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Cloning and characterization of SREBP-1 and PPAR-α in Japanese seabass *Lateolabrax japonicus*, and their gene expressions in response to different dietary fatty acid profiles

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ABSTRACT

In the present study, putative cDNA of sterol regulatory element-binding protein 1 (SREBP-1) and peroxisome proliferator-activated receptor α (PPAR-α), key regulators of lipid homoeostasis, were cloned and characterized from liver of Japanese seabass (*Lateolabrax japonicus*), and their expression in response to diets enriched with fish oil (FO) or fatty acids such as palmitic acid (PA), stearic acid (SA), oleic acid (OA), α-linolenic acid (ALA), and n−3 long-chain polyunsaturated fatty acid (n−3 LC-PUFA), was investigated following feeding. The SREBP-1 of Japanese seabass appeared to be equivalent to SREBP-1a of mammals in terms of sequence feature and tissue expression pattern. The stimulation of the mRNA expression level of SREBP-1 in liver of Japanese seabass by dietary fatty acids significantly ranked as follows: PA, OA > SA, ALA, and n−3 LC-PUFA > FO. A new PPAR-α subtype in Japanese seabass, PPAR-α2, was cloned in this study, which is not on the same branch with Japanese seabass PPAR-α1 and mammalian PPAR-α in the phylogenetic tree. Liver gene expression of PPAR-α1 of Japanese seabass was inhibited by diets enriched with ALA or FO compared to diets enriched with PA or OA, while the gene expression of PPAR-α2 of Japanese seabass was up-regulated by diets enriched with ALA or n−3 LC-PUFA compared to diets enriched with OA or FO. This was the first evidence for the great divergence in response to dietary fatty acids between PPAR-α1 and PPAR-α2 of fish, which indicated probable functional distribution between PPAR-α isotypes of fish.

1. Introduction

Sterol regulatory element binding proteins (SREBP) belong to the basic helix–loop–helix-leucine zipper (bHLH-Zip) family of transcription factors. The mammalian SREBP subfamily is encoded by two genes, SREBP-1 and SREBP-2, which are major regulators of mammalian fatty acid/lipid and cholesterol biosynthetic genes, respectively, including genes of long-chain polyunsaturated fatty acids (LC-PUFA) synthesis (Horton, 2002; Horton et al., 2002, 2003). The 5′ end of the mRNA encoding mammalian SREBP-1 exists in two forms, designated 1a and 1c. SREBP-la may be responsible for maintaining basal levels of cholesterol and fatty acid synthesis in vivo, while a number of studies in mammals have demonstrated that SREBP-1c predominantly acts to increase the expression of genes involved in fatty acid synthesis, including fatty acid synthase (FAS), fatty acid desaturase (Fad), and elongase of very long chain fatty acid (Elovl) genes (Matsuzaka et al., 2002; Kumadaki et al., 2008; Qin et al., 2009). In fish, however, so far only one single form of SREBP-1 gene has been characterized (Minghetti et al., 2011), and it is unknown whether it has functions of both SREBP-1a and c genes in mammals. Limited studies on fish have implied that SREBP-1 are important transcriptional regulators of salmon Δ6 Fad (Zheng et al., 2009) and the gene expression of salmon SREBP-1 could be regulated by DHA and EPA (Minghetti et al., 2011).

The peroxisome proliferator-activated receptors (PPAR) are members of the nuclear receptor superfamily of ligand activated transcription factors, which can bind to a specific DNA regulatory element (peroxisome proliferator response elements, PPREs) located in the promoter region or intronic sequence of the target genes (Desvergne and Wahli, 1999). Three PPAR isotypes, α, β (also known as δ), and γ, have been identified and these PPAR subtypes display distinct but overlapping expression and functions (Poulsen et al., 2012). Among the three PPAR isotypes, PPAR-α plays critical roles in fatty acid homeostasis, regulating the peroxisomal and mitochondrial fatty acid oxidation, and expressions of fatty acid desaturases, Δ5 and Δ6 Fad, i.e., having pleiotropic functions in the regulation of lipid metabolic pathways (Escher et al., 2001; Matsuzaka et al., 2002; Mandard et al., 2004; Nakamura et al., 2004; Oosterveer

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et al., 2009). In fish, two orthologs of human PPAR-α have been observed in fugu, zebrafish, Japanese medaka, turbot, and grass carp (Robinson-Rechavi et al., 2001; Maglich et al., 2003). However, in some fish such as olive flounder only one single form of PPAR-α gene has been observed (Cho et al., 2012). The exact number of genes and/or the presence of distinct PPAR-α isotypes, as well as the functional comparison of different PPAR-α in fish, have not been determined.

Based on the roles of SREBP-1 and PPAR-α in lipid/fatty acid metabolism, SREBP-1 and PPAR-α serve as major sensors of fatty acids, in particular n-3 PUFA, and thus have emerged as key mediators of gene regulation by fatty acids (Nakamura and Nara, 2003; Nakatani et al., 2003; Nakamura et al., 2004; Howell et al., 2009; Poulsen et al., 2012). PUFA are reported to be ligands of PPAR-α (Price et al., 2000) and suppressors of SREBP (Morton and Shimomura, 1999) in mammals and humans. In fish, considering that lipid homeostasis, in particular LC-PUFA homeostasis, is so important to fish products, and that the present expansion of aquaculture requires alternative lipid sources due to the decline in commercial fisheries and consequently the decreasing supply of fish oil, the research into the regulation of SREBP-1 and PPAR-α by dietary fatty acids will be critical for elucidating the mechanisms involved in the regulation of lipid homeostasis of fish, in particular the degradation and synthesis of LC-PUFAs, by dietary lipid sources. However, up to now, limited information is available about the expression pattern of fish SREBP-1 and PPAR-α in response to dietary fatty acids. It is also not clear whether fatty acids act simply by regulating SREBP/PPAR-DNA binding or if they can also regulate the biosynthesis of SREBP/PPAR themselves and consequently regulate the nuclear abundance of these nuclear factors (Di Nunzio et al., 2009).

