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Cloning, Expression and Polymorphism Analyses of PGC-1α Gene of *Schizothorax prenanti*

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ABSTRACT

PGC-1α (peroxisome proliferator-activated receptor γ coactivator-1α) is a master regulator of lipid metabolism and a candidate of flesh quality determinant. In this study, we characterized a cDNA of *Schizothorax prenanti* PGC-1α and investigated its tissue and developmental profiles, as well as the relationship between its developmental change and intramuscular fat (IMF) content. Cloning and sequencing analysis revealed that *S. prenanti* PGC-1α showed high similarity to those of other vertebrates with conservation of functional domain including Peroxisome Proliferator-Activated Receptor γ (PPARγ) binding site, RNA Recognition Motif (RRM) and serine-arginine repeats. The expression of PGC-1α in kidney, heart and intestine was significantly higher than in other tissues studied ($p<0.05$). Furthermore, the PGC-1α gene expression level in muscle increased with the growth of fish, showing higher mRNA level at 24 months than at other stages ($p<0.05$). PGC-1α mRNA levels were positively correlated with intramuscular fat (IMF) content ($R^2 = 0.714$, $p<0.01$). Allelic variation at nucleotide positions 588 of *S. prenanti* PGC-1α was detected by PCR-SSCP method and most samples examined were C/C homozygous. The results of this study will facilitate further investigation of fish PGC-1α function and the eventual control of cultured fish quality.

Key words: *Schizothorax prenanti*, peroxisome proliferator-activated receptor γ coactivator-1α, cloning, expression, polymorphism

INTRODUCTION

The peroxisome proliferator-activated receptor γ coactivator-1 (PGC-1) family is master regulators of energy metabolism which include three members termed PGC-1α, PGC-1β and PGC-1 related protein (PRC) (LeMoine *et al*., 2010). PGC-1α which is the first member discovered in this family, has been firstly characterized as an inducer of brown adipose tissue development in mouse (Puigserver *et al*., 1998). The subsequent analyses have revealed that the PGC-1α is involved in the regulation of fiber-type switching (Lin *et al*., 2002), mitochondrial biogenesis (Ventura-Clapier *et al*., 2008), oxidative metabolism (Summermatter *et al*., 2011), adaptive thermogenesis (Puigserver *et al*., 1998), glucose/fatty-acid metabolism (Zhu *et al*., 2009), peripheral circadian clock in skeletal muscle (Wende *et al*., 2007) and heart development (Sihag *et al*., 2009).

PGC-1α protein is composed of four main functional regions i.e., the Activation Domain (AD), the nuclear respiratory factor-1 (NRF-1) binding domain, the myocyte-specific enhancer factor 2C

(MEF-2C) binding domain and the RNA Binding Domain (RBD) (LeMoine *et al*., 2010). The C-terminus and N-terminus of PGC-1α contain the sequences that are responsible for binding of many nuclear receptors (Handschin, 2010). PGC1-α interacts with various members of the nuclear receptor superfamily, including Peroxisome Proliferator-Activated Receptors (PPARs), retinoid X receptor α (RXRα), hepatocyte nuclear factor 4α (HNF4α), as well as many non-nuclear receptor-type transcription factors such as MEF2, forkhead box1(FOXO1) and sirtuin 1 (Sirt1) (Handschin, 2010). Moreover, the PGC-1α transcriptional activator complex is able to displace repressor proteins such as histone deacetylase and small heterodimer partner and provides an alternative mechanism for gene activation (Puigserver *et al*.,1999). Among vertebrates, PGC-1α exhibits a high degree of sequence similarity with conservation of functional domains (LeMoine *et al*., 2010).

PGC-1α is a candidate of the determinant of meat quality. PGC-1α plays a key role in fiber-type switching by controlling the formation of type II fiber from type I in skeletal muscle and thereby PGC-1α influent meat quality such as color, juiciness and taste (Lefaucheur *et al*., 2004; Bowker *et al*., 2004). Besides, polymorphism analyses of PGC-1α gene in land animals revealed that it is a functional candidate gene for determining the body lipid content (Kunej *et al*., 2005; Wu *et al*., 2006). Whether PGC-1α polymorphism is present in fish and its association with fat deposition has not been reported at present. Therefore, we selected *S. prenanti* that is a unique cyprinid fish in Tibet plateau. We cloned *S. prenanti* PGC-1α gene, analyzed its tissue and developmental age expression profile and polymorphism. Present results will be helpful for elucidating the functions of PGC-1α gene in fish.

MATERIALS AND METHODS

Experimental fish: The experimental fish (*S. prenanti*) were purchased from a local dealer (Lushan farm, Yaan, China). The experimental fish were kept in our laboratory (Laboratory for genetic breeding of animals, Southwest University for Nationalities, Chengdu, China) for 1 week by feeding commercial diet. The details of the fish used in this study are presented in Table 1. This research project was conducted from 2009.10-2011.10.

