Aquaculture Research

Aquaculture Research, 2016, **47**, 445–459 doi: 10.1111/are.12505

Molecular cloning, tissue distribution and nutritional regulation of a Δ6-fatty acyl desaturase-like enzyme in large yellow croaker (*Larimichthys crocea*)

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Abstract

In this study, the full-length cDNA of a Δ6-fatty acyl desaturase-like (Δ6-Fad-like) enzyme was first cloned from large yellow croaker, *Larmichthys crocea*. The cDNA was 2049 bp, with a 107 bp 5′-UTR, a 604 bp 3′-UTR, and an ORF of 1338 that specified a protein of 445 amino acids. It contains two membrane-spanning domains, three histidine-rich regions and a cytochrome b_5 domain, which all align perfectly with the same domains located in other recently identified vertebrate Δ5 and Δ6 desaturases. Sequence comparison showed that the predicted protein revealed a high percentage identity (>70%) with Δ6 desaturases from other marine fish species. Tissue distribution analysis revealed that Δ6-Fad-like was expressed at highest level in brain, much lower level in liver and gill, and lowest level in spleen, heart, stomach and intestine. The hepatic mRNA levels of Δ6-Fad-like showed statistically negative relationship relative to increasing dietary n-3LC-PUFA and DHA/EPA ($P < 0.05$, $R > 0.6$). Transcription of Δ6-Fad-like in liver of fish fed diets with low and moderate level of n-3LC-PUFA (0.15%, 0.60% and 0.98% dry diet) was significantly increased by more than 20-fold than that with higher n-3LC-PUFA (1.37%, 1.79% and 2.25% dry diet; $P < 0.05$). The transcriptional levels of Δ6-Fad-like in the liver of fish fed diets with the ratio of 0.61, 1.54 and 2.17 (DHA/EPA) were significantly up-regulated by about 46-fold, 4.8-fold, and 1.2-fold compared with that in the control group (DHA/EPA = 3.88) respectively ($P < 0.05$). This could contribute to better understanding the process of n-3LC-PUFA biosynthesis in this fish species.

Keywords: large yellow croaker, Δ6-Fad-like, nutritional regulation, n-3 LC-PUFA, DHA/EPA

Introduction

Fish and seafood are the best source of health promoting n-3 LC-PUFA for humans (Agaba, Tocher, Zheng, Dickson, Dick & Teale 2005; Metcalf, Sanders, James, Cleland & Young 2008; Proudman, Cleland & James 2008; Smithers, Gibson, McPhee & Makrides 2008). Since wild fisheries are finite, normal development of aquaculture activities could be constrained due to FO shortage. Therefore, it is currently great urgency for the aquafeed industry to replace FO as much as possible. Among all alternative oils, vegetable oils are viewed a good choice due to its stable supply and relatively low cost over the past decades (Turchini, Torstensen & Ng 2009). However, high inclusion of vegetable oils could adversely affect the tissue n-3LC-PUFA level of marine fish due to its less ability of LC-PUFA biosynthesis from linolenic acid (18:3n-3, LNA) and linoleic acid (18:2n-6, LA) than the fresh water counterparts (Ghioni, Tocher, Bell, Dick & Sargent 1999; Sargent & Tacon 1999; Torstensen, Bell, Rosenlund, Henderson, Graff & Tocher 2005). Thus, there is great

interest in elucidating the LC-PUFA biosynthesis pathway and regulation mechanism in marine fish species.

Two categories of enzymes, fatty acid desaturase (Fads) and elongase (Elovl), were involved in LC-PUFA biosynthesis. Among them, Fads are more accepted as the rate-limiting enzyme during n-3LC-PUFA biosynthesis (González-Rovira, Mourente, Zheng, Tocher & Pendón 2009; Vagner & Santigosa 2011; Morais, Castanheira, Martinez-Rubio, Conceição & Tocher 2012). Until now, Δ6 Fads have been successfully cloned from a variety of teleost fish species (Seiliez, Panserat, Corraze, Kaushik & Bergot 2003; Zheng, Seiliez, Hastings, Tocher, Panserat, Dickson, Bergot & Teale 2004; Tocher, Zheng, Schlechtriem, Hastings, Dick & Teale 2006; González-Rovira *et al.* 2009; Zheng, Ding, Xu, Monroig, Morais & Tocher 2009; Monroig, Zheng, Morais, Leaver, Taggart & Tocher 2010; Ren, Yu, Xu & Tang 2012). Specially, some new functions of Fads, such as Δ5 in common octopus (*Octopus vulgaris*) (Monroig, Navarro, Dick, Alemany & Tocher 2011), Δ5 and Δ4 in white-spotted rabbitfish (*Siganus canaliculatus*) (Li, Monroig, Zhang, Wang, Zheng, Dick, You & Tocher 2010) and Δ4 in *Solea senegalensis* (Morais *et al.* 2012) have been reported recently. The expression of rate-limiting enzymes (Δ6/5/4 Fads and Elovl) of fish species could be regulated by developmental stage (Ishak, Tan, Khong, Jaya-Ram, Enyu, Kuah & Shu-Chien 2008; Tan, Chung & Shu-Chien 2010; Jaya-Ram, Ishak, Enyu, Kuah, Wong & Shu-Chien 2011; Morais *et al.* 2012), environment factors (salinity and water temperature) (Zheng, Torstensen, Tocher, Dick, Henderson & Bell 2005; Li, Hu, Zheng, Xia, Xu, Wang, Chen, Sun & Huang 2008) as well as diet nutrition (Zheng *et al.* 2005; Ling, Kuah, Sifzizul, Muhammad, Kolkovski & Shu-Chien 2006; Tocher *et al.* 2006; Jaya-Ram, Kuah, Lim, Kolkovski & Shu-Chien 2008; Morais, Mourente, Ortega, Tocher & Tocher 2011; Thanuthong, Francis, Manickam, Senadheera, Cameron-Smith & Turchini 2011; Morais *et al.* 2012).

Large yellow croaker, *Larimichthys crocea*, is an important carnivorous marine fish species widely cultured in southeast China. Recently, low retention of n-3 LC-PUFA has been observed after large proportion of fish oil was replaced by vegetable oil, which seriously affected fillet quality (Duan, Mai, Shentu, Ai, Zhong, Jiang, Zhang, Zhang & Guo 2014). During the past decade, numerous studies have been conducted to investigate nutrient requirement, metabolism and nutritional immunology in juvenile large yellow croaker (Ai, Mai, Tan, Xu, Duan, Ma & Zhang 2006; Ai, Mai, Zhang, Tan, Zhang, Xu & Li 2007; Ai, Zhao, Mai, Xu, Tan, Ma & Liufu 2008; Wang, Ai, Mai, Xu, Xu, Zhang, Wang & Liufu 2010; Ai, Xu, Mai, Xu, Wang & Zhang 2011; Zuo, Ai, Mai, Xu, Wang, Xu, Liufu & Zhang 2012a,b; Zuo, Ai, Mai & Xu 2013). However, as far as we know, no information was available about molecular basis of LC-PUFA biosynthesis in this fish species. Thus, this study was conducted to clone and detect mRNA profile of Δ6 Fad in response to dietary fatty acid. It was aimed to find some basic clues to better understand LC-PUFA biosynthetic process and potentially decrease reliance on fish oil for large yellow croaker.