The present study was aimed to clone and characterize the gene of SREBP-1 and PPAR-α in an important aquaculture species Japanese seabass *Lateolabrax japonicus*, as well as to investigate the gene expression patterns of Japanese seabass SREBP-1 and PPAR-α in response to different dietary fatty acid profiles. The liver was used as the tissue to extract and assay the quantitative expression of Japanese seabass SREBP-1 and PPAR-α mRNA considering its importance in lipid homoeostasis and the high expressions of these transcription factors in liver (Shimomura et al., 1997). A greater understanding of the molecular basis of lipid and fatty acid homeostasis in fish will enable efficient and effective use of sustainable dietary oils while maintaining the nutritional quality of farmed fish. Since it is still not clear whether SREBPs and PPARs have similar roles in teleosts and mammals and to what extent the regulation of SREBP and PPAR are shared between the two species (Rakhshandehroo et al., 2009), this study on Japanese seabass SREBP and PPAR-α will provide new information on the evolution of structure and function of these receptors, as well as a clearer understanding of the involvement of SREBPs and PPARs in lipid homeostasis of fish.

2. Materials and methods

2.1. RNA extraction and cDNA synthesis

Total RNA was extracted from liver using TransZol (TransGen, Beijing, China) and then electrophoresed on a 1.5% agarose gel to test the integrity. Then, 1 μg total RNA was subjected to TransScript ™ One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen, Beijing, China) in 20 μl volume for reverse transcription and DNA erasure.

2.2. Cloning and sequencing of SREBP-1 and PPAR-α2 cDNA fragment

Four specific primers (SREBP-1F, SREBP-1R, PPAR-α2F, and PPAR-α2R) (Table 1) were designed based on highly conserved regions from the genes of other fish available in the GenBank database and synthesized by Biosune (Shanghai, China). Liver cDNA were used as the template for amplification. PCR amplifications using the primers and Taq DNA Polymerase (Takara, Dalian, China) were performed with an initial denaturation at 95 °C for 3 min and 35 cycles of "95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min", followed by a final extension at 72 °C for 10 min. All PCR products were run on a 1.5% agarose gel, and then purified by SanPrep PCR Purification Kit (Sangon Biotech, Shanghai, China). PCR products were cloned into pEASY-T1 simple cloning vector (TransGen, Beijing, China) and sequenced in BioSune (Shanghai, China).

2.3. Rapid amplification of cDNA ends (RACE)

Four gene-specific primers of each gene were designed based on the known sequences of the internal fragments of SREBP-1 and PPAR-α2 cDNA singlet to clone the 3′- and 5′-end by rapid amplification of cDNA ends (RACE) using the SMARTer™ RACE cDNA Amplification Kit (Clontech, California, USA) (Table 1). The 3′- and 5′-end cDNA templates were synthesized according to the user's manual. For 3′ RACE, the first and nested PCR rounds were performed using primers SREBP-1-3F1, SREBP-1-3F2, PPAR-α2-3F1, and PPAR-α2-3F2, and for 5′ RACE, SREBP-1-5R1, SREBP-1-5R2, PPAR-α2-5R1, and PPAR-α2-5R2 were used (Table 1). All PCR products were purified, cloned into vector, and sequenced as described in Section 2.2.

2.4. Real-time quantitative-polymerase chain reaction (RT-qPCR) analysis and semi-quantitative PCR (Semi-qPCR)

Real-time fluorescent quantitative PCR (RT-qPCR) was used to assay the relatively quantitative mRNA expression of SREBP-1 and PPAR-α in tissues of Japanese seabass. β-actin (GenBank accession no. HE577671.1) was selected as the reference gene from a number of commonly used reference genes in Japanese seabass such as 18 s rRNA, β-actin, TUBA, ODC, GAPDH, and EF1α based on preliminary tests using geNorm (version 3.5) and NormFinder algorithms (Vandesompele et al., 2002; Andersen et al., 2004). Specific primers for SREBP-1 (SREBP-1-qF and SREBP-1-qR), PPAR-α1 (PPAR-α1-qF and PPAR-α1-qR), PPAR-α2 (PPAR-α2-qF and PPAR-α2-qR), and β-actin (β-actinF and β-actinR) were designed using Primer 5.0 based on the partial cDNA sequences of SREBP-1 and PPAR-α2 obtained previously, and the complete cDNA sequences of PPAR-α1 (GenBank accession no. FJ208989.1) and β-actin from NCBI (Table 1). To determine the amplification efficiency and linear range of the real-time PCR assay, standard curves of the template cDNA were generated. The generated standard curves showed linearity over the entire quantitation range (The coefficients of linear regression (R2) were more than 0.99). The amplification efficiency was 94.17%, 95.79%, 98.48%, and 92.42% for β-actin, SREBP-1, PPAR-α1, and PPAR-α2, respectively.

First strand cDNA was synthesized as described previously in Section 2.2 and then diluted by 4 times using sterilized double-distilled water. The real-time PCR was carried out in a quantitative thermal cycler (Mastercyclerep *realplex*, Eppendorf, Germany) in a final volume of 25 μl containing 2 × TransStart™ Green qPCR SuperMix (TransGen, Beijing, China), primer pairs, and cDNA. The program was 95 °C for 2 min followed by 40 cycles of "95 °C for 10 s, 58 °C for 10 s, and 72 °C for 20 s". Melting curve (1.85 °C increment/min from 58 °C to 95 °C) was performed after the amplification phase for confirmation. Each sample was run in triplicate. The gene expression levels of putative SREBP-1, PPAR-α1, and PPAR-α2 were studied by RT-qPCR method: $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001).

Semi-qPCR was also used to assay the mRNA expression of SREBP-1, PPAR-α, and β-actin in tissues of Japanese seabass. Semi-qPCR was performed with 1 μl of the first-strand cDNA and 12.5 μl TaKaRa Ex Taq mix (TaKaRa, Dalian, China). Semi-qPCR was carried out with an initial denaturation at 94 °C for 3 min, followed by 28 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 10 min. PCR products were visualized on 1% agarose gel. The expression of the housekeeping gene β-actin was used as internal control to check the efficiency of cDNA synthesis

Table 1
Sequences of the PCR primers used in this work.

