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Genetic polymorphisms and DNA methylation in exon 1 CpG-rich regions of *PACAP* gene and its effect on mRNA expression and growth traits in half smooth tongue sole (*Cynoglossus semilaevis*)

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Abstract The pituitary adenylate cyclase activating polypeptide (PACAP) is a new type of hypophysiotropic hormone and plays an important role in regulating the synthesis and secretion of growth hormone and gonadotropin. The research on the relationship between *PACAP* and different growth traits would contribute to explain its function during the process of growth. Moreover, epigenetic modifications, especially DNA methylation at the CpG sites of the SNPs, play important roles in regulating gene expression. The results suggest that a SNP mutation (c.C151G) in the *PACAP* gene of male half smooth tongue sole (*Cynoglossus semilaevis*) is significantly associated with growth traits and serum physiological and biochemical parameters such as inorganic phosphorus ($P < 0.05$). The SNP is located in a CpG-rich region of exon 1. Intriguingly, the transition (C→G) added a new methylation site of *PACAP* gene. This SNP was also significantly related to the expression and methylation level of *PACAP* ($P < 0.05$). Individuals with GG genotype had faster growth rates than those of CG and CC genotypes. Moreover, GG genotype had significantly higher *PACAP* expression level and lower methylation level than CG and CC genotypes. In the serum indexes, only inorganic phosphorus content within GG genotypes was significantly higher than CC genotypes. This implied that the mutation and methylation status of *PACAP* gene could influence growth traits and this locus could be considered as a candidate genetic or epigenetic marker for *Cynoglossus semilaevis* molecular breeding.

Keywords *PACAP* · SNP · DNA methylation · Growth · Molecular markers

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Introduction

The pituitary adenylate cyclase activating polypeptide (PACAP) is a new bioactive polypeptide originally isolated from ovine hypothalamus, and it belongs to the secreting/glucagon/VIP family (Miyata et al. 1989). In mammals, PACAP has two molecular variants, PACAP-38 and PACAP-27, resulting from different alternate splicing (Miyata et al. 1990). PACAP and its receptors are widely distributed in the central nervous system and peripheral organs

(Vaudry et al. 2009). By acting on PACAP receptors, PACAP activates adenylate cyclase, resulting in the higher level of the cAMP in target cells. PACAP, as a hypothalamic hormone, plays an important role in many aspects such as regulating pituitary hormone secretion and/or as regulatory proteins that control growth and differentiation of the pituitary glandular cells, and in controlling the synthesis and secretion of growth hormone (GH) and gonadotropin (GtH) (Vaudry et al. 2009). In teleosts, such as salmon (Parker et al. 1997), grass carp (Wong et al. 2005), European eel (Montero et al. 1998), goldfish (Wong et al. 1998), and turbot (Rousseau et al. 2001), PACAP has been identified as a new growth hormone-releasing factor. It can regulate growth hormone secretion at the pituitary level. PACAP is an upstream hormone of GH/IGF axis and plays a key role in regulating the growth process of animals. We have sought to find alleles of PACAP in GH/IGF growth axis that are related to high or low growth phenotypes.

Single nucleotide polymorphism (SNP) is a kind of gene marker, caused by a single base change. SNPs can greatly affect gene expression and protein functions in some cases and have been widely used for screening functional genes in many aspects of life such as health (Liu and Cordes 2004), growth (Amills et al. 2003; Bahrami et al. 2013; Ge et al. 2003; Tao and Boulding 2003; Zhang et al. 2009), and reproduction (Ding et al. 2012; He et al. 2008a, b; Shi et al. 2009). Some specific functional mRNAs or their proteins are important for controlling transcription and gene expression by combining to the specific site of DNA sequence, especially gene promoter or 5′-untranslated region (5′UTR) (Wilkie et al. 2003). Mutations occurring in the gene promoter or 5′UTR would influence the binding process of these transcriptional factors and then affect gene expression. Moreover, the SNP located in the coding regions (CDs) could promote or prevent gene expression (Ding et al. 2012). Growth is a sophisticated physiological process relying upon the interdependence of both gene expression pattern differences and their interaction with environmental parameters (Gui and Zhu 2012). It is essential to maintain high growth rate and avoid the loss of genetic diversity in fish farm. In teleosts, a few studies have been reported regarding the relationship between SNP and growth (Huang et al. 2014; Tian et al. 2014; Tsai et al. 2014; Wang et al. 2014). In half smooth tongue sole, Zhao et al. (2015) studied the polymorphisms of *GHR1* gene that might influence mRNA expression, growth traits and hormone level. *PACAP* gene (GenBank accession no. FJ608666) of half smooth tongue sole is composed of five exons, of which, exon 1 and exon 5 are 5′- and 3′-untranslated regions, respectively (Ji et al. 2011). Ma et al. (2011) studied sexual growth dimorphism associated with *GHRH* and *PACAP* gene. Interestingly, GHRH and PACAP mRNA were expressed differently during different stages of development. To date, few data are reported on the association between SNP and growth traits in half smooth tongue sole (*Cynoglossus semilaevis*).

Cytosine methylation in DNA sequences provides a layer of epigenetic control in many eukaryotes that is meaningful for the understanding of normal biology and disease (Laird 2010). Gardiner-Garden and Frommer (1987) defined a CpG-rich region as a sequence with (1) length more than 100 bp; (2) GC percent above 50.0; (3) ratio of observed-to-expected number of CpG dinucleotides above 0.6. There are four CpG-rich regions in *PACAP* gene of half smooth tongue sole, located in all five exons except exon 2. We hypothesized that mutations in the CpG context may play a vital role in regulating gene expression. Many studies have reported that DNA methylation could silence gene expression (Ding et al. 2012; Herman et al. 1994; Tachibana et al. 2008). Many mutations involved in A→G transition (Ding et al. 2012), as well as C→T and G→A transitions (Ding et al. 2012; Pfeifer 2000), could alter DNA methylation patterns in CpG sequences and then change gene expression level. The relationship between SNP and DNA methylation of *PACAP* gene in half smooth tongue sole is still unclear.

Cynoglossus semilaevis, a flatfish species, is an important commercial flatfish inhabiting seawater, which belongs to batch-spawning fish (Chen et al. 2010; Wen et al. 2014). The females of the species grow one to two times faster than males (Chen et al. 2007). Thus, identifying and selecting fast-growing males and females would be beneficial to genetic breeding and farming.

PACAP was chosen as an important candidate gene for the identification of genetic markers for growth in fish. The aim of the present study was the evaluation of the genetic variability of *PACAP* using a single-strand conformation polymorphism (SSCP) protocol. The associations between polymorphisms and some

growth traits, serum physiological and biochemical indexes were analyzed. DNA methylation patterns of all CpG sites including the new CpG position were identified, explaining the regulation mechanism of gene expression based on epigenetics. This will be a first-step study for the half smooth tongue sole breeding toward establishing a breeding program based on molecular marker-assisted selection.

