



# Molecular cloning and expression pattern of IGFBP-2a in black porgy (*Acanthopagrus schlegelii*) and evolutionary analysis of IGFBP-2s in the species of Perciformes

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**Abstract** Insulin-like growth factor-binding protein-2 (IGFBP-2) plays a key role in regulating growth and development by its affinity with insulin-like growth factors (IGFs). In this study, we cloned the coding sequence (CDS) of IGFBP-2a from the black porgy (*Acanthopagrus schlegelii*) muscle and identified that the full-length CDS of IGFBP-2a was 882 bp. Real-time quantitative PCR revealed that IGFBP-2a was most abundant in the liver of the black porgy and backcross breed (F1 ♀ × black porgy ♂) but remained lower in each tested tissue in self-cross breed (F1 ♀ × F1 ♂). In addition, the IGFBP-2a expression in the liver of three breeds showed a negative correlation with their growth rates, indicating that the IGFBP-2a played a growth-inhibiting role in the three breeds. We further identified 810 bp IGFBP-2b gene from the draft genome of black porgy. Finally, we examined the IGFBP-2a and IGFBP-2b genes by scanning the genomes of the species of Perciformes

and found the IGFBP-2 gene duplication took place earlier than the divergence of perciform species. Interestingly, six positively selected sites were detected in both Perciformes IGFBP-2 genes, although both genes were identified to be under purifying selection. Specially, these positively selected sites were located in the functional domains, suggesting these sites played key roles in the growth of Perciformes. Our study partially explains the molecular basis for the prepotency in black porgy hybrids, which will provide guidance for their cultivation in the future.

**Keywords** IGFBP-2 · *Acanthopagrus schlegelii* · Cloning · Tissue distribution · Gene evolution

## Introduction

In vertebrates, insulin-like growth factor (IGF) system, which comprises of ligands (IGF-I and IGF-II), IGF receptors, and IGF-binding proteins (IGFBPs), plays a crucial role in regulating their growth, metabolism, reproduction, and longevity. Among them, IGFBPs, which are under the modulation of their proteases, have the capability to bind ligands and thereby alter their bioavailability, distribution, stability, and/or interactions with IGF receptors. Also, IGFBPs can modulate cell growth or differentiation through ligand-independent manners via their putative receptor on cell membrane or direct nuclear transportation actions (Duan 2002; Firth and Baxter 2002; Schedlich et al. 2000). It has been widely recognized that all mature IGFBP peptides

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share a conserved structure consisting of three domains: N-terminal and C-terminal domains which both contribute to its high-affinity IGF binding and the variable central linker domain (L-domain) that plays a role in maintaining structural integrity (Allard and Duan 2018; Carrick et al. 2002). Although sharing related structures, six distinct IGFBPs (IGFBP-1 to IGFBP-6) have unique properties, activating or suppressing the biological activities of IGFs (Garcia de la Serrana and Macqueen 2018).

IGFBP-2, the second most abundant IGFBP in serum, is expressed highly in the fetus and continuously in several tissues of the adult (Shin and Kang 2017; Wheatcroft and Kearney 2009). Previous studies in teleost reported that IGFBP-2 mRNA was most abundant in the liver and was also detectable in the gonad, brain as well as many peripheral tissues, suggesting its autocrine/paracrine actions on regulating IGFs (Chen et al. 2010a; Funkenstein et al. 2002; Kamangar et al. 2006; Zhang et al. 2013). Researches in zebrafish and common carp revealed that IGFBP-2 acted as a negative growth regulator downstream of the growth hormone (GH) (Chen et al. 2009; Chen et al. 2014; Duan et al. 1999). In addition, early-stage zebrafish overexpressing IGFBP-2 showed a reduced growth and developmental rate (Wood et al. 2005; Zhou et al. 2008). IGFBP-2 expression was upregulated in fasted zebrafish (Duan et al. 1999) and decreased in Atlantic salmon upon post-fasting refeeding (Bower et al. 2008; Macqueen et al. 2013; Valente et al. 2012), both indicating its inhibitory effect on growth. Yet a few studies, on the contrary, reporting that such conclusion was not replicable using other nutritional conditions or other experimental species also exist (Amaral and Johnston 2011; Gabillard et al. 2006; Garcia de la Serrana et al. 2017; Pedroso et al. 2009; Safian et al. 2012; Valente et al. 2012).

Duplicate copies of IGFBP-2s (IGFBP-2a and IGFBP-2b) have been cloned in many teleosts, such as zebrafish (*Danio rerio*), medaka (*Oryzias latipes*), fugu (*Takifugu rubripes*), tetraodon (*Takifugu nigroviridis*), three-spined stickleback (*Gasterosteus aculeatus*) (Zhou et al. 2008), common carp (*Cyprinus carpio*) (Chen et al. 2010b), Japanese flounder (*Paralichthys olivaceus*) (Zhang et al. 2013), and goldfish (*Carassius auratus*) (Chen et al. 2014). However, only one IGFBP-2 has been found in some species, including gilthead seabream (*Sparus aurata*) (Funkenstein et al. 2002),

rainbow trout (*Oncorhynchus mykiss*) (Kamangar et al. 2006), orange-spotted grouper (*Epinephelus coioides*) (Chen et al. 2010a), yellowtail (*Seriola quinqueradiata*) (Pedroso et al. 2009), Atlantic croaker (*Micropogonias undulatus*) (Rahman and Thomas 2011), and fine flounder (*Paralichthys adspersus*) (Safian et al. 2012). So far, the duplicated IGFBP-2s display similar biological functions but distinct expression patterns (Zhang et al. 2013; Zhou et al. 2008), while their respective molecular evolution has not been well studied.

Black porgy (*Acanthopagrus schlegelii*), also known as black seabream, belongs to the family Sparidae in the order Perciformes. Owing to their delicious taste, euryphagous nature, and a capability to survive a wide range of temperature, as well as adapt to different salinity, black porgies have been successfully cultured as an important commercial species in China and Japan (Gonzalez et al. 2008; Hsu et al. 2008). In order to overcome the drawbacks of their slow growth and long breeding cycle, F1 hybridization has long been carried out between black porgy and red porgy (*Pagrosomus major*) since red porgy are known for their large size and rapid growth but poor stress resistance (Park et al. 2006; Zhu et al. 2016). Now, using hybrid F1 (black porgy ♀ × red porgy ♂) as a female parent, we have, for the first time, successfully cultured backcross breed (F1 ♀ × black porgy ♂) and self-cross breed (F1 ♀ × F1 ♂). These new breeds were developed in an attempt to further screen for new marine aquaculture species with optimal target traits. In terms of growth rate, self-cross breed are apparently better than parent black porgy, yet backcross breed are not so good as the self-cross ones (Table S1, Online Resource 1). The availability of these breeds not only provided basic knowledge for black porgy mariculture but also offered the unique opportunity to explore the impact of fish IGFBP-2s.