Materials and methods

Ethics statement

This study was conducted in Xiangshan Bay (Ningbo, China) under the permission of Ningbo Marine Fisheries Bureau. All procedures involving animals implemented during this experiment were approved by the Institutional Animal Care and Use Committee of the Ocean University of China (protocol number 20001001). All possible efforts were taken to minimize animal suffering.

Experimental designs and diets

Large yellow croaker (246.5 ± 4.8 g) were bought from a commercial farm in Xiangshan Bay, Ningbo, China and used for Δ6 Fad gene isolation and tissue-specificity expression detection. After being anaesthetized with eugenol (1:10 000; Shanghai Reagent, Shanghai, China), brain, kidney, spleen, liver, stomach, intestine and gill from five fish were collected, flash-frozen in liquid nitrogen and then stored at −80°C for RNA extraction and later analysis.

Then two feeding experiments were conducted to investigate the effects of dietary n-3 LC-PUFA (0.15%, 0.60%, 0.98%, 1.37%, 1.79% and 2.25% dry weight; Tables 1 and 2) and DHA/EPA (0.61%, 1.54%, 2.17%, 3.04% and 3.88; Tables 3 and 4) on hepatic mRNA profile of Δ6 Fad. Feeds formulation, pellets producing procedures and experimental conditions have been described in detail in our previous studies (Zuo *et al.* 2012a,b).

Table 1 Formulation and proximate analysis of the experimental diets with graded levels of n-3 LC-PUFA (% dry weight)

	Dietary n-3 LC-PUFA (% dry weight)					
Ingredients (%)	**0.15**	**0.60**	**0.98**	**1.37**	**1.79**	**2.25**
Defatted white fish meal*	15.00	15.00	15.00	15.00	15.00	15.00
Soybean meal	32.00	32.00	32.00	32.00	32.00	32.00
Casein†	12.00	12.00	12.00	12.00	12.00	12.00
Wheat meal	25.50	25.50	25.50	25.50	25.50	25.50
Mineral premix‡	2.00	2.00	2.00	2.00	2.00	2.00
Vitamin premix§	2.00	2.00	2.00	2.00	2.00	2.00
Attractant	0.30	0.30	0.30	0.30	0.30	0.30
Mould inhibitor	0.10	0.10	0.10	0.10	0.10	0.10
Lecithin	2.60	2.60	2.60	2.60	2.60	2.60
DHA-enriched oil¶	0.05	0.77	1.48	2.18	2.93	3.62
EPA-enriched oil**	0.00	0.45	0.90	1.36	1.79	2.26
Palmitin††	7.45	6.28	5.12	3.96	2.78	1.62
ARA-enriched oil‡‡	1.00	1.00	1.00	1.00	1.00	1.00
Total	100	100	100	100	100	100
Proximate analysis (n = 3)						
Crude protein (%)	41.27	41.21	40.99	42.08	41.42	41.36
Crude lipid (%)	11.37	11.37	11.29	11.03	11.18	10.98
n-3 LC-PUFA (% dry weight)	0.15	0.60	0.98	1.37	1.79	2.25

*Defatted fish meal: 79.1% crude protein and 1.6% crude lipid; white fish meal were defatted with ethanol (fish meal:ethanol = 1:2 (w:v)) at 37°C for three times.

†Casein: 93% crude protein and 1% crude lipid, Alfa Aesar, Avocado Research Chemicals, UK.

‡Mineral premix (mg or g kg^{-1} diet): $CuSO_4 \cdot 5H_2O$, 10 mg; Na_2SeO_3 (1%), 25 mg; $ZnSO_4 \cdot H_2O$, 50 mg; $CoCl_2 \cdot 6H_2O$ (1%), 50 mg; $MnSO_4 \cdot H_2O$, 60 mg; $FeSO_4 \cdot H_2O$, 80 mg; Ca $(IO_3)_2$, 180 mg; $MgSO_4 \cdot 7H_2O$, 1200 mg; zeolite, 18.35 g.

§Vitamin premix (mg or g kg^{-1} diet): vitamin D, 5 mg; vitamin K, 10 mg; vitamin B_{12}, 10 mg; vitamin B_6, 20 mg; folic acid, 20 mg; vitamin B_1, 25 mg; vitamin A, 32 mg; vitamin B_2, 45 mg; pantothenic acid, 60 mg; biotin, 60 mg; niacin acid, 200 mg; α-tocopherol, 240 mg; inositol, 800 mg; ascorbic acid, 2000 mg; microcrystalline cellulose, 16.47 g.

¶DHA-enriched oil: DHA content, 270.3 mg g^{-1} oil; EPA content, 6.5 mg g^{-1} oil; both in the form of methylester; Hubei Youzhiyou Biotechnology, China.

**EPA-enriched oil: EPA content, 301.2 mg g^{-1} oil; DHA content, 157.8 mg g^{-1} oil; both in the form of triglyceride; HEBEI HAIYUAN Health Biological Science and Technology, China.

††Palmitin: Palmitic acid content, 99.3% of TFA, in the form of methylester; Shanghai Dinghua Chemical, China.

‡‡ARA-enriched oil: ARA content, 348.1 mg g^{-1} oil; in the form of ARA-methylester; Hubei Youzhiyou Biotechnology, China.

At the termination of the experiment, the fish were fasted for 24 h before harvest. Liver from five fish in each cage was sampled and pooled together into 1.5 mL tube (RNAase-Free; Axygen, Tewksbury, MA, USA), frozen in liquid nitrogen and then stored at −80°C for the analysis of expression profiles of Δ6-Fad-like in response to dietary n-3 LC-PUFA and DHA/EPA.

RNA extraction and cDNA synthesis

Total RNA was extracted from liver using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) and electrophoresed on a 1.2% denaturing agarose gel to test the integrity. Then, RNA was treated with RNA-Free DNase (TaKaRa, Otsu, Japan) to remove DNA contaminant and reverse transcribed to cDNA by PrimeScript™ RT reagent Kit (TaKaRa) following the instructions.