Materials and methods

Experimental fish and data collection

Seventy adult male *Cynoglossus semilaevis* from the same batch were obtained from an aquatic farm. These samples were fostered for twenty months in commercial fish ponds, under the controlled conditions (20 ± 0.5 °C; ≥4 mg/L O_2; 14:10 h light: dark cycle), and fed with commercial feed of the appropriate size. After rearing these samples 2–3 days, gonad, liver, brain, pituitary, intestines, heart, kidney, spleen, stomach, head kidney, gill, and muscle were excised, immediately frozen in liquid nitrogen, and stored at −80 °C until some of them were selected for the extraction of genomic DNA and total RNA. Some growth traits, such as body weight, viscera weight, liver weight, gonad weight, body length, body height, head length, and diameter of eye were measured.

Hepatosomatic index (HSI) and Gonadosomatic index (GSI)

The HSI or GSI (%) of each animal was calculated as the ratio of the gonad or liver wet weight to the whole body net weight. Gonadosomatic or Hepatosomatic index = (Gonad or liver weight/(body weight − viscera weight)) × 100.

IGF-I, T3, and T4 radioimmunoassay (RIA)

Blood was collected from the caudal vein using a heparinized 1-ml syringe after anesthetizing the fish with MS-222 and centrifuged for 15 min at 4 °C at 10,000 rpm. Plasma was removed and stored at −20 °C. Plasma insulin-like growth factor-I (IGF-I), triiodothyronine (T3), and tetraiodothyronine (T4) levels were determined by ^{125}I radioimmunoassay, using diagnostic kits from Diagnostic Products Corporation (Tianjin Nine Tripods Medical & Bioengineering Co., Ltd., Sino–US jointventure enterprise).

Genomic DNA isolation and PCR-SSCP analysis of *PACAP*

Genomic DNA was extracted from muscle, liver, and brain samples using a phenol/chloroform isolation method. The DNA purity and concentration were measured using Ultramicro Nucleic Acid and Protein Analyzer BD-1000 (Beijing Oriental Science & Technology Development Co., Ltd., China), and their integrity was evaluated by agarose gel electrophoresis (Zhao et al. 2015). The DNA was stored at −20 °C until needed.

Five pairs of primers (Table 1) were designed to amplify five exons of half smooth tongue sole *PACAP* gene according to its cDNA sequence (GenBank accession no. FJ608666) using the Oligo 6.0 software. Among these exons, exon 1 belongs to 5′UTR, not encoding amino acids, and exon 5 has a sequence of poly A tail. PCRs were carried out in a total of 25 μl volume including 100 ng of genomic DNA, 0.2 mM each dNTP, 2.5 μl 10× PCR buffer, 0.2 mM primers, and 0.5 U Taq DNA polymerase. Amplification condition was 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, T_m for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 10 min. The amplification product was analyzed by single-stranded conformation polymorphism (SSCP). Five microliters PCR product was added to 9 μl denaturing buffer and then denatured at 98 °C for 10 min followed by a rapid chill on ice for 10 min. The denatured products of *CsPACAP* were analyzed using non-denaturing polyacrylamide gel at 120 V for 12–16 h at 4 °C. DNA bands were stained with silver. Individual genotypes were defined according to band patterns. PCR products from six individuals of each genotype were purified with EasyPure PCR Purification Kit (TransGen). The purified PCR products were ligated to PEasy-T1 vector (TransGen) and transferred into Trans-5α Chemically Competent Cell (TransGen). Positive recombinant colonies were sequenced by BGI company.

Total RNA extraction and Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from all tissues of *C. semilaevis* using RNAiso reagent (TaKaRa, Japan).

Table 1 Primers used for amplification exons of *PACAP* gene, DNA methylation, and quantitative PCR

Names	Sequences (5′–3′)	Length (bp)	*T* (°C)
EXON 1 (SNP)	F: AAAGCAGCGATGTGCAGACGGA	331	56
	R: AGCAGGGAGAGAGAGCGCTTTT		
EXON 2 (SNP)	F: GCCAGTTCGAGTAAAGCCACT	106	53
	R: CTGATCTTGGGGTAACTTAGTC		
EXON 3 (SNP)	F: ACTTGAAAACGACGCCTTCGATG	135	63
	R: TCTTCTCCGGGGGGTAGTACA		
EXON 4 (SNP)	F: TGAAGACAACAGCATGGAGG	254	53.9
	R: ATCTGAATGACTTTGATTTAACC		
EXON 5 (SNP)	F: TCTGACCAACCAGTGGATTG	305	57.8
	R: TCTGACCAACCAGTGGATTG		
Primer 6	F: GGGGATAAAAGTTTTAAAGTAG	202	52.8
	R: AAAAAAAAAAAATCTTCCTCTCC		
PACAP-RT(qPCR)	F: ACAGCATGGAGGACGAATCAG	190	62
	R: GCTCTGGAACAAGGCTACAAAT		
18S(qPCR)	F: CCTGAGAAACGGCTACCACATC	119	62
	R: CCAATTACAGGGCCTCGAAAG		

The concentration of extracted total RNA was spectrophotometrically measured. Reverse transcription was carried out using two-step method with PrimeScript^{TM}RT reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa, Japan). A 10 μl reverse transcriptase reaction mixture containing 5× gDNA Eraser Buffer 2 μl, gDNA Eraser 1 μl, total RNA 1 μg, and RNase-free dH_2O up to 10 μl at 42 °C was incubated for 2 min, and all the first-step reaction solution was added to the second-step reaction solution with 1 μl PrimeScriptRT Enzyme Mix I, 1 μl RT Primer Mix, 4 μl 5× PrimeScript Buffer2 (for Real Time), 4 μl RNase-free dH_2O and was incubated at 37 °C for 15 min and then at 85 °C for 5 s. The synthesized cDNA was stored at −20 °C for later use.

Quantitative real-time PCR (qPCR)

The relative expression of *PACAP* mRNA was determined using total RNA extracted from brains and livers of half smooth tongue sole. Quantitative PCR was performed via Roche LightCycler 480 (German) and SYBR Premix Ex Taq^{TM} (TliRNaseH Plus) Kit (TaKaRa, Japan, Code No. RR420A), according to the manufacturer's protocols. PACAP-RT-F, PACAP-RT-R and 18S-F, 18S-R were primers used for qPCR analysis, respectively (Table 1). The 20 μl mixture of PCR consisted of 10 μl SYBR®Premix Ex Taq (TliRNaseH Plus), 0.4 μl PCR forward primer, 0.4 μl PCR reverse primer, and 2 μl DNA template, and RNase-free water was added to it. The *PACAP* qPCR conditions were as follows: denaturation at 95 °C for