Primer	Sequences (5′-3′)	Sequence information
SREBP-1F	TCTGGAGRCAYCGCAARCAGGC	RT primer
SREBP-1R	GTGGGRCTGGCCCCNGCCATC	RT primer
PPAR-αF	TGGTCGGATGCCACAGGCGGAGAAGC	RT primer
PPAR-αR	GAACTGGAAYTTGGGCTCCATC	RT primer
SREBP-1-3F1	AGGCTCTTGGTCGTCCCTTACCCATCTC	3′RACE primer
SREBP-1-3F2	TGCCTGTGGGCTCTCTCGCTGAGGTCTA	3′RACE primer
PPAR-α2-3F1	GTGGAGAAAGAGGTGGCAAGTCCCATGC	3′RACE primer
PPAR-α2-3F2	TGAACAAGGCGAAAGCTCGGCTCATACT	3′RACE primer
SREBP-1-5R1	AGGAGGTGTTCTCTGAATGCCTGAGTCA	5′RACE primer
SREBP-1-5R2	AATAGCCCAATCCCCGTCACAAAGAAGC	5′RACE primer
PPAR-α2-5R1	TGAATGATGAAAGGCGGCTTGCTGGTCT	5′RACE primer
PPAR-α2-5R2	TGAGTATGAGCCGAGCTTTCGCCTTGTT	5′RACE primer
SREBP-1-qF	TGCTATCGGTTCTAACATGGCTAC	qPCR and *semi*-qPCR primer
SREBP-1-qR	AGTGCTCAACAGTCAGATACAGTC	qPCR and *semi*-qPCR primer
PPAR-α1-qF	AAGACCAGCACCCCTCCTTTCGT	qPCR and *semi*-qPCR primer
PPAR-α1-qR	CCGAACTTCTGCCTCCCTGTCCT	qPCR and *semi*-qPCR primer
PPAR-α2-qF	TTCCAGCTGGCAGAGAGGACGC	qPCR and *semi*-qPCR primer
PPAR-α2-qR	CACCCCACAGCCGGAACCACCT	qPCR and *semi*-qPCR primer
β-actin F	CAACTGGGATGACATGGAGAAG	qPCR and *semi*-qPCR primer
β-actin R	TTGG CTTTGGGGTTCAGG	qPCR and *semi*-qPCR primer

and cDNA integrity. The primer pairs used for Semi-qPCR were the same as RT-qPCR (Table 1).

2.5. Feeding procedure

Six diets with different characteristic fatty acid profiles but similar proximate compositions were used in the feeding trial (Tables 2, 3). The experimental diets contained approximately 42% crude protein and 11% crude lipid, which have been demonstrated to be suitable for Japanese seabass (unpublished data). Five different lipid sources, tripalmitin, tristearin, rapeseed oil, blended oil of linseed oil and perilla oil (linseed oil: perilla oil = 3:1), and n−3 LC-PUFA enriched oil, were supplemented separately to the basal diet at a ratio of 10% to obtain the dietary characteristic fatty acids, palmitic acid (PA, C16:0), stearic acid (SA, C18:0), oleic acid (OA, C18:1n−9), α-linolenic acid (ALA, C18:3n−3), and n−3 LC-PUFA (C22:6n−3 + C20:5n−3) respectively. The sixth diet was formulated with 10% fish oil (FO). The corresponding diets were named PA, SA, OA, ALA, n−3 LC-PUFA, and FO respectively. Juvenile Japanese seabass with initial average body weight of 29.53 ± 0.86 g were used in the feeding experiment. The feeding trial was conducted in sea floating net cages. Each diet was randomly assigned to triplicate cages, each stocked with 30 fish. Fish were hand-fed to apparent satiation twice daily (05:00 and 17:00). The feeding trial lasted 10 weeks. The detailed procedures of diet pelletization, fatty acid analysis, and feeding were according to our previous studies (Xu et al., 2014). At the end of the feeding trial, the fish were fasted for 24 h before harvest. Samples of liver from 5 fish from each cage were obtained, frozen with liquid nitrogen, and stored at −80 °C until use.

2.6. Statistical analysis

Similarity searches of the sequenced cDNA of SREBP-1 and PPAR-α2 were done by blastn (www.ncbi.nlm.nih.gov/blast/). The multiple-sequence alignments were performed using ClustalW. The deduced amino acid sequences were analyzed with DNAman and ExPASy Compute pI/MW (http://web.expasy.org/compute_pi/). SMART program (http://smart.emblheidelberg.de/) and PROSITE program (http://kr.expasy.org/prosite/) were used to predict the functional sites or domains in the amino acid sequence. Phylogenetic analyses were carried out based on amino acid sequences using the neighbor-joining method, and the trees were constructed using MEGA 4.1.

All data were subjected to one-way analysis of variance and correlation analysis where appropriate in SPSS 16.0 (SPSS Inc., Chicago, USA) for Windows. Differences between means were tested by Tukey's multiple range test. The level of significance was chosen at $P < 0.05$ and the results were presented as means ± standard error of the mean.

Table 2
Formulation (%) and proximate composition (%) of the experiment diets.

Gradients	PA	SA	OA	ALA	N−3 LC-PUFA	FO
Defatted fish meal[a]	26.00	26.00	26.00	26.00	26.00	26.00
Soybean meal[a]	34.00	34.00	34.00	34.00	34.00	34.00
Wheat meal	25.91	25.91	25.91	25.91	25.91	25.91
Tripalmitin[b]	10.00					
Tristearin[c]		10.00				
Rapeseed oil			10.00			
C18:3n−3 enriched oil [d]				10.00		
n−3 LC-PUFA enriched oil[e]					10.00	
Fish oil						10.00
Mineral premix[f]	2.00	2.00	2.00	2.00	2.00	2.00
Vitamin premix[g]	1.60	1.60	1.60	1.60	1.60	1.60
Mold inhibitor[h]	0.10	0.10	0.10	0.10	0.10	0.10
Attractant[i]	0.30	0.30	0.30	0.30	0.30	0.30
Ethoxyquin	0.05	0.05	0.05	0.05	0.05	0.05
Yttria	0.04	0.04	0.04	0.04	0.04	0.04
Proximate composition						
Crude protein	41.67	41.54	42.17	42.29	42.25	42.18
Crude lipid	11.36	11.16	10.93	10.89	11.26	11.51
Ash	11.06	10.47	11.16	10.46	10.84	11.25