Cloning and sequence analysis of *S. prenanti* PGC-1α gene: Total RNA was extracted from heart of *S. prenanti* with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the instruction manuals. cDNA was synthesized by reverse transcription from 2 μg of total RNA as described in the manufacturer's instruction (Fermentas Life Science, Hanover, MD, US). PCR amplification was performed in standard conditions: denaturation at 95°C for 5 min, then 38 cycles of amplification including 95°C for 45 sec, 62°C for 1 min and 72°C for 1.5 min. The amplification was followed by a final extension at 72°C for 10 min. The primers were designed to amplify the entire open reading frame of PGC-1α cDNA of *S. prenanti* using Primer Premier 5 software based

Table 1: The experimental fish

Experiments	Body weight (g)	No.
Cloning and sequence analysis of *S. prenanti*	350.0	1
Tissue distribution of PGC-1α gene in *S. prenanti*	349.8±0.70	6
The developmental changes of PGC-1α gene in *S. prenanti*		
3 months	10.1±0.30	10
12 months	70.6±0.60	10
18 months	132.2±0.90	10
24 months	351.8±1.40	10
Polymorphism assay by SSCP analyses	129.2±13.9	60

on the sequences of PGC-1α of *Danio rerio* (GenBank accession No. XM_002667531, AY998087, FJ710604 and DQ017637).The sequences of primers are as follows: F1: 5'-GGATGGCGTGGGACA GGTGTAATC-3', R1: 5'-GCTGGGGTGGTGCTGTCTCGTT-3', F2: 5'-CTGAGCAAGGCGTCCTCCA CTATG-3', R2: 5'-TTACCTTCTCAGGCTGTACTGGG-3'.

The PCR fragments were gel purified and cloned into pMD19-T vector (TaKaRa, Dalian, China) and transformed into *E. coli* DH5α. For each fragment, five clones were sequenced in both directions by Shanghai Sangon Biological Engineering Technology (Shanghai, China). The sequence, isoelectric point and molecular weight of the deduced amino acids were analyzed using ExPASy-Tools (http://www.expasy.org/tools). The amino acid sequence multiple alignment was constructed with the BioEdit software version 5.0.6 (Hall, 2001). The phylogenetic tree was generated using Neighbour-Joining (NJ) methods (Kimura two-parameter model, 10 000 replicates, bootstrap phylogeny test) based on PGC-1α amino acid sequences using MEGA software version 3.1 (Kumar *et al*., 2004).

Analysis of mRNA level of PGC-1α in tissues of *S. prenanti* and muscles of *S. prenanti* at different ages: Semi-quantitative RT-PCR was employed to reveal tissue and age differences of PGC-1α mRNA level. Total RNA was extracted as described above from the liver, heart , kidney, muscle, adipose tissue, intestine, brain and gill of *S. prenanti* (n = 6) and from muscles of *S. prenanti* at ages 3, 12, 18 and 24 months (n = 10 for each age). A pair of primers (PGC-1α-PF: GCTGCCTTGGTTGGTGAA, PGC-1α-PR: CCTTGCCACCTGGGTATTG) were designed to amplify a 439 bp fragment of *S. prenanti* PGC-1α cDNA. The primers for a reference gene β-actin (βF: GATTCGCTGGAGATGATGCT, βR: CGTTGTAGAAGGTGTGATGCC) were designed based on β-actin sequence of *S. prenanti* (GenBank accession No. JQ013000), the expected fragment size is 219 bp. The PCR condition was as follows: denaturation at 95°C for 5 min, then 32 cycles of amplification was performed and each cycle was consisted of denaturation step at 95°C for 30 sec, annealing step at 56.8°C (PGC-1α) or 54.5°C (β-actin) for 30 sec and extension step at 72°C for 30 sec. The amplification was followed by a final extension at 72°C for 5 min. The amplified fragments were separated by 1% agarose gel electrophoresis. The images of the RT-PCR stained with ethidium bromide were analyzed with Quantity One software (Bio-Rad, Hercules, CA, USA). The band intensity of the genes of interest was normalized to β-actin.

Intramuscular fat content assay: Intramuscular fat content in different ages fish (n = 10 for each age) muscle was measured by using Soxhlet petroleum-ether extraction.