In the present study, molecular and bioinformatic methods were applied to uncover the role of IGFBP-2 in perciform fish. First, we identified and characterized the coding sequence (CDS) of both IGFBP-2a and IGFBP-2b in black porgy. Second, the tissue distribution of IGFBP-2a was measured in different sea porgy breeds, whose result supported its growth inhibitory effect and partially revealed the prepotency of self-cross breed. Third, evolutionary analyses on Perciformes IGFBP-2s further affirmed the functional significance of IGFBP-2s in teleost and revealed their separate evolutionary patterns.

## Materials and methods

### Fish and sample collection

Healthy 8-month-old sea porgies were obtained from the Marine Fisheries Research Institute of Jiangsu Province (China). Before sampling, black porgy, back-cross breed, and self-cross breed were raised by single species culture, bred under the same outdoor pond condition and controlled to the same density. Samples of each breed were randomly selected in December 2017, with body lengths and body weights measured (Table 1). The muscle of black porgy was used for gene cloning of IGFBP-2a, while five tissues (liver, muscle, brain, heart, and gonad) from each breed were collected for tissue distribution analysis. All the tissue samples were isolated, soaked overnight in RNAlater RNA Stabilization Reagent (Qigan, China) at 4 °C, and stored at –20°C before RNA extraction.

### Cloning of IGFBP-2a cDNA in black porgy

Total RNA was extracted from the muscle samples of black porgy according to the manufacturer's instructions for TRIzol reagent. RNA samples were incubated in RNase-free DNase I (Promega, USA) to eliminate any genomic DNA contamination. First strand cDNA was synthesized from total RNA using PrimeScript® First Strand cDNA Synthesis Kit (Takara, Dalian, China) with an oligo(dT) primer following the manufacturer's instructions.

The initial RT-PCR was performed using degenerate primers (Table 2: *IGFBP-2-F1* and *IGFBP-2-R1*). PCR amplification was carried out using Ex Taq polymerase (Takara, Japan) under the following cycling conditions: an initial denaturation step (94 °C, 2 min), followed by 35 cycles of denaturation (94 °C, 30 s), annealing

(55 °C, 30 s), and extension (72 °C, 1 min), ending with a 10-min extension phase at 72 °C. The target PCR products were purified using OMEGA Gel Extraction Kit (OMEGA, USA) and then cloned into vector. After that, sequence analysis was conducted on the selected positive clones.

In order to obtain the full-length CDS of IGFBP-2, 3' rapid amplification of cDNA ends (RACE) was carried out. We synthesized first strand cDNA for 3' RACE from total RNA using 3' CDS primer A by SMARTScrib Reverse Transcriptase. The first PCR amplification for 3' RACE was performed using a universal primer together with a gene-specific primer *IGFBP-2-3gsp1* (Table 2), which was designed on the basis of the partial fragments sequenced above. The initial 3' RACE product was diluted 50 times and then mixed with a nested universal primer as well as the nested primer *IGFBP-2-3gsp2* (Table 2) for the purpose of nested PCR. The amplified fragments were separated and purified by agarose gel electrophoresis, cloned into pMD18-T easy vector, and subsequently sequenced. The 5' ends of black porgy IGFBP-2 were derived from its transcriptome data (unpublished data).

The prediction of full-length IGFBP-2 CDS was assembled by alignment of the partial cDNA fragments and 3' RACE fragment. Its initiation and termination codon positions were then analyzed using the ORF Finder in NCBI (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html/>). Using primers matching the respective start and end of the coding region (Table 2: *IGFBP-2-F2* and *IGFBP-2-R2*), the amplification of full-length CDS was carried out and later sequenced to confirm the former prediction of black porgy IGFBP-2 CDS. The PCR reaction contained 1 cycle of denaturation at 94 °C for 2 min, as well as 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min 30 s, followed by a 1-cycle final elongation step at 72 °C for 10 min. All the primers were designed by the Primer Premier 5.0 software (Premier Biosoft International, USA) and synthesized by Sangon Biotech (Shanghai) Co., Ltd. (China).

**Table 1** Body lengths and body weights of sea porgy in the present study

Sea porgy breed	<i>n</i>	Body weight (g)	Body length (cm)
Black porgy	3	75.47 ± 11.00	16.4 ± 0.6
Self-cross breed	3	108.39 ± 12.79	18.6 ± 0.5
Backcross breed	3	79.09 ± 4.29	17.0 ± 0.5

The values were expressed as means ± standard error of the mean (SEM)

### Sequence identification and analysis of black porgy IGFBP-2

The CDS of black porgy IGFBP-2b was derived from the CDS prediction in our recent research about black porgy genome (GigaDB DOI: <https://doi.org/10.5524/100409>). After the translation of the

IGFBP-2 genes into their amino acid sequences in MEGA 7 (Kumar et al. 2016), both cDNA sequence and the deduced amino acid sequence were again confirmed by comparison with the sequences in the GenBank database using NCBI-BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>).

Potential domains of the amino acid sequence were identified with the Simple Modular Architecture Research Tool server (SMART; <http://smart.embl-heidelberg.de/>) (Schultz et al. 2000) and Motifscan ([http://myhits.isb-sib.ch/cgi-bin/motif\\_scan](http://myhits.isb-sib.ch/cgi-bin/motif_scan)) (Hulo et al. 2008). The SignalP 4.1 Server (<http://www.cbs.dtu.dk/services/SignalP/>) was used to predict its signal peptide and the molecular mass of mature black porgy IGFBP-2a protein was estimated by DNAMAN 8 (<https://www.lynnon.com/>).

We then used IGFBP-2a or IGFBP-2b (Table 3) as queries to search for their homologous genes from the genome assembly of red porgy (GCA\_002897255.1), clown anemonefish (*Amphiprion ocellaris*, GCA\_002776465.1), and black rockcod (*Notothenia coriiceps*, GCA\_000735185.1) by Local Blast (BlastN) (Zhang et al. 2018) with the *E* value cutoff of  $10^{-5}$  (Johnson et al. 2008). The nucleotide and deduced amino acid sequence were aligned using both ClustalW and MUSCLE in MEGA 7, checked by visual inspection, and further verified through NCBI-BLAST. IGFBP-2 sequences of other Perciformes were downloaded from GenBank, with accession numbers listed in Table 3. Sequence alignments were performed according to their deduced amino acid sequences by ClustalW in MEGA 7.

