Molecular Characterization and mRNA Expression of ISP2 and ISP4 in the Large Yellow Croaker (Larimichthys Crocea) Under Acute Cold Stress

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Research Article

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Abstract

Ice structure proteins (ISPs), also known as antifreeze proteins, can lower the point of freezing by inhibiting the growth of ice crystals and protect organisms from freezing temperatures. The large yellow croaker (*Larimichthys crocea*) is an important warm-temperate marine fish in Chinese aquaculture. Only a few ISP studies have been reported in this fish to date. In this study, the cDNA of *ISP2* were cloned and characterized, and mRNA expression of *ISP2* and *ISP4* was assessed in different tissues of the large yellow croaker under different periods of acute cold stress (0, 6, 12, 24, 48 and 72 h, rewarming after 12 and 24 h). We found that *ISP2* cDNA is 861 bases in length, encoding a protein of 168 amino acid residues. The mRNA expression of *ISP2* and *ISP4* in tissues of large yellow croaker under different periods of acute cold stress changed significantly. In comparison with the control group, *ISP2* expression increased dramatically in the heart (1,976 fold) and intestine (26 fold) after 3 h of acute cold stress and increased 43 fold in the spleen after 6 h. *ISP4* expression was up-regulated significantly in the brain (43 fold) and gill (376 fold) at 1 h acute cold stress, and increased 2,774 fold in the intestine at 3 h, 64 fold in muscle and 141 fold in the spleen after rewarming for 1 h after 12 h acute cold stress. These results indicate that *ISP2* and *ISP4* may play an important role in the response of large yellow croaker to acute cold stress.

1. Introduction

Ice structure proteins (ISPs), also known as antifreeze protein, play a role in inhibiting the growth of ice crystals, modifying ice morphology and inhibiting the recrystallization of ice via adsorption–inhibition (DeVries and Wohlschlag, 1969; Lee and Kim, 2016). ISPs lower the point of freezing non-colligatively but do not change the point of melting through adsorption on the surface of ice crystals and then inhibit ice growth, which increases the temperature gap between melting point and freezing point, and this temperature gap is termed thermal hysteresis (TH) (Barrett, 2001). The larger the TH activity, the stronger the antifreeze activity of the ISPs. ISPs are vitally important for polar fish to prevent blood from freezing in subzero temperatures of seawater through depressing the freezing point from 1 to 1.2°C in the blood, allowing fish blood to flow during winter; simultaneously, other internal fluids are prevented from freezing by modifying the growth of ice crystals, which also protects cell membranes from ice crystal damage (DeVries, 1971; Hew and Yang, 1992; Fletcher et al., 2001; Harding et al., 2003). According to the composition of amino acids and structural characteristics, ISPs in fish are divided into five types, ISPI, ISP2, ISP3, ISP4 and antifreeze glycoproteins, and no homology exists between these types of ISPs (Zhong and Fan, 2002; Li and Ma, 2012).

ISP2 is found in Atlantic herring (*Clupea harengus harengus*), rainbow smelt (*Osmerus mordax*), Japanese smelt (*Hypomesus nipponensis*), longsnout poacher (*Brachyopsis rostratus*) and sea raven (*Hemitripterus americanus*) (Slaughter, et al., 1981; Ewart and Fletcher, 1990; Sorhannus, 2012). ISP2 is classified into two subtypes, Ca^{2+}-dependent and Ca^{2+}-independent, for instance, ISP2 produced by herring, rainbow smelt and Japanese smelt require Ca^{2+} for its antifreeze activity, while in the sea raven

and longsnout poacher, the antifreeze activity of ISP2 can be performed without Ca^{2+} (Ewart and Fletcher, 1990; Ewart et al., 1992; Ewart et al., 1996; Yamashita et al., 2003).

ISP4 was first isolated from the serum of the north-temperate coastal water fish longhorn sculpin (*Myoxocephalus octodecimspinosis*) (Deng et al., 1997). Subsequently, researchers found that the physiological concentration of ISP4 in the blood of adult longhorn sculpins and shorthorn sculpins was lower than any other type of ISP, and could not conduct antifreeze activity (Deng and Laursen, 1998; Zhao et al., 1998; Gauthier et al., 2008). Based on these findings, Gauthier et al. (2008) speculated that ISP4 may not be a key antifreeze protein in the blood of polar fish when other types of antifreeze protein are present. Surprisingly, in addition to polar fish, ISP4 is also found in warm-temperate fish and freshwater fish (Nishimiya et al., 2008; Zhang et al., 2009; Kim, 2015; Lee and Kim, 2016). These reports indicate that ISP4 may have other effects as well as antifreeze activity in warm-temperate fish.

