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Polymorphism in exons CpG rich regions of the *cyp17-II* gene affecting its mRNA expression and reproductive endocrine levels in female Japanese flounder (*Paralichthys olivaceus*)

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ABSTRACT

Cytochrome P450c17-*II* (*cyp17-II*) gene is an important factor affecting the growth, gonad differentiation and development, and other reproductive traits of fish. There are three CpG rich regions in the coding region of *cyp17-II* gene in Japanese flounder (*Paralichthys olivaceus*). The aim of this study was to understand whether mutations in exons of the *cyp17-II* gene occured at CpG sites, and mutations and methylation status of those CpG sites were involved in regulation of the expression level of *cyp17-II* gene and the reproductive endocrine of Japanese flounder. The results showed that three single nucleotide polymorphisms (SNPs) were identified. SNP1 [(c. G594A (p.Gly 188Arg)] located in exon 4 of L1 locus, and SNP2 (c.A939G) and SNP3 (c.C975T) of L2 locus located in CpG rich region of the exon 6 of *cyp17-II* gene. Furthermore, the A to G transition at 939 bp position added a new methylation site to the *cyp17-II* coding region. According to multiple-comparison analysis, two loci (L1 and L2) were significantly associated with serum testosterone (T) level ($P < 0.05$) and the expression of *cyp17-II* in ovary ($P < 0.01$). Intriguingly, individuals with GG genotype of L2 locus containing eight CpG methylation sites had significantly lower serum testosterone level and *cyp17-II* mRNA expression than those with AA genotype containing seven CpG methylation sites. Moreover, the CpG site was highly methylated ($\geqslant$77.8%) at 938 bp position of individuals with GG genotype of L2 locus. These implied that the mutation and methylation status of the coding region of *cyp17-II* could influence the gene expression and the reproductive endocrine levels in female Japanese flounder and L2 locus could be regarded as a candidate genetic or epigenetic marker for Japanese flounder breeding programs.

1. Introduction

Cytochrome P450c17, possessing 17α-hydroxylase and 17, 20 lyase activities, is an important enzyme participating in steroidogenic pathway for the production of C-18, −19 and −21 steroids biosynthesis in the gonads and head kidney of fish [7,26–28,47]. Unlike mammals and other lower vertebrates, two forms of P450c17, P450c17-*I* and P450c17-*II* encoded by *cyp17-I* and *cyp17-II* gene, respectively, are identified in some teleost species [48,49]. P450c17-*I* possesses both hydroxylase and lyase activities, while P450c17-*II* only has the hydroxylase activity. Furthermore, 17α-hydroxylase activity converts pregnenolone or progesterone (Prog) to 17α-hydroxypregnenolone or 17α-hydroxyprogesterone [29]. It is reported that P450c17-*II* is responsible for the production of 17α, 20β-dihydroxy-4-pregnen-3-one during oocyte maturation and cortisol production in the head kidney of Tilapias [48]. In barfin flounder (Verasper moseri),only P450c17-*II* is involved in the production of cortisol [16].

Single nucleotide polymorphisms (SNPs), a gene marker, have been widely used for screening functional genes in aquaculture species, and it also has been evidenced to have a great impact on gene expression and the function of proteins [2,4,24]. It is supposed that genetic polymorphisms influence the reproductive traits of fish. To date, some studies have reported the correlation analyses between polymorphisms and phenotype traits in fish. For instance, the mutations in *cyp17-II* coding region obviously affect the serum T level and hepatosomatic index (HSI) in male Japanese flounder [22]; SNPs in the promoter region and exons of *cyp19a* gene and the coding region of estrogen receptor α and β gene were significantly associated with 17β-estradiol (E_2) and gonadosomatic index (GSI) in some fish [9–11,36]. These suggest that the SNPs of the specific gene which are responsible for steroid hormones synthesis act on the fish reproductive traits. However, so far, no data is available for the association between genetic

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diversity of the *cyp17-II* gene and reproductive traits or gene expression in female Japanese flounder.

