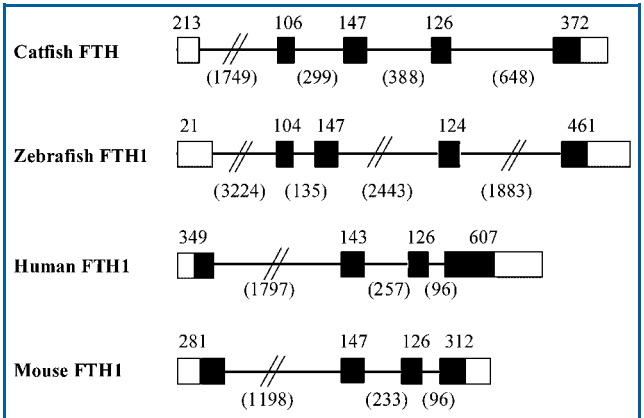

Figure 3. Schematic diagram of ferritin H gene structure. Exons are represented by boxes. Solid boxes indicate the coding region of the gene while open boxes indicate untranslated regions. The numbers that are indicated on the top of boxes are the length of the region in base pairs. The length of introns is represented in parentheses below each gene structure. Double slashes indicate nonproportional representation of the introns as specified.

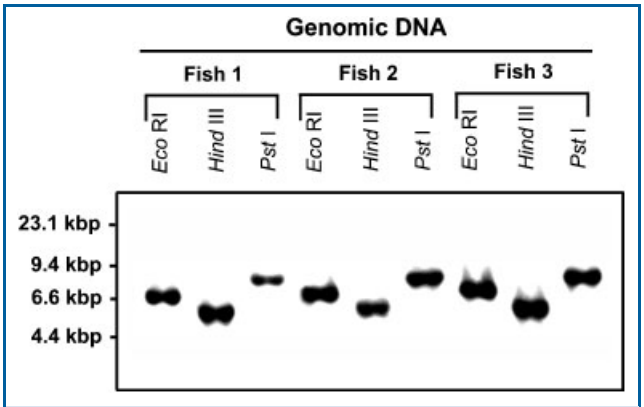

Figure 4. Southern blot analysis of catfish ferritin H using genomic DNA of three channel catfish individuals. Southern blot analysis procedures are detailed in the Materials and Methods section. Kb markers are indicated on the left margin.

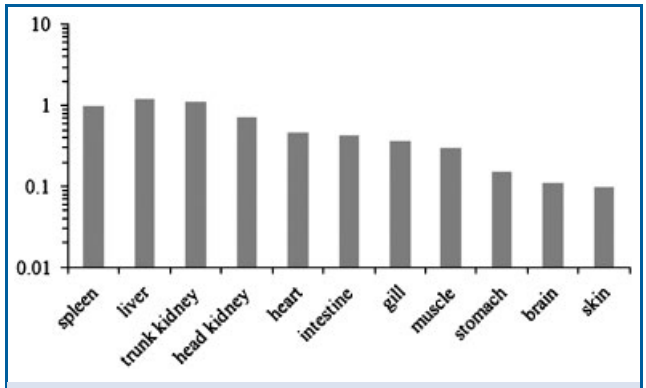

Figure 5. Relative expression of ferritin H in channel catfish determined using qRT-PCR. The *Y*-axis represents 18S-normalized relative expression values ($\log_{10}$) of catfish ferritin H. Tissue RNA samples are labeled along the *X*-axis. Expression levels in all tissues are presented relative to that in the spleen tissue (1 ×).

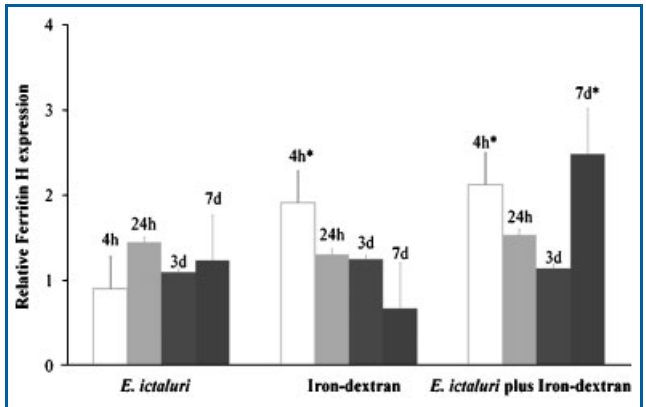

Figure 6. Fold induction ($\log_{10}$) of channel catfish ferritin H gene after *E. ictaluri* and/or iron dextran treatment in the liver. The RNA samples were collected at 4 hr, 24 hr, 3d, and 7d posttreatment. Relative ferritin H expression was expressed as fold change over control samples taken at the same time interval as normalized to change in expression in the 18S control. The bars indicate mean expression of 3 tested pools (15 fish each) ± SE. Asterisks indicate statistical significance at the level of $P < 0.05$.