Large yellow croaker is a warm-temperate marine fish that inhabits the north of the South China Sea and the East China Sea coastal waters of China. Since 1985, with the success of artificial breeding techniques in this species, it is now mainly farmed in single net cages (Hong and Zhang, 2003). The temperature of water is a key factor for fish and is closely related to growth, reproduction and immune metabolism. Extreme changes in water temperature are damaging for fish especially net cage farmed fish for they cannot escape from harmful regions of the sea. Large yellow croaker usually grow in water temperature from 10 ~ 32°C, rapid growth occurs in water temperatures 18 ~ 25°C, whereas survival may be threatened in water temperature higher than 34°C or lower than 7°C (Chen and Wu, 2011). On the basis of comparative transcriptome analysis in the liver of large yellow croaker under acute cold stress after 12 h conducted in our laboratory (data available at SRA http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE67756), we found that the expression of *ISP2* was significantly up-regulated and the expression of *ISP4* increased but with insignificance (Qian and Xue, 2016). In this study, we conducted a molecular characterization of *ISP2* and *ISP4*, mRNA expression of these two genes was observed in liver, muscle, gill, heart, spleen, intestine, brain and kidney of 1-year-old large yellow croaker under acute cold stress for 1, 3, 6, 12, 24, 48 and 72 h, rewarming 1 and 3 h after 12 h cold stress (12f1 h and 12f3 h) and rewarming 1 h after 24 h cold stress (24f1 h). Our objectives were (1) to analyze the molecular properties of type-II ISP in large yellow croaker and (2) investigate the spatiotemporal expression of *ISP2* and *ISP4* in large yellow croaker under acute cold stress.

2. Materials And Methods

2.1 Animals and acute cold stress

Large yellow croaker (mean weight 80 ± 0.7 g) were purchased from mariculture in Xiangshan Bay (Zhejiang, China) and maintained in a laboratory at the Ningbo Ocean and Fishery Science Technology Innovation Base. Fish were randomly divided into eight groups and acclimated in 500 L plastic aerated tanks with flow-through seawater at 28°C under 14 h light/10 dark photoperiod for 7 days (30 fish in each tank and eight tanks in total). They were fed with granulated feed for large yellow croakers twice per day

until 1 day before the experiment. A total of 150 fish in five tanks were treated with acute cold stress using a seawater chiller until the seawater temperature dropped to 14°C in 2 h (cold stress group). The other 90 fish with no treatment were cultured in another three tanks (control group). After 12 h and 24 h acute cold stress, 30 fish were immediately transferred to a tank with the water temperature at 28°C for the rewarming experiment. Tissues including liver, liver, muscle, gill, heart, spleen, intestine, brain and kidney were harvested from three fish at each time point (1, 3, 6, 12, 24, 48, 72, 12f1, 12f3 and 24f1 h) in the acute cold stress and control groups. All sampled tissues harvested in this experiment were snap-frozen in liquid nitrogen and stored at − 80°C. The protocols of all experiments meet the “Zhejiang Laboratory Animal Management” guideline established by the Zhejiang Provincial Department of Science and Technology on the Use and Care of Animals.

2.2 Total RNA extraction and cDNA synthesis

Total RNA was extracted from the tissues harvested in each time point (including three control and acute cold stressed fish) using a Tissue RNA Kit (Omega, Georgia, USA) following the manufacturer’s instructions. The total RNA was quantified with NanoDrop ND-1000 (Nanodrop Technologies) and RNA integrity (RIN) was assessed with an Agilent 2100 Bioanalyzer. The RIN values of all RNA samples were ≥ 8. All extracted RNA was stored at − 80°C. cDNA was synthesized using a PrimeScript RT reagent Kit with a gDNA Eraser (TaKaRa, Tokyo, Japan) according to the manufacturer’s instruction, and cDNA was stored at − 20°C for further experimental analysis.

2.3 Cloning of the full cDNA length of *ISP2* and *ISP4*

A partial cDNA length of *ISP2* was obtained from the liver transcriptome data of large yellow croaker, which was investigated earlier in our laboratory, and the detailed sequence information can be found at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE67756 (Qian and Xue, 2016). Gene-specific primers for 5'- and 3'-RACE were designed based on the aforementioned partial sequences (Table 1). 5'- and 3'-RACE was used to obtain sequences of the 5'- and 3'-untranslated terminal regions of *ISP2* using a SMARTer RACE 5'/3' Kit (TaKaRa, Tokyo, Japan) according to the kit manufacturer’s instructions. The full-length cDNA of *ISP2* was assembled using overlapped fragments.

Table 1
Primers for quantitative real time PCR.