The phylogenetic trees of IGFBP2s in Perciformes were reconstructed using Bayesian inference (BI) in MrBayes 3.2.3 (Ronquist and Huelsenbeck 2003) and Neighboring-Joining (NJ) methods in MEGA 7. GTR+GAMMA+I model was selected as the best-fit model of nucleotide substitution for BI tree reconstruction by MrModeltest 2.3 (Nylander 2004). BI tree was run for  $1 \times 10^6$  generations with four Markov chains and trees were sampled every 100 generations. NJ tree was reconstructed in MEGA 7 with 1000 bootstrap replicates (Kumar et al. 2016). The trees were rooted using IGFBP-2s from human (NC\_000002.12) and mouse (NC\_000067.6) as an outgroup (Fig. 4 and Online Resource 2).

Real-time quantitative PCR analysis of IGFBP-2a for tissue distribution

Relative IGFBP-2 mRNA expression levels were quantified by real-time quantitative PCR (RT-qPCR) in various tissues for each breed of sea porgy (Fig. 3). Total RNA was extracted from five tissues (liver, muscle, brain, heart, and gonad) of three 8-month-old sea porgy breeds (black porgy, backcross, and self-cross) according to the manufacturer's instructions for TRIzol reagent. A total of 500 ng RNA from each sample was reverse-transcribed with PrimeScript® First Strand cDNA Synthesis Kit (Takara, Dalian, China) with the Oligo(dT) Primer according to the protocol of the manufacturer. The housekeeping gene  $\beta$ -actin gene was used as an internal reference gene. Real-time PCR assays were carried out using SYBR

**Table 2** Sequence of primers used in the present study

Primer	Sequence(5'-3')	Application
<i>IGFBP-2-F1</i>	GGATTGCCTTCCATCGCC	<i>IGFBP-2</i> fragment cloning
<i>IGFBP-2-R1</i>	CTTTTCTTGTTTCACGCC	
<i>IGFBP-2-F2</i>	CCGGCTTCACCTGTTTCGC	
<i>IGFBP-2-R2</i>	ACGCCACACTATATCTGG	
<i>IGFBP-2-3gsp1</i>	AGAGGGGCGAGTGCTGGTGCGTC	<i>IGFBP-2</i> 3' RACE
<i>IGFBP-2-3gsp2</i>	CGACCCATCCCATCAGCCCCCACT	
<i>IGFBP-2-QRT-F</i>	GTCAGCGAGATCCCTGTGCATC	<i>IGFBP-2</i> qRT-PCR
<i>IGFBP-2-QRT-R</i>	TTCACCTCTCCACCTTGTTG	
<i><math>\beta</math>-actin-QRT-F</i>	TATCGTCATGGACTCCGGTG	<i><math>\beta</math>-actin</i> -qRT-PCR
<i><math>\beta</math>-actin-QRT-R</i>	TGATGTCACGCACGATTTC	

*F* forward primer, *R* reverse primer

**Table 3** Comparison of amino acid identities between the cloned black porgy IGFBP-2 and those of other perciform fish

Binomial name	Common name	GenBank accession no.	Identity (%)	Remark
<i>Seriola dumerili</i>	Greater amberjack	NW_019174274.1	96.2	
<i>Seriola lalandi dorsalis</i>		NW_019525183.1	96.2	IGFBP-2a
<i>Amphiprion ocellaris</i>	Clown anemonefish	NW_019372800.1	93.9	IGFBP-2a
<i>Larimichthys crocea</i>	Large yellow croaker	NW_017609283.1	93.5	
<i>Lates calcarifer</i>	Barramundi perch	NW_017366531.1	93.1	IGFBP-2a
<i>Notothenia coriiceps</i>	Black rockcod	NW_011360359.1	92.5	
<i>Haplochromis burtoni</i>	Burton's mouthbrooder	NW_005180140.1	90.6	
<i>Pundamilia nyererei</i>		NW_005187802.1	89.8	
<i>Oreochromis niloticus</i>	Nile tilapia	NC_031986.1	89.8	
<i>Neolamprologus brichardi</i>		NW_006272071.1	89.8	IGFBP-2a
<i>Maylandia zebra</i>	Zebra mbuna	NW_014447147.1	89.4	
<i>Epinephelus coioides</i>	Orange-spotted grouper	KR269815.1	56.6	
<i>Larimichthys crocea</i>	Large yellow croaker	NW_017608399.1	55.1	IGFBP-2b
<i>Lates calcarifer</i>	Barramundi perch	NW_017365854.1	55.0	IGFBP-2b
<i>Seriola lalandi dorsalis</i>		NW_019525242.1	53.8	IGFBP-2b
<i>Seriola dumerili</i>	Greater amberjack	NW_019174307.1	53.6	IGFBP-2b
<i>Haplochromis burtoni</i>	Burton's mouthbrooder	NW_005179473.1	51.9	IGFBP-2b
<i>Sparus aurata</i>	Gilthead seabream	AF377998.1	51.6	
<i>Pundamilia nyererei</i>		NW_005187472.1	51.5	IGFBP-2b
<i>Oreochromis niloticus</i>	Nile tilapia	NC_031987.1	51.5	IGFBP-2b
<i>Maylandia zebra</i>	Zebra mbuna	NC_036795.1	51.5	IGFBP-2b
<i>Neolamprologus brichardi</i>		NW_006272030.1	50.7	IGFBP-2b

The amino acid sequence identities were calculated with Multiple Sequence Alignment in NCBI-BLAST

Premix Ex Taq (Takara, Dalian, China) and performed on CFX Connect™ Real-Time PCR Detection System (Bio-Rad, USA). Specific primers (Table 2) of  $\beta$ -actin and IGFBP-2a were designed for RT-qPCR using Primer Premier 5.0 software.

