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Molecular cloning and functional characterization of the hypoxia-inducible factor-1α in bighead carp (*Aristichthys nobilis*)

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Abstract HIF-l is the earliest documented and most widely studied hypoxia-inducible factor (HIF) and plays a key role in the cell hypoxia signal transduction pathway. Particularly, the HIF-1α protein is sensitive to oxygen and plays a critical role in hypoxia regulation. This study is the first to report on the molecular cloning and characterization of HIF-1α in bighead carp (*Aristichthys nobilis*; *anHIF-1α*). The full-length cDNA of *anHIF-1α* was 2361 bp, and encodes an estimated 674 amino acids with a predicted molecular mass of 76.10 kDa and a theoretical isoelectric point of 7.72. Moreover, the conserved basic Helix-Loop-Helix domain along with two Per-ARNT-Sim domains (A/B), and C-TAD were identified in this protein. Interestingly, the tertiary structure of the anHIF-1α protein was found to be extremely similar to that of mice. Multiple comparison and phylogenetic tree results demonstrated that anHIF-1α was highly conserved. Under normoxic conditions, *anHIF-1α* mRNA transcripts could be detected in all tissues examined with the highest expression level in the heart. With gradually decreasing oxygen concentrations, *anHIF-1α* mRNA level was upregulated significantly in the gill, liver, kidney, spleen, intestine, brain, and muscle tissues ($P < 0.05$). Similarly, *anHIF-1α* was expressed in all examined bighead carp tissues, and the results suggested that the upregulation of *anHIF-1α* at the transcriptional level may be an important stress response adaptation to hypoxia in bighead carp. Finally, based on the tertiary structure comparative analyses between anHIF-1α with mouse HIF-1α, we think the physiological function, and protein structure of HIF-1α could be compared between fish and mammal in the future.

Keywords *HIF-1α* · Molecular cloning · Hypoxia stress · *Aristichthys nobilis*

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Introduction

Dissolved oxygen occurs in water in its molecular form and plays an extremely important role in aquaculture. Importantly, its dissolved concentrations are affected by various environmental factors such as water temperature, salinity, water eutrophication, and algae photosynthesis (Hosfeld et al. 2010; Lushchak and Bagnyukova 2006). Particularly, fish require dissolved oxygen levels to be maintained above 4.0 mg L^{-1} to grow normally, and given that fish inhabit aquatic environments, they are more susceptible to hypoxia than terrestrial organisms. Fish tend to float towards the surface when dissolved oxygen concentrations decrease below 2.0 mg L^{-1}. If the oxygen level decreases further to 1.0 mg L^{-1}, most fish tend to die from suffocation (Richards 2011).

However, most fish have adapted and developed different molecular strategies to cope with hypoxia, including increasing oxygen transfer and reducing oxygen consumption. For example, fish can shift from aerobic to anaerobic metabolism and possess molecular mechanisms to reduce energy consumption during biosynthetic pathways (Sun et al. 2016). HIF-l is the earliest documented and most widely studied hypoxia-inducible factor, and is a key transcriptional factor for a cascade of hypoxia-associated genes (e.g., vascular endothelial growth factor, erythropoietin, glucose transporter 1 (Glut 1)). Therefore, this factor plays a key role in the cell hypoxia signal transduction pathway (Semenza et al. 2006; Semenza and Wang 1992; Iyer et al. 1998). A series of biological processes related to hypoxia are mediated by the expression of related genes in the HIF signaling pathway (Zhu et al. 2013).

HIF-1 is a heterodimer and consists of α subunits and β subunits (Geng et al. 2014). HIF-1 activity mainly depends on the α subunit because the HIF-1α protein is sensitive to oxygen and acts as a key hypoxia regulator, whereas HIF-1β protein is not sensitive to oxygen. Under normoxic conditions, the newly synthesized HIF-1α is immediately hydrolyzed by the ubiquitin-proteasome pathway (Uchida et al. 2004). Moreover, the half-life of HIF-1α is less than 5 min, and its intracellular concentration is so low that it is difficult to detect. However, under hypoxic conditions, the HIF-1α degradation pathway is blocked, and therefore HIF-1α is accumulated and activated in the cytoplasm, after which it is translocated to the nucleus (Kallio et al. 1999; Cockman et al. 2000; Bruick and Mcknight 2001). At this time, HIF-1β is also translocated from cytoplasm to nucleus, where HIF-1α and HIF-1β are polymerized to form stable and active HIF-1. HIF-1 then binds with the hypoxia-response element (HRE) on the target gene's 3' promoter or enhancer to form a transcription initiation complex and initiate transcription (Koivunen et al. 2006; Lando et al. 2002). There are many studies on the *HIF-1α* gene in fish. The first fish *HIF-1α* gene sequence was cloned from rainbow trout (*Oncorhynchus mykiss*) by Soitamo et al. (2001). Scientists then cloned grass carp (*Ctenopharyngodon idellus*) (Law et al. 2006), crucian carp (*Carassius carassius*) (Rissanen 2006), Atlantic croaker (*Micropogonias undulatus*) (Rahman and Thomas 2007), zebrafish (*Danio rerio*) (Rojas et al. 2007), sea bass (*Dicentrarchus labrax*) (Terova et al. 2008), Chinese sucker (*Myxocyprinus asiaticus*) (Chen et al. 2012), Wuchang bream (*Megalobrama amblycephala*) (Shen et al. 2010), Indian catfish (*Clarias batrachus*) (Mohindra et al. 2013), channel catfish (*Ictalurus punctatus*) (Geng et al. 2014), *Fundulus heteroclitus* (Townley, et al. 2017) and Nile tilapia (*Oreochromis niloticus*) (Li et al. 2017) *HIF-1α* genes and have studied their role in hypoxia regulation.