some fish ferritin-M genes, formed the third clade in the phygenetic tree (Fig. 2). In mammals, ferritin molecules are composed of H and L chain subunits (Harrison and Arosio, '96). The ferritin M subunit was described for the first time in bullfrog *Rana catesbeiana* tadpoles (Dickey et al., '87). Subsequently, ferritin M genes were found in fish: *Lampetra fluviatilis* (Andersen et al., '98), *S. salar* (Andersen et al., '95), *T. bernacchii* (Mignogna et al., 2002), *T. bernacchii* and *T. newnesi* (Giorgi et al., 2008).

The three ferritin subunits from bullfrog (Dickey et al., '87) were named based on molecular mass rather than phylogenetic analysis based on protein sequence similarities. The placement of bullfrog ferritin sequences on the phylogenetic tree revealed that amphibian L subunit is likely intermediate to the H and M clades rather than grouping with mammalian L subunits. Interestingly, bullfrog H and M subunits form a distinct group from either of the larger H or M subunit clades. Sequences from African clawed frog (*Xenopus laevis*) were also placed in this amphibian group during phylogenetic analysis (data not shown). Although questions clearly remain concerning certain aspects of ferritin evolution, our analysis does suggest that the H subunit is the shared ancestral ferritin form. This H-chain is more closely

related to the M subunit observed in fish species than to the L subunit found only in higher vertebrates, potentially suggesting the earlier emergence of the M subunit in a common vertebrate ancestor. The L subunit seems to have evolved later in vertebrate evolution, after the divergence of teleost species, leading to the loss of the M subunit genes in mammalian species, perhaps owing to functional redundancy between L and M subunits.

The mammalian genomes have been shown to contain several copies of the ferritin H and L subunit sequences. The majority of these copies are apparently processed pseudogenes (Cragg et al., '85; Jain et al., '85; Lebo et al.,'85). A functional mitochondrial ferritin gene has also been described (Levi et al., 2001). In *Danio rerio* (zebrafish), the most evolutionarily similar species to catfish with a whole genome sequence, one ferritin subunit homologous to ferritin M is present in many copies (on chromosome 3, supplementary Table 1) with amino acid identity ranging from 88 to 98%, suggesting a tandem duplication of this ferritin subunit in the zebrafish genome. Similarly, in Atlantic salmon, there are also many ferritin M-like sequences deposited in GenBank with amino acid identity ranging from 97–99%. However, in contrast to the tandem duplication of ferritin genes observed in the diploid zebrafish genome, the multiple copies in salmonids may be owing to tetraploidy. In this study, we detected only a single copy of the channel catfish ferritin H gene in the catfish genome as evidenced by Southern blotting. To better understand the size and structure of the ferritin family in catfish, we also determined the genomic copy numbers of a channel catfish ferritin M gene by Southern blotting of 29 positive BAC clones. The catfish ferritin M gene, similar to the situation in zebrafish and salmon, seems to have many copies in the catfish genome based on preliminary Southern blot analysis (supplementary Fig. 1).

Iron is an essential mineral for nearly all living organisms. Its low solubility in the aerobic environment is often limiting for growth, and consequently, competition for iron can be a decisive factor in determining survival. Liver plays a central role in iron metabolism and is the major storage site for iron (Anderson and Frazer, 2005; Graham et al., 2007). Therefore, it was somewhat surprising that expression of ferritin H gene in liver was not significantly different in the *E. ictaluri* challenged group from the control group, differing from observations using microarrays (Peatman et al., 2007, 2008). Potentially, cross-hybridization between multiple expressed ferritin M transcripts and the ferritin H feature on the catfish microarray could account for these differing results. Similarly, the catfish transferrin gene was up-regulated in the liver of catfish after *E. ictaluri* challenge (Liu et al., 2010) but with lower fold changes compared with earlier observations with microarrays (Peatman et al., 2007, 2008). These differences may also result, at least in part, from differences in modes of pathogen exposure (immersion vs. injection) and fish sizes and genetic background between challenges. Further work is needed to obtain a comprehensive picture of ferritin expression across a variety of temporal and immune settings.