Cloning and sequencing of Fad cDNA fragment

Two specific primers (LycFad 01 and LycFad 02, Table 5) were designed to clone the middle fragment by polymerase chain reaction (PCR). Liver cDNA was used as a template for amplification. PCR was performed using primers LycFad 01 and LycFad 02, with one cycle of denaturation at 94°C for 3 min, 35 cycles of 94°C for 30 s, 58.0°C annealing for 30 s and 72°C for 60 s, followed by a 10 min extension at 72°C. All PCR products were cloned into pEASY-T1 simple cloning vector (Beijing TransGen Biotech, Beijing, China) and sequenced in BioSune (Shanghai, China).

Table 2 Fatty acid composition of the experimental diets with graded levels of n-3 LC-PUFA (% total fatty acids)*

	Dietary n-3 LC-PUFA (% dry weight)					
Fatty acid	**0.15**	**0.60**	**0.98**	**1.37**	**1.79**	**2.25**
14:0	0.57	1.03	1.79	2.23	2.80	3.53
16:0	70.55	64.25	60.40	48.67	39.77	31.16
18:0	2.71	2.67	2.58	2.75	2.87	2.91
20:0	0.43	0.48	0.49	0.66	0.73	0.84
∑SFA†	74.26	68.43	65.26	54.30	46.18	33.43
16:1	0.77	0.73	0.78	0.77	0.82	0.86
18:1	5.99	6.15	6.34	6.90	7.14	7.60
∑MUFA‡	6.76	6.88	7.12	7.67	7.96	8.46
18:2n-6	12.27	12.74	12.87	13.81	13.99	14.53
20:4n-6	3.37	3.65	3.46	4.23	4.39	4.61
∑n-6 PUFA§	15.64	16.40	16.33	18.04	18.38	19.14
18:3n-3	1.27	1.31	1.34	1.47	1.50	1.64
20:5n-3	0.48	1.71	2.79	4.86	6.32	8.05
22:6n-3	0.91	3.54	5.00	9.44	12.68	15.71
∑n-3 PUFA¶	2.66	6.56	9.14	15.78	20.51	25.39
n-3/n-6PUFA	0.17	0.40	0.56	0.87	1.11	1.33
n-3LC-PUFA**	1.39	5.25	7.79	14.31	19.01	23.75
DHA/EPA††	1.90	2.02	1.93	1.94	2.01	1.95

*Some fatty acids, of which the contents are minor, trace amount or not detected, such as C22:0, C24:0, C14:1, C20:1n-9, C22:1n-11, C20:2n-6, C20:3n-6, C22:5n-3, were not listed in the table.
†SFA: saturated fatty acids.
‡MUFA: mono-unsaturated fatty acids.
§n-6 PUFA: n-6 poly-unsaturated fatty acids.
¶n-3 PUFA: n-3 poly-unsaturated fatty acids.
**n-3 LC-PUFA: n-3 highly unsaturated fatty acids.
††DHA/EPA: 22:6n-3/20:5n-3.

Rapid amplification of cDNA ends

Based on the partial sequence of LycFad, the 3′ and 5′ ends were obtained by rapid amplification of cDNA ends (RACE) approaches. The 3′ end RACE PCR reaction was performed with liver cDNA template using the gene-specific primer LycFad 03 and the adaptor primer RIP following the instructions of a 3′-Full RACE Core Set Ver.2.0 kit (cat. no. D314; TaKaRa), and then a seminested PCR was performed using LycFad 04 and RIP (Table 5). The PCR amplification was performed using the same reaction system as described before with universal primer A mix (UPM; Table 5) and LycFad 05 by the 5′ RACE system (cat. no. 634923; Clontech, Mountain View, CA, USA), and then a semi-nested PCR was carried out using UPM and LycFad 06. PCR products were gel-purified, cloned and sequenced as described above.

Table 3 Formulation and proximate analysis of the experimental diets with graded ratios of DHA/EPA (% dry weight)

	Dietary DHA/EPA ratio				
Ingredients	**0.61**	**1.54**	**2.17**	**3.04**	**3.88**
White fish meal*	35.00	35.00	35.00	35.00	35.00
Soybean meal*	25.50	25.50	25.50	25.50	25.50
Wheat meal*	25.50	25.50	25.50	25.50	25.50
Mineral premix†	2.00	2.00	2.00	2.00	2.00
Vitamin premix‡	2.00	2.00	2.00	2.00	2.00
Attractant§	0.30	0.30	0.30	0.30	0.30
Mould inhibitor¶	0.10	0.10	0.10	0.10	0.10
Lecithin	2.60	2.60	2.60	2.60	2.60
DHA-enriched oil**	0.10	1.15	1.78	2.20	2.52
EPA-enriched oil††	1.68	1.05	0.67	0.42	0.25
Palmitin‡‡	3.82	3.40	3.15	2.98	2.83
ARA-enriched oil§§	1.40	1.40	1.40	1.40	1.40
Proximate analysis (n = 3)					
Crude protein (%)	41.40	41.31	41.19	41.08	41.42
Crude lipid (%)	11.22	11.27	11.19	11.03	11.38
DHA/EPA ratio	0.61	1.54	2.17	3.04	3.88
n-3 LC-PUFA (%)	1.07	1.04	1.02	1.04	1.03

*White fish meal: crude protein 74.3% dry matter, crude lipid 6.6% dry matter; soybean meal: crude protein 49.4% dry matter, crude lipid 0.9% dry matter; wheat meal: crude protein 16.4% dry matter, crude lipid 1.0% dry matter.
†Mineral premix (mg or g kg^{-1} diet): $CuSO_4{\cdot}5H_2O$, 10 mg; Na_2SeO_3 (1%), 25 mg; $ZnSO_4{\cdot}H_2O$, 50 mg; $CoCl_2{\cdot}6H_2O$ (1%), 50 mg; $MnSO_4{\cdot}H_2O$, 60 mg; $FeSO_4{\cdot}H_2O$, 80 mg; Ca $(IO_3)_2$, 180 mg; $MgSO_4{\cdot}7H_2O$, 1200 mg; zeolite, 18.35 g.
‡Vitamin premix (mg or g kg^{-1} diet): vitamin D, 5 mg; vitamin K, 10 mg; vitamin B_{12}, 10 mg; vitamin B_6, 20 mg; folic acid, 20 mg; vitamin B_1, 25 mg; vitamin A, 32 mg; vitamin B_2, 45 mg; pantothenic acid, 60 mg; biotin, 60 mg; niacin acid, 200 mg; α-tocopherol, 240 mg; inositol, 800 mg; ascorbic acid, 2000 mg; microcrystalline cellulose, 16.47 g.
§Attractant: glycine and betaine.
¶Mould inhibitor: contained 50% calcium propionic acid and 50% fumaric acid.
*DHA-enriched oil: DHA content, 270.3 mg g^{-1} oil; EPA content, 6.5 mg g^{-1} oil; both in the form of DHA-methylester; Hubei Youzhiyou Biotechnology, China.
††EPA-enriched oil: EPA content, 301.2 mg g^{-1} oil; DHA content, 157.8 mg g^{-1} oil; both in the form of triglyceride; HEBEI HAIYUAN Health Biological Science and Technology, China.
‡‡Palmitin: Palmitic acid content, 99.3% of total fatty acids, in the form of methylester; Shanghai Dinghua Chemical, China.
§§ARA-enriched oil: ARA content, 348.1 mg g^{-1} oil; in the form of ARA-methylester; Hubei Youzhiyou Biotechnology, China.