Gene	Primer sequence(5'—3')
ISP2	F: GCTGACTGTGTCCGCATTTC
ISP2	R: ACCCAGAGAGCGACAGTTTG
ISP4	F: TCTTGGAACTTAGCGGCCAG
ISP4	R: CTTTGCAGAAGAGCTCCCCA
B-actin	F: TCGGTATGGAATCTTGCG
β-actin	R: GTATTTACGCTCAGGTGGG
ISP2-GSP5	GATTACGCCAAGCTTGACCAGCCACCAGGACAAGATGCGG
ISP2-GSP3	GATTACGCCAAGCTTCCGTCCGTCTGTGCCAAGGAAATCT

2.4 Sequence and phylogenetic analysis of *ISP2*

The molecular analysis methods of Feng et al. (2019) were used in this study to analyze *ISP2*: 1) BLAST was used to analyze the nucleotide sequence and deduced amino acid sequence of *ISP2* (http://www.ncbi.nlm.nih.gov/blast); 2) SignaliP 4.1 Server was used to predict the ISP2 signal peptide (http://www.cbs.dtu.dk/services/SignalP); 3) SMART was used in domain prediction (http://www.cbs.dtu.dk/services/SignalP); 4) and MEGA 6.0 software was used to analyze the phylogenetic tree.

2.5 Spatiotemporal expression analysis of *ISP2* and *ISP4*

Quantitative real-time PCR (qRT-PCR) was used to investigate the spatial and temporal expression of *ISP2* and *ISP4*. The first-stand cDNA from different tissues of the control fish (n = 3) as well as acute cold stressed fish were diluted 1:5 with sterile and DNase/RNase free distilled water and used as qRT-PCR templates. Primers were designed for the qRT-PCR of *ISP2*, *ISP4* and *β-actin* (Table 1), and five 10-fold serial dilutions of cDNAs of all tissues were used to assess the amplification efficiency of the primers. Primers with an amplification efficiency between 0.90–1.05 were chosen for further experiments. The qRT-PCRs were performed in a total volume of 20 μl on a CFX96 Real-Time PCR System (Bio-Rad, California, USA), and the thermal cycling conditions were as follows: 95°C for 10 min, 40 cycles of 95°C for 15 s, 58°C for 20 s, 72°C for 20 s, followed by a melting curve. All samples were amplified in triplicate repeats, and the relative expression levels of *ISP2* and *ISP4* in each sample were normalized by *β-actin* quantification applying the method of $2^{-\Delta\Delta CT}$ (Schmittgen and Livak, 2008). One-way analysis of variance was used to determine the statistical significance by using SPSS software (Version 21), and a significant difference was considered at $P < 0.05$ and a highly significant difference was considered at $P < 0.01$

3. Results

3.1 Molecular characterization of *ISP2*

The full-length cDNA of *ISP2* (GenBank accession number: 2370265) was 861 bp, including a 77 bp 5'-untranslated region (UTR), a 301 bp 3'-UTR with an AATAAA signal and a poly (A) tail, and a 507 bp open reading frame (ORF) encoding a putative protein with 168 amino acid residues, which contains a putative signal peptide with 18 amino acid residues (Fig. 1).

By using SMART analysis, we deduced that the ISP2 amino acid sequence contains a C-type lectin (CLECT or CTL) domain or carbohydrate-recognition domain (CRD), which starts at position 40 and ends at position 165 aa. The ISP2 of the large yellow croaker has low homology in multiple sequence alignment with the ISP2 of 11 other fish species, and the highest homology has 76% identity (*Amphiprion ocellaris*) (Fig. 1). The phylogenetic tree constructed in this study was based on the ISP2 amino acid sequences of 12 fish species (Fig. 2). The ISP2 of the large yellow croaker was the furthest distance from that of other fish species.

3.2 *ISP2* mRNA spatiotemporal expression during the acute cold stress

The spatiotemporal expression levels of *ISP2* in large yellow croaker under acute cold stress are shown in Fig. 3. In liver tissue, *ISP2* mRNA expression increased approximately at 3, 6, 48, 72, 12f3 and 24f1 h after acute cold treatment in comparison to the control ($P < 0.01$). Especially at 3 h after cold treatment, the expression of *ISP2* increased to 8.23-fold in comparison to the control but decreased significantly at 12 h ($P < 0.01$). In muscle tissue, the initial expression of *ISP2* decreased significantly at 1 h after acute cold treatment and then was up-regulated remarkably at 3, 6, 12 h, and down-regulated again at 24 and 72 h ($P < 0.01$). In brain tissue, *ISP2* mRNA expression increased significantly at 1 h and 72 h in the cold stress group with 9.95 and 55.57-fold changes, respectively, compared to the control group ($P < 0.01$). *ISP2* mRNA expression decreased remarkably at 48 h and 12f1 h ($P < 0.01$), and no changes occurred throughout the other sampling time points in comparison to control ($P \geq 0.05$). In heart tissue, the expression of *ISP2* was up-regulated significantly at 1, 3, 6, 48, 72, 12f3 and 24f1 h ($P < 0.01$). In particular, at 3 h after cold treatment, the increased 1,976.29-fold compared to the control group. *ISP2* mRNA expression decreased at 12f1 h ($0.01 \leq P < 0.05$), and no expression occurred at 12 h (0.01-fold). In spleen tissue, at 6, 12f1 and 12f3 h after acute cold stress, the expression of *ISP2* increased significantly by 43.5, 8.41 and 10.99-fold ($P < 0.01$), respectively, but was down-regulated remarkably at 3 and 24 h ($P < 0.01$). In kidney tissue, there were no changes in the expression of *ISP2* from 1 to 72 h including 12f1 and 12f3 h after cold treatment ($P \geq 0.05$), but at 24f1, *ISP2* mRNA expression significantly increased 5.38-fold relative to the control group ($P < 0.01$). In gill tissue, the initial expression of *ISP2* was decreased significantly during 1 to 12 h, then increased remarkably during 24 to 72 h, and decreased significantly after rewarming (12f1, 12f3 and 24f1 h) ($P < 0.05$). In intestinal tissue, *ISP2* mRNA expression increased highly significantly by 26.62 and 6.09-fold at 3 and 12 h, respectively, and was highly significantly decreased at 6 and 24 h ($P < 0.01$). The expression of *ISP2* was increased significantly at 72, 12f1 and