Bighead carp (*Aristichthys nobilis*) typically inhabits the middle and upper layer of the water column and is docile by nature, which facilitates its management and transportation. Its meat is delicate and values for its pleasant taste. Moreover, bighead head is especially rich in unsaturated fatty acids such as phospholipid, DHA, and EPA, and therefore, it highly prized by consumers. Currently, research on bighead carp, both domestic and international, mainly focuses on genetics and breeding projects (Slechtova et al. 2010; Nagorniuk et al. 2015; Liu et al. 2013; Zhu et al. 2015; Zhu et al. 2014), food science (Gao et al. 2018; Liu et al. 2018; Liu et al. 2017), microcystin accumulation and toxicity (Ni et al. 2015; Li et al. 2014; Zhang et al. 2012), nitrite exposure (Lin et al. 2018a, 2018b), and others. However, studies on *HIF-1α* in bighead carp have currently largely focused on the effects of nitrite stress on the *NF-κB/HIF-1α* pathway in gill (Lin et al. 2018a). However, the bighead carp *HIF-1α* sequence has not been cloned, and no data have been published regarding its tissue expression patterns. Therefore, our study sought to clone *HIF-1α* and characterize *HIF-1α* gene expression patterns in the tissues of bighead carp exposed to different levels of oxygen, thereby providing a theoretical basis for future research on bighead carp optimal breeding conditions.

Materials and methods

Animals

Bighead carp were obtained from the Nanquan breeding base of the Freshwater Fisheries Research Center and temporarily reared in tanks (specifications:φ1000mm × 1000 mm, 785 L; water depth: 700 mm) connected to a temperature-controlled recirculating water system for 1 week. The experiment was conducted after selecting 45 healthy individuals with similar physical characteristics (initial weight: 133.15 ± 0.39 g).

Hypoxia

After an acclimation period prior to the experiments, 45 bighead carp were randomly distributed into three groups of 15 individuals each and held in three tanks (specifications:φ1000mm × 1000 mm, 785 L; water depth: 600 mm). The experiment was divided into two experimental groups and one control group. The initial dissolved oxygen concentration of the culture water in the three groups was 7.8 mg /L. In the two experimental groups, the breeding tanks were not subjected to oxygenation and circulation, and the mouth of the tanks was sealed with a thin film, so that the oxygen in the tanks would gradually decrease with the consumption of bighead carp. The control group was continuously oxygenated without circulation to ensure that dissolved oxygen in water remains at 7.8 mg/L. At the same time, the dissolved oxygen in water was measured every hour. After 10 h, the dissolved oxygen in water decreased to 3.15 mg/L in experimental group 1, and 10% of the bighead carp appeared floating head phenomenon. After 18 h, the dissolved oxygen in water decreased to 0.5 mg/L in experimental group 2, and all the bighead carp appeared floating head phenomenon. During the experiment, dissolved oxygen was continuously monitored with dissolved oxygen meters (YSI pro20i), water temperature was maintained at 23.0 ± 1.0 °C, NH_4-N at ≤ 0.2 mg/L, and pH between 7.2 and 7.8. No mortality was observed under experimental conditions.

Sampling

Nine individuals were randomly selected from control group (dissolved oxygen concentration: 7.8 mg/L), experimental group 1(dissolved oxygen concentration: 3.15 mg/L) and experimental group 2 (dissolved oxygen concentration: 0.5 mg/L) and were immediately euthanized using tricaine mesylate (MS-222; 200 mg/L). The kidney, gill, heart, spleen, muscle, brain, intestines, and liver tissues were obtained from each sample, flash-frozen with liquid nitrogen, and stored at − 80 °C for downstream analyses of molecular cloning and *HIF-1α* mRNA levels.

Molecular cloning of full-length cDNA of *HIF-1α* gene

The liver tissue (weight < 0.1 g) obtained from the experimental group at 0.5 mg/L was transferred into 1 mL of RNAiso Plus and rapidly and fully homogenized with a high-throughput tissue breaker. The supernatant was removed, and RNA was extracted according to the reagent manufacturer´s instructions. RNA mass and concentration were detected with a NanoDrop 2000 system and only samples with D260nm/D280nm ratios between 1.8 and 2.0 were selected. Using the Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) cDNA was synthesized according to the kit's specifications and stored for later use at − 20 °C.

HIF-1α gene sequence fragments were obtained from the bighead carp transcriptome data, and primers were designed for nonfull-length *HIF-1α* gene sequence fragments (Table 1). Using cDNA as a substrate, intermediate fragment sequences were obtained via polymerase chain reaction (PCR) amplification, and the PCR products were purified and sequenced with a gel recovery kit (Shanghai Biological Engineering co., LTD, China). Based on the obtained intermediate fragment sequences, specific 5' and 3' RACE (rapid amplification of cDNA ends) primers were designed (Table 1). Afterward, 5' and 3' terminus sequences were amplified with the RACE and SMARTer^{TM} RACE amplification kits. The PCR products were purified with a gel recovery kit (Shanghai Biological Engineering co., LTD, China), after which they were inserted into a PMD-18T carrier (TaKaRa, Japan) to be sequenced on a DNA analyzer system (ABI3730, USA). Based on the intermediate fragment sequences from the 5 'RACE and 3' RACE results, a full-length cDNA sequence of *HIF-1α* was spliced. All primers were designed using Primer 5.0 and synthesized by Shanghai Biological Engineering co., LTD, China.

HIF-1α sequence analysis

The DNAman software was used herein to derive the corresponding amino acid sequence from the full *HIF-1α* cDNA sequence. The ProtParam (http://web.expasy.org/ protparam/) tool was used to analyze the basic properties of the predicted protein. Afterward, signal peptide and transmembrane domain analysis were performed with Phobius (http://phobius.sbc.su.se), and NLStradamus (http://www.moseslab.csb.utoronto.ca/NLStradamus) was used to perform subcellular localization analysis. NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/ NetNGlyc/) was used to perform N-linked glycosylation site predictions, and a conserved domain database was analyzed with Interpro (http://www.ebi.ac.uk/ interpro/). Swiss-model

Table 1 Primers for amplification and characterization

Primers	Sequence (5' to 3')	Application
A-Forward primer	GATCCACTGAGAGAAGAGT	Partial sequence PCR
A- Reverse primer	AAGAATGTAGTTGATGCAG	Partial sequence PCR
B-1	CCTCCTTTTCTTCTGC	5'RACE PCR
B-2	AATATTTGCCTTCTTCTAGC	5'RACE PCR
B-3	TTCCATGGCACTCTTCTCTC	5'RACE PCR
C-1	CCATCTGACTGCCAACAAATCCGC	3'RACE PCR
C-2	TCGACTGGGAAATATCGGCTGCTG	3'RACE PCR

(https://swissmodel.expasy.org/ interactive) was used to conduct predictive tertiary structure prediction. Phosphorylation sites were predicted with the Netphos software (http://www.cbs.dtu.dk/services/NetPhos). HIF-1α phylogenetic analysis of bighead carp was performed via the Neighbor-Joining method in the MEGA7.0 software, and a bootstrap approach was used to test the support rate of each node of the phylogenetic tree 1000 times. All sequences used in the analysis are summarized in Table 2.