Sequence analysis and phylogenetic analysis

The cDNA sequence of LycFad was analysed for similarity with other known sequences using the BLAST program at web servers of NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). The deduced

Table 4 Fatty acid composition of the experimental diets with graded ratios of DHA/EPA (% total fatty acids)*

	Dietary DHA/EPA ratio				
Fatty acid	**0.61**	**1.54**	**2.17**	**3.04**	**3.88**
14:0	1.55	1.89	2.66	2.85	3.29
16:0	51.98	42.98	44.30	43.01	42.83
18:0	3.43	3.60	3.55	3.68	3.57
20:0	1.07	1.21	1.00	1.04	0.99
∑SFA†	58.03	49.68	51.51	50.58	50.68
16:1	2.02	1.70	1.78	1.85	1.94
18:1	9.71	9.54	9.27	9.34	8.86
∑MUFA‡	11.73	11.24	11.05	11.19	10.80
18:2n-6	13.30	13.33	13.45	13.45	13.56
20:4n-6	4.58	5.51	5.18	5.25	5.29
∑n-6 PUFA§	17.88	18.84	18.63	18.70	18.85
18:3n-3	1.41	1.45	1.48	1.45	1.57
20:5n-3	7.51	5.06	3.60	2.90	2.39
22:6n-3	4.58	7.79	7.82	8.81	9.27
∑n-3 PUFA¶	13.50	14.30	12.90	13.16	13.23
n-3/n-6PUFA	0.76	0.76	0.69	0.70	0.70
n-3LC-PUFA**	12.09	12.85	11.42	11.71	11.66
ARA/EPA††	0.61	1.09	1.44	1.81	2.21
DHA/EPA‡‡	0.61	1.54	2.17	3.04	3.88

*Some fatty acids, of which the contents are minor, trace amount or not detected, such as C22:0, C24:0, C14:1, C20:1n-9, C22:1n-11, C20:2n-6, C20:3n-6, C22:5n-3, were not listed in the table.
†SFA: saturated fatty acids.
‡MUFA: mono-unsaturated fatty acids.
§n-6 PUFA: n-6 poly-unsaturated fatty acids.
¶n-3 PUFA: n-3 poly-unsaturated fatty acids.
**n-3 LC-PUFA: n-3 highly unsaturated fatty acids.
††ARA/EPA: 20:4n-6/20:5n-3.
‡‡DHA/EPA: 22:6n-3/20:5n-3.

LycFad amino acid sequence was analysed with DNAstar. Alignment of multiple sequences was performed using the CLUSTALW program at the European Bioinformatics Institute (http://www.ebi.ac.uk/clustalw/) and Multiple Alignment show (http://www.bio-soft.net/sms/index.html). 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 and molecular evolutionary analyses were conducted according to the amino acid sequences of the selected HSP90s by programs of CLUSTAL X1.83 and MEGA 4.0 (Tamura, Dudley, Nei & Kumar 2007). An unrooted phylogenetic tree among these species was determined using the neighbour-joining distance method. The relative importance of branching order was evaluated by the bootstrapping method (1000 replications).

Real-time PCR analysis of LycFad mRNA expression

Real-time PCR was applied to evaluate the mRNA level of LycFad in different tissues as well as in livers of large yellow croaker fed diets with graded dietary n-3 LC-PUFA and DHA/EPA. First strand cDNA was synthesized as described in section 'Experimental designs and diets' and then diluted by four times using sterilized double-distilled water. LycFad QF and LycFad QR (Table 1) were used to amplify a

Table 5 Sequences of PCR primers utilized in the present study

Primers	Sequences (5′–3′)	Annealing temperature (°C)	Sequence information
LycFad 01	GGCCACCTGTCTGTCTTCAA	58.0	RT primer
LycFad 02	GCCACCAGGTGGTAGTTGTG	58.0	RT primer
LycFad 03	TGGACCACCGCTTCTCATTC	55.0	3′RACE primer
LycFad 04	ATTCGCTTCCTCTGCTGCTA	57.0	3′RACE primer
LycFad 05	GGGTACATAGCAGCAGAGGAAGCGAAT	69.0	5′RACE primer
LycFad 06	GAATGAGAAGCGGTGGTCCAACAAGAAG	75.0	5′RACE primer
RIP	CGCGGATCCTCCACTAGTGATTTCACTATAGG	–	3′RACE primer
UPM (short)	CTAATACGACTCACTATAGGGC	–	5′RACE primer
UPM (long)	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	–	5′RACE primer
LycFad QF	TTCGCTTCCTCTGCTGCTATG	57.5	qPCR primer
LycFad QR	CCAGTCACGGTGCTTCTCG	57.5	qPCR primer
β-actin F	TTATGAAGGCTATGCCCTGCC	57.5	Inner control
β-actin R	TGAAGGAGTAGCCACGCTCTGT	57.5	Inner control

fragment from the liver cDNA and the PCR product was sequenced to verify the specificity of RT-PCR. Two β-actin primers, β-actin F and β-actin R (Table 5) were used to amplify a fragment as an internal control to calibrate the cDNA template for corresponding samples.

Real-time RT-PCR was carried out in a quantitative thermal cycler (Mastercycler ep realplex, Eppendorf, Germany). The amplification was performed in a total volume of 25 μL, containing 1 μL of each primer (10 μM), 1 μL of the diluted first strand cDNA product, 12.5 μL of 2× SYBR® Premix Ex Taq™ II (TaKaRa) and 9.5 μL of sterilized double-distilled water. The real-time PCR programme was as follows: 95°C for 2 min, followed by 35 cycles of 95°C for 10 s, 57.5°C for 10 s and 72°C for 20 s. At the end of each PCR reaction, melting curve analysis was performed to confirm that only one PCR product was present in these reactions. Standard curves were made with five different dilutions (in triplicate) of the cDNA samples and amplification efficiency was analysed according to the following equation $E = 10^{(-1/\text{Slope})} - 1$. The primer amplification efficiency was 1.008 for LycFad, 1.073 for β-actin. The absolute ΔC_T value (Δ6 LycFad − β-actin) of the slope is 0.065, which is close to zero and indicate that $\Delta\Delta C_T$ calculation for the relative quantification of target genes can be used. To calculate the expression of Δ6 Fad, the comparative CT method ($2^{-\Delta\Delta t}$ method) was used as described by Yao, Kong, Wang, Ji, Cai, Liu and Han (2008).