RT-qPCR was performed in 10.0  $\mu$ l of reaction mix containing 1.0  $\mu$ l of cDNA sample, 5.0  $\mu$ l of 2 $\times$  SYBR® Premix Ex Taq II (Tli RNaseH Plus) (Takara, Dalian, China), 0.2  $\mu$ l of each primer (10  $\mu$ M), and 3.6  $\mu$ l of nuclease-free water. The qRT-PCR cycling conditions were as follows: 95 °C (3 min), followed by 40 cycles of 95 °C (10 s), 55 °C (30 s), and 72 °C (30 s). Finally, melting curve analysis was conducted. Each sample was performed in triplicate. Comparative  $C_T$  method was used to determine the relative abundance of IGFBP-2 mRNA according to the equation  $2^{-\Delta\Delta C_T}$ .  $\Delta C_T$  values were generated by normalizing the expressions of IGFBP-2 against the expressions of  $\beta$ -actin. The data were presented as the relative expression levels (means

$\pm$  SEM), and significant differences ( $P < 0.05$ ) were analyzed by one-way analysis of variance (ANOVA).

#### Selective pressure detection of IGFBP-2 genes in Perciformes

To test for the selective pressure for each IGFBP-2 gene, the nonsynonymous/synonymous substitution ratios ( $\omega = d_N/d_S$ ) were used as a measure in protein-coding sequences, where  $\omega > 1$ ,  $= 1$ , and  $< 1$  indicate positive selection, neutral selection, and purifying selection, respectively. The  $\omega$  ratios were estimated by codon-based maximum likelihood (ML) models (PAML release 4.7) (Yang 2007).

The phylogeny used as the input trees was reconstructed based on mitochondrial cytochrome c oxidase subunit I (COX1) genes of perciform fish with zebrafish (*D. rerio*) as the outgroup using Randomized Axelerated Maximum Likelihood (RAxML 8) (Stamatakis 2014), a

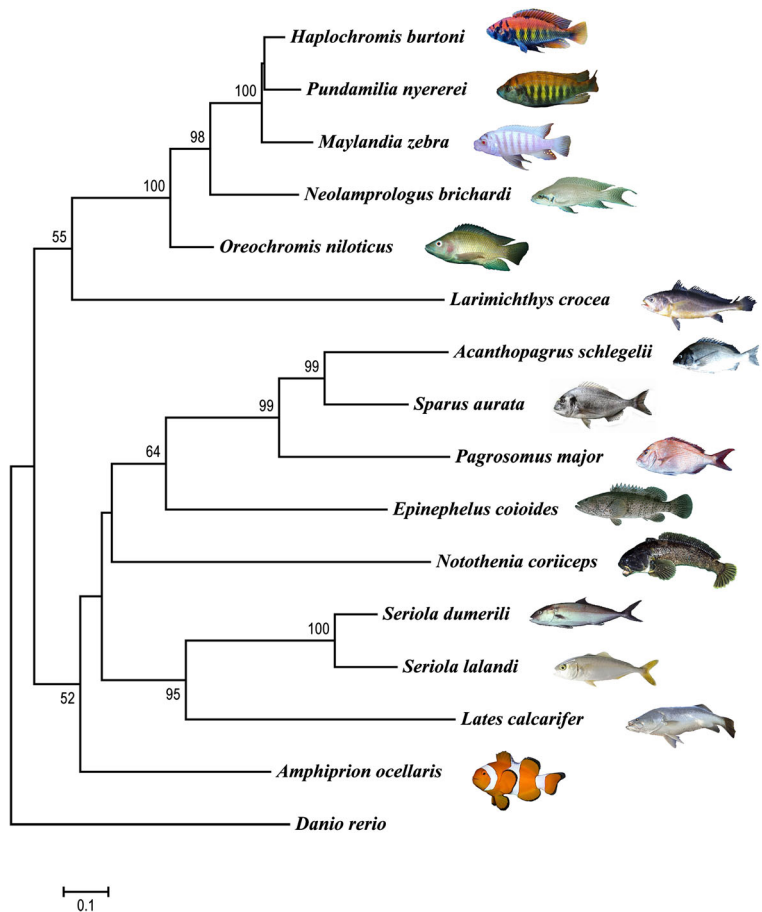
program for phylogenetic analyses of large datasets under maximum likelihood (Fig. 1). Its best-fit model was chosen as GTR+GAMMA+I with MrModeltest 2.3. The best-scoring ML tree was estimated with 1000 rapid bootstrapping replicates. The GenBank accession numbers for the COX1 sequences used are described in Table S2, Online Resource 1. Besides, in order to prevent false positive results, it is better to use high-quality alignment, stripping poorly aligned regions. Thus, the sequences were realigned by Prank-Codon, tested by alignment trimming (Sun 2017), and then treated with Gblocks (Castresana 2000) before conducting PAML tests.

Site models, where  $\omega$  could vary among sites (Nielsen and Yang 1998; Yang and Nielsen 2000), were implemented to examine the probabilities of sites under positive selection at each codon of IGFBP-2a or IGFBP-2b (Table 4). In particular, two pairs of models were tested: M0 (one-ratio) vs. M3 (discrete) and M7 ( $\beta$ ) vs. M8 ( $\beta$  and  $\omega$ ) (Yang

and Bielawski 2000). Among them, M0 had only one  $\omega$  ratio, whereas M3 was a discrete model which allows for three classes of sites with different  $\omega$  ratios. Both M7 and M8 assumed  $\beta$ -distribution among sites, but M8 differed from M7 in one additional class for positive selection with  $\omega > 1$ . Here, M0 and M3 models were implemented as a test of variable  $\omega$  among sites, while candidates for positively selected sites were identified in M8 by Bayes Empirical Bayes (BEB) analysis with posterior probabilities  $\geq 0.80$  (Yang 2007). The nested models were compared using a likelihood ratio test (LRT) with a  $\chi^2$  distribution at a threshold of  $P < 0.05$ .

To further investigate the functional significance of the putative sites under positive selection, we mapped the selected sites onto the three-dimensional (3D) structures of the IGFBP-2 proteins (Fig. 5). The 3D structures of black porgy IGFBP-2a and IGFBP-2b were deduced using Iterative Threading ASSEMBly Refinement (I-TASSER; [https://zhanglab.ccmb.med.umich.edu/I-](https://zhanglab.ccmb.med.umich.edu/I-TASSER/)

**Fig. 1** Phylogenetic trees reconstructed using maximum likelihood (ML) methods in RAxML 8 based on COX1 gene sequences of Perciformes. This tree was used as an input tree for selective pressure analysis. Numbers above the clades represent bootstrap values (%)



TASSER/), a hierarchical approach to protein structure and function prediction (Yang et al. 2015). The deduced positive sites were mapped on to the predicted structures by PYMOL (<http://pymol.org/2/>).