Many mutations were observed at CpG sequences and involved C → T and G → A transitions [33], as well as G → T transversion [46], which have been associated with cytosine methylation. In *p53*, all exonic CpG sequences are methylated [42] and thus prone to such mutations[39,46]. As an important epigenetic modification, DNA methylation plays profound role in gene expression, and this molecular mechanism is crucial for the understanding of most biological phenomena [30,47]. The cDNA sequence of Japanese flounder *cyp17-II* gene was formed by nine exons. According to the GardinerGarden sequence criteria with minor modification, a genomic region of an exon has to fulfill three conditions to be classified as a CpG rich region: (1) island size greater than 100 bp, (2) GC percent above 50.0, and (3) ratio of observed-to-expected number of CpG dinucleotides above 0.6. There are three CpG rich regions in the coding region of *cyp17-II* gene in Japanese flounder, located in exon 1, exon 4 and exon 6, respectively. We hypothesized that mutations in the CpG context within the coding region (gene body) may play an important role in regulating gene expression.

As a member of the flatfish species, the Japanese flounder has become an economically important cultivated marine species in China. And it is also a multiple spawner with oocytes showing asynchronous development, spawns almost daily during the spawning season [23,32]. Moreover, the Japanese flounder is a teleost fish with an XX/XY sex determination system. XX flounders can be sex-reversed to a male phenotype by rearing the larvae at high water temperatures [17,41,45]. Thus, the sex of the flounder is determined by genotype and temperature effects [31]. Hence, the Japanese flounder provides an excellent model to study the molecular mechanism of fish reproduction and endocrine physiology.

In this study, we examined the SNPs of *cyp17-II* gene of female Japanese flounder, analyzed the associations of the gene polymorphisms on the *cyp17-II* gene expression and reproductive traits: T level, E_2 level, GSI and HSI. In addition, we identified the DNA methylation patterns of the CpG site at 938 bp position, where occurred base substitution, for elucidating the regulation mechanism of gene expression based on epigenetic.

2. Materials and methods

2.1. Animals and data collection

Adult Japanese flounder, with an average wet weight of 685.5 ± 0.5 g, were obtained from a local fish farmer. They were reared in commercial fish pond for twenty months, under the controlled conditions (20 ± 0.5 °C; ⩾ 4 mg/L O_2; 14:10 h light: dark cycle), and fed on a commercially prepared diet. Fifty female fish were firstly anesthetized, and then wet weights and body lengths were measured. Later, the blood was extracted from the fish tail,and gonads and livers were taken out and weighed. Finally, the ovaries were bisected, and one part was fixed in Bouin's solution for hematoxylin and eosin (HE) staining to identify the ovarian developmental stages, according to the method as previously described [37]. The remaining gonad, liver and muscle samples were immediately frozen in liquid nitrogen and then stored at −80 °C until further processing.

2.2. HSI and GSI

The HSI or GSI of each fish 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.

2.3. Steroid radioimmunoassay (RIA)

Blood samples were stored at 4 °C for 8 h, then centrifugated at 12,000 g for 10 min. The serum was obtained and transferred into 1.5 ml plastic microfuge tubes and then stored at −40 °C. The serum concentrations of T and E_2 were determined by ^{125}I radioimmunoassay, according to the protocol provided by Wen et al. [43].

2.4. Genomic DNA isolation

Genomic DNA was isolated from muscle samples by the phenol chloroform method. The DNA purity and concentration were measured using an UV spectrotometer (Amersham, American), and their integrity was evaluated by normal and denaturing agarose gel electrophoresis, respectively.

2.5. PCR–SSCP analysis

According to the cDNA sequence (GenBank ID: FJ 6135292), eight pairs of primers were designed using Oligo 6 software to amplify the single exon of *cyp17-II* gene (Table 1) from 50 female Japanese flounder, except the primers of exon 5, because their sequences were less than 100 base pairs (bp). PCR reactions were carried out in a total of 25 μl volume containing 100 ng of genomic

Table 1
Primers used for amplification exons of *cyp17-II* gene and quantitative PCR.