Statistical analysis

All data were subjected to a one-way ANOVA and differences between means were tested by Tukey's multiple range test. Transcriptional levels of Δ6-Fad-like in liver were analysed by linear regression relative to increasing dietary n-3 LC-PUFA and DHA/EPA. The level of significance was chosen at $P < 0.05$ and the results were presented as means ± SEM. All statistical analyses were performed by SPSS 16.0 for Windows (SPSS Incorporation, Chicago, IL, USA).

Results

Sequence analyses of large yellow croaker LycFad cDNAs

The cDNA fragment of 884b was amplified by the specific primers LycFad 01 and LycFad 02 and its nucleotide sequence was homogeneous to other known fatty acid Δ6 Fads. Then specific primers were designed based on the known sequence of Fad and used for the full-length cloning of this gene based on the RACE technology. Two end fragments were amplified by 3′-RACE and 5′-RACE PCR respectively. The complete cDNA sequence of the LycFad was obtained by overlapping these fragments mentioned above. The full-length sequence of LycFad was deposited in Genbank under the accession no. JX434611. The complete sequence of LycFad mRNA and the deduced amino acids are shown in Fig. 1. The full-length cDNA sequence of LycFad was shown to be 2049 bp with a 5′-UTR of 107 bp and 3′-UTR of 604 bp. Analyses by DNAstar indicated that cDNA included an open reading frame of 1338 bp, which encoded a polypeptide of 445 amino acids with predicted molecular mass of 52.041 kDa, theoretical isoelectric point of 8.47. The protein sequence included all the characteristic features of microsomal fatty acyl desaturases, including two transmembrane regions, three histidine boxes and an *N*-terminal cytochrome b5 domain containing the haem-binding motif, HPGG (Fig. 2).

Multiple sequences alignment and phylogenetic analysis

The deduced amino acid sequences of LycFad in large yellow croaker were analysed using BLAST and results showed that it shared higher homologies with Δ6 Fads from teleosts, such as Nibe croaker (*Nibea mitsukurii*, 99%), Gilthead seabream (*Sparus aurata*, 85%), European seabass (*Dicentrarchus labrax*, 85%), Orange-spotted grouper (*Epinephelus coioides*, 82%), Cobia (*Rachycentron canadum*, 79%), Turbot (*Scophthalmus maximus*, 78%), Senegalese sole (*S. senegalensis*, 74%), Snakedhead murrel (*Channa striata*, 71%).

To reveal the molecular phylogenetic position of the LycFad, a phylogenetic tree was constructed using the programs of CLUSTAL X1.83 and MEGA 4.0. The relationship which was observed in the phylogenic tree was in a good agreement with traditional taxonomy. The LycFad belongs to the branch of Δ6 Fad, which were separated from branches of other Fad subclass in the tree. In the branch of Δ6 Fad, all marine fish species and freshwater fish species were clustered together and formed a sister group to the branch of other

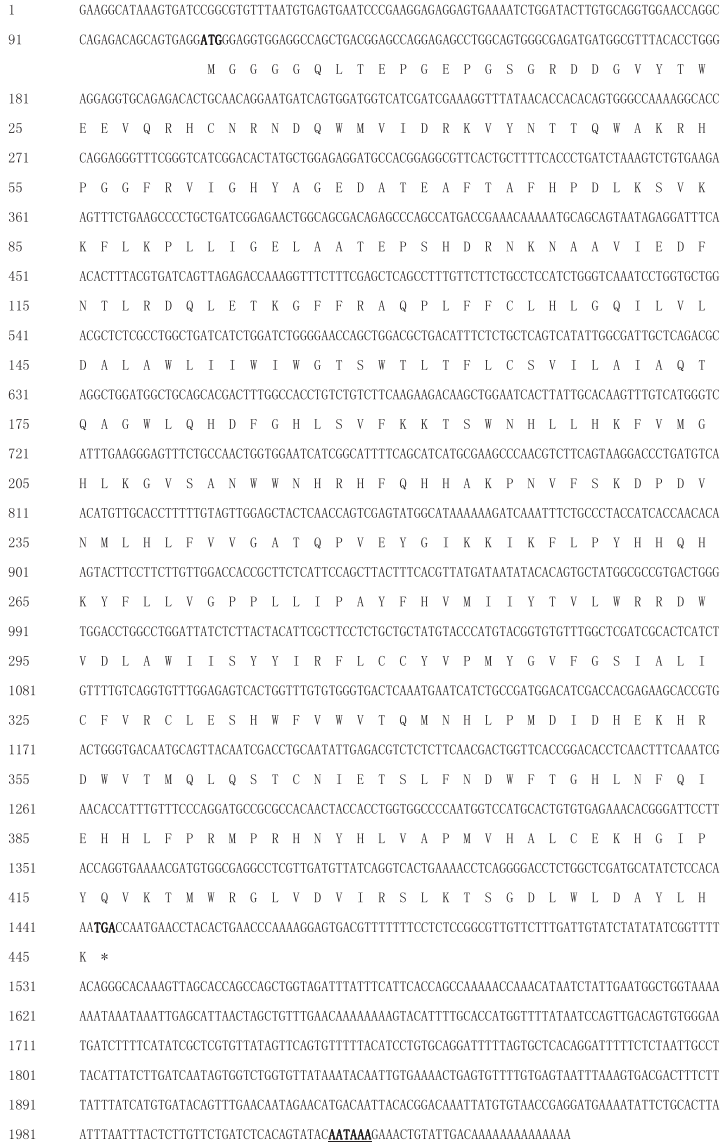

Figure 1 Nucleotide and deduced amino acid sequences of Δ6-Fad-like. The nucleotides and amino acids are numbered along the left margin respectively. The start (ATG) and stop (TGA) codons are marked in bold. Highly conserved motifs are boxed, and the ER retention signal is bold shaded.

vertebrates, including fog and some mammal species such as human and mouse (Fig. 3).

Tissue expression of large yellow croaker Δ6-Fad-like

The constitutive expressions of Δ6-Fad-like in tissues were confirmed by quantitative real-time RT-PCR using the specific primers (LycFad QF/QR and β-actin F/R, Table 5) and β-actin was used as an internal control. The transcription of Δ6-Fad-like was detected in liver, brain, intestine, gill, heart, stomach and spleen. Results indicated that Δ6-Fad-like was strongly expressed at highest level in brain, much lower level in liver and gill, and lowest level in spleen, heart, stomach and intestine. The transcriptional levels of Δ6-Fad-like in brain and liver was more than 500-fold than that in spleen, heart and intestine ($P < 0.01$; Fig. 4).

