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Characterisation, mRNA expression and nutritional regulation of a Δ6 fatty acyl desaturase-like gene by dietary DHA levels of *Perinereis aibuhitensis* (Gube, 1878)

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ABSTRACT

The Δ6 fatty acyl desaturase (Δ6 Fad) enzyme plays an important role in the pathways for the biosynthesis of the highly unsaturated fatty acids (HUFA) by converting linoleic (LA, 18:2n-6) and α-linolenic (LNA, 18:3n-3) acids to 18:3n-6 and 18:4n-3, respectively. In the present study, a Δ6 Fad-like gene was cloned and its characterisation, tissue expression and nutritional regulation by dietary DHA (docosahexaenoic acid) levels were analysed in the clamworm *Perinereis aibuhitensis*. The full-length cDNA was 1535 bp including a 1335 bp open reading frame (ORF) encoding a polypeptide of 444 amino acids, a 113 bp 50'-UTR and an 87 bp 3'-UTR with a poly (A) tail. Sequence comparison revealed that the predicted protein shared higher homologies with Δ6 Fad from *Apostichopus japonicus* with 56% identify. The tissue distribution of *P. aibuhitensis* Δ6 Fad-like mRNA was found predominantly in intestine, followed by head, esophagus and stomach. Quantitative PCR showed that the Δ6 Fad-like transcriptional levels in intestine gradually decreased with increased content of dietary DHA.

Keywords: Δ6 fatty acyl desaturase-like gene, Gene cloning, Nutritional regulation, *Perinereis aibuhitensis*

Introduction

The long-chain polyunsaturated fatty acids (LC-PUFAs), which include PUFAs with ≥20 carbon chain length and ≥3 double bonds, play crucial roles in growth, normal development and functioning of nervous system and sensory organs (Bell *et al.,* 2001; Li *et al.,* 2014). The roles of LC-PUFAs in human pathologies such as cardiovascular, inflammatory and neurological diseases are increasingly being recognised (Awada *et al.,* 2013). In vertebrates, the LC-PUFAs are synthesized from C_{18} polyunsaturated fatty acids (PUFA) such as linoleic (LA, 18:2n-6) and α-linolenic (LNA, 18:3n-3) acids and two main types of enzymes are involved in these conversions, *i.e.* the elongases of very long fatty acids (Elovl) and fatty acyl desaturases (Fad) (Cook and Mcmaster, 2002). Elovl enzymes are responsible for the condensation reaction of activated fatty acids with malonyl-CoA in the elongation multi-enzyme complex and Fad enzymes could introduce double bonds in the fatty acyl chain at carbon 6 (Δ6 Fad) or carbon 5 (Δ5 Fad) from the carboxyl group (Cook and Mcmaster, 2002). Therefore, the content and activity of Elovl and Fad determine the amount of LC-PUFAs, among which the Δ6 Fad is the rate-limiting enzyme involved in LC-PUFAs biosynthetic pathway (Hastings *et al.,* 2004; Tocher *et al.,* 2006).

The Δ6 Fad enzyme converts LNA (18:3n-3) and LA (18:2n-6) to 18:4n-3 and 18:3n-6, respectively in the biosynthetic pathway of LC-PUFAs (Monroig *et al.,* 2010). The Δ6 Fad enzyme was first reported in cyanobacterium *Synechocystis* in 1993 (Reddy *et al.,* 1993) and now the Δ6 Fads have been successfully cloned from many vertebrates, such as *Perca fluviatilis* (Geay *et al.,* 2016), *Dicentrarchus labrax* L. (Santigosa *et al.,* 2011), *Cyprinus carpio* and *Psetta maximus* (Zheng *et al.,* 2004). As far as invertebrates are concerned, a few Δ6 Fads have been reported, except for the *Antheraea pernyi* (Wang *et al.,* 2010), *Caenorabditis elegans* (Napier *et al.,* 1998), *Eriocheir sinensis* (Yang *et al.,* 2013) and *Scylla paramamosain* (Lin *et al.,* 2017).

Historically, wild fish are the major source of dietary HUFA especially n-3 HUFA (Lin *et al.,* 2017). In recent years, the rapidly increasing demands and slowly rising production of wild fish stocks have promoted aquaculture production. The Food and Agriculture Organisation (FAO) of the United Nations has estimated that by 2030, over half of the fish consumed by the world's people will be produced by aquaculture (Tidwell and Allan, 2001). For aquaculture, it is critical to prepare a suitable feed, which contained not only the nutrients needed for fish growth but also the nutrients essential for human health who consume fish. As a candidate bait for fish and as a food supply for broodstock diets in aquaculture, the clamworm *Perinereis aibuhitensis* gets more and more attention.

P. aibuhitensis is one of the most important commercially important polychaete that colonises estuarine and shallow soft-bottom environments in the temperate and tropical zones of the North-west Pacific (Sun and Yang, 2004). Being rich in protein, unsaturated fatty acids, vitamins, nereid fibrinolytic enzymes and other active substances, *P. aibuhitensis* has been extensively used in aquatic feed, recreational fisheries and medicine (Gu *et al.,* 2002; Deng *et al.,* 2007). In a previous study, we found that more 18C FA (oleic, linoleic and linolenic acid) in the experimental diets led to more HUFAs such as C20:3n-3, EPA and DHA in *P. aibuhitensis,* which suggested that *P. aibuhitensis* had the ability to synthesise HUFAs with 18C FA (Lv *et al.,* 2017). In order to further investigate the synthesis mechanism of HUFAs in *P. aibuhitensis*, the gene coding for the rate-limiting enzyme Δ6 Fad was cloned and its tissue expression and nutritional regulation were analysed in the present study.

Materials and methods

Experimental diets

Seven isonitrogenous and isolipidic experimental diets with added DHA (docosahexaenoic acid) algal oils ranging from 0 to 3% in 0.5% increments were formulated, which were named Diet 1 to Diet 7 correspondingly. The algal oils contained only 50% DHA and in turn the experimental diets were enriched with 0 to 1.5% DHA in 0.25% increments. Graded doses of algal oil were added to the basal diet and mixed manually. Composition of the basal diet is given in Table 1. The diets were prepared as per the method described by Fu Lv *et al.* (2017) and were air-dried to about 10% moisture and stored in plastic bags at 4^0C. The fatty acid (FA) composition of the experimental diets is shown in Table 2.