Excess iron can contribute to the formation of reactive oxygen species, leading to protein, lipid, and DNA damage (Crichton et al., 2002). When the iron level is high, transferrin receptor synthesis expression is repressed and ferritin synthesis is stimulated. Conversely, under low iron conditions, ferritin synthesis is repressed and transferrin receptor synthesis is stimulated (Aisen and Listowsky, '80). Ferritin H expression was found to be induced 4h post-injection with iron dextran in this study, suggesting a conserved role for catfish ferritin in coordinating iron metabolism. The increased ferritin expression during iron overload reflects the need to store excess iron, minimizing free iron toxicity. In the group with coinjection of *E. ictaluri* and iron dextran, ferritin H was found to be induced in the liver at 4h and again at 7d post-injection. One potential explanation for this pattern may be that the initial upregulation in ferritin expression was chiefly due to iron overload alone and that the latter upregulation was in response to the bacterial proliferation caused, at least in part, by iron overload. It seems that iron overloading heightened the induction of ferritin-mediated responses of catfish to bacterial infection. Similarly, the intelectin 2 gene response to infection was more dramatic when catfish was treated with both bacteria and iron than with bacteria or iron alone (Takano et al., 2008). Further characterization of ferritin family member roles in homeostatic and disease processes is needed to better understand the complex functions of these genes.

ACKNOWLEDGMENTS

This project was supported in part by a Specific Cooperative Agreement with USDA ARS Aquatic Animal Health Laboratory, Auburn, Alabama, under Contract Number 58-6420-5-030, in part by a grant from USDA NRI Animal Genome Basic Genome Reagents and Tools Program (USDA/NRICGP award# 2009-35205-05101), and in part by the National Natural Science Foundation of China; Grant number: 30871941. We thank Jeffery Terhune for providing the catfish; Fei Chen, Jianguo Lu, Lilian Wong, Ludmilla Kaltenboeck, Parichat Ninwichian, Shikai Liu, Wenqi Wang, Yanliang Jiang, and Yoona Lee for their help with taking the tissue samples.

LITERATURE CITED

Aisen P, Listowsky I. 1980. Iron transport and storage proteins. Annu Rev Biochem 49:357–393.

Anderson GJ, Frazer DM. 2005. Hepatic iron metabolism. Semin Liver Dis 25:420–432.

Andersen O, Dehli A, Standal H, Giskegjerde TA, Karstensen R, Rorvik KA. 1995. Two ferritin subunits of Atlantic salmon (*Salmo salar*): cloning of the liver cDNAs and antibody preparation. Mol Mar Biol Biotechnol 4:164–170.

Andersen O, Pantopoulos K, Kao HT, Muckenthaler M, Youson JH, Pieribone V. 1998. Regulation of iron metabolism in the sanguivore

lamprey *Lampetra fluviatilis*—molecular cloning of two ferritin subunits and two iron-regulatory proteins (IRP) reveals evolutionary conservation of the iron-regulatory element (IRE)/IRP regulatory system. Eur J Biochem 254:223–229.

Cao D, Kocabas A, Ju Z, Karsi A, Li P, Patterson A, Liu Z. 2001. Transcriptome of channel catfish (*Ictalurus punctatus*): initial analysis of genes and expression profiles of the head kidney. Anim Genet 32:169–188.

Cragg SJ, Drysdale J, Worwood M. 1985. Human ferritin H-chain sequences are present on several chromosomes. Br J Haematol 61:566.

Crichton RR, Charloteauxwauters M. 1987. Iron transport and storage. Eur J Biochem 164:485–506.

Crichton RR, Wilmet S, Legssyer R, Ward RJ. 2002. Molecular and cellular mechanisms of iron homeostasis and toxicity in mammalian cells. J Inorg Biochem 91:9–18.