Polymorphism assay by PCR-SSCP analyses: Genomic DNA was extracted from muscle of *S. prenanti* (n = 60) by Ausubel method (Ausubel, 1992). PCR primers (SSCP-F: AAACCCCTG GAACAGCAA (Res. 185-Res. 190), SSCP-R: AGGACGATGGAGAGGAAGAA (Res. 247-Res.252) were designed for 206 bp amplification. This part corresponds to the exon 5 of zebrafish PGC-1α (XM_002667531). A 50 ng of genomic DNA was subjected to PCR in 25 μL reaction volume. The composition of PCR mixture was described above. The thermal condition was as follows: the first denaturation was carried out at 95°C for 3 min. A 32 cycles of amplification was performed and each cycle was consisted of denaturation step at 95°C for 30 sec, annealing step at 56°C for 30 sec and extension step at 72°C for 10 sec.

One microliter of PCR product was added to 20 μL of dye solution (10% saccharose, 0.01% bromophenol blue and 0.01% xylene cyanol FF) and incubated for 2 min at 97°C. The 10 μL of the mixture was applied to a 12% polyacrylamide gel electrophoresis. Following pre-run at 200 V for 10 min, the electrophoresis was carried out in 45 mM tris-borate (pH 8.0)/1 mM EDTA on ice at

180 V for 10 min and then 150 V for 4 h. After electrophoresis, the gel was subjected to silver staining. PCR products with different SSCP patterns were subjected to the direct sequence analyses by Shanghai Sangon Biological Engineering Technology (Shanghai, China).

Statistics: Data were expressed as Mean±SE and statistically analyzed using SPSS 13.0 for Windows Software (SPSS, Chicago, IL, USA). Differences of the IMF content and the gene expression level among tissues and different ages were analyzed by one-way ANOVA and independent-sample t-test, respectively. Significant differences were set at $p<0.05$.

RESULTS

Cloning and sequence analysis of *S. prenanti* PGC-1α gene: Two fragments (approximately 1.4 kb) were amplified by RT-PCR and a 2,633 bp of *S. prenanti* PGC-1α nucleotide sequence was obtained (GenBank accession No. JN195738). The obtained nucleotide sequence covered an entire ORF of 2,631 bp encoding 876 amino acids (Fig. 1). The predicted PGC-1α protein has a molecular weight and isoelectric point of 96.78 kDa and 6.11, respectively.

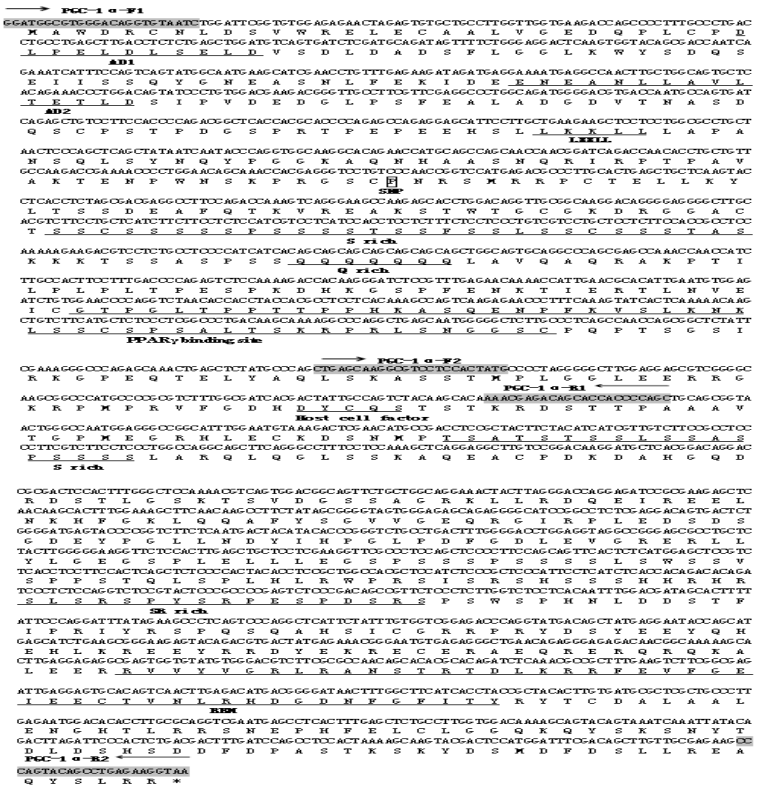

Fig. 1: The cDNA sequence of PGC-1α and the deduced amino acid sequence of *S. prenanti*, Grey bases: The primers were used for the cloning, *A terminal codon, AD1 and AD2: The activation domain 1 and 2, LXXLL: Motif, PPARγ: Host cell factor binding sites, RRM: RNA recognition motif, SR rich: Sequential series of serine-arginine repeats, S-rich: Fish specific serine rich sequence, Q rich: Fish specific glutamine rich sequence, SNP: Single nucleotide polymorphic site. This sequence was submitted to the NCBI GenBank with accession No. JN195738