Experimental animal and feeding trial

The clamworm used in the present study was incubated in our laboratory according to the local standard of Jiangsu (DB32/T2450-2013) for *P. aibuhitensis* artificial breeding. Sixty days old *P. aibuhitensis* obtained from the same batch of fertilised eggs were collected to analyse nutritional regulation of Δ6 Fad gene. Clean tidal flat soil which has been elutriated and dried was ground, passed through a 10-mesh sieve and baked at 60°C for 8 h. Then the soil was distributed into 21 polyethylene tanks (60 × 40 × 25 cm, Zhongkehai, Qingdao, China) where the soil was approximately 10 cm in height and completely immersed with clean artificial seawater (salinity 24‰) to generate water-saturated soil.

Prior to initiation of the experiment, 2000 juvenile clamworms with an average body weight of 51.5±1.2 g were dug out and acclimated in artificial seawater (salinity 24‰, 25°C) for 24 h. Then 1050 healthy clamworms were selected and randomly allocated into 21 groups (50 clamworms per group) and the other healthy clamworms were stored at -80°C as initial samples. For each group, the clamworms were weighed to record total initial body weight (IBW) and placed in polyethylene tank and fed one type of experimental diet. There were

Table 1. Feed ingredients and composition of the experimental diets (Dry matter basis, g kg^{-1})

Ingredients	Diet1	Diet2	Diet3	Diet4	Diet5	Diet6	Diet7
Fish meal [a]	20.00	20.00	20.00	20.00	20.00	20.00	20.00
Bean flour [a]	25.00	25.00	25.00	25.00	25.00	25.00	25.00
Peanut meal [a]	18.00	18.00	18.00	18.00	18.00	18.00	18.00
Soybean oil [b]	8.00	7.50	7.00	6.50	6.00	5.50	5.00
DHA algae oils[c]	0.00	0.50	1.00	1.50	2.00	2.50	3.00
Corn starch [a]	27.5	27.5	27.5	27.5	27.5	27.5	27.5
Choline chloride [a]	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Mix vit-min [d]	1.00	1.00	1.00	1.00	1.00	1.00	1.00

[a] Procured from Hengxing Feed Industry Co., Ltd., Yancheng, China

[b] Procured from local edible oil processing plant, Yancheng, China

[c] Procured from Several Billion Biologigal Technology Co., Ltd., Zhejiang, China

[d] Premix supplied the following minerals (g kg^{-1}) and vitamins (IU or mg or g per kg):

CuSO4•5H2O 13.5 g, FeSO4•7H2O 58 g, ZnSO4•7H2O 65 g, MnSO4•4H2O 16 g, Na2SeO3•5H2O 10.8 g, KI 12.64 g, CoCl2•6H2O 21.5 g, VA 1600000 IU, VD3 500000 IU, VE 9000 mg, VK3 450 mg, VB1 700 mg, VB2 2500 mg, VB6 1000 mg, VB12 3.2 mg, VC 18000 mg, pantothenate 2000 mg, folic acid 650 mg, niacin 1500 mg, biotin 400 mg, myoinositol 300 mg

Table 2. Fatty acid composition (% total fatty acids) of experimental diets

Fatty acid [a]	Experimental diets						
	Diet1	Diet2	Diet3	Diet4	Diet5	Diet6	Diet7
C12:0	0.01	0.01	0.01	0.02	0.02	0.02	0.02
C14:0	0.09	0.13	0.15	0.17	0.21	0.22	0.23
C15:0	0.02	0.03	0.03	0.03	0.04	0.04	0.04
C16:0	10.82	12.00	12.38	13.25	14.50	16.53	15.98
C17:0	0.09	0.09	0.09	0.10	0.11	0.11	0.12
C18:0	4.24	4.04	3.92	3.77	3.89	3.96	3.27
C20:0	0.32	0.31	0.32	0.31	0.32	0.34	0.31
C22:0	0.32	0.36	0.35	0.34	0.35	0.36	0.30
C16:1	0.15	0.16	0.20	0.16	0.20	0.17	0.15
C18:1	22.84	21.42	20.68	19.38	19.10	18.56	15.36
C20:1	0.21	0.21	0.17	0.18	0.18	0.17	0.13
C22:1	-	0.08	0.07	0.07	0.13	0.23	0.05
C18:2n-6	53.6	49.57	47.80	45.06	41.90	38.31	36.12
C18:3n-6	0.01	0.01	0.02	0.02	0.03	0.03	0.04
C18:3n-3	7.28	6.72	6.49	6.18	5.59	5.00	5.00
C20:2	-	0.06	0.05	0.04	0.05	0.06	0.05
C20:3	-	0.05	0.05	0.06	0.07	0.08	0.09
C20:4n-6	-	0.05	0.07	0.08	0.12	0.13	0.22
C20:5n-3	-	0.05	0.06	0.09	0.11	0.18	0.16
C22:3	-	0.09	0.07	0.07	0.08	0.14	0.09
C22:4	-	0.87	1.42	2.15	2.70	3.20	4.52
C22:5n-3	-	0.11	0.11	0.11	0.11	0.12	0.18
C22:6n-3	-	3.59	5.48	8.36	10.19	12.05	17.58
∑SFA[b]	15.91	16.97	17.25	17.99	19.44	21.58	20.27
∑MUFA[c]	23.2	21.87	21.12	19.79	19.61	19.13	15.69
∑PUFA[d]	60.89	61.17	61.62	62.22	60.95	59.30	64.05
∑n-3 PUFA	7.28	10.47	12.14	14.74	16.00	17.35	22.92
∑n-6 PUFA	53.61	49.63	47.89	45.16	42.05	38.47	36.38
DHA/EPA	-	71.80	91.33	92.89	92.64	66.94	109.88

[a] Data expressed as area % of FAME, Represent mean±SD, n=3. Fatty acids present at ≤0.1 % of total fatty acids were not included.