## Results

### Identification and characterization of black porgy IGFBP-2s

Using two degenerate primers, a cDNA fragment, 924 bp in length, was amplified from total RNA of black porgy. Subsequently, 3' RACE revealed products of 600 bp by two rounds of PCR. Assembly of the two products resulted in a 1403-bp cDNA of the black porgy IGFBP-2 containing a 501-bp 3'-untranslated region (UTR) and an 882-bp complete open-reading frame (ORF). The cDNA sequence of the black porgy IGFBP-2 was further verified through full-length CDS cloning using primers matching the potential start and end of the coding region. The ORF encodes a predicted polypeptide of 293 amino acid residues with a putative signal peptide of 33 amino acids. Thus, the mature protein has 260 amino acid residues and an estimated molecular mass of 29.5 kDa. The CDS of black porgy IGFBP-2a has been submitted to GenBank under the accession number of MH794248.

Homology analysis revealed that the deduced IGFBP-2 protein shared high similarities with other Perciformes IGFBP-2as (Table 3), the greatest of which was 96.2% with *Seriola dumerili* or *Seriola lalandi dorsalis*. It was no less than 89.4% similar to other identified IGFBP-2a sequences, while showing relatively lower identities to IGFBP-2bs (50.7–56.6%), indicating that it belongs to the IGFBP-2a subfamily.

We also searched the CDS of black porgy IGFBP-2b from its draft genome (Zhang et al. 2018), which turns out to be 810-bp long. Similar to IGFBP-2a, a putative signal peptide of 22 residues lies in the front of its deduced 271-aa polypeptide. Thus, the mature protein is predicted to have 249 amino acid residues and a 27.7-kD molecular mass.

Like other vertebrate IGFBPs, mature IGFBP-2 proteins of black porgy have highly conserved cysteine-rich domains both in N-terminal (12 cysteine residues) and C-terminal (6 cysteine residues) (Fig. 2). Also, a typical IGFBP motif (GCGCCXXC) exists within N-domain, and the characteristic Arg-Gly-Asp (RGD) sequence

together with a thyroglobulin type-1 motif is found in C-domain. On the other hand, the putative heparin-binding motif (HBD) (PKKXRP), which is identical to all the known mammalian IGFBP-2s, is absent in the L-domain of both black porgy IGFBP-2s.

### Phylogeny of Perciformes IGFBP-2s

To explore the phylogenetic relationship of IGFBP-2s among species of Perciformes, we first identified the IGFBP-2a and IGFBP-2b genes by scanning the genomes in the species of Perciformes. Then, we reconstructed the BI tree and NJ tree. The BI tree revealed that the IGFBP-2s of Perciformes constituted two separated clades (i.e., IGFBP-2a and IGFBP-2b) with a bootstrap value of 99% (Fig. 4). This is the evidence that IGFBP-2 gene duplicated earlier than perciform species diverged.

Also, the cloned and predicted CDS of IGFBP-2a, 2b of the black porgy grouped into the clade of Perciformes IGFBP-2a, 2b with a bootstrap value of 99% and 100%, respectively (Fig. 4). Similar results were found in the NJ tree (Online Resource 2). This result suggested that IGFBP-2a, 2b of the black porgy were indeed orthologous to the Perciformes IGFBP-2s gene.

### Tissue distribution of IGFBP-2a in three sea porgy breeds

The mRNA expression patterns of IGFBP-2a in five tissues (including liver, muscle, brain, heart, and gonad) of three sea porgies breeds were determined by RT-qPCR analysis (Fig. 3). Extremely high expression of IGFBP-2a was detected in the liver of black porgy and backcross breed. IGFBP-2a was also detectable in self-cross's liver and the brain, muscle, and gonad of all three breeds. However, no expression was detected in the heart. Interestingly, the IGFBP-2a expression level in the livers of each breed showed extremely significant differences from each other, where the expression in black porgy was up to 12-fold more than the self-cross breed.

### Analysis of positive selection on Perciformes IGFBP-2 genes

Based on the reconstructed BI tree, we estimated the selective pressure acting on IGFBP-2 genes by two pairs of site models (Table 4). We first used M0 model to estimate the  $\omega$  value for IGFBP-2s across all the

lineages. The result showed that  $\omega$  values were 0.101 for IGFBP-2a and 0.201 for IGFBP-2b, indicating purifying selection acting on Perciformes IGFBP-2s. We then compared the M0 model with the M3 model to test the variability of  $\omega$  among sites. In both Perciformes IGFBP-2 genes (IGFBP-2a and IGFBP-2b), M0 were respectively rejected by a big margin when compared with M3 ( $P < 0.001$ ), indicating that the selective pressure was highly variable among sites. Finally, a pair of models, i.e., M7 vs. M8, were tested to determine whether positive selection pattern occurred in perciform fish. The result revealed that M8, which incorporated positive selection, fitted the data significantly better than the neutral model (M7) for both IGFBP-2a and IGFBP-2b genes ( $P < 0.05$ ). Using M8, the most stringent model carried out in PAML, four and two codons were estimated to be under selection with average  $\omega$  values of 1.810 and 1.526 at the IGFBP-2a and IGFBP-2b genes.

#### Spatial distribution of positively selected sites in 3D structures

To gain a deeper understanding of their functional significance, these positively selected sites were then mapped onto the predicted 3D structures of IGFBP-2a and IGFBP-2b proteins (Fig. 5). It revealed that these sites were primarily located in functional regions, like signal peptide, which are necessary for transportation and exocytosis, and the L-domain (central linker domain), which contains sites for proteolytic cleavage and potential glycosylation or phosphorylation (Bach et al. 2005; Berg et al. 2007; Forbes et al. 2012; Russo et al. 2005). For IGFBP-2a, three sites were situated in signal peptide (sites 5, 9, and 10), and one site was located in L-domain (site 140). For IGFBP-2b, two selected sites scattered in its N-domain (site 97) and L-domain (site 187), respectively.