Real-time quantitative RT-PCR analysis

The total RNA from all samples was uniformly diluted to 20 ng/μL using double-distilled nuclease-free water, after which *HIF-1α* and *β-actin* expression levels were measured in different tissues via RT-PCR using the method provided by the One-Step SYBR Prime Script TM PLUS RT-PCR kit (Takara, Japan). All assays were conducted in an ABI 7500 Real-time PCR System. After designing RT-PCR specific primers based on sequences from the NCBI database, the primers were synthesized by Shanghai Biological Engineering co., LTD (Table 3). The $2^{-\Delta\Delta Ct}$ method was employed to characterize *HIF-1α* and *β-actin* mRNA relative expression in different tissues in response to different oxygen concentrations.

Statistical analyses

Statistical analyses were carried out using the SPSS software (version 20.0). The differences in the level of *HIF-1α* mRNA in different tissues in response to

Table 2 HIF-1α protein sequences used for multiple sequence alignment and phylogenetic tree construction

Species	Accession no.	Species	Accession no.
Homo sapiens	EAW80809.1	*Scleropages formosus*	XP_018607083.1
Cyprinus carpio	XP_018929377.1	*Galeopterus variegatus*	XP_008564341.1
Carassius auratus	XP_026051762.1	*Salmo salar*	XP_013984964.1
Danio rerio	NP_001012371.2	*Bos taurus*	NP_776764.2
Anabarilius grahami	ROL47699.1	*Drosophila melanogaster*	NP_001263100.1
Astyanax mexicanus	XP_007244540.1	*Xenopus laevis*	NP_001080449.1
Ictalurus punctatus	XP_017348520.1	*Rattus norvegicus*	NP_077335.1
Esox lucius	XP_010893542.1	*Mus musculus*	NP_001300848.1
Oncorhynchus mykiss	CDQ63418.1	*Lipotes vexillifer*	XP_007471763.1
Acipenser baerii	ATY75506.1	*Helicoverpa armigera*	AMH87782.1
Ctenopharyngodon idella	AAR95697.2	*Hypophthalmichthys molitrix*	ADJ67806.1
Cynoglossus semilaevis	NP_001281116.1	*Megalobrama amblycephala*	ADF50043.1
Scylla paramamosain	ARO76395.1	*Siniperca chuatsi*	QDA76729.1

Table 3 PCR primers

Gene	GenBank Accession no.:	Primer	Primer sequences (5'-3')	Length (bases)
HIF-1α	MT740326	Forward primer	CTGACTGCCAACAAATCCGC	20
		Reverse primer	GAAGGGGCAGGATGGGATTC	20
β-action	AY170122.2	Forward primer	TCGTCCACCGCAAATGCTTCTA	22
		Reverse primer	CCGTCACCTTCACCGTTCCAGT	22

different oxygen concentrations were assessed using one-way ANOVA. If significant differences were found, Tukey's multiple range tests were used to determine the differences between means. The data were expressed as mean ± standard error (SE), $P < 0.05$ indicated significant differences.