Table 2 Means and standard errors of growth traits and blood measurements

Traits	Mean	SE[a]
Body weight (g)	211.1950	5.2489
Net weight[b] (g)	189.1068	4.8956
Body length (cm)	32.1900	0.2841
Liver weight (g)	1.7452	0.0574
Gonad weight (g)	0.9279	0.0738
Body height (cm)	8.8775	0.1096
Head length (cm)	6.9075	0.0666
Diameter of eye (cm)	0.9275	0.0171
HSI[c]	0.0093	0.0003
GSI[d]	0.0050	0.0004
IGF-I (pg/mL)[e]	5.9074	0.0281
T_3 (pg/mL)[f]	2.9373	0.1044
T_4 (pg/mL)[g]	1.5167	0.0172

[a] Standard error

[b] Body weight − viscera weight

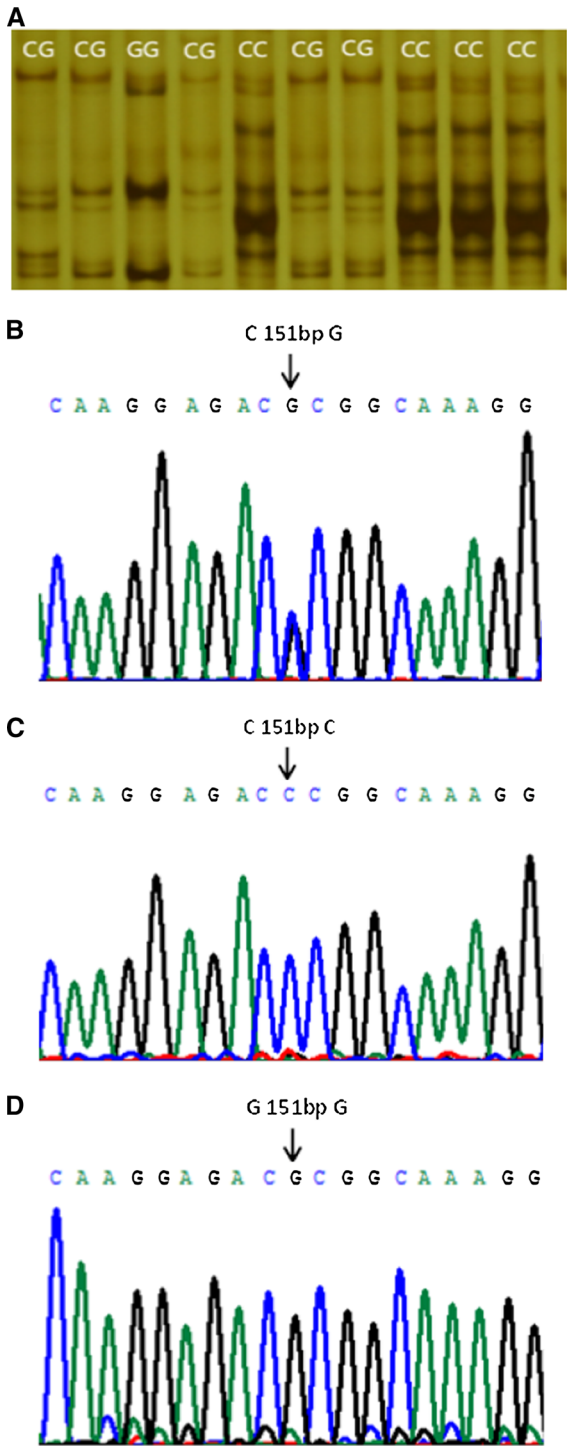
[c] Hepatosomatic index

[d] Gonadosomatic index

[e] Insulin-like growth factor-I

[f] Triiodothyronine

[g] Tetraiodothyronine



◀**Fig. 1** *PACAP* genotyping and sequencing confirmation. **a** Band pattern for the SNP in exon 1. CC and GG were the homozygote, and CG was heterozygote. The chromatograms show sequences of this SNP (c. C151G): the number indicates the position of mutation site; **b** sequence of CG genotype in exon 1; **c** sequence of CC genotype in exon 1; **d** sequence of GG genotype in exon 1

Table 3 Frequencies of alleles and genotypes of SNP site of *Cynoglossus semilaevis PACAP* gene (%)

Locus	Genotype frequency (%)			Allele frequency (%)	
	CC	CG	GG	C	G
SNP	27.5	55	17.5	55	45

Table 4 Associations between SNP within *Cynoglossus semilaevis PACAP* gene and growth index by one-way ANOVA

Traits/SNP	*F* value	*P* value
Body weight	3.24	0.05
Body length	4.42	0.02*
Liver weight	3.57	0.04*
Net weight	3.72	0.03*
HSI	2.58	0.05*
GSI	0.38	0.68
IGF-I	0.08	0.92
T3	0.70	0.50
T4	0.27	0.76

* $P < 0.05$; ** $P < 0.01$

30 s, 40 cycles of denaturation at 95 °C for 5 s, annealing at 62 °C for 30 s, and extension at 72 °C for 30 s. *18S* ribosomal RNA, as a reference gene, was amplified under the same conditions. Each individual had three repetitions. As a negative control, all the PCR reagents were used but without cDNA added. Relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001).

DNA bisulfite modification and analysis

Genomic DNA was extracted from brain, liver, and muscle tissues using Marine Animals DNA Kit according to the protocol (CWBIO). DNA samples (200 ng) were sodium bisulfite-modified using the Methylamp™ DNA Modification Kit (QIAGEN) following the manufacturer's instructions. The CpG-rich regions of