Nutritional regulation of large yellow croaker Δ6-Fad-like

Transcriptional levels of Δ6-Fad-like in liver showed statistically significant negative linear relationship relative to increasing dietary n-3LC-PUFA, with an $R = 0.648$ and $P < 0.01$ ($Y = 48.84 - 22.35X$, Fig 5a). Transcription of Δ6-Fad-like in

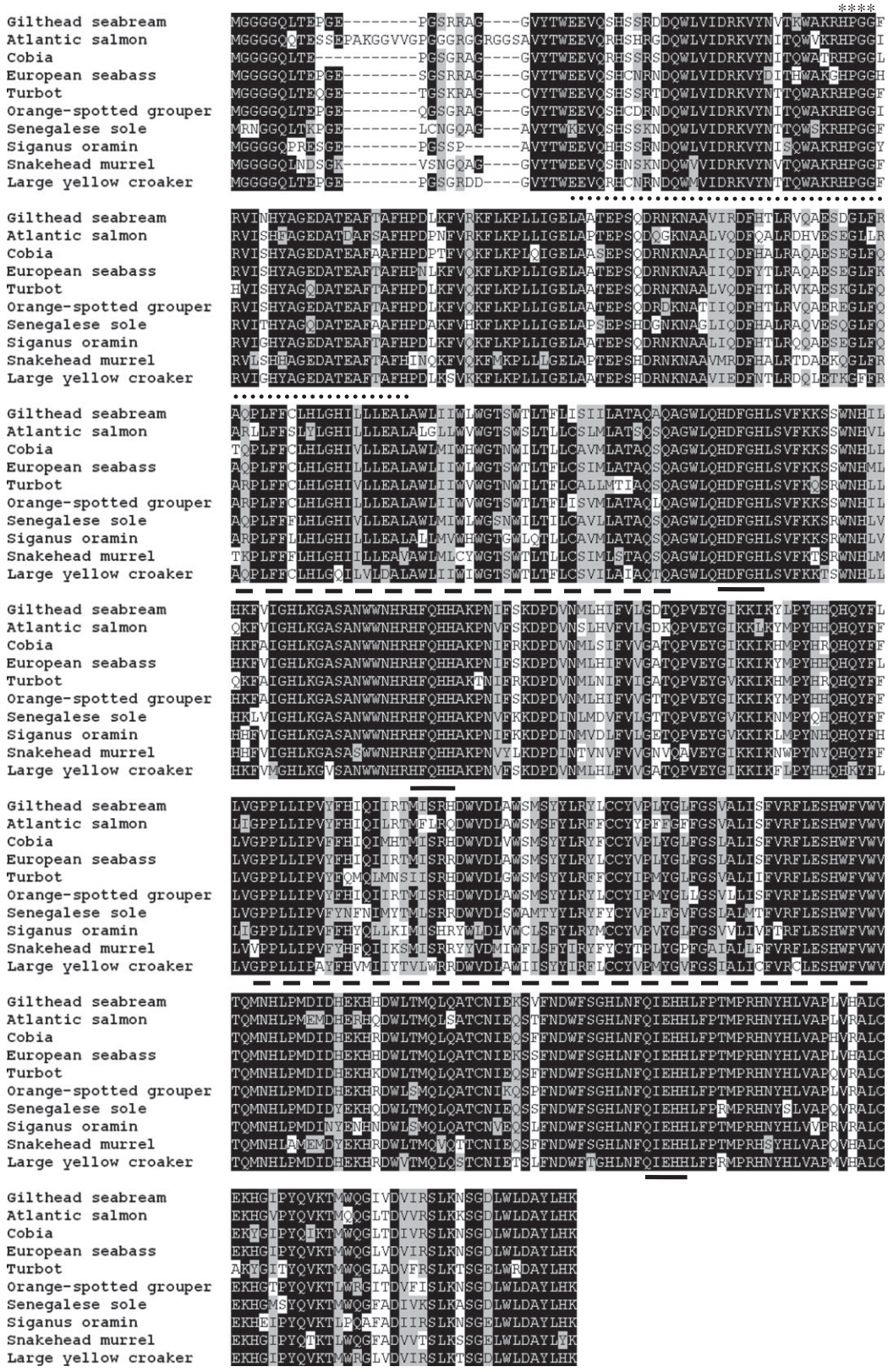
Gilthead seabream
Atlantic salmon
Cobia
European seabass
Turbot
Orange-spotted grouper
Senegalese sole
Siganus oramin
Snakehead murrel
Large yellow croaker

Figure 2 A deduced amino acid sequence comparison of the Δ6-Fad-like from large yellow croaker (*Larmichthys crocea*) with Δ6 desaturases from Gilthead seabream (*Sparus aurata*, ADD50000), Atlantic salmon (*Salmo salar*, NP_001165752), Cobia (*Rachycentron canadum*, ACJ65149), European seabass (*Dicentrarchus labrax*, ACD10793), Turbot (*Scophthalmus maximus*, AAS49163), Orange-spotted grouper (*Epinephelus coioides*, ACJ26848), Senegalese sole (*Solea senegalensis*, AEQ92868), Snakedhead murrel (*Channa striata*, ACD70298). The amino acid sequences were aligned using ClustalW Multiple alignment. Identity/similarity shading was based on the BLOSUM62 matrix, and the cutoff for shading was 75%. The cytochrome b5–like domain is dot-underlined, the two transmembrane regions are dash-under-lined, and the three histidine-rich domains are solid–underlined. The asterisks on the top mark the haem-binding motif, HPGG.

liver of fish fed diets with low and moderate level of n-3LC-PUFA (0.15%, 0.60% and 0.98%) was significantly increased by more than 20-fold than that with higher n-3LC-PUFA (1.37%, 1.79% and 2.25%; $P < 0.05$; Fig. 5a). The hepatic mRNA level of Δ6-Fad-like also showed significantly negative linear relationship relative to increasing ratio of DHA/EPA, with an $R = 0.699$ and $P < 0.05$ ($Y = 32.39 - 9.11X$, Fig 5b). The mRNA level of Δ6-Fad-like in the liver of fish fed diets with the ratio of 0.61, 1.54 and 2.17 (DHA/EPA) was significantly up-regulated by about 46-fold, 4.8-fold, and 1.2-fold compared with the control group (DHA/EPA = 3.88) respectively ($P < 0.05$; Fig. 5b).