12f3 ($0.01 \leq P < 0.05$), and no changes occurred throughout the other sampling times relative to the control fish ($P < 0.05$).

3.2 *ISP4* mRNA spatiotemporal expression during acute cold stress

The spatiotemporal expression levels of *ISP4* in large yellow croaker under acute cold stress are shown in Fig. 4. In liver tissue, *ISP4* mRNA expression increased significantly at 48 and 24f1 h and decreased significantly at 6 and 72 h after acute cold stress ($P < 0.05$). There were no changes in the expression of *ISP4* at other sampling time points in comparison to the control ($P \geq 0.05$). In muscle tissue, there was no *ISP4* mRNA expression at 1, 24 and 24f1 h, but expression increased dramatically at 3, 48 and 12f1 h ($0.01 \leq P < 0.05$), particularly at 12f1 h after acute cold treatment, the expression of *ISP4* was up to 64.02-fold relative to the control. In brain tissue, the expression of *ISP4* was up- or down-regulated significantly throughout sampling time points except at 12f3 h there was no change compared to the control ($P < 0.05$). At 1 h after acute cold treatment, *ISP4* mRNA expression was increased by 42.81-fold relative to the control. In heart tissue, *ISP4* mRNA expression decreased significantly during the first 1 to 3 h compared to the control but increased significantly at 48 to 72 h, including the sampling time point 12f1 and 12f3 h ($P < 0.01$). In spleen tissue, the expression of *ISP4* significantly changed at sampling time point 6 and 12f3 h ($P < 0.05$), particularly at 12f1 h, the expression of *ISP4* mRNA was up to 141.27-fold that of the control group. In kidney tissue, *ISP4* mRNA expression was significantly increased at 6, 12, 48 and 72 h, and decreased significantly at 3, 24, 12f3 and 24f1 h in comparison to the control fish ($P < 0.05$). In gill tissue, *ISP4* mRNA expression increased by 375.84-fold that of the control, and increased significantly at 12, 24, 72 and 24f1 h, but decreased significantly at 3, 48 and 12f3 h ($P < 0.05$). In intestinal tissue, the expression of *ISP4* decreased significantly at 6, 12, 24, 12f1 and 12f3 h, and increased significantly at 1, 3, 48 and 24f1 h, especially at the sampling time point 3 h, *ISP4* mRNA expression was up to 2,774.02-fold that of the control group ($P < 0.05$).

4. Discussion

Cold stress, especially in winter, is a key limiting factor in warm-temperate marine fish, including the large yellow croaker, which is cultured in net cages. In recent years, researchers have sought to understand the molecular response to cold stress in the large yellow croaker, in studies including the gene expression of *CIRP* (Miao et al, 2017), *HSP27*, *HSP30*, *HSP47*, *HSP90*, *caspase-1* and *caspase-7* (Yang, 2011). Our previous study found that *ISP2* mRNA expression was significantly increased after 12 h cold stress in the liver of large yellow croaker, but there was no significant change in *ISP4* compared to the control group (Qian and Xue, 2016). In this study, mRNA expression of *ISP2* and *ISP4* were investigated in eight tissues of the large yellow croaker during periods of cold stress (1, 3, 6, 12, 24, 48 and 72 h) and rewarming (12f1, 12f3 and 24f1 h). Results showed that the expression changes of these two genes during stress are tissue and time-dependent.