Phylogenetic analysis revealed that the *S. prenanti* PGC-1α was closely related to the PGC-1α of other vertebrates (Fig. 2). The *S. prenanti* PGC-1α amino acid exhibits high degrees of sequence identities with cyprinidae but low with mammals and birds. Amino acid sequence analysis revealed that the functional domains of PGC-1α including canonical LXXLL (a.a. 142-146) motifs, PPARγ binding site (a.a. 333-382), RNA Recognition Motif (RRM) (a.a. 755-822) and sequential series of serine-arginine repeats (a.a. 646-677) were conserved among species (Fig. 3). In addition, the fish specific serine and glutamine rich sequences were found in *S. prenanti* PGC-1α (Fig. 1, 3).

Expression patterns of PGC-1α gene in tissues of *S. prenanti*: By RT-PCR, the expression of PGC-1α gene was detected in eight kinds of tissues of adult *S. prenanti* (Fig. 4). The expression

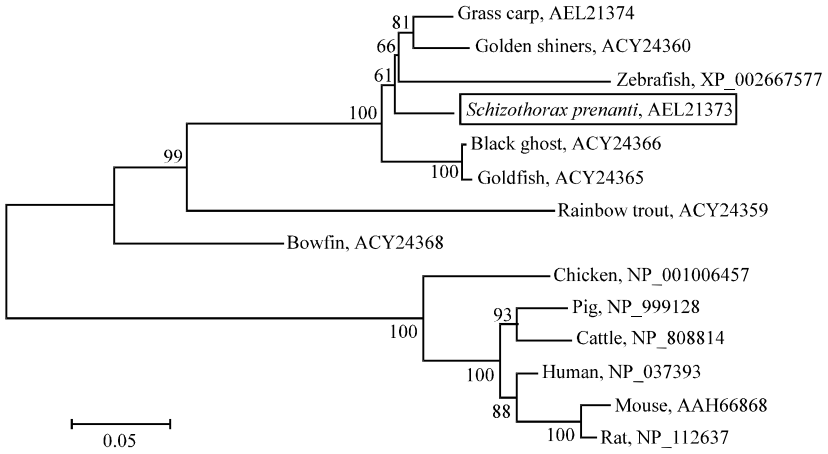

Fig. 2: Phylogenetic analysis of PGC-1α amino acid sequences, The phylogenetic tree was generated using neighbour-joining (NJ) methods (Kimura two-parameter model, 10,000 replicates, bootstrap phylogeny test) based on PGC-1α amino acid sequences using MEGA software version 3.1. Bootstrap values and genetic distance are shown