[b] SFA: 12:0, 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 22:0

[c] MUFA: 16:1, 18:1, 20:1, 22:1

[d] PUFA: 18:2, 18:3n-6, 18:3n-3, 20:2, 20:3, 20:4, 20:5, 22:3, 22:4, 22:5, 22:6

seven dietary treatments (Diets 1 to 7), each with three replicates. The clamworms were fed the diets twice daily: at 08:00 and 19:00 hrs, at approximately 1~2 and 2~3% of their body weight, respectively. Each diet was mixed well with a little freshwater to supplement the evaporated water and splashed unevenly into the tanks. Uneaten feed was collected 1.5-2.0 h after every meal to estimate the feed intake (FI). A photoperiod of 14:10 light:dark (05:30-19:30 h, light period) was maintained with the soil temperature ranging from 25 to 28°C during the feeding trial. The clamworms were checked daily and dead clamworms were removed immediately and recorded. The 70 days feeding trial was carried out at the Laboratory of Tidal flats benthos at Yancheng Institute of Technology, Jiangsu, China.

Growth performance and feed utilisation analysis

On termination of the feeding trial, all clamworms were removed and placed in tanks containing clean artificial seawater (salinity 24‰) for 24 h to empty their intestines. Individuals were dried using a soft cloth and the total final body weight (FBW) for each group was recorded. The specific growth rate (SGR), feed conversion ratio (FCR) and protein efficiency ratio (PER) were calculated using the following formulae:

Specific growth rate (% day^{-1}) = 100 × (ln FBW - ln IBW) / No. of days

Feed conversion ratio = Feed consumed in g / Weight gain in g

Protein efficiency ratio = (FBW -IBW) / Protein intake

To further analyse the effects of dietary DHA content on the growth trait of *P. aibuhitensis*, the regression between DHA content and SGR, FCR and PER were conducted respectively using Microsoft Excel 2010 and the regression equations were applied to derive the level of DHA in diet that would lead to the maximum value of SGR and PER and the minimum FCR value in *P. aibuhitensis*.

RNA isolation, cDNA synthesis and full-length cDNA cloning

Total RNA was extracted from the muscle tissue using Trizol (Invitrogen, USA), according to the manufacturer's instructions. The quality and concentration of RNA was assessed using a BioPhotometer (Eppendorf, Germany). First strand cDNA was synthesised with Revert Aid First Strand cDNA synthesis kit (Fermentas, USA).

Degenerate primers PaFadF1 and PaFadR1 were designed based on the conserved sequence of Δ6 Fad gene in other species, including *Caenorhabditis elegans* (AAC15586), *Lates calcarifer* (ACS91458), *Rachycentron canadum* (ACJ65149), *Oreochromis niloticus* (BAB62850) and *Homo sapiens* (AAD31282.1). The primers were designed using the CODEHOP PCR primer design method (http://blocks.fhcrc.org/codehop.html) and were applied to amplify part of the Δ6 Fad gene of *P. aibuhitensis*. The PCR products were gel purified, cloned into the pMD19-T vector (TaKaRa, Japan), and sequenced at Shenggong Biotech Co. Ltd. (Shanghai, China). Based on the partial Δ6 Fad gene sequence obtained for *P. aibuhitensis*, the primers PaFadF2 and PaFadR2 were designed and SMART™ RACE cDNA Amplification Kit (Clontech, Heidelberg, Germany) were used to amplify the full-length PaFad cDNA sequence. Details of all the primers used in the present study are provided in Table 3. The 5'/3'RACE products were purified, cloned and sequenced by Shenggong Biotech Co. Ltd., Shanghai, China.

To determine the tissue-specific distribution of Δ6 Fad transcript in *P. aibuhitensis*, seven tissues including head, pharynx, esophagus, esophageal gland, stomach, intestine and muscle were collected from four clamworms selected randomly from initial samples. Intestinal tissues of clamworms fed with experimental diets were also collected to determine the Δ6 Fad transcript in response to different dietary DHA levels. Total RNA from each sample was extracted as described above and PrimeScript® RT reagent Kit with gDNA Eraser (TaKaRa, Japan) was used to synthesize cDNA. The quality and concentration of RNA and cDNA were assessed using a BioPhotometer (Eppendorf, Germany).

Sequence analysis

The similarity of the resulting sequence was analysed using the BLASTX programs (http://www.ncbi.nlm.nih.gov/blast/). The partial fragment, as well as 3'- and 5' end sequences, were assembled into the complete Fad cDNA with DANMAN 7.0 software package. The nucleotide sequence was translated into amino acid sequence with the ORF Finder (http://www.ncbi.nlm.nih. gov/projects/gorf/) program and the functional domains were predicted using the SMART program (http://smart.embl-heidelberg.de/). The isoelectric point and molecular weight of the deduced protein were determined using the "Compute PI/MW" tool on the ExPASY Server (http://www.expasy.org/tools). Based on amino acid alignments, multiple sequence alignments and a neighbor-joining (NJ) phylogenetic tree (Saitou and Nei, 1987) was constructed using ClustalX2 and MEGA 5.0, respectively. The robustness of each topology was checked by 1000 bootstrap replications. The sequences used for analysis were retrieved from NCBI GenBank database.