As mentioned earlier, type-II ISPs are only found in a few fish species (Yang, 2016), and almost no studies focus on ISP2 in warm-temperate fish. The brain, neural centre, is the tissue in fish that responds to changes in temperature the fastest and is an important tissue in the regulation of temperature (Crawshaw et al, 1985; Xu, 2011). In this study, encephalic mRNA levels of *ISP2* increased significantly at 1 h cold stress, while no changes in *ISP2* expression occurred in other tissues such as liver, heart, kidney and intestine. This seems to confirm that compared to other tissues the brain in fish responds first to cold stress. At 3 h of cold stress, *ISP2* mRNA expression does not change in the brain but increased dramatically in the liver (8.23-fold), muscle (21.6-fold), and intestine (26.62-fold), especially in the heart, mRNA levels of *ISP2* increased by 1796.29-fold compared to the control group. A possible reason for this result could be that cardiomyocytes receive certain signals from central nerves, which promote *ISP2* expression dramatically to alleviate the damage of 3 h acute cold stress on cardiomyocytes, and a similar response mechanism occurs in other tissues. It should be noted that, in this study, prolonged cold stress has no effect on the expression of *ISP2* in the brain of large yellow croaker until 48 h, and on the contrary, significant changes occurred in *ISP2* mRNA expression from 3 to 48 h in other tissues including liver, muscle, spleen, heart, gill and intestine. It is possible that ISP2 synthesized in the brain of large yellow croaker at 1 h cold stress is enough to protect nerve cells from subsequent cold stress, and in other tissues, ISP2 must be synthesized constantly to achieve protection in prolonged cold stress. In our study, a strange phenomenon occurred after rewarming, the expression of *ISP2* in some tissues including liver (12f3 and 24f1), muscle (12f3 and 24f1), heart (12f3 and 24f1), spleen (12f1 and 12f3), kidney (24f1) and intestine (12f1 and 12f3) were significantly increased. It is not clear why rewarming should have such an effect in these tissues. Maybe it is a compensation mechanism in response to the warmer water temperature.

ISP4 (also known as AFP IV) is different from ISP2 and has been detected in warm-water fishes as well as cold-water species, its function is controversial (Mao et al, 2018). In addition, the mRNA expression of *ISP4* has only been investigated in the liver of large yellow croaker, and the protein of ISP4 has no remarkable antifreeze activity (Zhang et al, 2009). Unlike in the research by Zhang et al., we found that the mRNA expression of *ISP4* was significantly increased in tissues of large yellow croaker that were subjected to acute cold stress and rewarming, including the liver (48 h cold stress and rewarmed 1 h after 24 h cold stress), muscle (3 and 48 h cold stress and rewarmed 1 h after 12 h cold stress), brain (1, 6, 12, 24 and 72 h cold stress and rewarmed 1 h after 24 h cold stress), spleen (12, 24, 48 and 72 h cold stress and rewarmed 1 h after 12 and 24 h cold stress), gill (1, 12, 24 and 72 h cold stress and rewarmed 1 h after 24 h cold stress), kidney (6, 12, 48 and 72 h cold stress), intestine (1, 3 and 48 h cold stress and rewarmed 1 h after 24 h cold stress) and heart (48 and 72 h cold stress and rewarmed 1and 3 h after 12 h cold stress). Interestingly, *ISP4* expression was remarkably increased in brain, gill and intestine at 1 h acute cold stress, especially in gill tissue, mRNA expression was increased by 375.84-fold compared to the control group. This could indicate that the brain, gills and intestine were more sensitive to cold stress, and ISP4 may play an important role in the protection of these three tissues from the influence of cold at 1 h. A number of studies have observed an evolutionary relationship between ISP4 and apolipoproteins (for example ApoA and ApoE) (Deng et al, 1997; Gauthier et al, 2008; Zhang et al, 2009; Mao et al, 2018).

In our study, the mRNA expression of *ISP4* was highly up-regulated (2774.02-fold) in the intestine at 3 h, but there were no significant effects or it was significantly decreased in other tissue including liver, brain, spleen, gill, kidney and heart. It is not clear why changes in *ISP4* mRNA expression are different in these tissues in the same fish treated with acute cold stress after 3 h. One possibility could be that ISP4 functions as an apolipoprotein in the intestine after 3 h of cold stress.

In summary, the expression of *ISP2* and *ISP4* mRNA changed in many tissues of the large yellow croaker after acute cold stress and was time and tissue-dependent. The brain, a high-level central neural system, was a sensitive tissue which responded to acute cold stress within a short time (1 h). The expression of *ISP2* and *ISP4* were both significantly increased at 1 h in the brain, which may indicate that ISP2 and ISP4 proteins could be synthesized in large yellow croaker while the fish is undergoing acute cold stress and protect the central neural system from cold stress. It should be noted that some differences occurred in the level of mRNA expression of these two genes at the same stress time in the same tissue. We do not know why cold stress and rewarming should have such effects in mRNA expression of *ISP2* and *ISP4* within the same tissue at the same duration of cold stress. Could it mean that ISP2 and ISP4 have a synergistic effect to protect fish from cold stress of extreme differences in temperature? Further studies should be conducted to fully understand the function of ISP2 and ISP4 in this warm temperature fish.

Declarations

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Declarations

We declare that we have no conflicts of interest.