Primer name	Primer sequence	Product size (bp)	Tm (°C)	Accession no
Exon 1	For5′-ACATGGGATTTCATTTTCTCTTTGTT-3′ Rev5′-CCGGGTGAAGAGCAGGT-3′	279	60	FJ613529.2
Exon 2	For5′-GGTTAACAGGTACGGCTCTCTG-3′ Rev5′-CTCGGTCGTCCAGCAAAGT-3′	125	60	
Exon 3	For5′-TGACCTGTTGACCAGAGGAGGT-3′ Rev5′-GATGTCCTGCAGACGACTGGTT-3′	130	61	
Exon 4	For5′-GTCTCTGTGTCGAGTTGTTGTCCAG-3′ Rev5′-CCTTCATCCAGGGATAAATGTCC-3′	213	62	
Exon 6	For5′-CTTCTGGACGCCTTGCTG-3′ Rev5′-AGCAGGTAGGCCAGGATC-3′	158	60	
Exon 7	For5′-AGTGCAGAAGGAGCTGGACGAA-3′ Rev5′-ATAGCGGTGTGTGGGATCAGGA-3′	147	62.5	
Exon 8	For5′-TATTGGAGGTCACTCTGTCGGT-3′ Rev5′-CTGGGTTGAGGAGGTCCGGT-3′	104	62.5	
Exon 9	For5′-ATGACCAGGGTCAGCGGGTCAC-3′ Rev5′-TGGTTGCAGGACCACGCCAAGG-3′	197	66	
cyp17-II (qPCR)	For5′-GAGCGAGCAGTGAGCGTGTC-3′ Rev5′-ACGAGTCCCAGGACCGACAG-3′	159	62	
18S (qPCR)	For5′-ATTGACGGAAGGGCACCAC-3′ Rev5′-ATGCACCACCACCCACAGA-3′	134	62	EF126037.1

DNA isolated from muscle, 0.2 mM each dNTP, 2.5 μl 10 × PCR buffer, 0.20 mM primers and 0.5 U Taq DNA polymerase (rTaq TaKaRa). Amplification conditions were 94 °C for5 min followed by 35 cycles of 94 °C for 30 s, 60–66 °C (Table 1) for 30 s, 72 °C for 30 s; a final extension was performed at 72 °C for 10 min. A 5 μl aliquot of each PCR product was electrophorized in a 1.5% agarose gel containing ethidium bromide and then visualized under a UV transilluminator.

The PCR products of *cyp17-II* were genotyped by single-stranded conformation polymorphism (SSCP) method as previously described [10]. 3 μl PCR products from each individual were mixed with 9 μl denaturing buffer (98% formamide, 0.09% xylene cyanole FF, and 0.09% bromophenol blue). Each mix was denatured at 98 °C for 10 min followed by a rapid chill on ice for 10 min. The denatured PCR products of *cyp17-II* gene were separated in 10–12% non-denaturing polyacrylamide gel at 120 V for 12–14 h at room temperature. SSCP patterns on the gels were visualized by silver staining [34]. The PCR products of different genotypes samples were purified with DNA Fragment Quick Purification/Recover Kit (TIANGEN), then inserted into the PGM-T vector (TIANGEN) and transferred into