Quantitative real-time PCR analysis of Δ6 Fad expression

Quantitative real-time PCR was used to determine the tissue-specific distribution of Δ6 Fad transcript in *P. aibuhitensis*, including head, pharynx, esophagus, esophageal glands, stomach, intestine and muscle. Also, intestine mRNA levels of Δ6 Fad in response to different dietary DHA levels were evaluated by qRT-PCR. qRT-PCR was carried out using SYBR Premix Ex Taq^{TM} II (Tli RNaseH Plus) Kit (TaKaRa, Japan) according to the manufacturer's protocol. The sequence-specific primers PaFadF3 and PaFadR3 were applied and the qRT-PCRs were conducted in 25 µl reactions containing 12.5 µl SYBR Premix Ex Taq^{TM} II, 2 µl template cDNA, 8.5 µl RNase-free water and 2 µl each primer. The amplifications were carried out on a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA), with the following parameters: initial heat inactivation at 95^{0}C for 30 s followed by 40 cycles of denaturation at 95^{0}C for 5 s and combined annealing/extension at 60^{0}C for 30 s. The melting curve analyses were performed to verify the specificity of the products at the end of each round of PCR. The *P. aibuhitensis* β-actin (Chen *et al.,* 2012) was used as an internal control gene for sample loading and normalisation and the qRT-PCRs were conducted as described above with primers Actin-F and Actin-R. Three clamworms were used and the measurements were performed with 4 replicates, respectively.

Statistical analysis

Comparative Ct (2-ΔΔCt) method was employed to determine the relative gene expression as described previously (Livak and Schmittgen, 2001). The data were statistically analysed by one-way analysis of variance (ANOVA) and further assessed with Duncan's multiple range test and when $p<0.05$, difference was considered statistically significant. All analyses were performed using SPSS 21.0 software (SPSS Inc., Chicago, Illinois, USA).

Results

Growth performance and feed utilisation

Clamworms in all the treatments appeared normal and did not exhibit any overt pathology. The final body weight (FBW), specific growth rate (SGR), feed conversion ratio (FCR) and protein efficiency ratio (PER) showed significant difference in *P. aibuhitensis* fed with different diets which are displayed in Table 4. FBW, SGR and PER increased with increase in dietary DHA from 0% (Diet 1) to 0.5% (Diet 3) and thereafter decreased, while the FCR displayed opposite trend. More concretely, *P. aibuhitensis* fed with

Table 3. Primers used in the study

Primer	Sequence (5'-3')	Application
PaFadF1	CAACTGGTGGAACCACAGACAYTTYCARCA	Partial cDNA cloning
PaFadR1	GGAACAGGTGGTGCTCGATYTGRAARTT	Partial cDNA cloning
PaFadF2	TGCCCGAAACCATGTTGAGT	3'-RACE
PaFadR2	CCGTACCTGGCATCCCTTT	5'-RACE
PaFadF3	TCCCGTACCTGGCATCCCTTTC	Realtime PCR
PaFadR3	CACCAATCACTGCTCGCTCCCT	Realtime PCR
Actin-F	GGGCTACTCCTTCACCACCA	Realtime PCR
Actin-R	CGAAGTCCAGAGCAACATAGCA	Realtime PCR

Diet 2-4 showed the highest value of FBW and SGR and *P. aibuhitensis* fed with Diet 3 had the highest value of PER and lowest value of FCR among all the treatments.

To further analyse the effects of dietary DHA content on the growth trait of *P. aibuhitensis*, the curvilinear regression between DHA content and SGR, FCR and PER were conducted respectively (Fig. 1). Based on regression equation, the maximum value of SGR and PER appeared when the DHA content was 0.4677 and 0.5405 respectively, while the minimum FCR value appeared when the DHA content was 0.4997.

Sequence analysis of Δ6 Fad cDNA

The full-length cDNA sequence of Δ6 Fad gene in *P. aibuhitensis* (GenBank Accession No. KT445964.1.) was 1535 bp including a 1335 bp open reading frame (ORF) encoding a polypeptide of 444 amino acids, a 113 bp 5'-UTR and an 87 bp 3'-UTR with a poly(A) tail. The nucleotide and putative amino acid sequences are shown in Fig. 2. Analysis of the deduced polypeptide sequence showed that it has a theoretical isoelectric point of 8.93 and molecular weight of 52.32 kDa. The protein sequence included all the characteristic features of microsomal fatty acyl front-end desaturase, including an N-terminal cytochrome b5 domain containing the heme-binding motif (H-P-G-G), three histidine-rich boxes and three transmembrane regions. For the three histidine-rich domains, domains HGFGH and HYQHH were located between the two transmembrane regions and domain QVEHH was located near the carboxyl terminus of the peptide.

Multiple sequence alignment and phylogenetic analysis

Multiple sequence comparison of amino acid sequences of *P. aibuhitensis* Δ6 Fad with Δ6 Fads from other representative vertebrate and invertebrate species using Clustal X2 software showed that it shared higher homologies with Δ6 Fads from *Apostichopus japonicus* with 56% identify. Compared with crustaceans (*Macrobrachium nipponense*, 24%; *Eriocheir sinensis*, 24%), the Δ6 Fad from *P. aibuhitensis* shared higher homologies with fish species and mammals, such as *Rattus norvegicus* (52%), *Mus musculus* (52%), *Lates calcarifer* (51%) and *Rachycentron canadum* (50%).

Neighbor-joining tree constructed using MEGA 5.1 software showed that the showed that the vertebrate branch is much more expansive than the invertebrate branch. In the branch of vertebrates, there were mammals and fish species and in the invertebrate branch, there was a crustaceans group. The Δ6 Fad genes from similar species clustered into same group which implied that this gene is relatively conservative. The Δ6 Fad gene of *P. aibuhitensis* clustered together with Δ6 Fad gene derived from *A. japonicus* which belonged to invertebrate branch (Fig. 3).

Tissue expression of Δ6 Fad in P. aibuhitensis.

The constitutive expressions of Δ6 Fad in tissues were confirmed by qRT-PCR using the specific primers (PaFad F3/R3 and Actin-F/R, Table 3) and β-actin was used as an internal control. The transcription of Δ6 Fad gene in *P. aibuhitensis* was detected in head, pharynx, esophagus, esophageal gland, stomach, intestine and muscle. Results indicated that Δ6 Fad was strongly expressed at highest level in intestine, at much lower level in head, esophagus and stomach and at the lowest levels in pharynx, esophageal gland and muscle (Fig. 4).