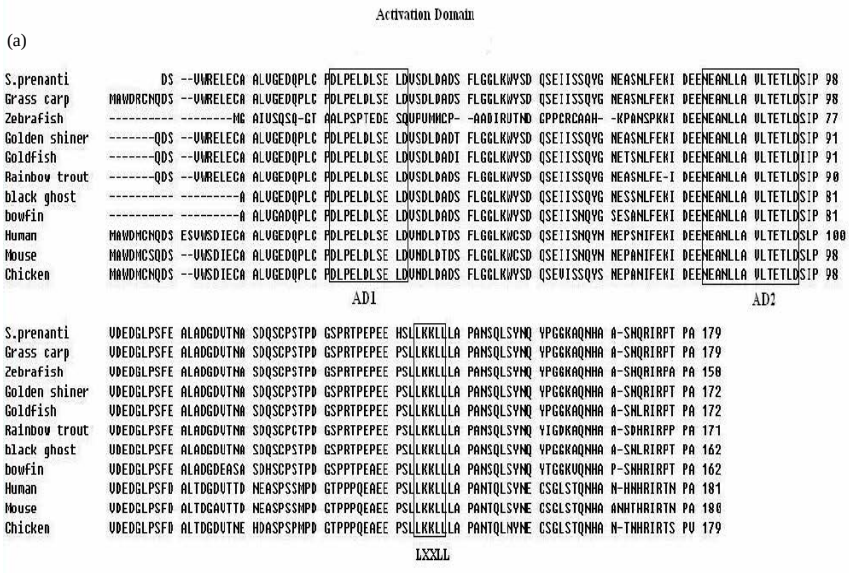

Fig. 3(a-d): Continue

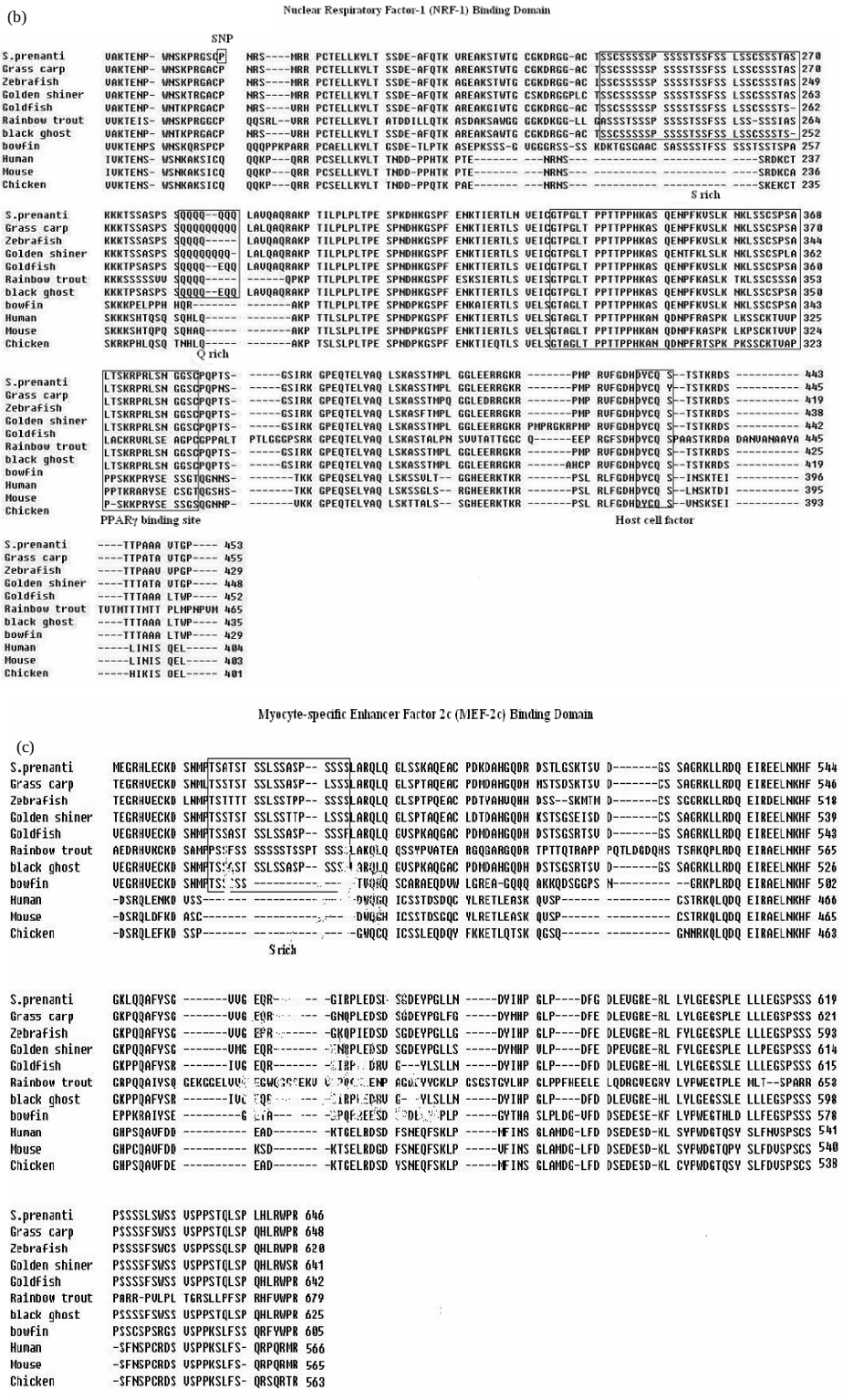

Fig. 3(a-d): Continue

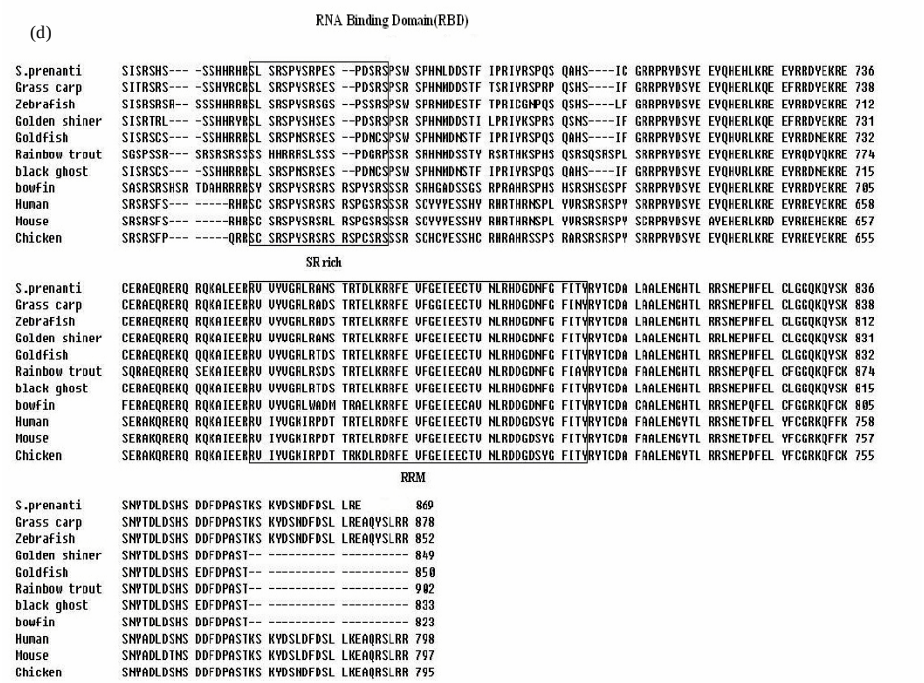

Fig. 3(a-d): Multiple alignment of the PGC-1α amino acid sequences, AD1 and AD2: The activation domain 1 and 2, LXXLL: Motif, PPARγ: Host cell factor binding sites, RRM: RNA Recognition Motif, SR-rich: Sequential series of serine-arginine repeats, S rich: Fish specific serine rich sequence, Q rich: Fish specific glutamine rich sequences, SNP: A single nucleotide polymorphic site, GenBank accession number used in this analysis are *Schizothorax prenanti*: AEL21373, Grass carp: AEL21374, Zebrafish: XP_002667577, Golden shiners: ACY24360, Goldfish: ACY24365, Black ghost: ACY24366, Bowfin: ACY24368, Rainbow trout: ACY24359, Human: NP_037393, Mouse: AAH66868, Chicken: NP_001006457

levels of PGC-1α in kidney and head were greater than the other tissues. However, the expression of PGC-1α was not detected in adipose tissue and gill.