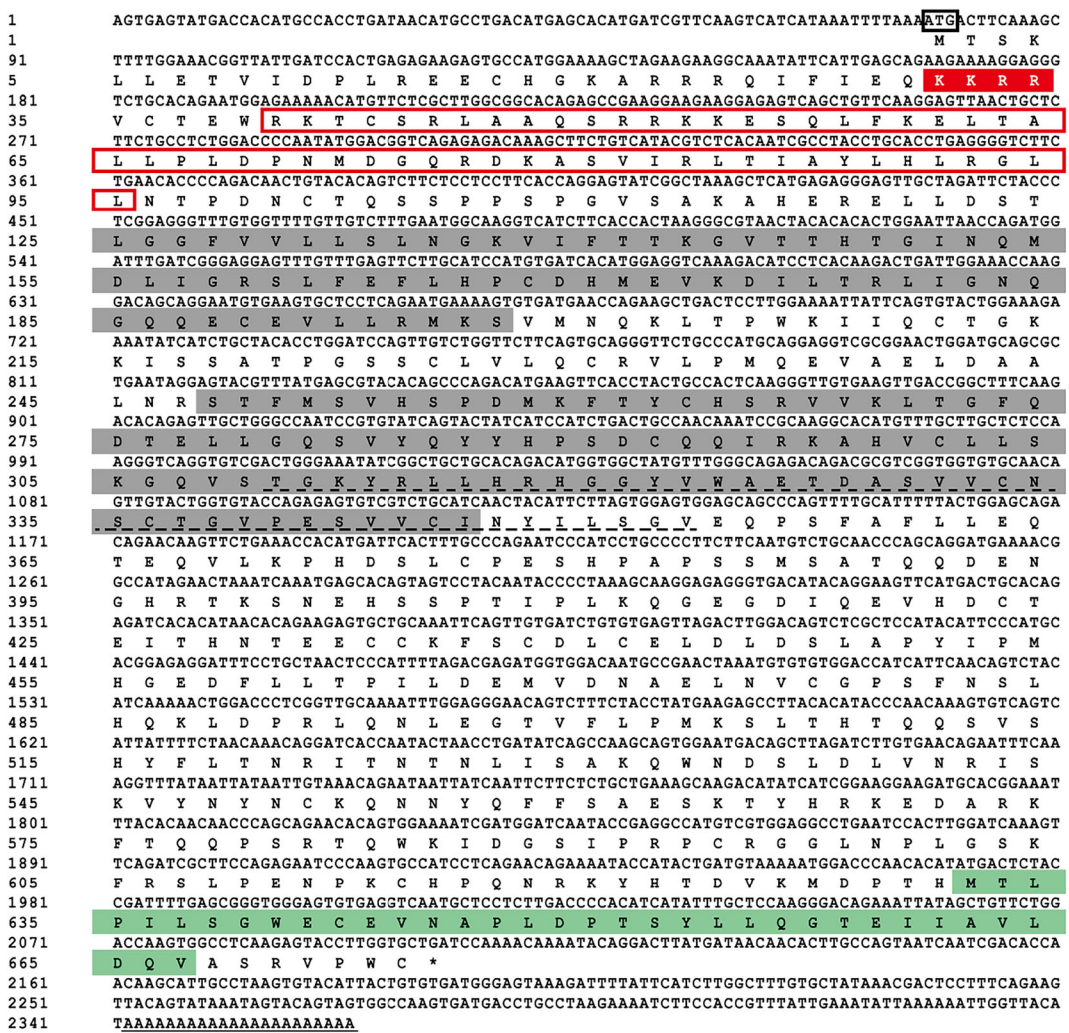

Fig. 1 Sequencing results and amino acid sequence analysis of anHIF-1α. Notes: The black box indicates the start codon (ATG) and the asterisk (*) indicates the termination codon (TGA), double underlines indicate poly (A) structures, red boxes denote possible bHLH domains, the gray shading indicates the two possible PAS domains, green shading indicates the possible HIF-1α C-terminal transcriptional activation domain, and the red-shaded fragment denotes the possible nuclear localization signal

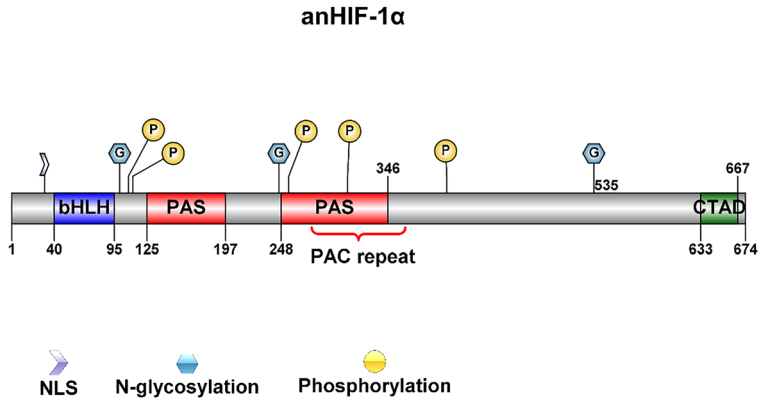

Fig. 2 Location of conserved domains, nuclear localization sequence (NLS), N-glycosylation, and phosphorylation of the HIF1α protein cloned from bighead carp

Results

Molecular cloning and structural analysis of *anHIF-α*

As shown in Fig. 1 and Fig. 2, the *anHIF-1α* sequence length of bighead carp was 2361 bp-long and encoded 674 amino acids. The predicted molecular weight of the encoded protein is 76.10 kDa, and its isoelectric point (pI) is 7.72. The total number of negatively charged residues (Asp + Glu) is 73, and the total number of positively charged residues (Arg + Lys) is 75. The predicted protein formula is $C_{3327}H_{5294}N_{948}O_{1011}S_{42}$. The instability index was computed to be 49.64, indicating that the protein is likely unstable. Using the NLStradamus software, the nuclear localization signal sequence of the anHIF-1α protein was located in amino acids 31–34 (KKRR).

Moreover, the anHIF-1α has four conserved domains, namely bHLH (40–95), two PAS (125–197, 248–346) domains, and C-TAD (633–667). Transmembrane domains and signal peptides were not identified, but three N-linked glycosylation sites and five phosphorylation were predicted. Subcellular localization analyses revealed the presence of nuclear localization signals.

HIF-α amino acid sequence alignment and phylogenetic tree construction

Comparative analyses of anHIF-α amino acid sequence similarity with other species were conducted via the NCBI smartBlast tool. Table 4 summarizes the species with high similarity to the bighead carp anHIF-α amino acid sequence, including *Anabarilius graham*, *Cyprinus carpio*, *Carassius auratus*, *Danio rerio*, *Triplophysa tibetana*, and *Chanos chanos*. Comparative sequence analysis showed that the bighead carp HIF-α protein shares the highest identity (92.66 %) with *Anabarilius grahami* among the listed HIF-1αs proteins (*E* value = 0).

Using the Clustal Omega software, the anHIF-1α sequence with that of other species was compared (Fig. 3), and phylogenetic trees were constructed with MEGA 7.0 (Fig. 4). The results of the established phylogenetic tree using drosophila HIF1α as an outgroup demonstrated that bighead carp HIF-1α clustered into a branch with members of the *Cypriniformes* order including *Anabarilius graham*, *Carassius auratus*, *Cyprinus carpio*, and *Danio rerio*.

Table 4 Similarity analysis of HIF-1α amino acid sequences between *Aristichthys nobilis* and other species

Protein	Species	GenBank Accession no.	Identity	Query cover
HIF-1α	*Anabarilius grahami*	ROL47699.1	92.66%	93%
	Cyprinus carpio	XP_018929377.1	79.09%	100%
	Carassius auratus	XP_026051762.1	77.06%	100%
	Danio rerio	NP_001012371.2	73.52%	100%
	Triplophysa tibetana	KAA0720175.1	65.58%	99%
	Chanos chanos	XP_030638909.1	50.07%	99%

Prediction of anHIF-1α protein structure

AnHIF-1α secondary structure analysis indicated that it contains 30 α-helixes and 13 β-strands. The tertiary structure of the anHIF-1α protein was also predicted in bighead carp with the SWISS-MODEL software. On the left side of Fig. 5, the green box represents the helix-loop-helix structure at the N-terminal end, the yellow box represents the first PAS domain at the N-terminal end, and the blue box represents the second PAS domain at the C-terminal end. The right side of Fig. 5 illustrates the result of the tertiary structure comparison between bighead carp HIF-1α (red) and mouse HIF1-α (cyan). It can be seen from the results that bighead carp HIF1α and mouse HIF1α were remarkably similar in spatial structure, number of HLH, number of ligands, and number of lanyard folds; the main difference is indicated with an arrow.