Nutritional regulation of Δ6 Fad expression

Transcriptional levels of Δ6 Fad gene in *P. aibuhitensis* fed with Diets 1-7 showed significant difference ($p<0.05$) (Fig. 5). More concretely, the transcription of Δ6 Fad gene in *P. aibuhitensis* fed Diet 1 and 2 showed higher levels compared to other groups, while the transcription of Δ6 Fad gene in *P. aibuhitensis* fed Diet 5-7 showed the lowest levels among all the groups. The transcription of Δ6 Fad gene in *P. aibuhitensis* fed Diet 3-4 were not significantly different from other groups ($p>0.05$).

Discussion

The cloning and characterisation analysis of genes involved in LC-PUFA biosynthesis is an essential step in the understanding of variable endogenous LC-PUFA synthesis capacities among vertebrate and invertebrate species. In order to use plant oils to replace fish oil in

Table 4. Growth performance and feed utilisation of *P. aibuhitensis* fed with different diets

	Experimental diets						
	Diet1	Diet2	Diet3	Diet4	Diet5	Diet6	Diet7
IBW (mg)	51.4 ± 0.3	51.4 ± 0.2	51.4 ± 0.2	51.3 ± 0.5	51.5 ± 0.3	51.6 ± 0.2	51.6 ± 0.3
FBW (mg)	759.4 ± 10.7^{bc}	822.5 ± 24.9^{d}	850.8 ± 8.5^{d}	820.8 ± 20.5^{d}	781.9 ± 15.6^{c}	735.4 ± 17.4^{ab}	712.6 ± 24.8^{a}
SGR (day^{-1})	4.48 ± 0.04^{bc}	4.60 ± 0.04^{d}	4.65 ± 0.02^{d}	4.60 ± 0.05^{d}	4.52 ± 0.02^{c}	4.42 ± 0.04^{ab}	4.35 ± 0.06^{a}
FCR	0.87 ± 0.01^{c}	0.83 ± 0.03^{b}	0.77 ± 0.01^{a}	0.81 ± 0.02^{b}	0.84 ± 0.03^{b}	0.91 ± 0.01^{c}	0.95 ± 0.02^{d}
PER	3.59 ± 0.03^{b}	3.80 ± 0.14^{c}	4.07 ± 0.06^{d}	3.88 ± 0.09^{c}	3.86 ± 0.14^{c}	3.46 ± 0.05^{b}	3.29 ± 0.08^{a}

Values represent Mean±SD, n=3. Means in the same line with different superscripts are significantly different ($p<0.05$).
IBW: Initial body weight; FBW: Final body weight; SGR: Specific growth rate; FCR: Feed conversion ratio; PER: Protein efficiency ratio

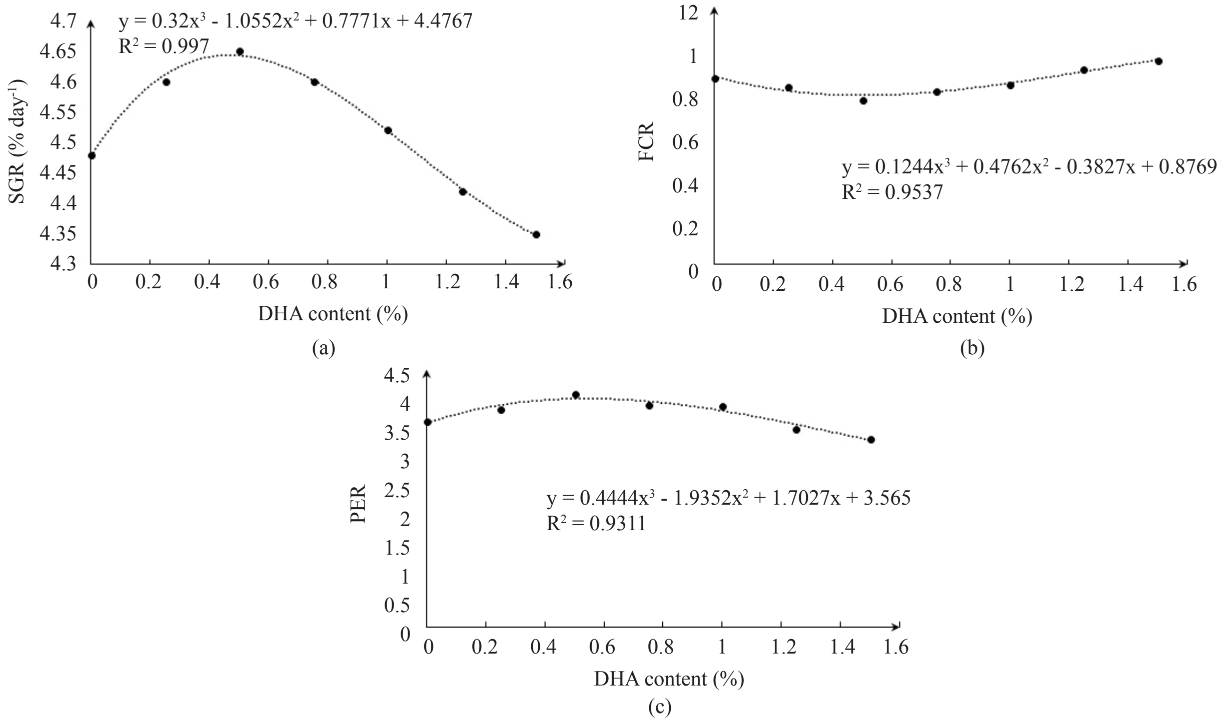
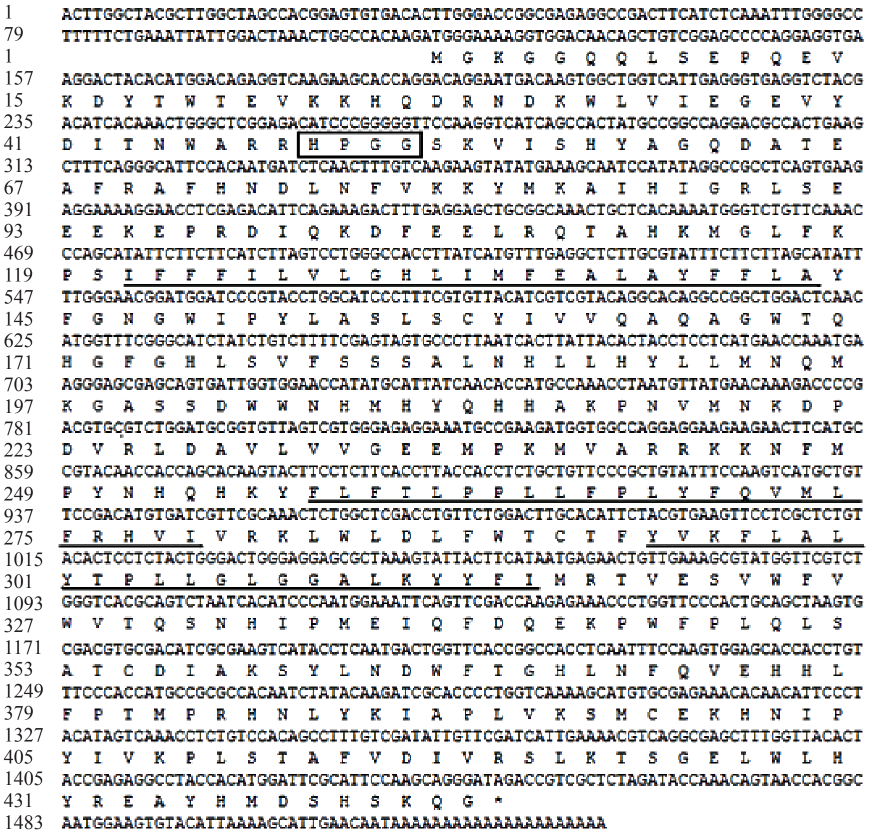