## Discussion

### Characterization of IGFBP-2 cDNA in Perciformes

In the present study, we cloned, predicted, and characterized the full-length cDNAs of two IGFBP-2 genes from black porgy, an important commercial fish species in China. The overall structure of the IGFBP-2 genes in the black porgy was highly similar to other species of

**Table 4** Positive selection on Perciformes IGFBP-2 genes detected by Site Models

Gene	Site Model	#p <sup>a</sup>	ln L	2ΔlnL	P value <sup>b</sup>	Parameter estimates	Positively selected sites <sup>c</sup>
IGFBP-2a	M0 (one-ratio)	1	-2467.074			$\omega = 0.101, K = 3.738$	None
	M3 (discrete)	5	-2440.902	26.172	<0.001 (vs. M0)	$p_0 = 0.580, p_1 = 0.392, p_2 = 0.028, K = 3.782, \omega_0 = 0, \omega_1 = 0.200, \omega_2 = 1.798$	Not allowed
	M7 ( $\beta$ )	2	-2445.580			$p = 0.129, q = 0.776, K = 3.734$	Not allowed
	M8 ( $\beta$ and $\omega$ )	4	-2441.030	4.549	0.011 (vs. M7)	$p_0 = 0.973, p_1 = 0.027, K = 3.780, p = 0.204, q = 1.723, \omega = 1.810$	5 (0.809), 9 (0.865), 10 (0.908), 140 (0.822)
IGFBP-2b	M0 (one-ratio)	1	-3036.969			$\omega = 0.201, K = 2.724$	None
	M3 (discrete)	5	-2985.081	51.889	<0.001 (vs. M0)	$p_0 = 0.370, p_1 = 0.550, p_2 = 0.080, K = 2.870, \omega_0 = 0, \omega_1 = 0.229, \omega_2 = 1.479$	Not allowed
	M7 ( $\beta$ )	2	-2993.900			$p = 0.294, q = 0.855, K = 2.841$	Not allowed
	M8 ( $\beta$ and $\omega$ )	4	-2985.512	8.388	<0.001 (vs. M7)	$p_0 = 0.927, p_1 = 0.073, K = 2.872, p = 0.549, q = 1.526$	97 (0.909), 187 (0.861)

<sup>a</sup>#p is the number of free parameters in the distribution

<sup>b</sup>Likelihood ratio test (LRT). P values were adjusted for multiple testing with a Benjamini and Hochberg's procedure

<sup>c</sup>Codons were identified using Bayes Empirical Bayes (BEB) analysis with posterior probabilities  $\geq 0.80$ . Numbers in parentheses represent the posterior probabilities of corresponding sites



Perciformes counterparts (Fig. 2). The black porgy IGFBP-2s also have conserved N- and C-terminal domains that are rich in cysteine. Twelve and six cysteine residues were identified in the N-terminal domain and C-terminal domain in the black porgy IGFBP-2, respectively, which are highly conserved across species of Perciformes. It was suggested by previous studies that conserved N- and C-terminal domains of IGFBP-2s are required for IGF binding (Hwa et al. 1999).

A typical IGFBP motif (GCGCCXXC) was observed in the N-terminal domain across species of Perciformes IGFBP-2s, which were considered to play a key role in interactions with IGFs (Hwa et al. 1999; Kim et al. 1997). It was reported that residue Tyr-60 in the N-domain of bovine IGFBP-2 has been proven a determinant for the IGF binding function (Hobba et al. 1998). This residue is also found in perciform species (Tyr-83) and in zebrafish (Duan et al. 1999), suggesting its functional conservation. The C-domain of black porgy IGFBP-2s contained a thyroglobulin type 1, which was hypothesized to inhibit certain types of proteinases (Fowlkes et al. 1997; Molina et al. 1996) and had other untraditional functions, like binding IGFBPs with IGFs and extracellular matrix (Zhou et al. 2008). An RGD motif was also present in the C-domain of the black porgy IGFBP-2s. RGD motif in human IGFBP-1 and IGFBP-2 was reported to mediate IGF's action on cell migration through binding to  $\alpha 5 \beta 1$  integrin (Jones et al. 1993; Wang et al. 2006). However, IGFBP-2 cell surface association was not dependent on the RGD motif in rat and zebrafish (Hoeflich et al. 2002; Zhou et al. 2008). In addition, a stretch of amino acids (K<sup>222</sup>HGLYNLKQCKMSLN<sup>236</sup>) in the C-domain of bovine IGFBP-2 played a key role in IGF binding (Forbes et al. 1998). These residues were conserved in the mature protein of black porgy IGFBP-2s (IGFBP-2a: K<sup>233</sup>RGQYNLKQCKMSLN<sup>247</sup>, IGFBP-2b: K<sup>233</sup>HGLYNLKQCNMSTH<sup>247</sup>), which was also detected in zebrafish (Duan et al. 1999), common carp (Chen et al. 2009), and Japanese flounder (Zhang et al. 2013). A putative N-glycosylation site was found in the C-terminal of black porgy IGFBP-2s (IGFBP-2a: N<sup>275</sup>CSQ, IGFBP-2b: N<sup>243</sup>MST). Like Chinook salmon (Shimizu et al. 2011), goldfish, zebrafish, common carp, and tetraodon (Chen et al. 2014), the N-glycosylation site in black porgy IGFBP-2a was adjacently downstream to the RGD motif. Without affecting IGF binding, N-glycosylation of IGFBPs exerts physiological function by modulating

their susceptibility to proteolysis, interaction with cell surface, and half-life in circulation (Bach et al. 2005; Miller et al. 2009; Russo et al. 2005; Wang et al. 2006). Besides, the conserved C<sup>253</sup>WCV motif in all known vertebrate IGFBPs was also found in the species of Perciformes (Daza et al. 2011). In the L-domain of black porgy IGFBP-2s, HBD was not detectable. HBD in IGFBPs was suggested to change the configuration of IGFBPs through binding to heparin or glycosaminoglycans, lowering their affinity for IGF-I and promoting the association of IGFBP-2 with the cell surface or the extracellular matrix (Russo et al. 2005; Zhou et al. 2008). The lack of the HBD is one of the possible mechanisms for the growth inhibitory effect of IGFBP-2s (Duan et al. 1999). This suggestion was in agreement with the fact that the mutation of the HBD caused notable changes to the growth inhibitory action of IGFBP-2 in zebrafish (Pozios et al. 2001; Zhou et al. 2008). Also, L-domain is the region susceptible to post-translational modification and proteolytic cleavage. Phosphorylation in L-domain was proved in IGFBP-1, IGFBP-3, and IGFBP-5, modulating their IGF-I binding affinity as well as IGF-I action (Bach et al. 2005; Dolcini et al. 2009; Firth and Baxter 2002; Gupta 2015), susceptibility to proteolysis, association to cell surface (Kamei et al. 2008), and even IGF-independent apoptosis (Pozios et al. 2001). Notably, limited proteolysis of IGFBPs was also described in their L-domains (Berg et al. 2007; Bunn and Fowlkes 2003), reducing their inhibitory effect on IGF action, which indicates a potential mechanism to release free IGFs from IGF-IGFBP complexes (Bach et al. 2005).