Expression level of PGC-1α gene in muscle of *S. prenanti* at different ages: Semi-quantitative RT-PCR analyses revealed that PGC-1α mRNA level in muscle increased with growth in *S. prenanti* (Fig. 5). The significantly higher mRNA level than those in other ages was observed at 24 months ($p<0.05$). PGC-1α mRNA levels were positively correlated with IMF content ($R^2 = 0.714$, $p<0.01$).

Polymorphism of exon 5 of *S. prenanti* PGC-1α gene: PCR-SSCP analysis revealed two SSCP patterns (C/C and CT) in exon 5 of *S. prenanti* PGC-1α gene in 60 samples, originated from two alleles (C/T single nucleotide polymorphism at nt588) as shown by direct sequencing of PCR product (Fig. 6). The allelic and genotype frequencies in PGC-1α gene are shown in Table 1. CC is the dominate genotype.

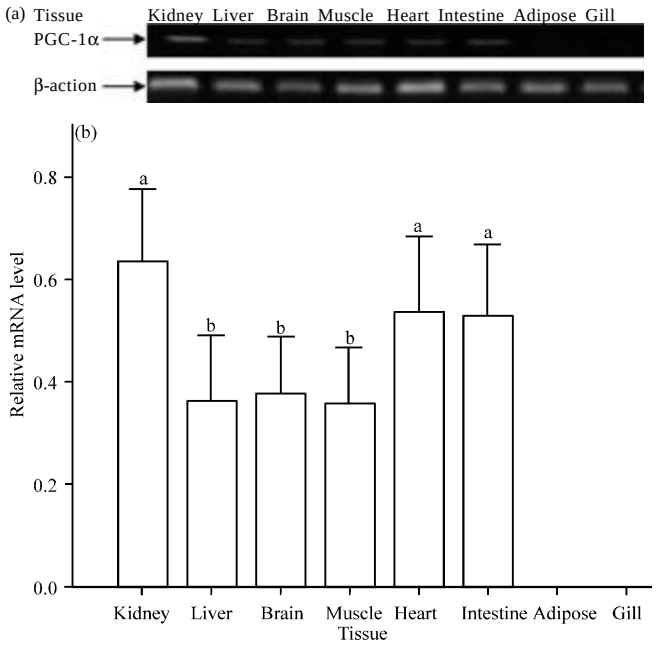

Fig. 4(a-b): Semi-quantitative RT-PCR analysis of PGC-1α mRNA level in tissues of *S. prenanti*, (a) Typical results of RT-PCR and (b) Relative level of PGC-1α mRNA, The values are the means of six independent experiments, Error bars represent the SE, Values without the same superscript are significantly different at $p<0.05$