Fig. 1. Regressions between the DHA content in diet and (a) Specific growth rate (SGR), (b) Feed conversion ratio (FCR) and (c) Protein efficiency ratio (PER)



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1     ACTTGGCTACGCTTGGCTAGCCACGGAGTGTGACACTTGGGACCGGCGAGAGGCCGACTTCATCTCAAATTTGGGGCC
79    TTTTTCTGAAATTATTGGACTAAACTGGCCACAAGATGGGAAAAGGTGGACAACAGCTGTCGGAGCCCCAGGAGGTGA
1                                        M  G  K  G  G  Q  Q  L  S  E  P  Q  E  V
157   AGGACTACACATGGACAGAGGTCAAGAAGCACCAGGACAGGAATGACAAGTGGCTGGTCATTGAGGGTGAGGTCTACG
15    K  D  Y  T  W  T  E  V  K  K  H  Q  D  R  N  D  K  W  L  V  I  E  G  E  V  Y
235   ACATCACAAACTGGGCTCGGAGACATCCCGGGGGTTCCAAGGTCATCAGCCACTATGCCGGCCAGGACGCCACTGAAG
41    D  I  T  N  W  A  R  R  H  P  G  G  S  K  V  I  S  H  Y  A  G  Q  D  A  T  E
313   CTTTCAGGGCATTCCACAATGATCTCAACTTTGTCAAGAAGTATATGAAAGCAATCCATATAGGCCGCCTCAGTGAAG
67    A  F  R  A  F  H  N  D  L  N  F  V  K  K  Y  M  K  A  I  H  I  G  R  L  S  E
391   AGGAAAAGGAACCTCGAGACATTCAGAAAGACTTTGAGGAGCTGCGGCAAACTGCTCACAAAATGGGTCTGTTCAAAC
93    E  E  K  E  P  R  D  I  Q  K  D  F  E  E  L  R  Q  T  A  H  K  M  G  L  F  K
469   CCAGCATATTCTTCTTCATCTTAGTCCTGGGCCACCTTATCATGTTTGAGGCTCTTGCGTATTTCTTCTTAGCATATT
119   P  S  I  F  F  F  I  L  V  L  G  H  L  I  M  F  E  A  L  A  Y  F  F  L  A  Y
547   TTGGGAACGGATGGATCCCGTACCTGGCATCCCTTTCGTGTTACATCGTCGTACAGGCACAGGCCGGCTGGACTCAAC
145   F  G  N  G  W  I  P  Y  L  A  S  L  S  C  Y  I  V  V  Q  A  Q  A  G  W  T  Q
625   ATGGTTTCGGGCATCTATCTGTCTTTTCGAGTAGTGCCCTTAATCACTTATTACACTACCTCCTCATGAACCAAATGA
171   H  G  F  G  H  L  S  V  F  S  S  S  A  L  N  H  L  L  H  Y  L  L  M  N  Q  M
703   AGGGAGCGAGCAGTGATTGGTGGAACCATATGCATTATCAACACCATGCCAAACCTAATGTTATGAACAAAGACCCCG
197   K  G  A  S  S  D  W  W  N  H  M  H  Y  Q  H  H  A  K  P  N  V  M  N  K  D  P
781   ACGTGCGTCTGGATGCGGTGTTAGTCGTGGGAGAGGAAATGCCGAAGATGGTGGCCAGGAGGAAGAAGAACTTCATGC
223   D  V  R  L  D  A  V  L  V  V  G  E  E  M  P  K  M  V  A  R  R  K  K  N  F  M
859   CGTACAACCACCAGCACAAGTACTTCCTCTTCACCTTACCACCTCTGCTGTTCCCGCTGTATTTCCAAGTCATGCTGT
249   P  Y  N  H  Q  H  K  Y  F  L  F  T  L  P  P  L  L  F  P  L  Y  F  Q  V  M  L
937   TCCGACATGTGATCGTTCGCAAACTCTGGCTCGACCTGTTCTGGACTTGCACATTCTACGTGAAGTTCCTCGCTCTGT
275   F  R  H  V  I  V  R  K  L  W  L  D  L  F  W  T  C  T  F  Y  V  K  F  L  A  L
1015  ACACTCCTCTACTGGGACTGGGAGGAGCGCTAAAGTATTACTTCATAATGAGAACTGTTGAAAGCGTATGGTTCGTCT
301   Y  T  P  L  L  G  L  G  G  A  L  K  Y  Y  F  I  M  R  T  V  E  S  V  W  F  V
1093  GGGTCACGCAGTCTAATCACATCCCAATGGAAATTCAGTTCGACCAAGAGAAACCCTGGTTCCCACTGCAGCTAAGTG
327   W  V  T  Q  S  N  H  I  P  M  E  I  Q  F  D  Q  E  K  P  W  F  P  L  Q  L  S
1171  CGACGTGCGACATCGCGAAGTCATACCTCAATGACTGGTTCACCGGCCACCTCAATTTCCAAGTGGAGCACCACCTGT
353   A  T  C  D  I  A  K  S  Y  L  N  D  W  F  T  G  H  L  N  F  Q  V  E  H  H  L
1249  TTCCCACCATGCCGCGCCACAATCTATACAAGATCGCACCCCTGGTCAAAAGCATGTGCGAGAAACACAACATTCCCT
379   F  P  T  M  P  R  H  N  L  Y  K  I  A  P  L  V  K  S  M  C  E  K  H  N  I  P
1327  ACATAGTCAAACCTCTGTCCACAGCCTTTGTCGATATTGTTCGATCATTGAAAACGTCAGGCGAGCTTTGGTTACACT
405   Y  I  V  K  P  L  S  T  A  F  V  D  I  V  R  S  L  K  T  S  G  E  L  W  L  H
1405  ACCGAGAGGCCTACCACATGGATTCGCATTCCAAGCAGGGATAGACCGTCGCTCTAGATACCAAACAGTAACCACGGC