#### Differential expression of IGFBP-2a mRNAs in sea porgy breeds

Far-origin hybridization is often used to select and obtain new breeds for cultivation. So far, numerous studies have focused on the advantages of F1 hybridizations between black porgy and red porgy (Chen et al. 2017; Jiang et al. 2010; Wang et al. 2016). However, their low survival rates have always restricted the amplification of mariculture (Jiang and Wu 2011). The mature of gonad in reverse-cross F1 (black porgy♀×red porgy♂) enabled us to carry out trials for new breeds with optimal target traits. We have already developed backcross breed (F1♀×black porgy♂) and self-cross breed (F1♀×F1♂). Recently, their different growth rates caught our eyes. It has been



**Fig. 2** Deduced amino acid sequence of black porgy IGFBP-2 aligned with other Perciformes IGFBP-2s. The sequences were aligned using ClustalW in MEGA 7. The conserved cysteine residues are shaded with gray. The signal peptides are underlined. The IGFBP motif, thyroglobulin-1 motif, and RGD motif are boxed. Missing or ambiguous data are represented by “question marks,” gaps are noted with “hyphens,” and “periods” means the amino acid matches that in the first line

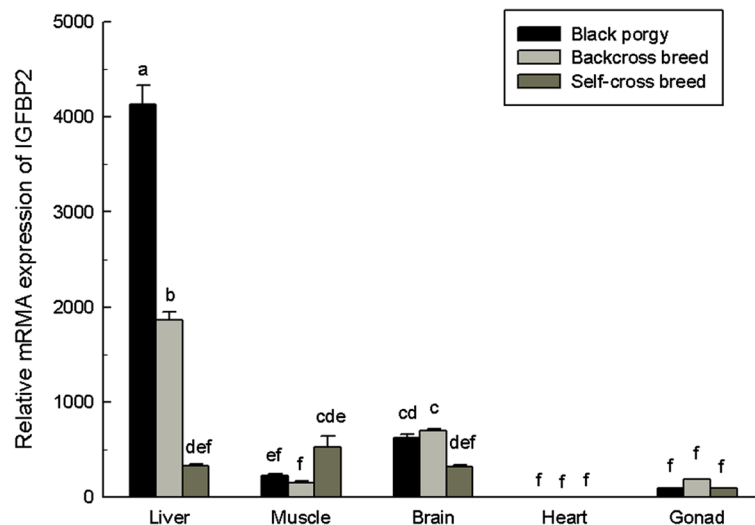
noticed that the self-cross breed grow apparently faster than black porgy and backcross breed (Table S1, Online Resource 1). In the present study, the three breeds (black porgy, backcross breed, and self-cross breed) served as excellent raw materials to explore the role of IGFBP-2 in these fish growths. The results would help us to understand the molecular mechanism of growth difference of sea porgy breeds.

Previous studies showed that IGFBP-2 was widely expressed in various tissues in teleost fish, such as gilthead seabream, zebrafish, common carp, orange-spotted grouper, Japanese flounder, and goldfish (Chen et al. 2009, 2010a, 2014; Funkenstein et al. 2002; Zhang et al. 2013; Zhou et al. 2008), suggesting that IGFBP-2 was synthesized not only in the liver but also in extra-hepatic tissues. However, in the present study, the expression of IGFBP-2a mRNA was extremely abundant in the liver of the black porgy and backcross breed, relatively lower in the brain, muscle, and gonad but none in the heart of the three breeds. Different

expressions of IGFBP-2a mRNAs in sea porgy breeds were well matched with their growth. As can be seen in Fig. 3 and Table S1, self-cross breed grew fastest and had the lowest IGFBP2 expression in the liver. In addition, we developed hybridization breeds in order to improve the growth speed of black porgy. Although similar growth speed was examined in the backcross breed and the black porgy, the second lowest expression found in the backcross breed indicated its prepotency in the growth of hybrid breed. This expression result was in agreement with the mainstream view that IGFBP-2 played a growth-inhibiting role in teleost, for overexpressing IGFBP-2 in early-stage zebrafish reduced their growth and developmental rate (Wood et al. 2005; Zhou et al. 2008). Also, fasted zebrafish showed higher IGFBP-2 expression (Duan et al. 1999), and post-fasting refeeding in Atlantic salmon decreased the IGFBP-2 level (Bower et al. 2008; Macqueen et al. 2013; Valente et al. 2012).

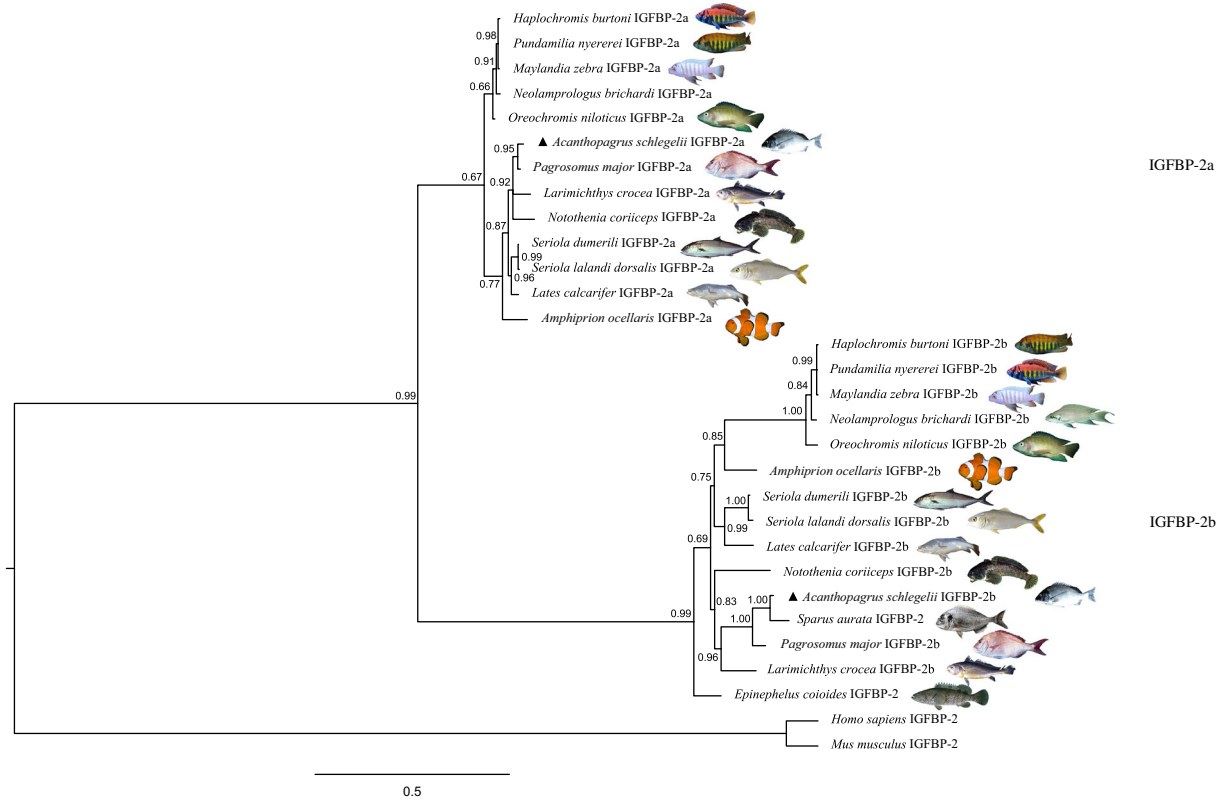
#### IGFBP-2 gene evolution in Perciformes