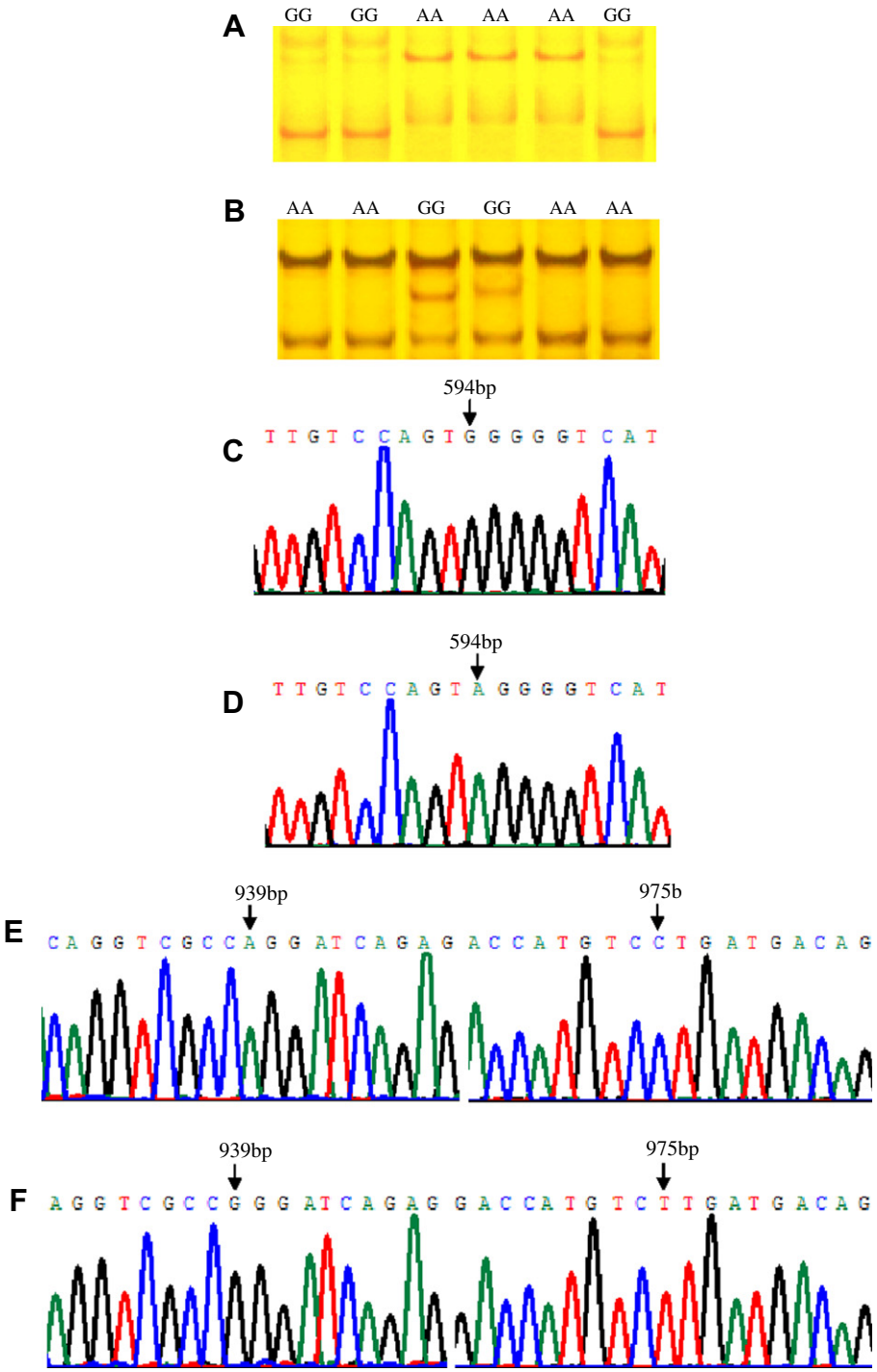

Fig. 1. *cyp17-II* genotyping and sequencing confirmation. (A) Band pattern for genotypes (AA and BB) of L1 locus in exon 4; (B) band pattern for genotypes (AA and BB) of L2 locus in exon 6. The chromatograms show sequences of the three SNPs(c. G594A. c.A939G and c.C975T): the number indicates the position of mutation site; (C) sequence of GG genotype in exon 4; (D) sequence of AA genotype in exon 4; (E) sequence of AA genotype in exon 6; (F) sequence of GG genotype in exon 6.