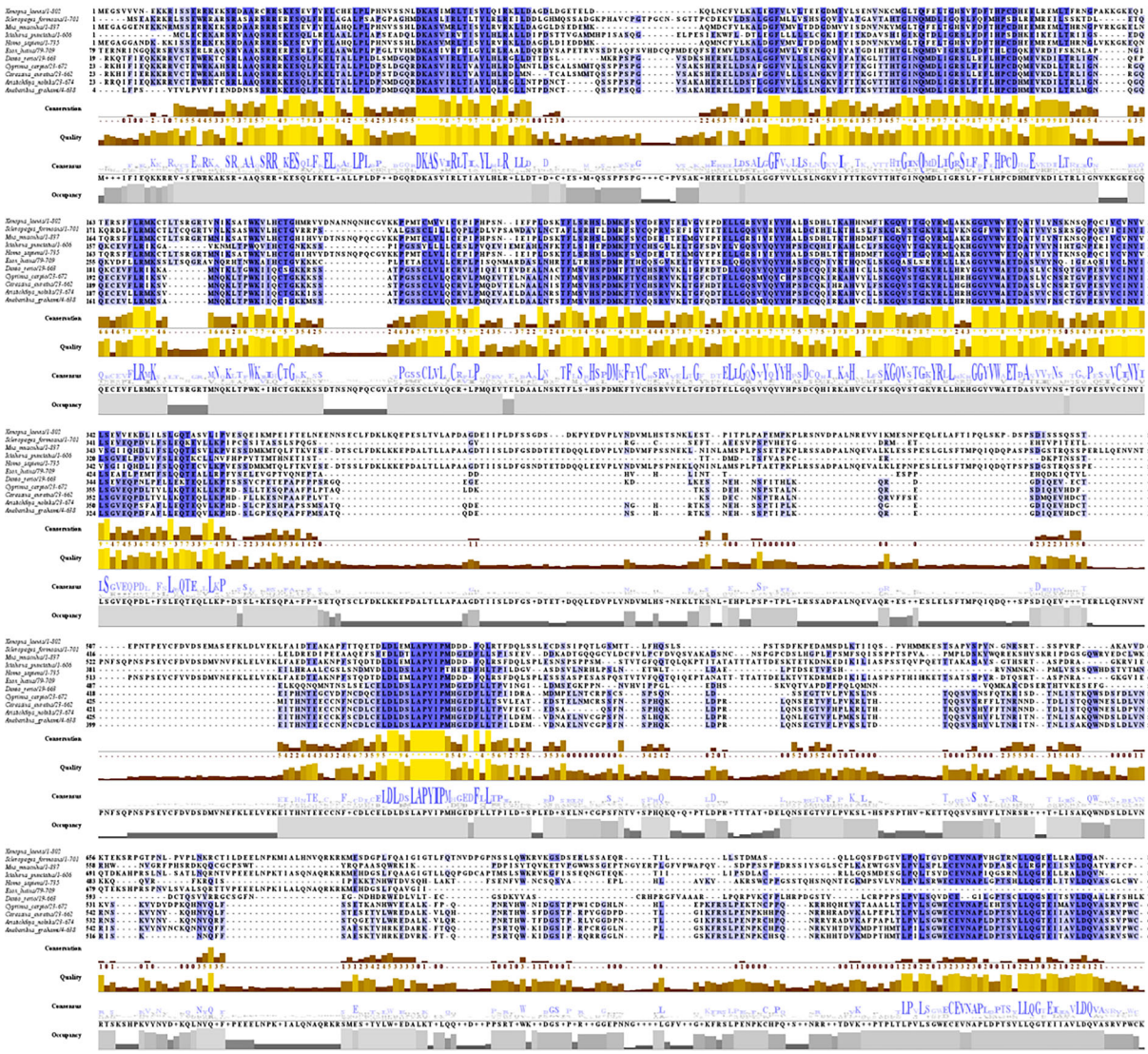

Fig. 3 Multiple-sequence alignment between the predicted bighead carp HIF-1α amino acid sequence and known HIF-1α sequences in other species. Notes: anHIF-1α sequences were aligned with those of *Homo sapiens, Mus musculus*, *Xenopus laevis*, *Scleropages formosus*, *Esox Lucius*, *Ictalurus punctatus*, *Anabarilius_grahami*, *Danio rerio*, *Carassius auratus*, and *Cyprinus carpio*. Gaps were inserted to maximize alignment and filled up with dashed lines. The "conservation" row column height indicates the conservation degree of corresponding amino acid sequences. The "consensus" row indicates the amino acids with the highest frequency in the corresponding column. Taller letters indicate higher frequencies, and therefore more conserved sequences

anHIF-1α mRNA expression in different tissues

As shown in Fig. 6, through the determination of *anHIF-1α* mRNA expression in different tissues in the 7.8 mg/L oxygen concentration group, the *anHIF-1α* mRNA expression level was highest in the heart and significantly higher than in other tissues ($P < 0.05$). The expression level in the liver and spleen was significantly higher than that in the gills, kidneys, intestines, and muscles ($P < 0.05$). Furthermore, *anHIF-1α* mRNA expression level in the brain was significantly higher than that in the kidneys, intestines, and muscles ($P < 0.05$).

Changes in *anHIF-1α* mRNA expression in response to different oxygen concentrations

As shown in Fig. 7, with decreased oxygen concentrations, *anHIF-1α* expression levels in the gill, liver, kidney, spleen, and intestine in the 3.15 mg/L and 0.5 mg/L oxygen concentration groups were significantly higher than that in the control group ($P < 0.05$). In brain and muscle tissues, the *anHIF-1α* expression levels in the 3.15 mg/L group were significantly higher than those in the 0.5 mg/L group and control groups ($P < 0.05$). Moreover, *anHIF-1α* expression levels of the 0.5 mg/L group was significantly higher than that in the control group ($P < 0.05$). However, heart transcription levels in the 3.15 mg/L group were significantly higher than those of the 0.5 mg/L group and the control group ($P < 0.05$).

Discussion and conclusion

Scientists first cloned the *HIF-1α* of fish from rainbow trout (Soitamo et al. 2001). Their results demonstrated that HIF-1α was present in fish, and identified the conserved bHLH domain (basic helix–loop–helix domain), as well as the PAS A/B (Per-ARNT-Sim A/B domain) region. Since then, scientists have been increasingly studying HIF-1α in fish because these organisms are easily affected by water hypoxia. In the introduction, we mentioned that *HIF-1α* had been cloned from various fish species and that its gene expression patterns had been studied. Here, we cloned, identified, and