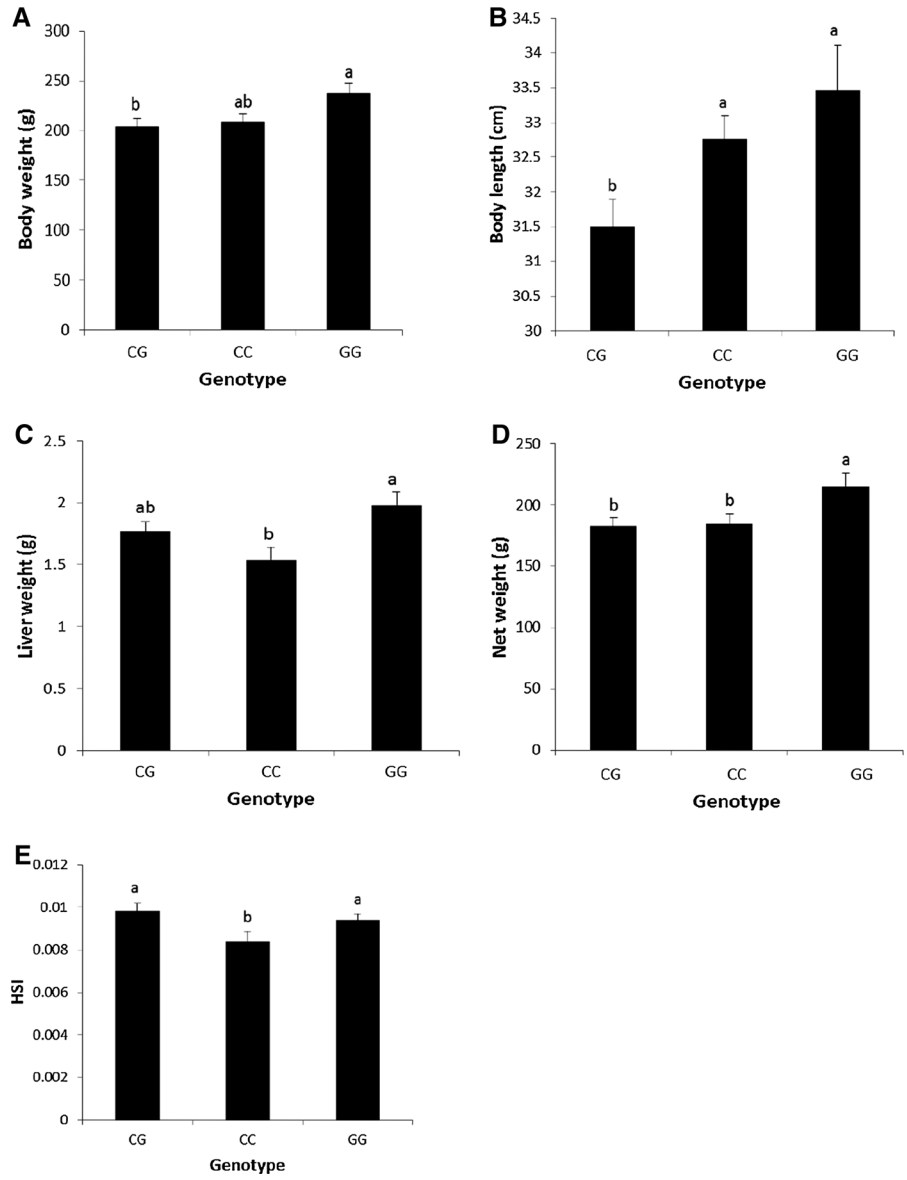


Fig. 2 Associations between SNP in half smooth tongue sole *PACAP* gene and growth traits. **a–e** The relationship between three genotypes on body weight, body length, liver weight, net weight and HSI, respectively. Statistical relationships between groups are indicated by *letters* where significant differences were detected ($P < 0.05$)

PACAP were identified by online MethPrimer design software (http://www.urogene.org/methprimer/). Primer 6 was designed according to the mutation sequence using Oligo 6.0 software (Table 1). Cycling conditions were 94 °C for 5 min followed by 40 cycles of 94 °C for 30 s, 52.6 °C for 30 s, and 72 °C for 30 s with a final extension of 10 min at 72 °C. The 25 μl reaction system included TaKaRa EpiTaq HS 0.125 μl, 10× EpiTaq PCR buffer 2.5 μl, $MgCI_2$ 2.5 μl, dNTP mixture 3 μl, template 3 μl (<100 ng), BS-F, and BS-R primers 1 μl, respectively, and dH_2O was added to it. PCR product was purified and cloned into PEasy-T1 vector, sequenced, and analyzed using DNAMAN software. Non-CpG cytosines of each sequence served as internal controls to verify bisulfite DNA modification efficiency ($P > 95\%$ in all samples).

Blood serum physiological and biochemical indexes analysis

Serum biochemical parameters including glucose (Glu), total protein (TP), serum albumin (ALB), blood

Table 5 Associations between genotypes and physiological and biochemical indexes by ANOVA

genotypes/indexes	CC	CG	GG
TP (g/L)	35.7636 ± 6.39316	36.7476 ± 3.49537	38.7 ± 3.07463
ALB (g/L)	10.8727 ± 3.92405	11.019 ± 2.97298	12.7286 ± 1.08277
UA (mmol/L)	3.0182 ± 4.58777	3.5762 ± 5.67	2.0714 ± 3.20765
UREA (mmol/L)	2.2682 ± 0.89207	2.6048 ± 0.73881	2.4057 ± 0.83817
Glu (mmol/L)	2.0155 ± 1.34813	1.7838 ± 1.18462	2.3686 ± 1.66814
TC (mmol/L)	8.3218 ± 4.23635	10.019 ± 3.69289	11.5571 ± 3.22706
TG (mmol/L)	6.6273 ± 3.14119	6.9348 ± 2.64248	8.4586 ± 2.13292
Ca (mmol/L)	2.68 ± 0.31711	2.6371 ± 0.25852	2.6929 ± 0.13462
P (mmol/L)	3.0336 ± 1.04531[a]	3.2343 ± 0.56323[ab]	3.529 ± 0.34884[b]

Means with a different letter in superscript were significantly different ($P < 0.05$)

uric acid (UA), urea, total cholesterol (TC), triglyceride (TG), Ca^{2+}, and P were determined with an automated biochemistry analyzer (Mindray BS-180, China) using kits (Shenzhen Mindray Bio-Medical Electronics Co., Ltd., China) according to the instructions.

Statistical analysis

All the data were calculated by Microsoft Excel. The results were expressed as mean ± SE. To evaluate the effects of *PACAP* gene polymorphisms on growth traits, statistical analysis of variance (ANOVA) and Duncan's multiple comparison tests were performed using GLM procedure of SPSS 17.0 (SPSS Co., Ltd., Chicago).

The statistical model of the genotype was as follows:

$$y_i = x_i + e(y_i : \text{the growth trait measured on the individuali}; x_i : \text{genotype}; e : \text{random error})$$

Associations between genotypes and *PACAP* mRNA expression or methylation level were examined by Spearman's tests using SPSS 17.0.

Statistical significance was determined at P value <0.05. Microsoft Excel is used to analyze the correlation relationship between gene expression and methylation level.

Results

The analysis of growth traits and polymorphisms within exons of *PACAP* gene

The growth traits and relative hormone data were collected and calculated and are shown in Table 2. Among the five pairs of primers, only one primer pair for exon 1 of *PACAP* was polymorphic in SSCP pattern. We obtained the 331 bp fragment with one mutation (c. C151G) in exon 1. Exon 1 belongs to 5′-untranslated region. Via online software (http://www.urogene.org/methprimer/), this mutation site was found located in a CpG-rich region. Three SSCP patterns were detected on the gel (Fig. 1a). There was a double peak in Fig. 1b, showing that heterozygote CG existed, while Fig. 1c and d reflected that there were two homozygotes CC and GG. We found that the shift C→G resulted in a new CpG methylation site. Two allelic frequencies (C and G) were identified, resulting in three genotypes (GG, CG, and CC) with the frequency of 17.5, 55, and 27.5%, respectively (Table 3).

Association between *PACAP* gene polymorphisms and growth traits, blood serum physiological and biochemical indexes

Growth indexes including biological traits (body weight, body length, body height, liver weight, net weight, gonad weight, head length, diameter of eye, HSI, and GSI) and physiological indexes (T3, T4, and IGF-I) were selected. Table 4 shows that SNP site was significantly associated with body weight, body length, liver weight, net weight, and HSI in male tongue sole ($P < 0.05$), respectively. However, the locus showed no significant effects on physiological indexes such as T3, T4, and IGF-I in this population.