431   Y  R  E  A  Y  H  M  D  S  H  S  K  Q  G  *
1483  AATGGAAGTGTACATTAAAAGCATTGAACAATAAAAAAAAAAAAAAAAAAAAA
```

Fig. 2. Nucleotide and deduced amino acid sequences of Δ6 Fad from *P. aibuhitensis* (GenBank Accession No. KT445964.1). The heme-binding motif "HPGG" of the cytochrome b5 domain is boxed. Three conserved regions with known histidine-rich motifs are shaded and the three transmembrane domains are underlined. Double-underlined letters indicate the polyadenylation signal (ATTAAA).

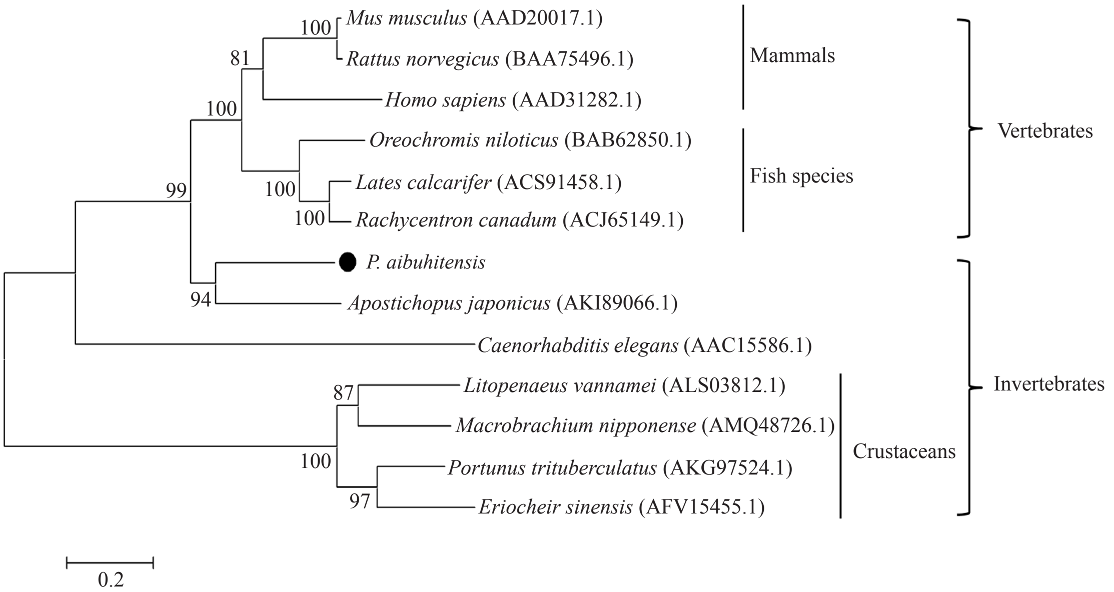

Fig. 3. Neighbor-joining phylogenetic tree relating *P. aibuhitensis* Δ6 Fad with 12 other Δ6 Fad proteins from selected vertebrate and invertebrate species. The numbers represent the frequencies (%) with which the tree topology presented was replicated after 1000 iterations

aquafeeds, previous studies mainly focused on fish species of interest in aquaculture such as marine carnivorous species (Zheng *et al.,* 2004; Tocher *et al.,* 2006; Gonzalez-Rovira *et al.,* 2009; Yamamoto *et al.,* 2010) and freshwater species (Ren *et al.,* 2012; Tanomman *et al.,* 2013). Results showed that freshwater species exhibit higher abilities in LC-PUFA endogenous synthesis than marine carnivorous species. However, the endogenous capacity of invertebrate species like *P. aibuhitensis* to biosynthesise LC-PUFA is poorly documented. As *P. aibuhitensis* is a candidate bait for fish and shrimps and other marine economic species, it is of great importance to study the ability to synthesise PUFA in *P. aibuhitensis*. Thus, this work represents a first step in the characterisation of the endogenous potential of *P. aibuhitensis* to bioconvert PUFA into LC-PUFA.

To study the effects of dietary DHA content on growth trait of *P. aibuhitensis*, seven isonitrogenous and isolipidic experimental diets with DHA contents ranging from 0 to 1.5 in 0.25% increments were formulated. Growth performance and feed utilisation indicated that *P. aibuhitensis* fed with Diet3 (with 0.5% DHA content) recorded highest value of FBW, SGR and PER and lowest value of FCR among all the treatments. These results suggested that *P. aibuhitensis* needs moderate dietary DHA content to support normal growth performance and the optimal dietary DHA content in *P. aibuhitensis* was estimated to be 0.4677% based on SGR. Studies have shown that too high levels of DHA in the diet would inhibit growth in many aquatic organisms and the optimum dietary levels of DHA varies with different species and different aquaculture environments (Sui *et al.,* 2007; Mansara *et al.,* 2015).

In the present study, the Δ6 Fad gene was cloned from *P. aibuhitensis*, which possesses all the characteristic features of microsomal fatty acyl front-end desaturase, including an N-terminal cytochrome b5 domain containing

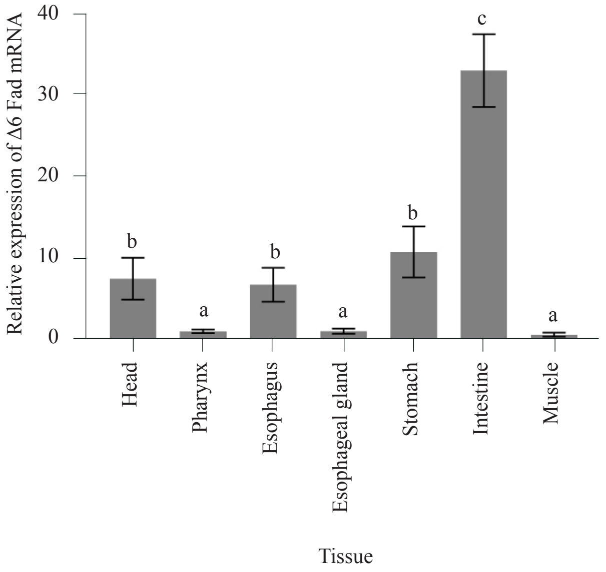

Fig. 4. Relative expression of *P. aibuhitensis* Δ6 Fad gene in different tissues estimated by qRT-PCR. Groups not sharing a common alphabet are significantly different ($p<0.05$)

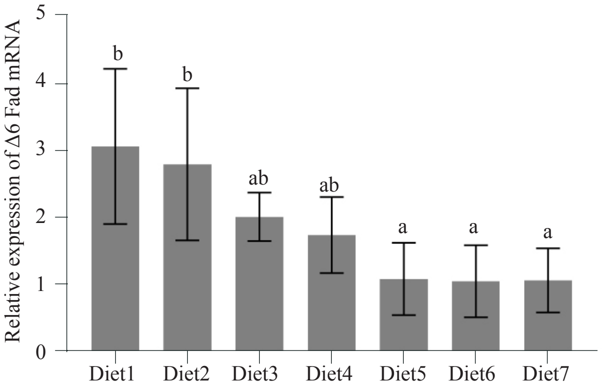