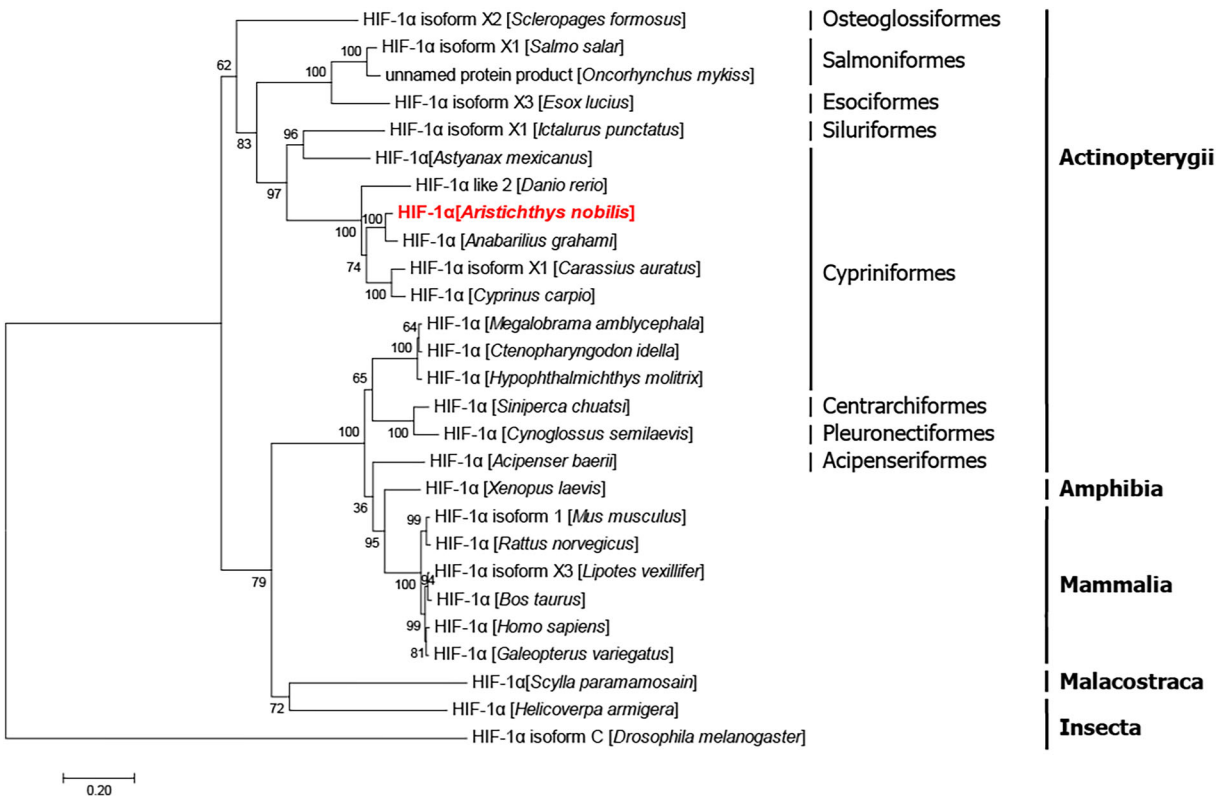

Fig. 4 Phylogenetic tree based on anHIF-1α amino acid sequences

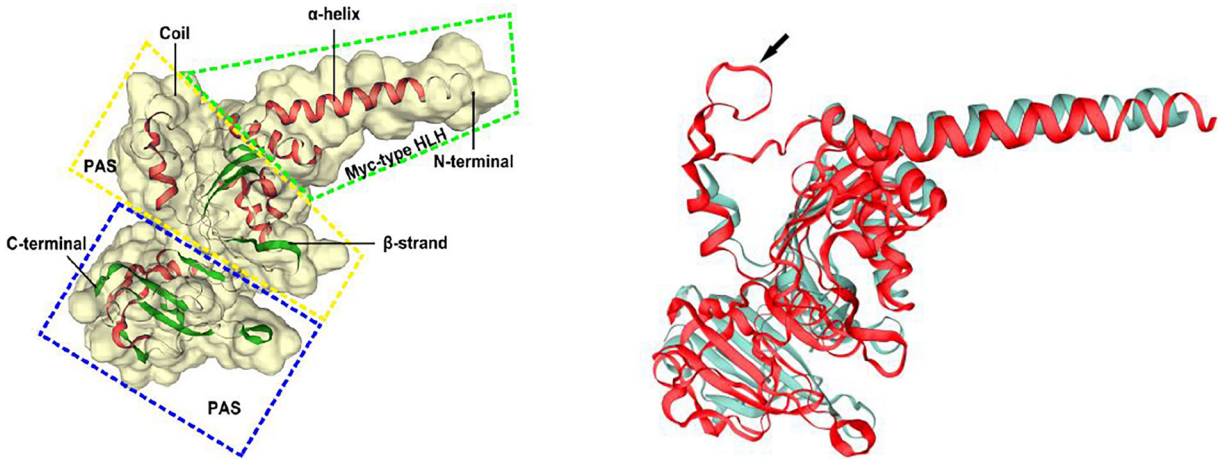

Fig. 5 *Aristichthys nobilis* HIF1α protein 3D structure prediction

characterized the *anHIF-1α* gene from the bighead carp. The full-length cDNA of *anHIF-1α* was 2,361 bp and it encoded a polypeptide of 674 amino acids. Our predicted amino acid sequence contains the conserved bHLH domain, as well as PAS A/B and C-TAD. Similar studies have been conducted in sea bass (Terova et al. 2008) and grass carp (Law et al. 2006). The conserved domain ensures the formation of HIF dimers that activate the transcription of downstream oxygen-sensitive genes in the predicted amino acid sequence. We also predicted the possible tertiary structure of anHIF-1α. The N-terminal of anHIF-1α has a typical MYB-type helix-loop-helix (HLH) structure, which can cooperate with the distal PAS domain to bind anoxic response elements on the DNA and act as transcription factors to activate downstream gene expression (Kajimura et al. 2005). Additionally, we also conducted a tertiary structure comparison between anHIF-1α and mouse HIF-1α (Wu et al. 2015). It could be seen from the results that anHIF-1α and mice HIF-1α shared important similarities in spatial structure, as well as in the number of HLH, α-helixes, and β-strands. The main difference was that the anHIF-1α protein possesses an additional random coil; however, this structure has little relation with the MYB-type HLH structure or the PAS domain and DNA binding sensitive sites. Therefore, we can compare the physiological function and protein structure of HIF-1α between fish and mammal in the future. Moreover, we found that anHIF-1α had no transmembrane domains or signal peptides. Subcellular localization analysis determined that anHIF-1α contains a nuclear localization signal. This was also confirmed by functional prediction analysis, which identified anHIF-1α as a transcription factor.