Furthermore, multiple comparison analysis was performed in different genotypes (Fig. 2). Figure 2a showed that body weight of GG genotype was significantly higher than that of CG genotype ($P < 0.05$). In Fig. 2b, body length of GG and CC genotypes was significantly greater than that of CG

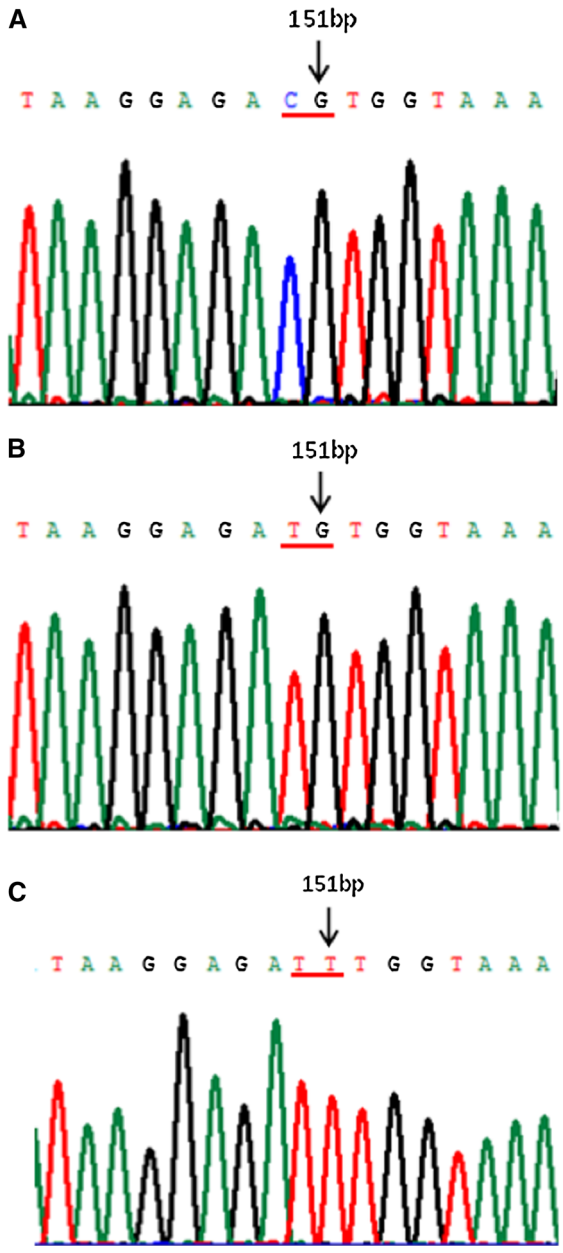


Fig. 3 Part of exon 1 sequence of *PACAP* gene was amplified from bisulfate treatment DNA. The *number* indicates the position of mutation (C/G) site: **a** there was a novel CpG dinucleotide due to guanine substitute for cytosine at 151 bp position in CG genotype, and the cytosine at 151 bp position was converted; **b** the cytosine at 151 bp position was converted to thymine in CC genotype; **c** there was a novel CpG dinucleotide due to guanine substitute for cytosine at 151 bp position, and the cytosine of CpG dinucleotide was methylated in GG genotype

genotype ($P < 0.05$). The liver weight of GG genotype was higher than that of CC genotype significantly ($P < 0.05$) (Fig. 2c). The fish with GG genotype had significantly higher net weight ($P < 0.05$) than CC and CG genotypes (Fig. 2d). HSI of the individuals with genotypes GG and CG was significantly higher than that of those with genotype CC ($P < 0.05$) (Fig. 2e).

The means and standard errors of the blood measurements by genotype are reported in Table 5.In all the parameters tested, the individual serum P concentrations were found to be highly variable. The value of P within GG genotype was significantly higher than that of CG and CC genotypes ($P < 0.05$). There were no significant associations between genotypes and the level of Glu, TP, ALB, UA, urea, TC, TG, and Ca^{2+}. However, the level of TP, ALB, Glu, TC, TG, and Ca^{2+} of GG genotype was higher than those of CC and CG genotypes.

Relationship between the DNA methylation level in exon 1 and mRNA expression of *PACAP* gene in individuals of different genotypes

More than 99 % of cytosines had been transformed by bisulfite treatment in all the samples of CpG dinucleotide sequence analyzed. This implied that the procedure of DNA modification was very efficient. We examined *PACAP* exon 1 methylation of 11 CpG (CC genotype) or 12 CpG (GG and CG genotypes) sites in brain and liver. GG and CG genotypes have one more CpG dinucleotide than CC genotype due to the substitution from C to G at nucleotide 151 bp in exon 1 of *PACAP* gene (Fig. 3). The cytosine base of the CpG site at 150 bp position was found to be lower methylated in the individuals with GG genotype, and the other non-mutation CpG sites were also hypomethylation (Fig. 4). The whole CpG dinucleotides were lower methylated in GG genotype than in CC and CG genotypes in both liver (Fig. 5a) and brain (Fig. 5b) ($P < 0.05$).

One-way ANOVA demonstrated that there was a significant association between the locus and *PACAP* gene expression of male half smooth tongue sole in brain ($P < 0.05$) (Table 6). Additionally, multiple comparisons showed that fish with GG genotype had a