Discussion

The study reported here revealed that large yellow croaker express a fatty acyl desaturase. Comparing the protein sequence with sequences of a range of other desaturases of fish and human showed the LycFad sequence in large yellow croaker to be more similar to the human Δ6 than to the human Δ5, but most similar to the Δ6 desaturases previously cloned from other marine fish, particularly Nibe croaker (*N. mitsukurii*), gilthead sea bream (*S. aurata*) European seabass (*D. labrax*, 85%), Orange-spotted grouper (*E. coioides*, 82%), Cobia (*R. canadum*, 79%), Turbot (*S. maximus*, 78%), Senegalese sole (*S. senegalensis*, 74%), Snakedhead murrel (*C. striata*, 71%). This was consistent with the findings of previous studies which found that Δ6 in most marine teleosts have high similarity (Tocher *et al.* 2006). Phylogenetic analysis of the desaturase sequences reflected classical phylogeny, showing the large yellow croaker branching from the Acanthopterygia (cichlids, perciformes and pleuronectiformes) line, and further separated from both the carp and zebrafish (Ostariophysi; cyprinids), and salmonids (Salmoniformes; salmonidae) (Tocher *et al.* 2006). However, in this study, no functional characterization has been done to detect whether it possessed the function of Δ6-Fad or not. Thus, this gene was putative only and named as Δ6-Fad-like.

Tissue expression study showed that Δ6-Fad-like was constitutively expressed in all detected tissues, with the highest expression level in brain, followed by liver and gill, and the lowest level in heart, spleen and intestine. It was also acknowledged that Fads and Elovl were highly expressed in brain and liver (Zheng *et al.* 2005; Tocher *et al.* 2006). High brain transcriptional levels of Δ6 in this and some previous studies could imply the importance of this enzyme in maintaining normal LC-PUFA levels of cell membranes, especially those in the neutral tissues (Zheng *et al.* 2005; Tocher *et al.* 2006). Neural tissue phospholipids of vertebrates are rich in DHA, which plays a critical role in visual and learning processes (Neuringer, Connor, van Petten & Bastad 1984; Neuringer, Anderson & Connor 1988; Rodríguez, Pérez, Díaz, Izquierdo, Fernández-Palacios & Lorenzo 1997). A diet lacking DHA or with a low ratio of DHA/EPA could result in the visual development problems which would then lead a decrease in hunting efficiency and consequently a reduction in growth rate of marine fish larvae (Watanabe, Izquierdo, Takeuchi, Satoh & Kitajima 1989; Rodríguez *et al.* 1997) and juveniles such as striped jack (Watanabe, Takeuchi, Arakawa, Imaizumi, Sekiya & Kitajima 1989), red seabream (Takeuchi, Toyota, Satoh & Watanabe 1990), grouper (Wu, Ting & Chen 2002) and barramundi (Glencross, Rutherford & Jones 2011).

In this study, Δ6-Fad-like expression was also nutritionally regulated, with transcription enhanced by low dietary n-3 LC-PUFA and DHA/EPA. This was consistent with the findings of some previous studies, which found that expression levels of both Δ6 and Δ5 desaturases were increased in

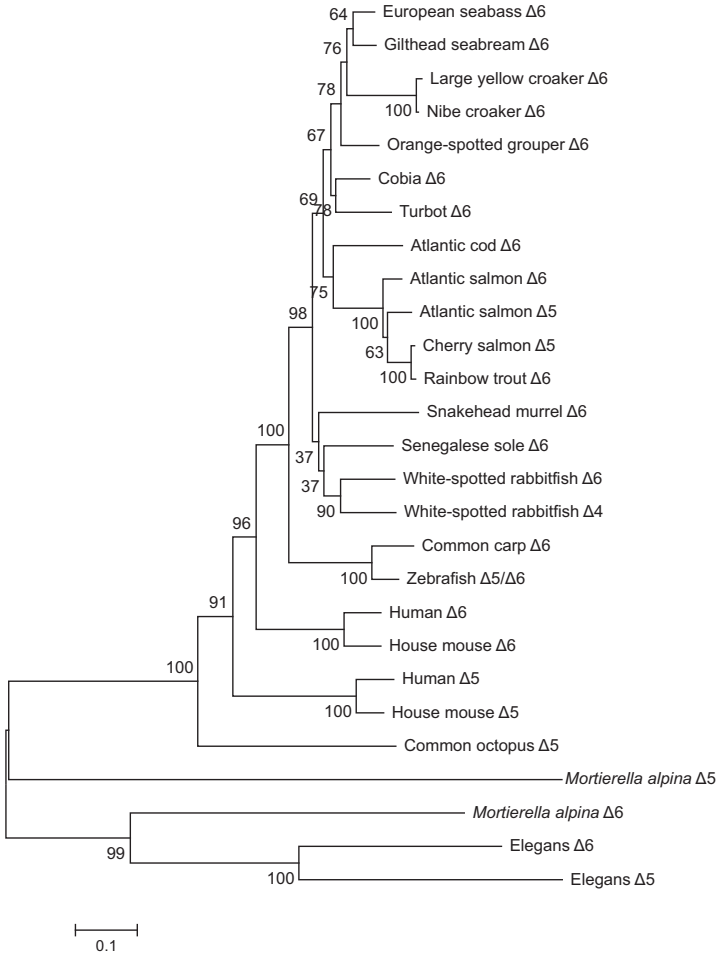

Figure 3 Phylogenetic relationship between the amino acid sequences of Δ6-Fad-like from large yellow croaker (*Larmichthys crocea*) and 26 available desaturases. The amino acid sequences were derived from the GenBank under the following accession numbers (in parentheses): European seabass (*Dicentrarchus labrax*): Δ6 Fad (ACD10793); Gilthead seabream (*Sparus aurata*): Δ6 Fad (ADD50000); Nibe croaker (*Nibea mitsukurii*): Δ6 Fad (ACX54437); Orange-spotted grouper (*Epinephelus coioides*): Δ6 Fad (ACJ26848); Cobia (*Rachycentron canadum*): Δ6 Fad (ACJ65149); Turbot (*Scophthalmus maximus*): Δ6 Fad (AAS49163); Atlantic cod (*Gadus morhua*): Δ6 Fad (AAY46796); Atlantic salmon (*Salmo salar*): Δ6 Fad (NP_001165752), Δ5 Fad (AAL82631), Cherry salmon (*Oncorhynchus masou*): Δ5 Fad (ABU87822); Rainbow trout (*Oncorhynchus mykiss*): Δ6 Fad (AAK26745); Snakehead murrel (*Channa striata*): Δ6 Fad (ACD70298); Senegalese sole (*Solea senegalensis*): Δ6 Fad (AEQ92868); White-spotted rabbitfish (*Siganus canaliculatus*): Δ6 Fad (ABR12315), Δ4 Fad (ADJ29913); Common carp (*Cyprinus carpio*): Δ6 Fad (AAG25711); Zebrafish (*Danio rerio*): Δ6/Δ5 Fad (AAG25710); Human (*Homo sapiens*): Δ6 Fad (AAD20018), Δ5 Fad (AAF29378); House mouse (*Mus musculus*): Δ6 Fad (AAD20017), Δ5 Fad (BAB69894); Common octopus (*Octopus vulgaris*): Δ5 Fad (AEK20864); Mortierella alpine (*Mortierella alpina*): Δ6 Fad (AAF08685), Δ5 Fad (AAC72755); Elegans (*Caenorhabditis elegans*): Δ6 Fad (AAC15586), Δ5 Fad (AAC95143).

liver of salmonids fed a VO blend (rich in C18 PUFA) compared with levels in fish fed FO (Seiliez, Panserat, Kaushik & Bergot 2001; Zheng *et al.* 2004, 2005; Ling *et al.* 2006; Tocher *et al.* 2006; Turchini & Francis 2009). However, hepatic n-3LC-PUFA retention in 0.15% n-3LC-PUFA treatment was significantly lower than that with moderate or high intake of n-3LC-PUFA (Zuo *et al.* 2012a). This indicated that very limited content of n-3LC-PUFA was biosynthesized.