Multiple comparisons between the anHIF-1α and the HIF-1α from other vertebrate species evidently indicated that anHIF-1α is a member of the HIF-1α gene family, indicating that anHIF-1α might share similar biological functions to those of mammalian HIF-1αs. The same research indicated that the HIF-1α from crucian carp (Rissanen 2006) has a high homology with other cyprinid fish but a poor homology with rainbow trout. Moreover, HIF-1α from zebrafish (Rojas et al. 2007) was highly homologous with those of other vertebrates. In our study, phylogenetic tree analyses showed that anHIF-1α clustered into a branch with several *Cypriniformes* species including *Anabarilius graham*, *Carassius auratus*, *Cyprinus carpio*, and

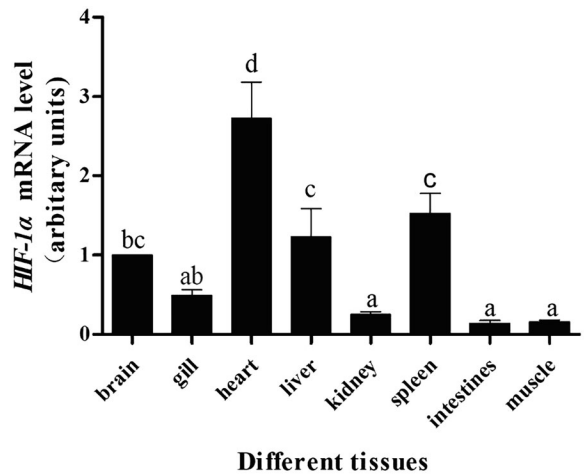

Fig. 6 Expression of *anHIF-1α* mRNA level in different tissues. Notes: Data are presented as means ± SE (n = 9). Values in the different tissues sharing different superscript letters indicate a significant difference, as determined by Tukey's test ($P < 0.05$)

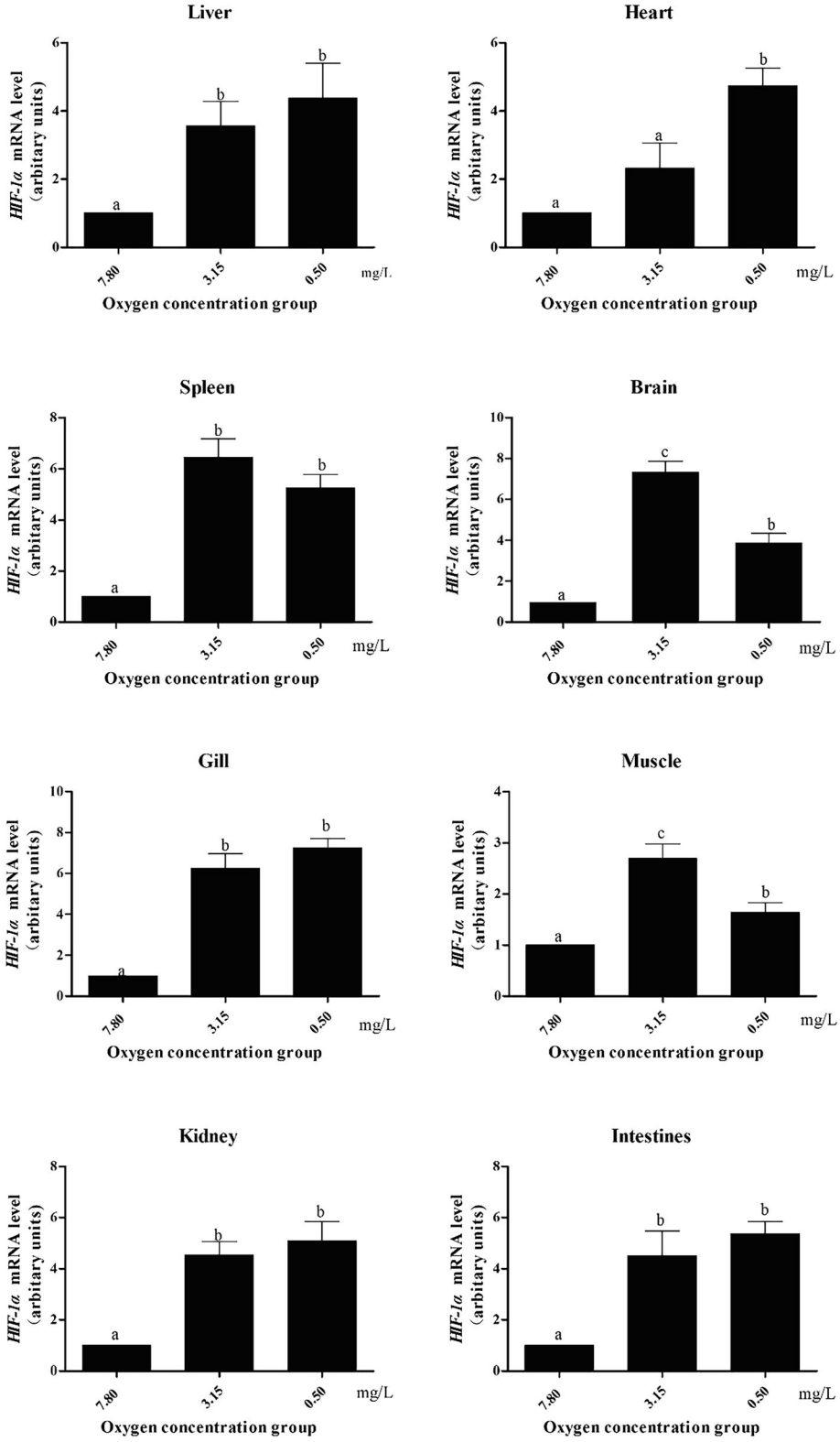
Liver
Heart
Spleen
Brain
Gill
Muscle
Kidney
Intestines
HIF-1α mRNA level (arbitary units)
Oxygen concentration group
7.80
3.15
0.50
mg/L

Fig. 7 Changes in *anHIF-1α* mRNA expression level at different oxygen concentrations. Notes: Data are presented as means ± SE (n = 9). Values in the different oxygen concentration groups sharing different superscript letters indicate a significant difference as determined by Tukey's test ($P < 0.05$)