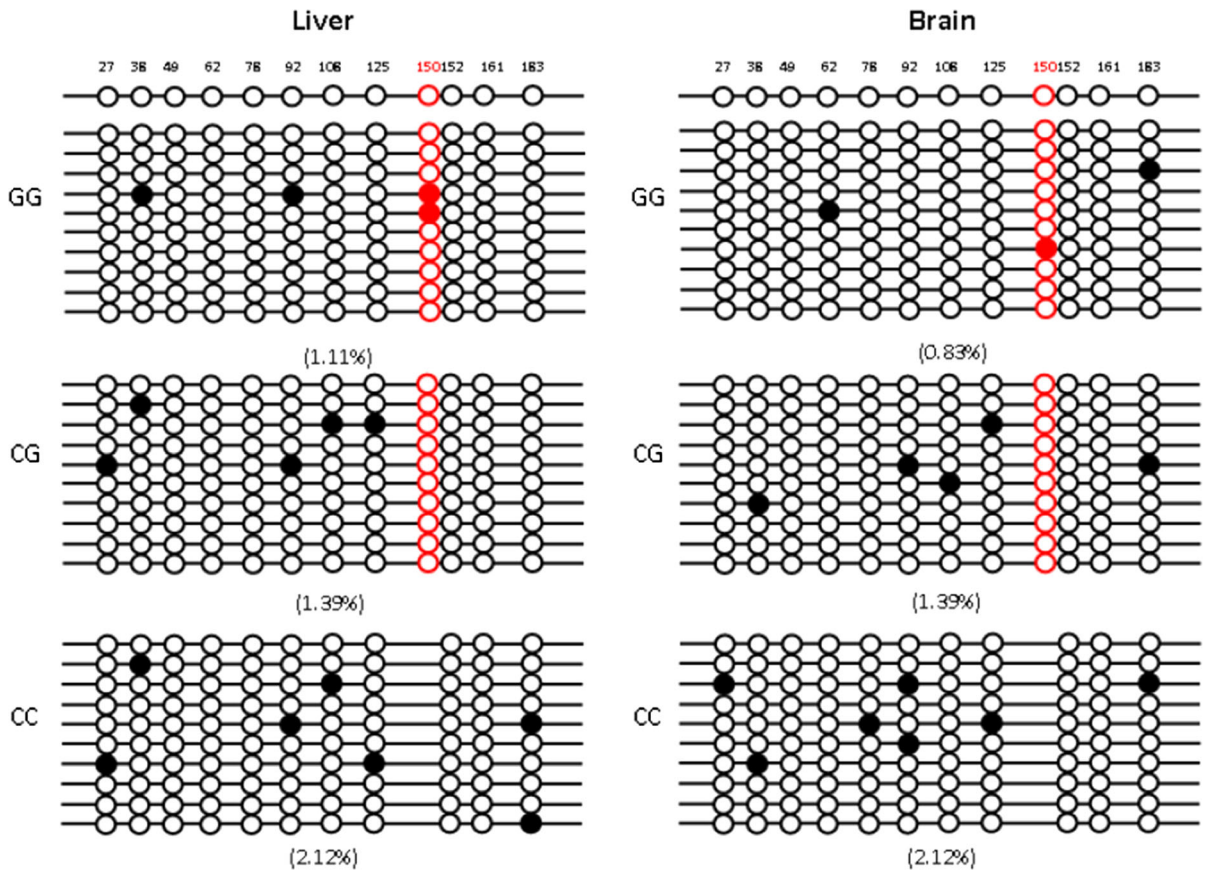


Fig. 4 DNA methylation patterns of exon 1 in *PACAP*. *Filled* and *open circles* denote methylated and unmethylated sites, respectively. *Each line* represents one sequenced clone. The *first line* indicates the localizations of studied CpG sites related to the sequence of *PACAP* coding region. Each genotype has 3 repetitions. **a**, **b** Represents methylation status in liver and brain, respectively. The *percentage* indicates the methylation level (*under the line*), calculated as the number of methylated CpG per total number of CpG sequences

significantly higher gene expresses level ($P < 0.05$) than CC genotype (Fig. 6). However, *PACAP* mRNA was weakly expressed in the liver and was barely detectable.

Figure 7a showed that *PACAP* gene expression level had the opposite change trend with the level of DNA methylation in three different genotypes. DNA methylation was negatively correlated to *PACAP* gene expression (Fig. 7b). GG genotype had lower DNA methylation level and also had higher *PACAP* mRNA expression level. Moreover, there are no significant differences between each CpG site at methylation status in both brain (Fig. 8a) and liver (Fig. 8b).

Discussion

SNPs of gene exons could influence some traits in fishes. Previous studies on Japanese flounder have shown that gene polymorphisms including cytochrome *P450-c19a* gene, estrogen receptor alpha gene, *GnRHR* gene, and *cyp17-I* gene are associated with reproduction traits (He et al. 2008a, b, 2011; Ma et al. 2012). Zhao et al. (2015) reported that coding variants in exon 8 of *Cynoglossus semilaevis GHR1* gene were associated with growth traits such as body weight and gonad weight. In Nile tilapia, two *GH* genotypes are compared and GH double-band geno-

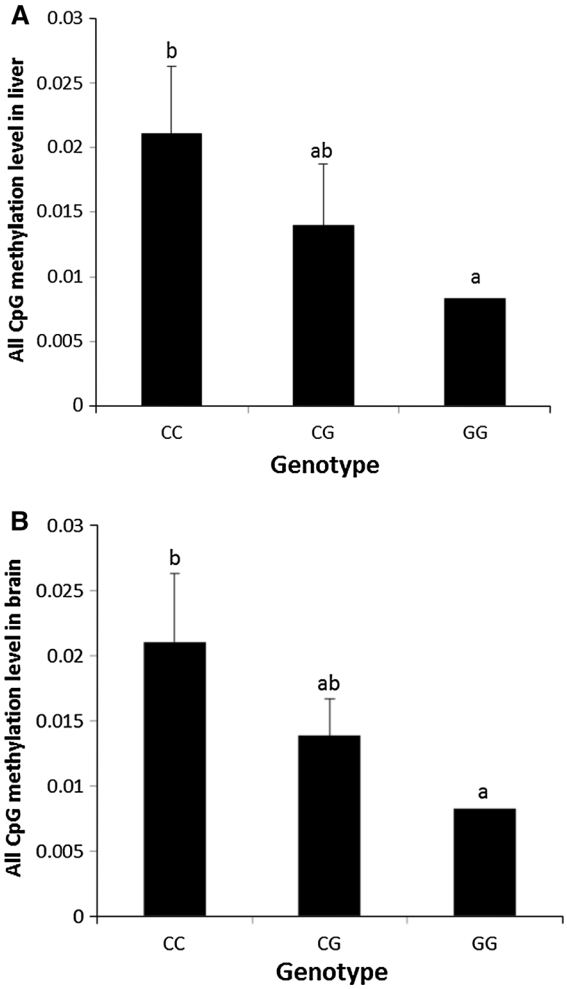


Fig. 5 DNA methylation level of the whole CpGs of *PACAP* exon 1 in liver (**a**) and brain (**b**). The value was calculated as the percentage of methylated CpG sites of sequenced copies in every genotype. *Bars* with *unshared letters* are significantly different from each other ($P < 0.05$)

Table 6 Associations between SNP of half smooth tongue sole *PACAP* gene and expression in brain by one-way ANOVA

Locus	Gene expression of PACAP	
	F value	*P* value
SNP	7.046	0.017*

* $P < 0.05$; ** $P < 0.01$

type individuals presented the best performance characteristics (Tanamati et al. 2015). Similarly, in this study, *PACAP* gene mutations of half smooth

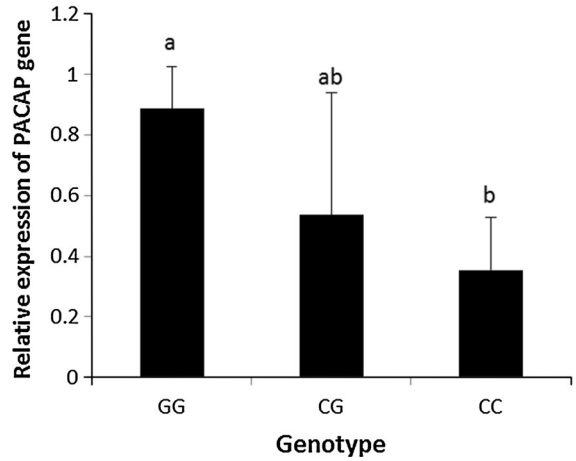


Fig. 6 Relative abundance of *PACAP* gene of CC, CG, and GG genotype was quantified by quantitative PCR. *Bars* with *unshared letters* are significantly different from each other ($P < 0.05$)

tongue sole were identified and were associated with growth traits. We found that there were three genotypes, GG, CG and CC, due to the C to G transition of *PACAP* gene exon 1. GG genotype had the best production performance in these three genotypes.