[a] Defatted fish meal: 75.6% crude protein, 1.8% crude lipid; soybean meal: 51.7% crude protein, 2.0% crude lipid of dry matter.
[b] Shanghai Zhixin chemical Co., Ltd., Shanghai, China.
[c] HUDONG article of everyday use Co., Ltd., Jiaxing, China.
[d] Blended oil of linseed oil and perilla oil (Linseed oil: Perilla oil = 3:1).
[e] n−3 LC-PUFA enriched oil: DHA content, 37.1% of total fatty acid (TFA); EPA content, 20.8% of TFA; both in the form of triglyceride; HEBEI HAIYUAN Health biological Science and Technology Co., Ltd., Cangzhou, China.
[f] Mineral premix (mg or g/kg diet): $MgSO_4 \cdot 7H_2O$, 1200 mg; $CuSO_4 \cdot 5H_2O$, 10 mg; $ZnSO_4 \cdot H_2O$, 50 mg; $FeSO_4 \cdot H_2O$, 80 mg; $MnSO_4 \cdot H_2O$, 45 mg; $CoCl_2 \cdot 6H_2O$ (1%), 50 mg; $NaSeSO_3 \cdot 5H_2O$ (1%), 20 mg; $Ca(IO_3)_2 \cdot 6H_2O$ (1%), 60 mg; $CaH_2PO_4 \cdot H_2O$, 10 g; and zoelite, 8.485 g.
[g] Vitamin premix (mg or g/kg diet): thiamin 25 mg; riboflavin, 45 mg; pyridoxine HCl, 20 mg; vitamin B_{12} (1%), 10 mg; vitamin K_3, 10 mg; inositol, 800 mg; pantothenic acid, 60 mg; niacin, 200 mg; folic acid, 20 mg; biotin (2%), 60 mg; retinol acetate, 32 mg; cholecalciferol, 5 mg; alpha-tocopherol (50%), 240 mg; ascorbic acid, 2000 mg; choline chloride (50%), 4000 mg; and wheat middling, 8.47 g.
[h] Mold inhibitor: contained 50% calcium propionic acid and 50% fumaric acid.
[i] Attractant: glycine and betaine.

Table 3
Fatty acid profiles of experimental diets (of total fatty acid)[a].

Fatty acid	PA	SA	OA	ALA	N−3 LC-PUFA	FO
C14:0	0.79	1.94	0.29	0.38	0.65	4.13
C16:0	56.30	25.64	7.69	10.08	6.17	20.92
C18:0	18.60	55.78	3.62	4.99	7.13	14.25
∑SFA[b]	75.69	83.36	11.60	15.45	13.95	39.31
C16:1n−7	1.09	0.32	tr	tr	2.59	4.89
C18:1n−9	10.03	2.62	41.06	29.00	7.05	8.96
C20:1n−9	1.36	0.89	6.64	1.11	2.36	4.40
C22:1n−11	0.64	0.59	tr	0.22	0.57	4.93
C22:1n−9	nd	nd	15.97	nd	nd	nd
∑MUFA[c]	13.12	4.42	63.66	30.33	12.57	23.18
C18:2n−6	6.37	5.88	16.74	14.81	14.91	10.69
C20:4n−6	0.07	0.08	0.10	0.10	2.73	0.73
∑n−6[d]	6.54	6.32	16.85	14.91	17.86	11.64
C18:3n−3	0.80	1.04	5.48	35.38	2.17	2.13
C18:4n−3	0.11	0.09	0.11	0.15	0.93	1.36
C20:5n−3	0.74	0.63	0.67	0.87	15.19	6.03
C22:5n−3	0.08	0.07	0.08	0.09	1.19	0.70
C22:6n−3	0.87	0.84	0.83	1.18	28.80	9.12
∑n−3[e]	2.60	2.67	7.18	37.68	48.28	19.33
∑PUFA[f]	9.15	9.00	24.02	52.59	66.14	30.97
∑n−3LC-PUFA	1.69	1.55	1.58	2.15	45.18	15.84
∑n−3/∑n−6	0.40	0.42	0.43	2.53	2.70	1.66
C18:1n−9/∑n−3	3.85	0.98	5.72	0.77	0.15	0.46

[a] Some fatty acids, of which the contents are minor, trace amount or not detected, such as C22:0, C24:0, C14:1, C20:2n−6, and C20:3n−6, were not listed in the table. tr: trace; nd: non-detected.
[b] SFA: saturated fatty acid.
[c] MUFA: monounsaturated fatty acid.
[d] n−6: n−6 unsaturated fatty acid.
[e] n−3: n−3 unsaturated fatty acid.
[f] PUFA: polyunsaturated fatty acid.

3. Results

3.1. Cloning and characterization of SREBP-1 and PPAR-α2

The full length of SREBP-1 cDNA from Japanese seabass was 3797 bp (GenBank accession no. KM502869), including a 5′- untranslated region (UTR) of 98 bp, a 3′-UTR of 105 bp, and an open reading frame of 3594 bp encoding a polypeptide of 1197 amino acids with predicted molecular weight of 128.285 KDa and theoretical isoelectric point of 8.24 (Fig. 1). The deduced protein sequence of SREBP-1 displayed a typical structure of SREBP, a bHLH-zip domain (Fig. 1).

The full length sequence of PPAR-α2 cDNA from Japanese seabass was 1895 bp (GenBank accession no. KM502870) with a 5′-UTR of 241 bp, a 3′-UTR of 220 bp, and an open reading frame of 1434 bp, which encoded a polypeptide of 477 amino acids with predicted molecular weight of 52.831 KDa and theoretical isoelectric point of 5.82. The deduced protein sequence of PPAR-α2 displayed a typical structure of PPAR, which contains a DNA binding domain (C4-type zinc finger) and ligand binding domains (Fig. 1).

3.2. Multiple sequences alignment and phylogenetic analysis

The BLAST analysis revealed that SREBP-1 and PPAR-α2 of Japanese seabass shared high identity to known SREBP-1 and PPAR-α of teleost, respectively. For SREBP-1: rabbitfish (*Siganus canaliculatus*), 88%; zebra mbuna (*Maylandia zebra*), 87%; Nile tilapia (*Oreochromis niloticus*), 87%; Atlantic salmon (*Salmo salar*), 76%; and zebrafish (*Danio rerio*), 70%. For PPAR-α2: European sea bass (*Dicentrarchus labrax*), 96%; gilthead seabream (*Sparus aurata*), 94%; common dentex (*Dentex dentex*), 92%; rabbitfish (*S. canaliculatus*), 91%; Nile tilapia (*O. niloticus*), 91%; rainbow trout (*Oncorhynchus mykiss*), 76%; and Atlantic salmon (*S. salar*), 75%. Comparing the N-terminal protein sequence of Japanese seabass SREBP-1 with sequences of a range of other SREBP-1 from fish and human showed the SREBP-1 sequence in Japanese seabass to be more similar to human SREBP-1a than to human SREBP-1c (Fig. 1). There was no obvious difference in sequence conservation among PPAR-α from Japanese seabass, other fish, and humans. They enjoy a high sequence conservation in DNA binding domain (Fig. 1).