Fig. 5. Relative expression of Δ6 Fad gene in *P. aibuhitensis* fed with different diets estimated by qRT-PCR. Error bars are SD. Groups not sharing a common alphabet are significantly different ($p<0.05$)

the heme-binding motif (H-P-G-G), three histidine-rich boxes and three transmembrane regions. In addition, the first two histidine-rich domains were located between the two transmembrane regions and the third histidine-rich domain was located near the carboxyl terminus of the peptide (Fig. 2). These results are in accordance with other Δ6 Fad genes reported by Li *et al.* (2014) in grouper *Epinephelus coioides* and Lin *et al.* (2017) in mud crab *Scylla paramamosain*. The cytochrome b5 domain has been identified to play essential roles as a heme-binding electron donor in desaturation reactions of fatty acids and the conserved heme-binding motif (H-P-G-G) is found in the cytochrome b5 superfamily domain at the N-terminus of known Fad gene (Hastings *et al.,* 2004; Zheng *et al.,* 2004; Tanomman *et al.,* 2013). Phylogenetic analysis revealed that the Δ6 Fad in *P. aibuhitensis* gene was clustered with other invertebrate Δ6 Fads, which had the highest similarity with Δ6 Fad derived from *A. japonicus*. All the above sequence similarity, conserved domains and phylogenetic analysis support that the cloned gene is a Δ6 Fad-like cDNA.

The tissue expressions of Δ6 Fad gene in *P. aibuhitensis* showed highest level in intestine, followed by head, esophagus and stomach and lowest level in pharynx, esophageal gland and muscle. The high expression of Δ6 Fad mRNA in the intestine possibly indicated the importance of fatty acid metabolism and PUFA biosynthesis in the tissue. Previous studies showed that the highest expressions of Δ6 Fad gene were detected in hepatopancreas in mud crab (Yang *et al.,* 2013; Lin *et al.,* 2017) and in liver in some freshwater fish, such as rainbow trout (Ren *et al.,* 2012) and common carp (Ren *et al.*, 2012). In marine fish, the Δ6 Fad gene showed highest expression in brain among all the detected tissues, in Atlantic cod (Tocher *et al.,* 2006), *R. canadum* (Zheng *et al.,* 2009) and *Larimichthys crocea* (Zuo *et al.,* 2016). Besides, the highest expression was found in the heart of European seabass, *Dicentrarchus labrax* (Gonzalezrovira *et al.,* 2010). These differences may be due to the multiple subtypes of Fad and different functions in different species.

Transcriptional levels of Δ6 Fad gene in *P. aibuhitensis* fed with Diets 1-7 showed significant difference ($p<0.05$) (Fig. 5) and suggested that Δ6 Fad gene expression in intestine is regulated by dietary DHA content. Specifically, the expression level of Δ6 Fad gene in *P. aibuhitensis* decreased significantly with the increase of dietary DHA content from 0-17.58% or the ∑n-3 PUFA content from 7.28 to 22.92%. The effect of dietary DHA content on expression level of Δ6 Fad gene as observed in the study indicates that this gene plays an important role in the synthesis of DHA. Similar results have been observed in *E. coioides* (Li *et al.,* 2014), large yellow croaker (Zuo *et al.,* 2016), rainbow trout (Seiliez *et al.,* 2001) and Atlantic salmon (Zheng *et al.,* 2005).

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