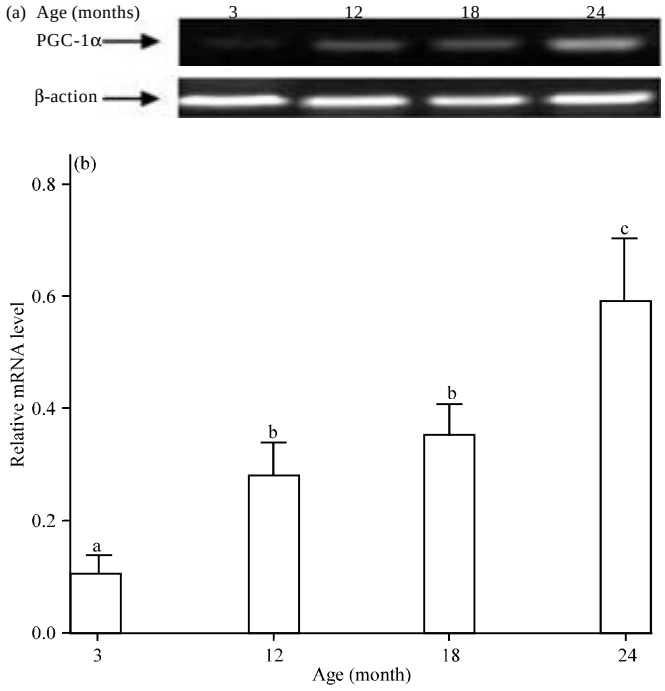

Fig. 5(a-b): Semi-quantitative RT-PCR analysis of PGC-1α mRNA level in muscle of *S. prenanti* at different ages, (a) Typical results of RT-PCR and (b) Relative level of PGC-1α mRNA in muscle, The values are the means of 10 independent experiments, Error bars are the SE, Values without the same superscript are significantly different at $p<0.05$

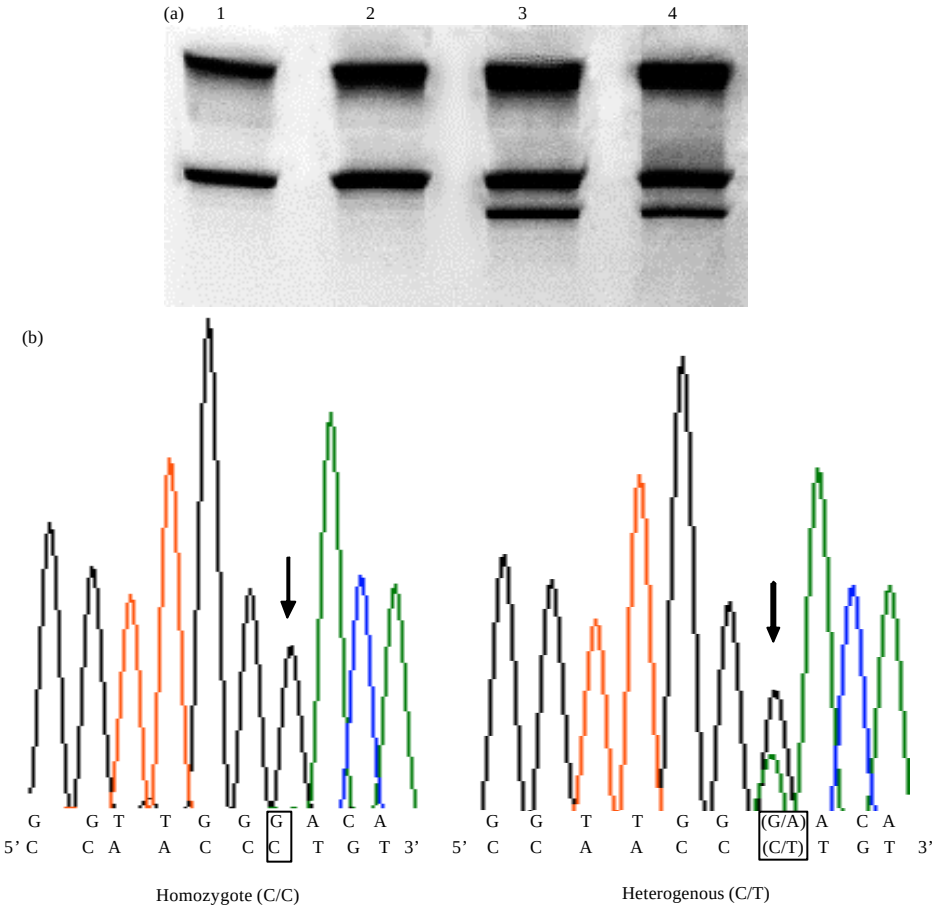

Fig. 6(a-b): Detection of allelic variation at nucleotide positions 588 of *S. prenanti* PGC-1α gene by PCR-SSCP, (a) Typical results of PCR-SSCP of *S. prenanti* PGC-1α, Two samples were electrophoresed for each genotype. Lane 1 and 2: C/C homogeneous genotype, Lane 3 and 4: C/T heterogeneous genotype, The 206 bp PCR products of PGC-1α fragment were separated by 12% PAGE, followed by silver staining and (b) Sequence analyses of two genotypes by direct sequencing at nucleotide position 588 with homogeneous and heterogeneous genotype, The 206 bp products of *S. prenanti* genomic PCR were subjected to the direct sequence analyses