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Figures

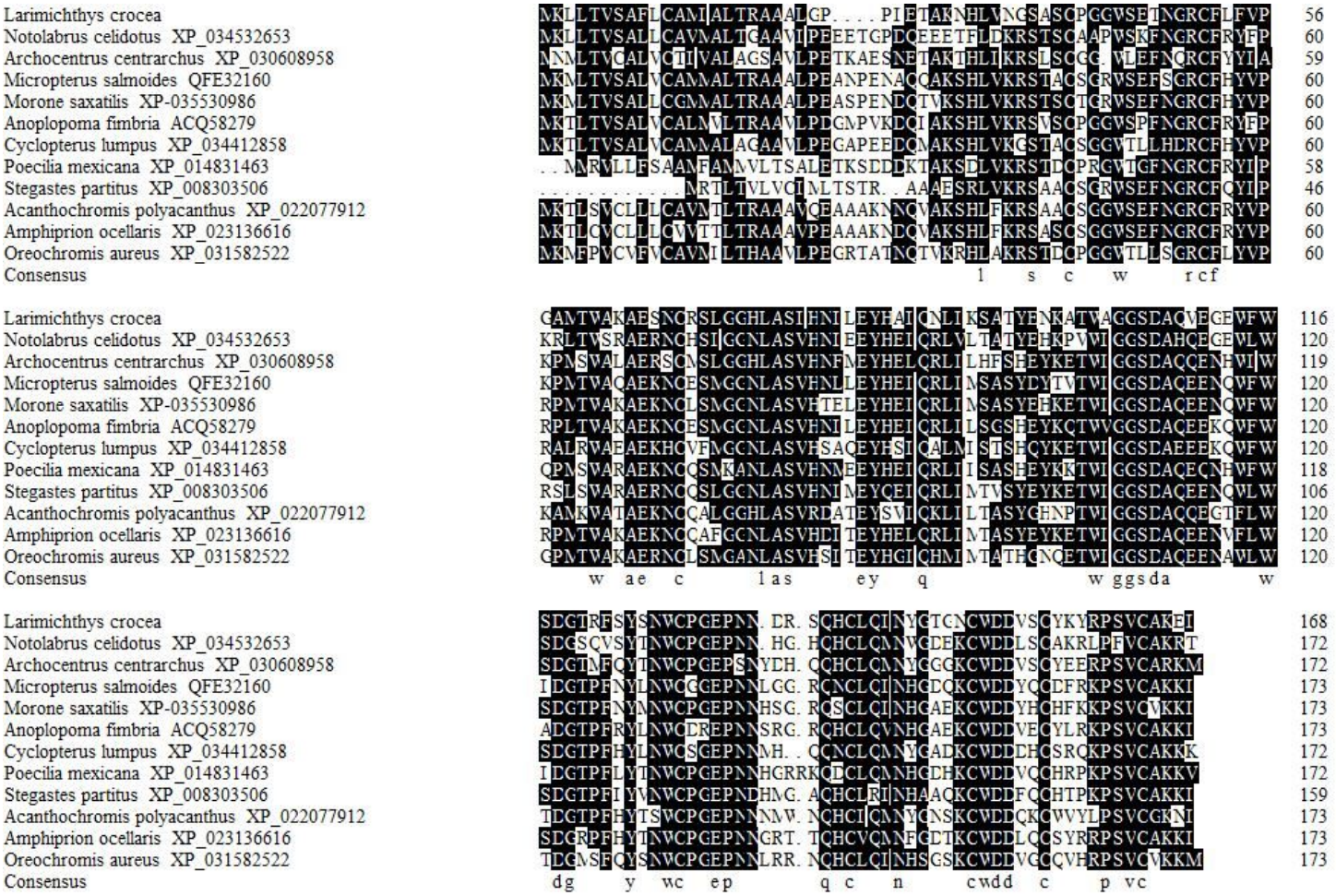

Figure 1

The full-length cDNA of ISP2 (GenBank accession number: 2370265) was 861 bp, including a 77 bp 5'-untranslated region (UTR), a 301 bp 3'-UTR with an AATAAA signal and a poly (A) tail, and a 507 bp open reading frame (ORF) encoding a putative protein with 168 amino acid residues, which contains a putative signal peptide with 18 amino acid residues