Many genes with statistically significant association of single nucleotide polymorphisms (SNPs) with expression variation were reported. However, gene mutation can change the transcription activity, such as the base change from A to G at 939 bp in exon 6 of cyp17-II gene (Ding et al. 2012); this SNP generates potential GATA-1 and GATA-2 binding sites, which is thought to increase cyp17 gene expression level. One-way ANOVA manifested that there were significant associations between the two loci and cyp17-II gene expression ($P < 0.05$). Furthermore, multiple comparisons showed that individuals with AA genotype of both loci have significantly higher gene expresses level ($P < 0.05$) than GG genotype (Ding et al. 2012). *PACAP* gene is known as a candidate growth-related gene. Any transcriptional regulation of *PACAP* gene is critical to GH release (Montero et al. 1998). Thus, sequence variation in *PACAP* gene may affect growth hormone secretion, thus indirectly affecting growth and development traits in animals. An analysis of the intron sequence between *GHRH-like* exon and *PACAP* exon from individuals of Arctic charr found a substitution from G to A. The results suggested G allele at this SNP marker could cause a

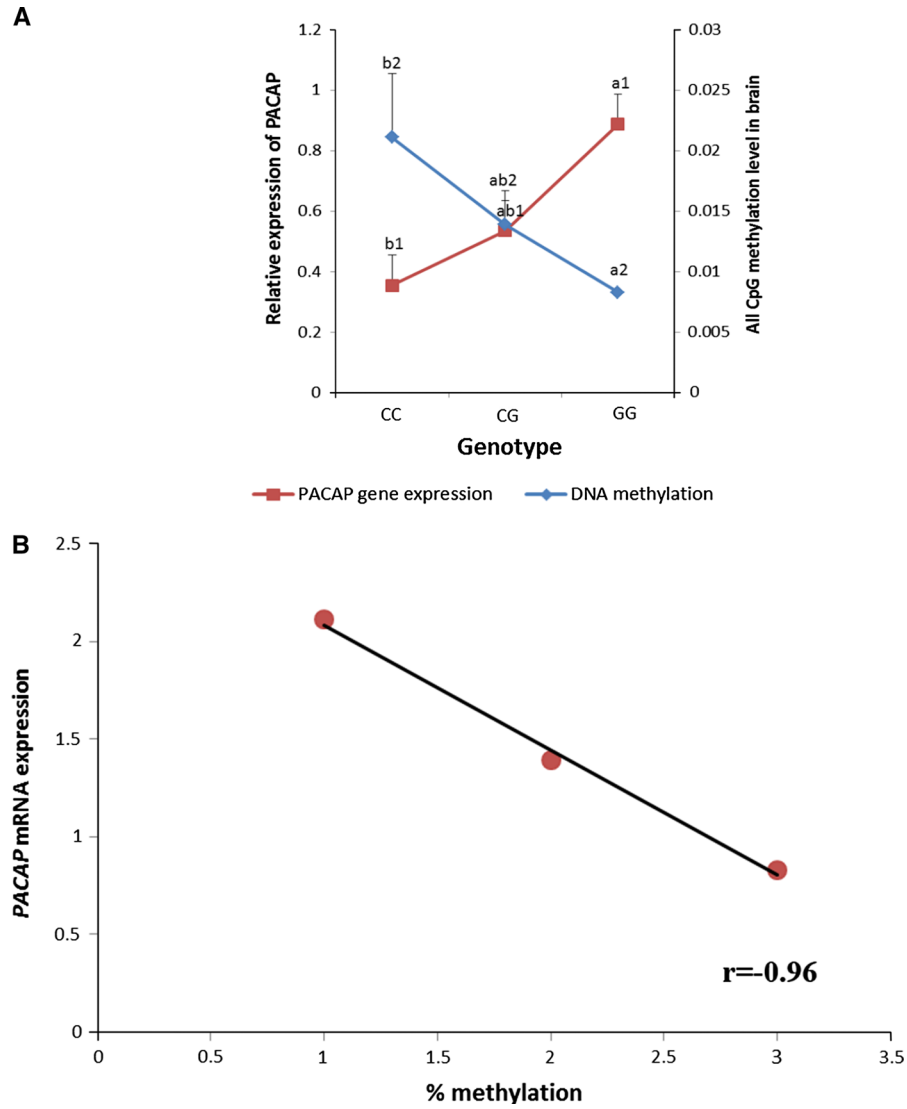


Fig. 7 Correlation of brain *PACAP* mRNA level and *PACAP* methylation level. **a** The changes in trend of the *PACAP* mRNA level and all CpG sites methylation level in different genotypes in half smooth tongue sole. *Different letters* indicate significant difference ($P < 0.05$). **b** The correlation of brain *PACAP* mRNA level and the methylation level *PACAP* exon 1

greater expression of *PACAP* by biasing splicing of mRNA to favor the short mRNA (Tao and Boulding 2003). Similarly, the expression of three genotypes by real-time quantitative PCR showed that GG genotype had significantly higher expression level than that with CC genotype ($P < 0.05$). We proposed that this mutation observed might promote the gene transcription and then impact the growth.

5′UTR and exon 1 play important roles in regulating gene expression. In addition, most CpG islands are located in these areas. Many studies suggested that transcription rate was altered by methylated CpG sites in 5′-untranslated region (5′UTR). For instance, 5′UTR methylation resulted in absence or reduction in CDX1 expression in colorectal carcinomas, while there were no associations between CDX1 mRNA expression and different polymorphic genotypes; further, the loss of CDX1 expression is attributable to 5′UTR methylation but not mutation or loss of heterozygosity (Wong et al. 2004). Si et al. (2015) detected the methylation levels of *cyp19a1a* promoter and exon 1. Results showed the methylation levels were negatively associated with their expression levels during the ovarian development. In conclusion, DNA methylation in 5′-untranslated region leads to transcriptional suppression. In the

present study, this mutation site happened in CpG-rich region of *PACAP* gene and resulted in a new CpG dinucleotide at the 150 bp. Results displayed that the CpG site at 150 bp position of *PACAP* gene was lower methylated in GG and CG genotypes in brain and liver, the other eleven CpG dinucleotides also had lower methylation level, and no significant difference was found between AA and GG genotype in each single CpG dinucleotides. However, GG genotype had lower methylation level and higher gene expression than CG and CC genotypes. This demonstrated that it is methylation not the mutation regulating gene expression. Furthermore, statistic results showed that body weight, body length, liver weight, net weight, GSI, and mRNA expression of GG genotype (containing twelve CpG dinucleotide) were significantly higher than that of CC genotypes (containing eleven CpG dinucleotide) ($P < 0.05$). As a result, we have reason to conclude: epigenetic modification of this mutation could regulate the *PACAP* gene transcription, and this SNP could be regarded as an important genetic or epigenetic marker.