The phylogenic tree of SREBP-1 was in good agreement with traditional taxonomy. All marine fish species and freshwater fish species clustered together and formed a sister group to the branch of mammal species such as human and mouse (Fig. 2). The phylogenic tree of PPAR-α obviously divided into two branches, each with a PPAR-α isoform. Japanese seabass PPAR-α was clustered closest to PPAR-α of marine fish such as European sea bass (*D. labrax*) and European plaice (*Pleuronectes platessa*), and distant from PPAR-α from freshwater fish, and further still from PPAR-α of mammals (Fig. 2).

3.3. Tissue distribution of SREBP-1 and PPAR-α in Japanese seabass

The expression levels of SREBP-1 and PPAR-α varied among different tissues. SREBP-1 had the highest mRNA expression levels in the brain and eye, much lower levels in the intestine, stomach, and gill, and the lowest level in the muscle. PPAR-α1 had the highest mRNA expression level in the intestine and adipose tissue, moderate levels in the eye, brain, muscle, and heart, and the lowest level in the spleen and gill, while PPAR-α2 gene was strongly expressed in the liver, with much lower expression levels in the stomach, adipose tissue, muscle, and brain, and the lowest level in the kidney, eye, and spleen (Fig. 3).

3.4. SREBP-1 and PPAR-α mRNA expression in response to dietary fatty acid profiles

The stimulation of the mRNA expression level of SREBP-1 in the liver of Japanese seabass by dietary fatty acids significantly ($P < 0.05$) ranked as follows: PA, OA > SA, ALA, N−3 LC-PUFA > FO. The expression level of PPAR-α1 showed a similar trend with SREBP-1. Fish fed diets enriched with PA or OA showed significantly ($P < 0.05$) higher PPAR-α1 gene expression than fish fed diets enriched with ALA or FO, while no significant difference was observed either among fish fed diets enriched with PA, SA, OA, and N−3 LC-PUFA respectively or among fish fed diets enriched with SA, ALA, N−3 LC-PUFA, and FO respectively. The highest PPAR-α2 mRNA level in the liver of Japanese seabass was observed in fish fed the diet enriched with ALA, significantly ($P < 0.05$) higher than those of other treatments. Fish fed the diet enriched with N−3 LC-PUFA showed the second high PPAR-α2 gene expression level, significantly ($P < 0.05$) higher than those in fish fed diets enriched with OA or FO. Moderate PPAR-α2 gene expression levels were observed in fish fed diets enriched with PA or SA and no significant difference was observed either among fish fed diets enriched with PA, SA, and N−3 LC-PUFA respectively or among fish fed diets enriched with PA, SA, OA, and FO respectively (Fig. 4).

In addition, the fatty acid composition in the liver, as well as in the muscle, serum, and gut, in response to the experimental fatty acids profiles can be obtained from a supplementary material (Xu et al., 2014, doi: 10.1371/journal.pone.0087726). Briefly, the liver fatty acid compositions closely reflected those of the diets.

4. Discussion

The regulation of fatty acid metabolism is of great importance to nutrition and quality of farmed fish since fish are unique sources of n−3 LC-PUFA. The critical roles of SREBP-1 and PPAR-α in lipid/fatty acid homeostasis makes the study on fish SREBP-1 and PPAR-α indispensable for the research into lipid and fatty acid nutrition of fish (Chinetti et al., 2000; Horton, 2002; Horton et al., 2002; Ibabe et al., 2002; Nakamura et al., 2004; Minghetti et al., 2011; Cunha et al., 2013).

In the preset study, the cDNA of SREBP-1 in Japanese seabass were cloned and characterized for the first time. No evidence of alternatively spliced mRNAs of SREBP-1 was found in Japanese seabass, although this cannot be excluded since SREBP-1 cDNA was only synthesized from