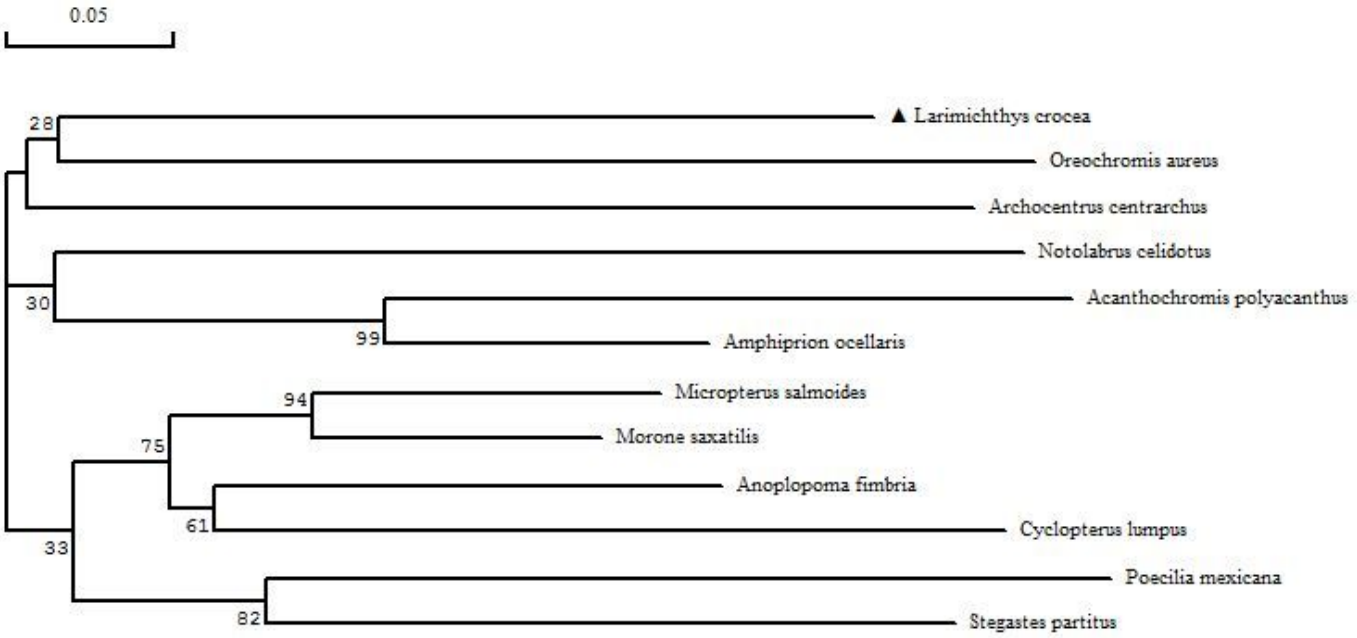

Figure 2

The phylogenetic tree constructed in this study was based on the ISP2 amino acid sequences of 12 fish species