Dickey LF, Sreedharan S, Theil EC, Didsbury JR, Wang YH, Kaufman RE. 1987. Differences in the regulation of messenger RNA for housekeeping and specialized-cell ferritin. A comparison of three distinct ferritin complementary DNAs, the corresponding subunits, and identification of the first processed in amphibia. J Biol Chem 262:7901–7907.

Dunham R. 1984. Ancestry and breeding of catfish in the United States, Cir. 273. Alabama Agricultural Experiment Station. Auburn, AL: Auburn University.

Giorgi A, Mignogna G, Bellapadrona G, Gattoni M, Chiaraluce R, Consalvi V, Chiancone E, Stefanini S. 2008. The unusual co-assembly of H- and M-chains in the ferritin molecule from the Antarctic teleosts *Trematomus bernacchii* and *Trematomus newnesi*. Arch Biochem Biophys 478:69–74.

Graham RM, Chua ACG, Herbison CE, Olynyk JK, Trinder D. 2007. Liver iron transport. World J Gastroenterol 13:4725–4736.

Harrison PM, Arosio P. 1996. The ferritins: molecular properties, iron storage function and cellular regulation. Biochim Biophys Acta 1275:161–203.

Harrison PM, Ford GC, Smith JMA, White JL. 1991. The location of exon boundaries in the multimeric iron-storage protein ferritin. Biol Met 4:95–99.

Jain SK, Barrett KJ, Boyd D, Favreau MF, Crampton J, Drysdale JW. 1985. Ferritin-H and ferritin-L chains are derived from different multigene families. J Biol Chem 260:1762–1768.

Ju Z, Karsi A, Kocabas A, Patterson A, Li P, Cao D, Dunham R, Liu Z. 2000. Transcriptome analysis of channel catfish (*Ictalurus punctatus*): genes and expression profile from the brain. Gene 261:373–382.

Karsi A, Cao D, Li P, Patterson A, Kocabas A, Feng J, Ju Z, Mickett KD, Liu Z. 2002. Transcriptome analysis of channel catfish (*Ictalurus punctatus*): initial analysis of gene expression and microsatellite-containing cDNAs in the skin. Gene 285:157–168.

Lebo RV, Kan YW, Cheung MC, Jain SK, Drysdale J. 1985. Human ferritin light chain gene-sequences mapped to several sorted chromosomes. Hum Genet 71:325–328.

Levi S, Corsi B, Bosisio M, Invernizzi R, Volz A, Sanford D, Arosio P, Drysdale J. 2001. A human mitochondrial ferritin encoded by an intronless gene. J Biol Chem 276:24437–24440.

Li P, Peatman E, Wang S, Feng J, He C, Baoprasertkul P, Xu P, Kucuktas H, Nandi S, Somridhivej B, Serapion J, Simmons M, Turan C, Liu L, Muir W, Dunham R, Brady Y, Grizzle J, Liu Z. 2007. Towards the ictalurid catfish transcriptome: generation and analysis of 31, 215 catfish ESTs. BMC Genomics 8:177.

Li M, Saren G, Zhang S. 2008. Identification and expression of a ferritin homolog in amphioxus *Branchiostoma belcheri*: evidence for its dual role in immune response and iron metabolism. Comp Biochem Physiol B 150:263–270.

Liu H, Jiang Y, Wang S, Ninwichian P, Somridhivej B, Xu P, Abernathy J, Kucuktas H, Liu Z. 2009. Comparative analysis of catfish BAC end sequences with the zebrafish genome. BMC Genomics 10:592.

Liu H, Takano T, Abernathy MJ, Wang S, Sha Z, Jiang Y, Terhune J, Kucuktas H, Peatman E, Liu Z. 2010. Structure and expression of transferrin gene of channel catfish, *Ictalurus punctatus*. Fish & Shellfish Immunol 28:159–166.

Mignogna G, Chiaraluce R, Consalvi V, Cavallo S, Stefanini S, Chiancone E. 2002. Ferritin from the spleen of the Antarctic teleost *Trematomus bernacchii* is an M-type homopolymer. Eur J Biochem 269:1600–1606.

Neves JV, Wilson JM, Rodrigues PNS. 2009. Transferrin and ferritin response to bacterial infection: the role of the liver and brain in fish. Dev Comp Immunol 33:848–857.