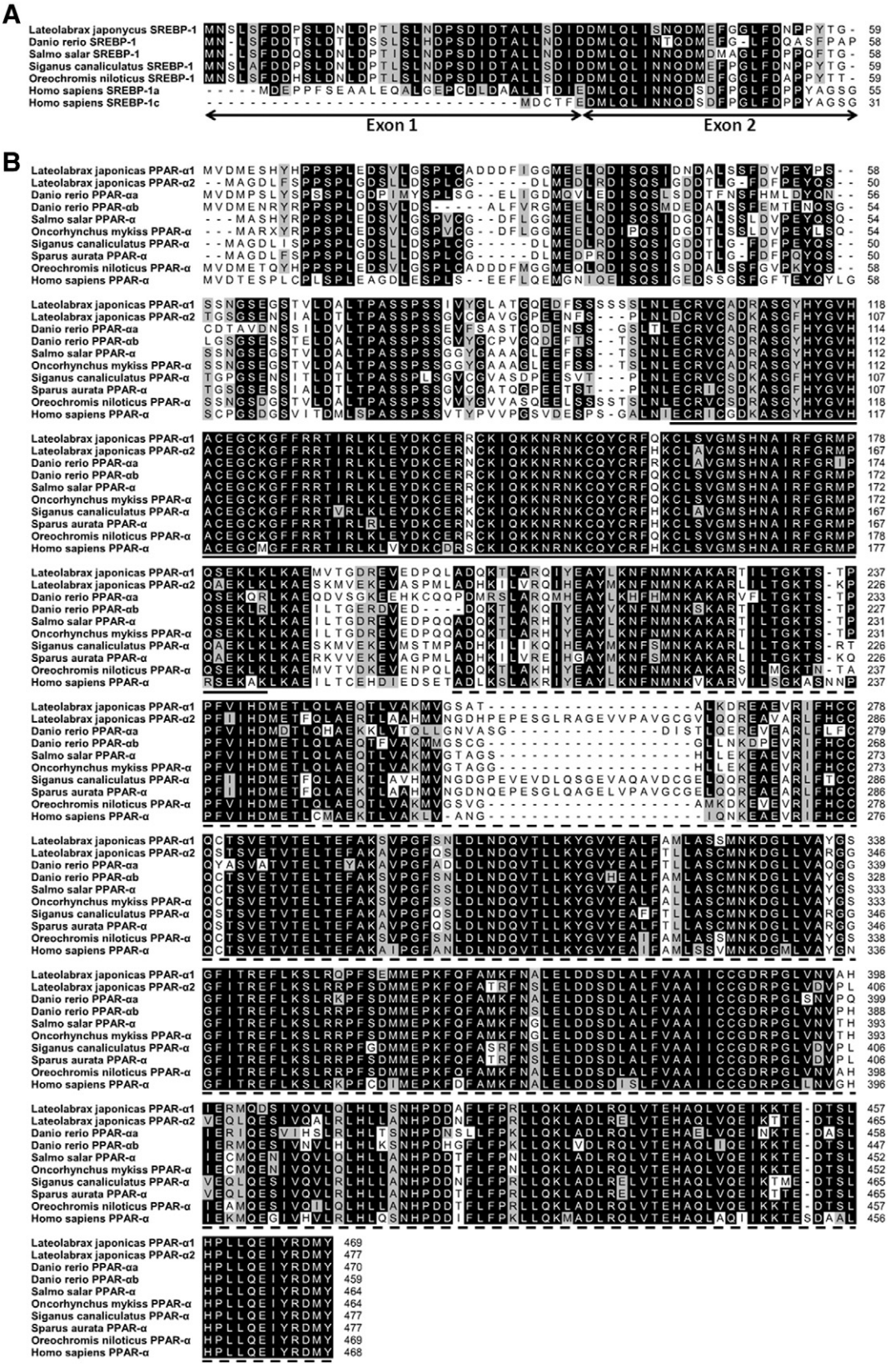

Fig. 1. Comparison of the deduced amino acid sequences of SREBP-1 (A, with parts of the sequences) and PPAR-α (B) from Japanese seabass, other fish, and humans. The DNA-binding domain and ligand-binding domain is solid and dash underlined respectively.

liver RNA in this study. Similar findings were also observed in other teleost, such as Atlantic salmon (Minghetti et al., 2011) and zebrafish (Thomas et al., 2013). The alignments analysis of the deduced amino acid sequences of SREBP-1 from Japanese seabass, other fish, and humans suggested that the SREBP-1 sequences in Japanese seabass, as well as those from other fish, were more similar to human SREBP-1a than to

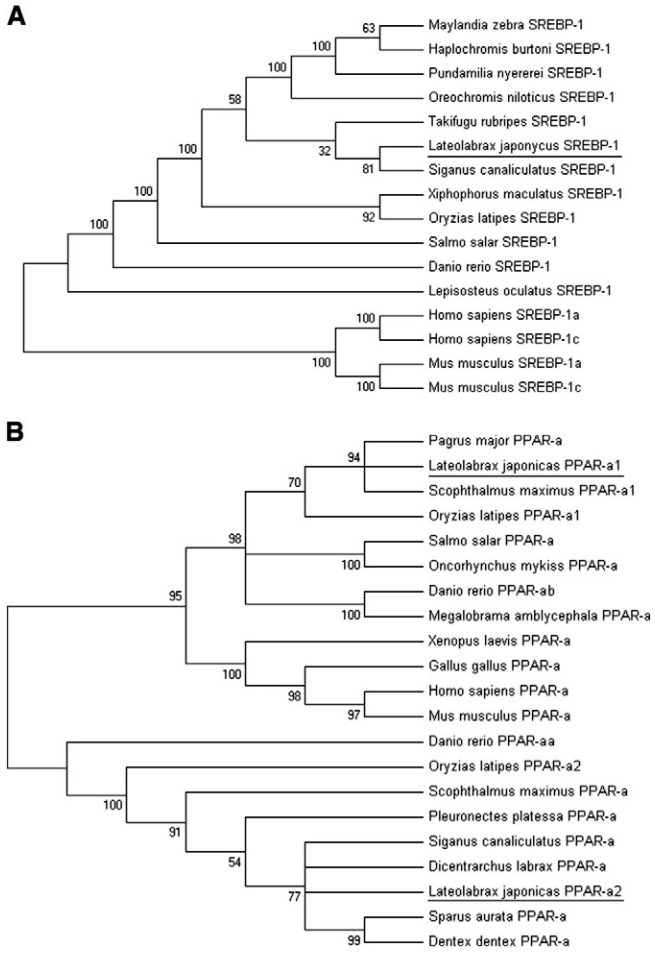

Fig. 2. Phylogenetic tree of SREBP-1 (A) and PPAR-α (B). The amino acid sequences of genes used in the phylogenetic tree included those from teleost, frog, nematode, chicken, and mammals. The horizontal branch length is proportional amino acid substitution rate per site. The numbers represent the frequencies with which the tree topology presented here was replicated after 1000 bootstrap iterations.

human SREBP-1c, indicating that fish SREBP-1 probably had functions similar to human SREBP-1a. Phylogenetic analysis of the SREBP-1 sequences reflected classical phylogeny, which showed that the SREBP-1 sequence in Japanese seabass was similar to rabbitfish *S. canaliculatus* and tiger puffer *Takifugu rubripes*, and the furthest related with mammals, only in which two forms of SREBP-1, SREBP-1a and 1c, have been observed.

The PPAR-α cDNA cloned in the present study (named PPAR-α2 here) was different from the one reported previously (GenBank accession no. FJ208989.1, named PPAR-α1 here), indicating that a new subtype of Japanese seabass PPAR-α was observed. Two PPAR-α genes also appeared in zebrafish, Japanese medaka, turbot, and grass carp, but not in olive flounder in which only one single form of PPAR-α gene has been observed (Cho et al., 2012). The deduced amino acid sequence of PPAR-α2 in Japanese seabass shared a high homology with Japanese seabass PPAR-α1, and the alignments analysis of the deduced amino acid sequences of PPAR-α proteins derived from Japanese seabass, other fish species, and human showed areas of strong sequence conservation, especially in the sequences of DNA binding domain. However, the phylogenetic analysis revealed that PPAR-α1 and PPAR-α2 of Japanese seabass were on different branches, with Japanese seabass PPAR-α1 and mammal PPAR-α on the same branch. This phenomenon also appeared in other fish species such as zebrafish, Japanese medaka, turbot, and grass carp. To date two PPAR-α subtypes have been observed only in fish species, while other species such as xenopus, jungle fowl, mouse, and humans, possess only one PPAR-α gene. Gene duplication might occur in the evolution of PPAR-α (Jaillon et al., 2004), resulting in two PPAR-α genes with diverged functions in some fish.