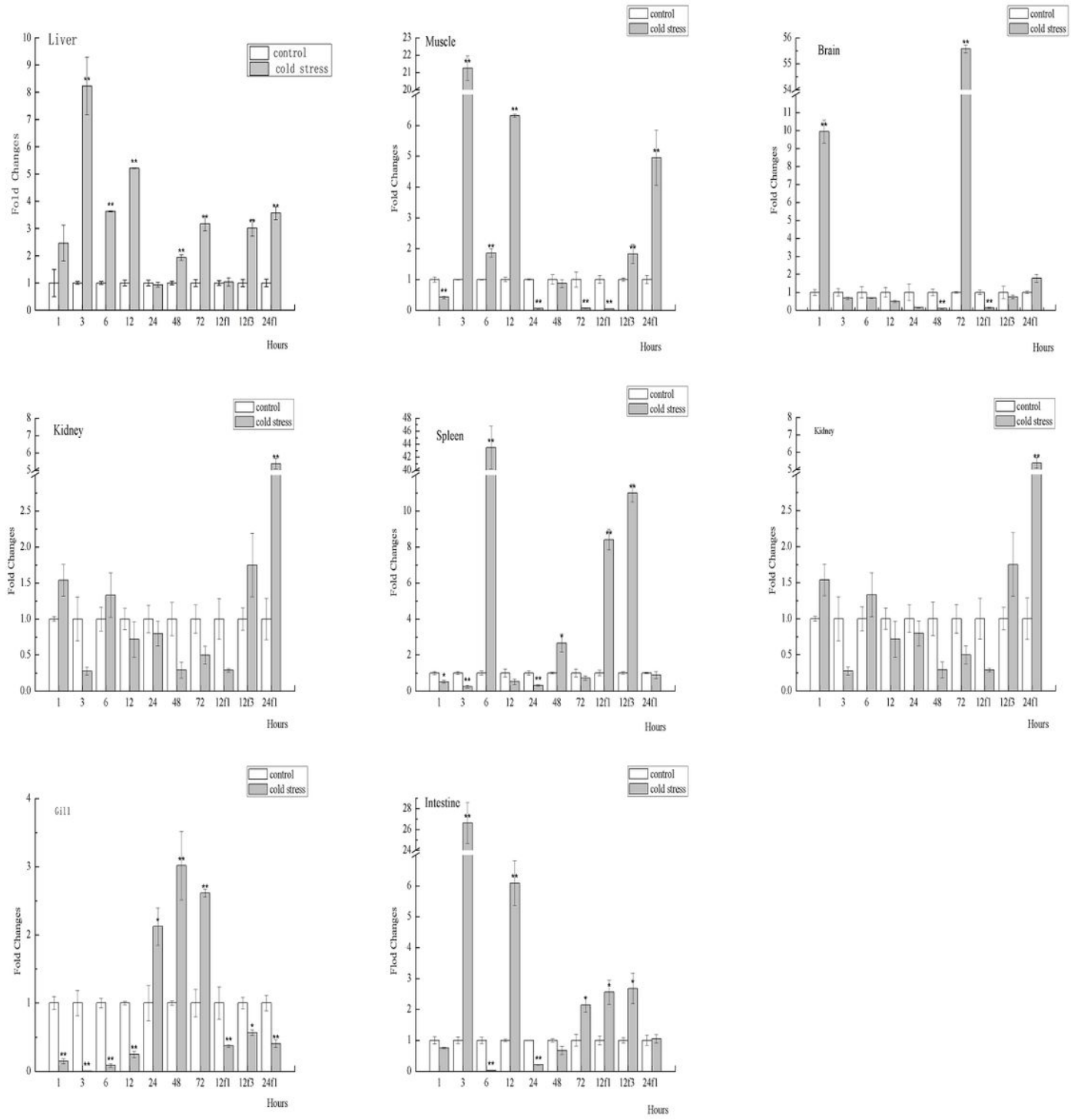

Figure 3

The spatiotemporal expression levels of ISP2 in large yellow croaker under acute cold stress

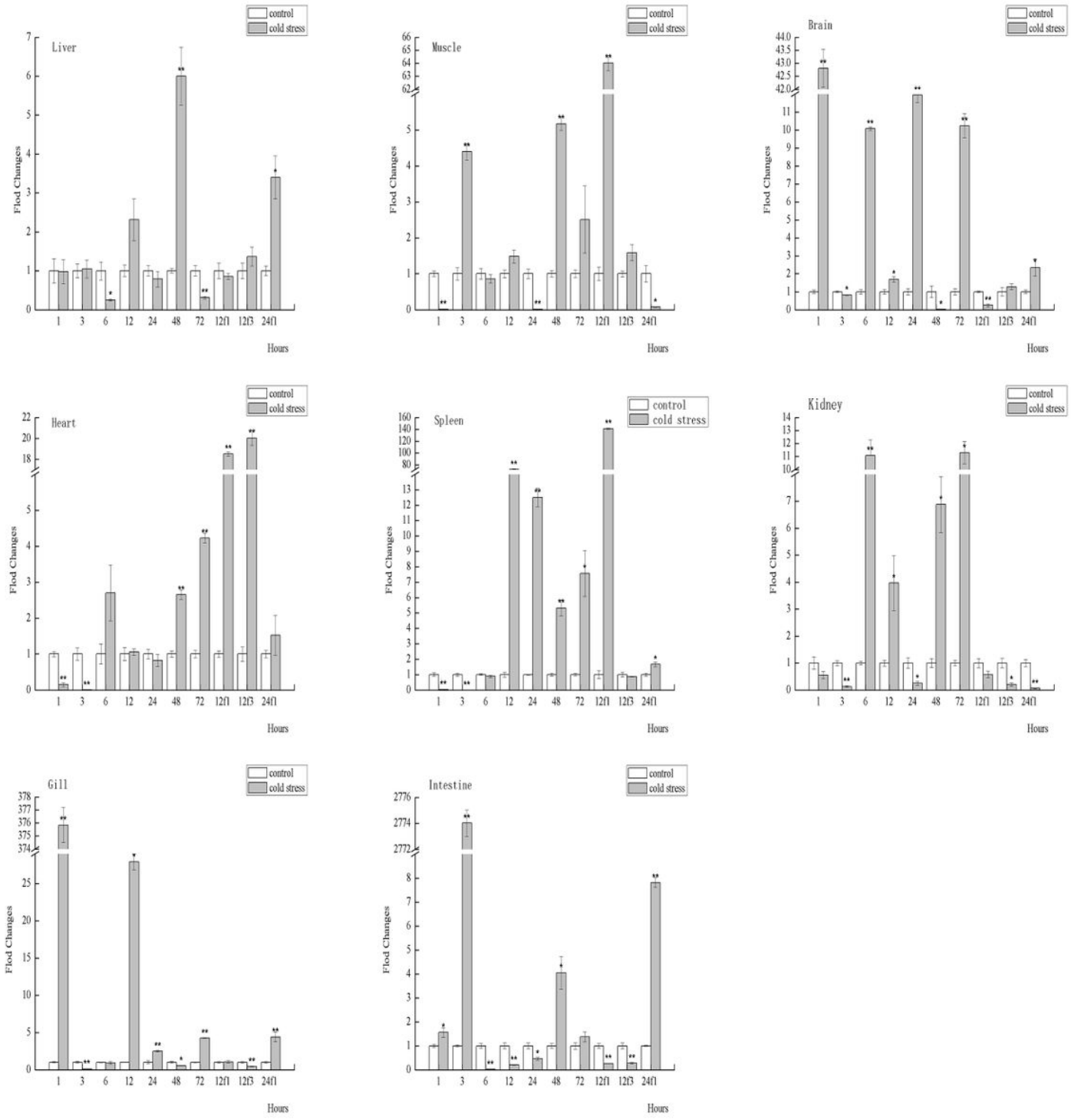

Figure 4

The spatiotemporal expression levels of ISP4 in large yellow croaker under acute cold stress