Previous studies have reported that IGFBP-2 has duplicated in the teleost fish (Kamei et al. 2008), which may take place no later than the divergence of ray-fin fish and tetrapods (Zhou et al. 2008). The epoch of this duplication is in accord with a genome duplication event in the teleost lineage, which occurred about 350 million years



**Fig. 3** RT-qPCR analysis of IGFBP-2a mRNA expression in the different tissues of black porgy, backcross breed, and self-cross breed. Three 8-month-old individuals were analyzed for each group. Transcript abundances were calibrated with  $\beta$ -actin as an internal control. The values are compared to the average heart expression

level in self-cross breed and subjected to natural logarithmic transformation before statistical analysis (comparative  $C_T$  method). The statistical comparisons were made breed-wise. Vertical bars represent means  $\pm$  SEM. Values with different letters are significantly different (one-way ANOVA and Tukey test;  $P < 0.05$ )



**Fig. 4** Phylogenetic tree of Perciformes IGFBP-2s reconstructed by Bayesian inference (BI). Numbers on branches indicate the Bayesian posterior possibilities. The black porgy IGFBP-2s are

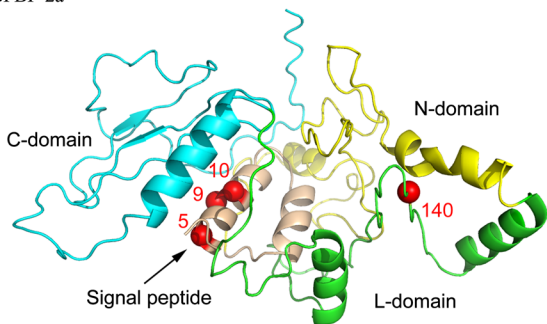
noted by a black up-pointing triangle. The accession numbers of downloaded IGFBP-2s are described in Table 3

ago, prior to the beginning of the teleost radiation (Amores et al. 1998; Postlethwait et al. 2004; Taylor et al. 2003). In the present study, IGFBP-2 gene duplication was also found in the black porgy and red porgy. Furthermore, the full length of two IGFBP-2 CDSs was also obtained either from GenBank or by Local Blast in

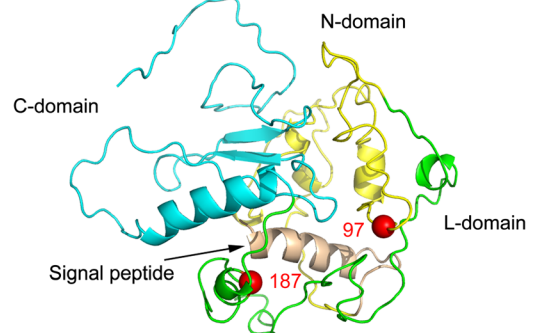
all the perciform fish whose genome assemblies were accessible. The gene tree of IGFBP-2s also revealed that the gene duplication took place earlier than the divergence of perciform species (Fig. 4).

We further investigated the evolution of two IGFBP-2 genes in perciform fish to explore the genetic basis of

IGFBP-2a



IGFBP-2b



**Fig. 5** The distribution of positively selected sites of Perciformes IGFBP-2s in the three-dimensional (3D) structure of black porgy IGFBP-2s. The signal peptide is colored with wheat, while N-, L-,

and C-domains are yellow, green, and cyan, respectively. Sites identified in M8 are marked by red balls

their potential divergence and functional evolution. Selection test showed that both IGFBP-2 genes were identified to be under purifying selection, suggesting their critical importance in fish growth and development. Interestingly, six codons (four in IGFBP-2a and two in IGFBP-2b) were detected to be under positive selection in perciform lineages by site-specific model. Importantly, these positively selected amino acid sites were localized within the functional region of the predicted 3D structures of the two IGFBP-2 genes (Fig. 5). For example, positively selected sites in both IGFBP-2s were located in the L-domain which is the essential region for post-translational modification and proteolytic cleavage. In addition, three selected sites were localized in the signal peptide region of IGFBP-2a, while none is found in IGFBP-2b. The different distributions of positively selected sites in two IGFBP-2 genes suggest that IGFBP-2a and IGFBP-2b may have different functions in IGF-dependent or IGF-independent actions. Of course, the gene function of IGFBP2a and 2b should be further examined in the Perciformes in the future.

In conclusion, the present study firstly cloned the coding sequence of IGFBP-2a from the black porgy and identified IGFBP-2b from its draft genome. We then determined the expression of the IGFBP-2a in five tissues in the black porgy and two new cultured breeds—backcross breed ( $F1♀ \times \text{black porgy}♂$ ) and self-cross breed ( $F1♀ \times F1♂$ )—and found a negative correlation with their growth rates. Finally, we identified the IGFBP-2a and IGFBP-2b genes by scanning the genomes of the species of Perciformes and found the IGFBP-2 gene duplication took place earlier than the divergence of perciform species. Additionally, the six positively selected sites were identified in the species of Perciformes, suggesting these sites played key roles in the growth in the Perciformes.

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