The tissue expression analysis in Japanese seabass showed that SREBP-1 was expressed in all detected tissues. The highest expression level of SREBP-1 was observed in the brain, which was in accordance with the high gene expression of Δ6 Fad in the brain of Japanese seabass observed in our previous studies (Xu et al., 2014). This to some extent provided new evidence for the role of SREBP-1 in regulating fish Fad (Matsuzaka et al., 2002). However, in Atlantic salmon the highest gene expression of SREBP-1 was observed in the intestine, followed by the brain and gill (Minghetti et al., 2011). In mammals, SREBP-1a and SREBP-1c are differentially expressed across tissues. SREBP-1c is highly expressed in liver whilst in other tissues, particularly lipogenic tissues, SREBP-1a predominates (Shimomura et al., 1997). This was in accordance with the results of multiple-protein sequence alignments that the deduced amino acid of Japanese seabass SREBP-1 was more similar to mammal SREBP-1a than to mammal SREBP-1c.

PPAR-α were expressed in the adipose tissue, heart, intestine, stomach, liver, brain, and muscle of Japanese seabass. This was consistent with the findings on other fish species and mammals. In

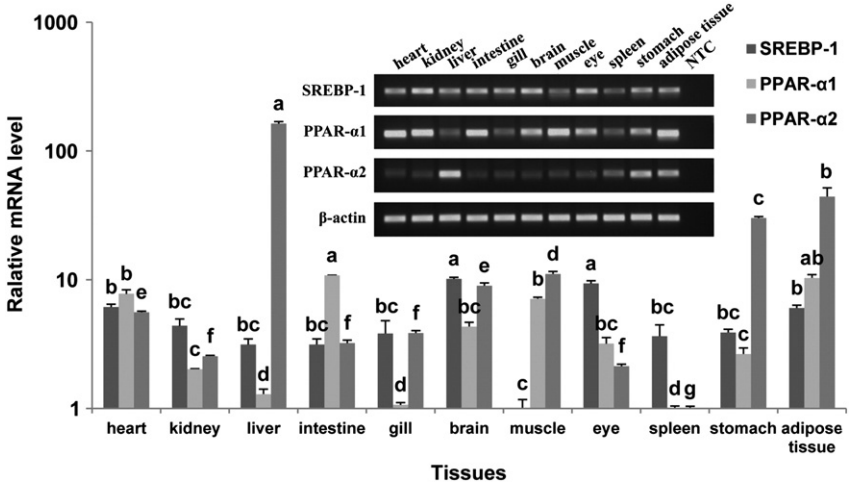

Fig. 3. Tissue distribution of SREBP-1, PPAR-α1, and PPAR-α2 in Japanese seabass. Relative mRNA levels of SREBP-1, PPAR-α1, and PPAR-α2 were expressed relative to β-actin levels. For each tested gene, the tissue with the lowest ΔCt value was used as the control to calculate the ΔΔCt value. Results are expressed as mean ± standard error (n = 5). Different letters above the bars denote significant ($P < 0.05$) differences among tissues. The electrophoretogram shows the semi-quantitative PCR assay of tissue expression of SREBP-1, PPAR-α1, PPAR-α2, and β-actin in Japanese seabass. NTC: No template control.

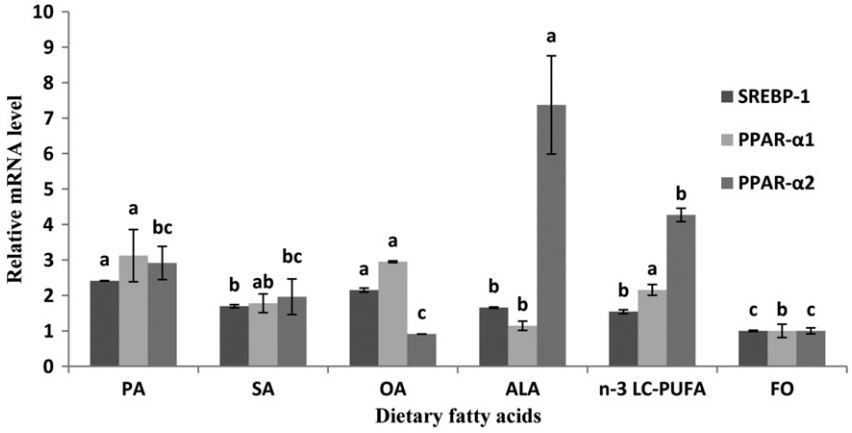

Fig. 4. Relative mRNA levels of SREBP-1, PPAR-α1, and PPAR-α2 in liver of experimental fish. Relative mRNA levels of SREBP-1, PPAR-α1, and PPAR-α2 were expressed relative to β-actin levels. For each tested gene, group FO was used as the control to calculate the ΔΔCt value. Results are expressed as means ± standard error (n = 3). Different letters above the bars denote significant ($P < 0.05$) differences among dietary groups.

Japanese flounder and zebrafish, PPAR-α was highly expressed in the stomach, liver, and intestine (Ibabe et al., 2002; Cho et al., 2012), while mammalian PPAR-α was mainly expressed in the liver (Wang et al., 2003; Mandard et al., 2004). On the other hand, the two subtypes of PPAR-α in Japanese seabass, PPAR-α1 and PPAR-α2, were differentially expressed across tissues. PPAR-α1 was highly expressed in the intestine, eye, muscle, and brain but PPAR-α2 was preferentially expressed in the liver, adipose tissue, and stomach. This indicated the possible functional differentiation between Japanese seabass PPAR-α1 and PPAR-α2. The tissues with high PPAR-α1 expression are the main places for LC-PUFA synthesis in fish, indicating the possible involvement of fish PPAR-α1 in LC-PUFA synthesis, while the tissue expression pattern of PPAR-α2 could be related to its roles in hydrolysis of triglyceride and phospholipid (Cunha et al., 2013).