Terrestrial carnivores appeared to lack or express low Δ5 and Δ6 Fads (Rivers, Sinclair & Crawford

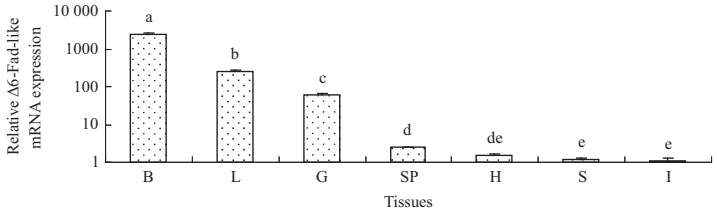

Figure 4 Relative expression of Δ6-Fad-like in different tissues of *Larmichthys crocea*. The transcriptional levels of Δ6-Fad-like in brain (B), stomach (S), spleen (SP), gill (G), heart (H), intestine (I) and liver (L) were normalized to that of intestine (I). Bars bearing with different letters are significantly different by Tukey's test ($P < 0.05$).

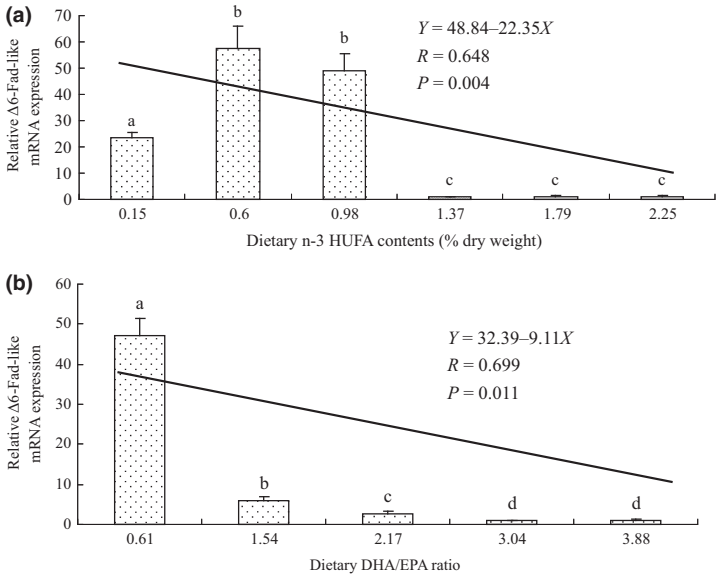

Figure 5 Relative hepatic Δ6-Fad-like mRNA expression of large yellow croaker, *Larmichthys crocea* fed diets with graded levels of dietary n-3 LC-PUFA (a) and DHA/EPA (b). Values are means ± SEM ($n = 3$). Bars bearing with different letters are significantly different by Tukey's test ($P < 0.05$). A line with P and R values was shown across the five or six bars if significant linear relationship was detected relative to increasing dietary n-3 LC-PUFA and DHA/EPA.

1975). This led to the proposition that carnivorous marine fish may be deficient in desaturase activity (Mourente & Tocher 1993). During the past decade, numerous studies have proven the existence of Δ6 (Seiliez *et al.* 2003; Zheng *et al.* 2004; Tocher *et al.* 2006; González-Rovira *et al.* 2009; Zheng, Ding *et al.* 2009; Monroig *et al.* 2010; Ren *et al.* 2012), Δ5 (Li *et al.* 2010; Monroig *et al.* 2011), and even Δ4 Fads (Li *et al.* 2010; Morais *et al.* 2012) in marine fish species. Furthermore, several Elovls have also been cloned from marine fish species (Agaba *et al.* 2005; Hastings, Agaba, Tocher, Zheng, Dickson, Dick & Teale 2005; Morais, Monroig, Zheng, Leaver & Tocher 2009; Zheng, Ding *et al.* 2009; Gregory, See, Gibson & Schuller 2010; Carmona-Antoñanzas, Tocher, Taggart & Leaver 2013). This further verified that low LC-PUFA biosynthesis could not be due to deficiencies in these steps (desaturasion and elongation). Thus, further studies should be emphasized to find practical means of elevating the activities of crucial enzymes (Δ6, Δ5 Fads, Elovl5 and Elovl2), such as promoters (Zheng, Leaver & Tocher 2009) and transcription factors (e.g. sterol regulatory element-binding protein and peroxisome proliferator-activated receptor) involved in LC-PUFA metabolism (Li, Mai, He, Ai, Zhang, Xu, Wang, Liufu, Zhang & Zhou 2013).

Recently, the first Fad showing Δ4 activity has been cloned from a herbivorous marine fish species, *S. canaliculatus* (Li *et al.* 2010), which revealed the possibility that docosapentaenoic acid (DPA; 22:5n-3) could be desaturated directly to DHA under the help of Δ4 desaturase and not need the extra 'elongation-Δ6 desaturation-chain short' steps during the synthesis of DHA from EPA (Agaba *et al.* 2005). In this study, expression of Δ6-Fad-like could be significantly decreased with the increase in dietary DHA/EPA. Thus, it is speculated that Δ6 Fad rather than Δ4 Fad could participate in the bioconversion from EPA to DHA in large yellow croaker. Further investigations are needed to ascertain whether Δ4 Fad existed in large yellow croaker.

To conclude, a Δ6-Fad-like enzyme was cloned and investigated in large yellow croaker in the present study. Δ6-Fad-like was broadly expressed in most tissues with the highest level in brain, followed by liver and gill, and lowest level in spleen, heart, stomach and intestine of large yellow croaker. The hepatic expression of Δ6-Fad-like could be up-regulated by low dietary n-3 LC-PUFA and DHA/EPA. Future studies are needed to functionally characterize this gene and other critical enzymes involved in LC-PUFA biosynthesis for this fish species.

Acknowledgments

This research was supported by the National Natural Foundation of China (grant no. 30871930). We thank M. Zhao, W.J. Mu and Y.W. Luo for their selfless help in gene clone and expression analysis. Thanks are also due to J. Wang, H.G. Xu, H. Asino and K.K. Zhang for their help in the feeding experiment. There are no conflicts of interest to report.

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