Danio rerio. However, it is worth noting that not all *Cypriniformes* HIF-1αs were clustered together. For example, *Astyanax mexicanus*, which belongs to the *Cypriniformes*, was closer to the HIF-1α of *Siluriformes* such as *Ictalurus punctatus*. Further, the HIF-1α of *Megalobrama amblycephala*, *Ctenopharyngodon idella*, and *Hypophthalmichthys molitrix*, all of which also belong to the *Cypriniformes*, clustered into one branch with species belonging to the *Centrachiformes*, *Pleuronectiformes*, and *Acipenseriformes*, and shared stronger evolutionary relationships with HIF-1α of amphibians, crustaceans, and mammals. At this point, HIF-1α seems to have two distinct ancestral genes in fish that evolved separately but ultimately performed the same function. Many fishes have evolved the ability to survive extended periods of hypoxia (Zeraik et al. 2013). However, according to our analysis, blunt-snout bream, grass carp, and silver carp still have poor oxygen tolerance. Nonetheless, the results of Li et al. (2017) showed that the HIF-1α of fish and land animals were markedly divided into two branches, which may not be consistent with the fish selected in this experiment. Moreover, the aforementioned study did not include blunt-snout bream, grass carp, and silver carp in the pertinent analyses.

Under normoxic conditions, *anHIF-1α* mRNA expression was highest in the heart, followed by the spleen, liver, and brain. However, *anHIF-1α* exhibited low expression in the gills, kidneys, intestines, and muscle. In Chinese sucker (Chen et al. 2012), *HIF-1α* mRNA was most abundantly expressed in the liver and gonads, with lower expression levels being detected in spleen and muscle. A study in grass carp (Law et al. 2006) found that *HIF-1α* mRNA was highly expressed in the eye and kidney, with lower expression levels in the brain, gill, heart and liver, and muscle under normoxic conditions. *HIF-1α* mRNA was highly expressed in the liver, gill, and testis in *Megalobrama amblycephala* (Shen et al. 2010). A study in *Penaeus japonicus* demonstrated that *HIF-1α* mRNA was more highly expressed in the intestine than in any other organ tissues (Okamura et al. 2018). Importantly, these studies demonstrated that the expression patterns of *HIF-1α* in tissues of different species are different under normal oxygen conditions.

In our study, *anHIF-1α* mRNA levels were upregulated in the brain, muscle, gill, liver, kidney, spleen, and intestine tissues when oxygen concentration decreased from 7.8 mg/L to 3.15 and 0.5 mg/L. However, in brain and muscle tissues, the expression levels in the 3.15 mg/L group were also significantly higher than that of the 0.5 mg/L group. The heart expression levels in the 3.15 mg/L group were significantly higher than in the 0.5 mg/L and control groups. It has been suggested that hypoxia not only inhibits the degradation of HIF-1α but also induces the expression of *anHIF-1α* in various tissues of bighead carp. We speculated that hypoxia inhibits hydroxylation of proline residues in the oxygen- dependent degradation domain, and that HIF-1α is thus stabilized by the ubiquitin protease system, which does not recognize and degrade HIF-1α (Fandrey et al. 2006). Upon regulation of *anHIF-1α* expression, *anHIF-1α* potentially induces anaerobic metabolism by regulating cell metabolism to adapt to hypoxic conditions (Fraga et al. 2009; Papandreou et al. 2006), inducing angiogenesis to increase oxygen supply (Chin et al. 2007; Riddle et al. 2009; Shi 2009), and reducing the damage caused by hypoxia to tissues and cells (Adams et al. 2009) to protect them against hypoxia-induced damage. Another study (Law et al. 2006) also found that grass carp *HIF-1α* was significantly expressed in gill and kidney tissues after exposure to hypoxia for 4 h, whereas *HIF-1α* expression was seemingly downregulated in the brain, heart, and liver, and appeared unchanged in the eye. Moreover, *HIF-1α* mRNA was upregulated in the liver after 3 h of acute hypoxia (0.5 mgO_2/L) in Oscar (Baptista et al. 2016), whereas significant changes in *HIF-1α* mRNA levels were detected in muscle after chronic hypoxia exposure. Short-term hypoxia exposure (2.2, 2.8, and 3.2 mg/L dissolved oxygen for 24 h) also increased mRNA levels of *HIF-1α* in Chinese sucker (Chen et al. 2012). Kuruma shrimp (Okamura et al. 2018) intestinal expression levels of *HIF-1α* increased significantly after 24 h of hypoxia. Atlantic croaker (Rahman and Thomas 2007) HIF-1α mRNAs were highly expressed in the brain, heart, liver, and gonads under hypoxic conditions. In summary, studies on different fish species have demonstrated that low oxygen levels can upregulate the expression of the *HIF-1α* gene in different tissues.

In conclusion, the full-length sequence of *anHIF-1α* was cloned for the first time in bighead carp, and its amino acid sequence was predicted and analyzed. Our predicted amino acid sequence contains the conserved bHLH domain, as well as PAS A/B and C-TAD. Multiple comparison and phylogenetic tree results demonstrated that anHIF-1α was highly conserved. Based on the tertiary structure comparative analyses between anHIF-1α with mouse HIF-1α, we suggest that the physiological function and protein structure of HIF-1α could be compared between fish and mammal in the future. *anHIF-1α* expression levels in different tissues and at different oxygen concentrations were also quantified via qRT-PCR. The results suggest that *anHIF-1α* mRNA transcripts could be detected in all tissues examined with the highest expression level in the heart, and hypoxia induces the expression of *anHIF-1α* in various tissues of bighead carp. Molecular cloning analysis of *anHIF-1α* and the determination of mRNA level were carried out to characterize the molecular mechanisms of hypoxia-induced *anHIF-1α* expression, thereby providing a theoretical basis for healthy bighead carp cultivation.

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Authors' contributions Yan Lin, Ling-Hong Miao, and Xian-Ping Ge conceived the idea and designed the project. Bo Liu, Bing-Wen Xi, and Liang-Kun Pan performed the experiments. Yan Lin analyzed the data and wrote the manuscript. All authors have read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethics approval All experimental protocols were approved by the Bioethical Committee of Freshwater Fisheries Research Center (FFRC) of Chinese Academy of Fishery Sciences (CAFS) (BC 2013863, 9/2013)

Consent to participate All authors agree to participate.

Consent for publication All authors agree to publish.

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