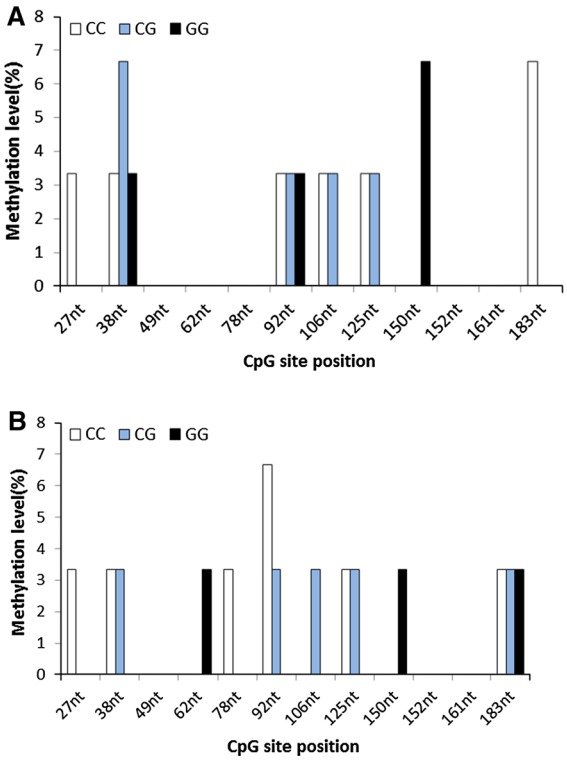


Fig. 8 DNA methylation level of the twelve CpGs (at position of 27nt, 38nt, 49nt, 62nt, 78nt, 92nt, 106nt, 125nt, 150nt, 152nt, 161nt and 183nt) of *PACAP* gene in liver (**a**) and brain (**b**)

Intriguingly, all the CpG sites of exon 1 were analyzed for putative transcription factor binding sites by TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html). Silico analysis founded some potential regulatory elements including MZF1, TATA, SRY, CdxA, NKx-2, p300, and AP-1 (Fig. 9). Among them, TATA boxes and AP-1 were reported to be the binding sites of PACAP in other species (Tao and Boulding 2003). TATA boxes are normally required for accurate initiation of transcription, indirectly controlling the mRNA expression. TATA motifs in the 5′UTR of *PACAP* were widely distributed within fish (Tao and Boulding 2003), mouse (Cummings et al. 2002), and human (Vaudry et al. 2009). However, there were no reports describing that TATA boxes involved in the regulation of *PACAP* gene in teleosts. AP-1 (activating protein-1) is a component including Jun, Fos, or ATF (activating transcription factor) subunits that bind to a common DNA site, the AP-1-binding site (Karin et al. 1997).

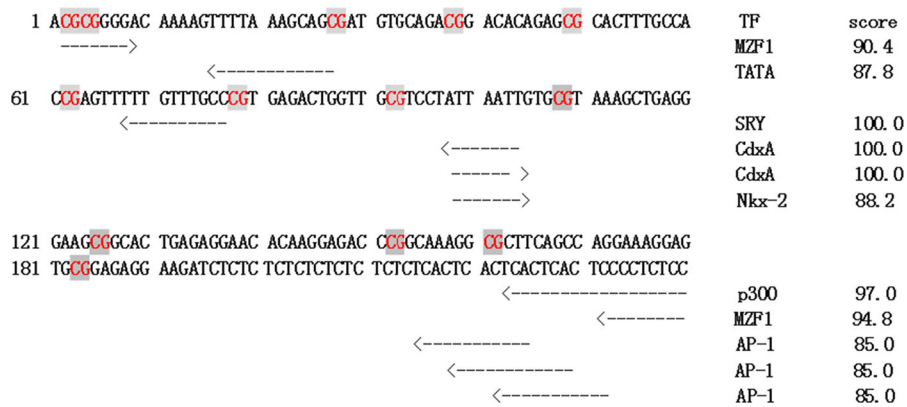


Fig. 9 Part of sequence of *PACAP* exon 1 is shown, the "TF" in the right of the nucleotide is the abbreviation of transcription factor. The *dotted line* indicates the consensus binding sequence of the transcription factor

The previous study demonstrated that AP-1 complexes containing c-Fos mediated the effect of PACAP on *Bcl-2* gene expression in cerebellar granule neurons (Aubert et al. 1996). In parallel to c-Fos/c-Jun induction, PACAP rapidly activates the heterodimeric transcription factor AP-1 (Schäfer et al. 1996). Thus, it is possible that methylation status of CpG site at 263, 268, and 272 bp position alters the protein structure and subsequently prevents or enhances AP-1 transcription factors from binding to their consensus sequence. This may reduce the transcriptional activity of *PACAP* gene, but there were no reports describing that AP-1 transcription factors had association with the regulation of *PACAP* gene in teleosts. Therefore, further experiments are needed to prove that the TATA boxes and AP-1 are on the sequence of *PACAP* gene and involved in the regulation of *PACAP* gene expression. Epigenetic modifications (especially DNA methylation) play important roles in influencing growth traits in fish, and more attention should be paid to interpret the molecular mechanism of fish hypothalamus-pituitary growth axis physiology (Dai et al. 2015).

The phosphorus is an essential component of cells and participates in almost all the important organics synthesis and degradation pathways. The high-energy phosphate compound plays an extremely important role in energy storage, release, and transformation, influencing body growth indirectly in mammals (Luo et al. 2004). Phosphorus plays an important role in regulating body fluids in the forms of phosphate salts. But inorganic phosphorus's effect on growth in teleosts is still unknown. In our study, inorganic phosphorous content in serum of GG genotype is significantly higher than CC genotype, and we deduced that inorganic phosphorous level in serum may reflect fish growth condition.

Conclusions

In this study, we identified one SNP in *PACAP* gene of male half smooth tongue sole which was significantly associated with growth traits including body weight, body length, liver weight, net weight, and HSI. Interestingly, the mutation (c.C151G) was located in CpG-rich region of *PACAP* gene. The individuals of GG genotype with one more CpG site had lower methylation level, higher gene expression level, and fast growth traits than those of CC and CG genotypes. These results provided important evidences for interpreting the molecular mechanism of endocrine physiology in fish and could also support the role of this locus as a candidate genetic or epigenetic marker for half smooth tongue sole breeding programs.

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