DISCUSSION

In this study, we characterized PGC-1α gene of *S. prenanti* by the sequence and expression analysis. We determined the nucleotide sequence of the most part of coding region of *S. prenanti* PGC-1α (Fig. 1). The deduced amino acid sequence of *S. prenanti* PGC-1α is similar to those of other vertebrates (Fig. 3) but possesses the structural features unique for fish. S. prenanti PGC-1α contains only one Serine-arginine Repeats (SR) whereas mammalian PGC-1α contains two (Fig. 3). Moreover, fish specific serine and glutamine rich sequences were found in *S. prenanti* PGC-1α (Fig. 3). Thus the primary structure of *S. prenanti* PGC-1α is similar to but differs in several aspects from those of mammals and avian.

PGC-1α is expressed in high energy demand tissues such as mitochondria-rich tissues. The human PGC-1α is highly expressed in heart, kidney, liver and skeletal muscle but expressed at very low level in intestine and white adipose tissue (Larrouy *et al*., 1999). The mouse PGC-1α is highly expressed in brown adipose tissue, heart, kidney and brain (Puigserver *et al*., 1998). In this study, we determined the tissue distribution of PGC-1α mRNA in *S. prenanti* (Fig. 4). As in mammals (Larrouy *et al*., 1999; Puigserver *et al*., 1998), high level of PGC-1α mRNA was observed in kidney of *S. prenanti* (Fig. 4). Kidney of fresh water fish has a function as an osmoregulatory organ as well as a urinary organ (Lin, 1999). The high level of PGC-1α mRNA in kidney of *S. prenanti* may reflect the high energy demand in this tissue. PGC-1α showed high mRNA level in kidney of *S. prenanti*, quite different from that in mammals. The intestine of cyprinid fish (stomach less fish) is a main organ for digestion and absorption of diet (Lin, 1999). The difference in the intestinal PGC-1α gene expression level between *S. prenanti* and mammals may suggest the functional difference of intestine among species. Moreover, the muscular PGC-1α expression was increased with the growth of *S. prenanti* (Fig. 5). Although the detailed mechanisms remains to be elucidated, the results suggest that PGC-1α is most likely involved in fish growth.

IMF is an important factor affecting meat flavor, tenderness and juiciness (Wang *et al*., 2005) and thus it is of significance to discover the candidate genes associated with IMF. This study examined the expression pattern of PGC-1α gene in different developmental stages of Schizothorax and analyzed correlation with IMF content. The results indicate that PGC-1α expression was increased with the growth of Schizothorax and positively correlated with IMF content, suggesting that the gene may be associated with IMF deposition but the detailed mechanism needs further study.

It has been reported that Single Nucleotide Polymorphisms (SNPs) of PGC-1α gene is associated with metabolic disorder (Hara *et al*., 2002). A Gly482Ser polymorphism in the human PGC-1α gene has been reported as a risk factor for development of type 2 diabetes (Pratley *et al*., 1998; Kunej *et al*., 2004) which is associated with obesity indices in middle-aged women (Esterbauer *et al*., 2002). Furthermore, in domestic animals, it has been reported that SNPs in PGC-1α gene affect economically important traits as one of the Quantitative Trait Loci (QTL). A Cys430Ser polymorphism in PGC-1α gene has been proposed as a candidate for determining breed specific phenotypes (fat and lean) in pig (Kunej *et al*., 2005). Weikard *et al*. (2005) have indicated that PGC-1α is involved in the genetic variation of milk fat synthesis which is determined by QTL and they identified the gene locus on bovine chromosome (Weikard *et al*., 2005). Wu *et al*. (2006) have reported the existence of a SNP in exon 5 of PGC-1α gene in chicken which cause amino acid change from Asp to Asn at codon 216 and this SNP is associated with abdominal fatness (Wu *et al*., 2006). In the current study, a SNP (C and T at position nt588) in the open reading frame of *S. prenanti* PGC-1α gene was identified which causes amino acid change from Pro to Ser at codon 196 (Fig. 3). This position locates in the NRF-1 binding domain which contains PPARγ and host cell factor binding sites (Fig. 1, 3). Furthermore, we found a SNP in exon 5 of *S. prenanti* PGC-1α gene in 60 samples, however, whether it correlates with IMF content needs further investigation.

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