The quantitative analysis of SREBP-1 and PPAR-α gene expression in fish fed diets with different fatty acid profiles showed that these genes were highly influenced by dietary fatty acids. Diets enriched with C16:0 or C18:1n − 9 significantly up-regulated SREBP-1 gene expression in the liver of Japanese seabass compared to the diet enriched with fish oil, while fish fed diets enriched with C18:0, C18:3n − 3, or n − 3 LC-PUFA showed moderate SREBP-1 mRNA levels. This was in great accordance with the regulation of Δ6 Fad gene expression in the liver of Japanese seabass by these diets, observed in a previous study of ours (the correlation coefficient (r) between SREBP-1 and Δ6 Fad gene expression regulated by these diets was 0.834, $P = 0.000$) (Supplementary Fig. 5 in Xu et al., 2014, online at doi: 10.1371/journal.pone.0087726). This result provided a new evidence for the involvement of SREBP-1 in the regulation of fish Fad by dietary fatty acids. Studies on Japanese flounder and Atlantic salmon also showed that fish fed diets supplemented with vegetable oils had higher gene expression levels of SREBP-1 compared with fish fed diets supplemented with fish oil (Morais et al., 2011; Limtipsuntorn et al., 2014). In mammal, it has been well known that SREBP-1 in particular SREBP-1c activity can be inhibited by PUFA such as α-linolenic acid (18:3n − 3), γ-linolenic acid (18:3n − 6), ARA (20:4n − 6), EPA (20:5n − 3), and DHA (22:6n − 3) (Deng et al., 2002; Yoshikawa et al., 2002; Sekiya et al., 2003; Jump et al., 2005, 2008; Espenshade, 2006; Leaver et al., 2008; Lecker et al., 2011; Lee and Song, 2012; Fukumitsu et al., 2013). Results of the present study further showed that the inhibition of SREBP-1 by PUFA increased with increasing carbon length and desaturation degree of fatty acids, as well as that even among saturated and monounsaturated fatty acids; the regulation of SREBP-1 varied. However, it remains not clear why the diet enriched with C18:0 down-regulated Japanese seabass SREBP-1 gene expression compared to the diet enriched with C16:0 or C18:1n − 9.

The gene expression pattern of Japanese seabass PPAR-α1 in response to dietary fatty acid profiles was very similar to that of SREBP-1. A significantly positive correlation was also observed between the Japanese seabass PPAR-α1 gene expression level and the previously observed Δ6 Fad transcript level in the liver of Japanese seabass in response to same dietary fatty acid profiles ($r = 0.650$, $P = 0.004$) (Supplementary Fig. 5 in Xu et al., 2014, online at doi: 10.1371/journal.pone.0087726). This indicated that PPAR-α1 was probably also involved in the regulation of fish Fad by dietary fatty acids. The gene expression of Japanese seabass PPAR-α1 in response to dietary fatty acid profiles was in agreement with what was observed in studies on European sea bass larvae and rainbow trout, which have showed that diets low in n − 3 LC-PUFA up-regulated the PPAR-α transcription compared to diets higher in n − 3 LC-PUFA (Vagner et al., 2009; Vestergren et al., 2013).

Interestingly, however, the gene expression pattern of Japanese seabass PPAR-α2 in response to dietary fatty acids was totally different from that of PPAR-α1. The diets enriched with n − 3 PUFA, in particular C18:3n − 3, significantly up-regulated the PPAR-α2 transcript level compared to diets enriched with monounsaturated fatty acids. This was consistent with the stimulation of mammalian PPAR-α by PUFA, in particular C18:3n − 3 (Sekiya et al., 2003; Gani, 2008; Harnack et al., 2009; Hajjar et al., 2012; Lee and Song, 2012; Schmidt et al., 2012; Strand et al., 2012; Devarshi et al., 2013). PPAR-α2 of Japanese seabass was possibly primarily involved in fatty acid oxidation, as have been demonstrated in mammals. The higher stimulating activity of C18:3n − 3 compared to n − 3 LC-PUFA on PPAR-α2 of Japanese seabass could be due to C18:3n − 3 being preferentially used as substrate for PPAR-mediating β-oxidation (Harnack et al., 2009). C18:3n − 3 could also stimulate PPAR-α expression as a high-affinity ligand. Preliminary evidence have suggested that preferred ligands for PPAR activation are 18 C and 20 C fatty acids, as fatty acids longer than 20 C will not fit correctly into the PPAR binding pocket and stabilize the AF-2 helix (Sprecher, 2000; Jump, 2002). This probably accounted for the reason that in the present study the diet enriched with fish oil, which is also characterized by high LC-PUFA contents, led to significantly lower PPAR-α2 gene expression than the diet enriched with n − 3 LC-PUFA, since the latter diet had a much higher C20:5n − 3 content (15.9% of total fatty acids) than the diet enriched with fish oil (6.03% of total fatty acids). Regarding findings on other fish species, PPAR-α expression in Atlantic salmon was also down-regulated by diets supplemented with vegetable oils, but only in lean fish (Morais et al., 2011).

The difference between the gene expression patterns of PPAR-α1 and PPAR-α2 in response to dietary fatty acid profiles indicated functional distribution of PPAR-α in Japanese seabass. This was in accordance with the pleiotropic functions of mammalian PPAR-α. The function of Japanese seabass PPAR-α1 and PPAR-α2 appears to be equivalent to the function of mammalian PPAR-α in regulating LC-PUFA synthesis and

fatty acid oxidation respectively. However, the DNA-binding domain, which determines the target gene and consequently the function of PPAR, was highly conserved between Japanese seabass PPAR-α1 and PPAR-α2, indicating that even a slight variation in the critical area of gene sequence may lead to functional distributions of PPAR-α proteins. The studies on turbot larvae also suggested that the different transcription profile observed in the two PPARα isoforms is probably indicative of a gene sub-functionalization (Cunha et al., 2013).

In conclusion, in the present study, SREBP-1 and a new PPAR-α from Japanese seabass, PPAR-α2, were first cloned and characterized. The gene expression of SREBP-1 and PPAR-α1 in liver of Japanese seabass was down-regulated by dietary PUFA compared to dietary saturated or monounsaturated fatty acids, while PPAR-α2 gene expression was stimulated by dietary n−3 PUFA, in particular α-linolenic acid. This study provided basic information for future studies on teleost SREBP-1 and PPAR-α, as well as further insight into the mechanism involved in the regulation of fish lipid homeostasis by alternative lipid sources.

(Supplementary materials related to this article can be found online at doi: 10.1371/journal.pone.0087726).

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