Peatman E, Baoprasertkul P, Terhune J, Xu P, Nandi S, Kucuktas H, Li P, Wang S, Somridhivej B, Dunham R, Liu Z. 2007. Expression analysis of the acute phase response in channel catfish (*Ictalurus punctatus*) after infection with a Gram-negative bacterium. Dev Comp Immunol 31:1183–1196.

Peatman E, Terhune J, Baoprasertkul P, Xu P, Nandi S, Wang S, Somridhivej B, Kucuktas H, Li P, Dunham R, Liu Z. 2008. Microarray analysis of gene expression in the blue catfish liver reveals early activation of the MHC class I pathway after infection with *Edwardsiella ictaluri*. Mol Immunol 45:553–566.

Pfaffl MW, Horgan GW, Dempfle L. 2002. Relative expression software tool (REST (c)) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 30:e36.

Piccinelli P, Samuelsson T. 2007. Evolution of the iron-responsive element. RNA 13:952–966.

Recalcati S, Invernizzi P, Arosio P, Cairo G. 2008. New functions for an iron storage protein: the role of ferritin in immunity and autoimmunity. J Autoimmun 30:84–89.

Scudiero R, Trinchella F, Riggio M, Parisi E. 2007. Structure and expression of genes involved in transport and storage of iron in red-blooded and hemoglobin-less antarctic notothenioids. Gene 397:1–11.

Takano T, Sha Z, Peatman E, Terhune J, Liu H, Kucuktas H, Li P, Edholm ES, Wilson M, Liu Z. 2008. The two channel catfish intelectin genes exhibit highly differential patterns of tissue

expression and regulation after infection with *Edwardsiella ictaluri*. Dev Comp Immunol 32:693–705.

Tamura K, Dudley J, Nei M, Kumar S. 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599.

Thomson AM, Rogers JT, Leedman PJ. 1999. Iron-regulatory proteins, iron-responsive elements and ferritin mRNA translation. Int J Biochem Cell Biol 31:1139–1152.

Torti FM, Torti SV. 2002. Regulation of ferritin genes and protein. Blood 99:3505–3516.

Torti SV, Kwak EL, Miller SC, Miller LL, Ringold GM, Myambo KB, Young AP, Torti FM. 1988. The molecular-cloning and characterization of murine ferritin heavy-chain, a tumor necrosis factor-inducible gene. J Biol Chem 263:12638–12644.

USDA. 2006. Catfish Processing Report, National Agriculture Statistics Service, Service USDA Washington, D.C; (October 10, 2006).

Wang S, Xu P, Thorsen J, Zhu B, de Jong PJ, Waldbieser G, Kucuktas H, Liu Z. 2007. Characterization of a BAC library from channel catfish *Ictalurus punctatus*: indications of high levels of chromosomal reshuffling among teleost genomes. Mar Biotechnol NY 9:701–711.

Wang S, Peatman E, Abernathy J, Waldbieser G, Lindquist E, Richardson P, Lucas S, Wang M, Li P, Thimmapuram J, Liu L, Vullaganti D, Kucuktas H, Murdock C, Small BC, Wilson M, Liu H, Jiang Y, Lee Y, Chen F, Lu J, Wang W, Somridhivej B, Baoprasertkul P, Quilang J, Sha Z, Bao B, Wang Y, Wang Q, Takano T, Nandi S, Liu S, Wong L, Kaltenboeck L, Xu P, Quiniou S, Bengten E, Miller N, Trant J, Rokhsar D, Liu Z, Catfish Genome Consortium. 2010. Assembly of 500,000 inter-specific catfish expressed sequence tags and large scale gene-associated marker development for whole genome association studies. Genome Biol 11:R8.

Xu P, Wang S, Liu L, Peatman E, Somridhivej B, Thimmapuram J, Gong G, Liu Z. 2006. Channel catfish BAC-end sequences for marker development and assessment of syntenic conservation with other fish species. Anim Genet 37:321–326.

Xu P, Wang S, Liu L, Thorsen J, Kucuktas H, Liu Z. 2007. A BAC-based physical map of the channel catfish genome. Genomics 90: 380–388.

Yamashita M, Ojima N, Sakamoto T. 1996. Molecular cloning and cold-inducible gene expression of ferritin H subunit isoforms in rainbow trout cells. J Biol Chem 271:26908–26913.