Table 2
Associations between SNPs of Japanese flounder *cyp17-II* gene and reproductive traits by ANOVA.

Traits	T		E_2		GSI		HSI	
Locus	*F* value	*P* value	*F* value	*P* value	*F* value	*P* value	*F* value	*P* value
L1	5.99	0.0193*	0.45	0.5089	0.11	0.7457	0.62	0.4363
L2	58.44	<.0001**	0.40	0.5317	0.74	0.3938	0.52	0.4756

* $P < 0.05$.
** $P < 0.01$.

Table 3
Associations between SNPs of Japanese flounder *cyp17-II* gene and expression by ANOVA.

Locus	Gene expression of *cyp17-II*	
	F value	*P* value
L1	5.65	0.035*
L2	12.76	0.003*

* $P < 0.05$.

Trans-5α Chemically Competent Cell (Beijing TransGen Biotech Co, Ltd). Positive recombinant colonies were sequenced by the ABI 377 sequencer.

2.6. RNA isolation and Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from ovary using RNAiso reagent (TaKaRa, Japan) following manufacturer's instructions. The ovary tissues were obtained from three different individuals of each genotype of SNPs loci. Complimentary DNA was synthesized with PrimeScript® RT reagent Kit (TaKaRa). To generate cDNA of mRNA, a 10 μl reverse transcriptase reaction mixture containing 500 ng of the RNA sample, 2 μl 5 × PrimeScript® Buffer (for Real Time), 0.5 μl PrimeScript® RT Enzyme Mix I, 0.5 μl Oligo dT Primer (50 μM), 0.5 μl Random 6 mers (100 μM) and RNase Free dH_2O up to 10 μl at 37 °C was incubated for 15 min, then at 85 °C for 5 s. The RT-PCR products were used for quantitative PCR reaction.

2.7. Quantitative PCR (qPCR)

Quantitative PCR was performed using Roche LightCycler480 (German), according to the manufacturer's instructions. SYBR Premix Ex Taq™ IIKit (TaKaRa, Bio, Kyoto, Japan) was used for the reaction. qPCR primers for *cyp17-II* and 18S ribosomal RNA were designed by Oligo 6 software (Table 1). The 25 μl mixture of PCR consisted of 12.5 μl SYBR Green supermix, 9.5 μl RNase-free water, 0.5 μl of each primer and 2 μl reverse transcribed product. The following cycling conditions were used: pre-incubation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 20 s, annealing at 62 °C for 15 s and extension at 72 °C for 15 s. At the end of PCR run, a melt curve analysis was performed to ensure that only a single product was amplified. The experiment was repeated in triplicate. Meanwhile, 18S ribosomal RNA gene was used as an internal control for expression analysis and amplified under the same reaction conditions with *cyp17-II*. For a PCR negative control, all other PCR reagents were used but without cDNA added. Relative expression was normalized to that of 18S ribosomal RNA ($2^{-\Delta Ct}$; where ΔCt = [Ct(cyp17-*II*)-Ct(18S ribosomal RNA)]). *cyp17-II* mRNA expression levels in both genotypes of each SNPs locus were compared using paired *t*-test (GraphPad Prism version 5.3). A probability level of $P < 0.05$ was considered statistically significant.

2.8. Analysis of DNA methylation pattern

2.8.1. Purification of DNA and bisulphate conversion of DNA

Marine animals DNA kit (CWBIO) was used for extracting high purity genomic DNA from ovary tissues of three individuals (the same ovary tissues were used for RNA isolating) with each genotype, according to the manufacturer's instructions. The DNA concentration and the purity of the samples were determined by the method as described above. Then the genomic DNA (200 μg) was bisulfate-treated using the Methylamp™ DNA Modification Kit (QIAGEN) following the manufacturer's instructions. Bisulfite treated DNA was eluted in 16 μl of elution buffer.

2.8.2. Identification of CpG islands and bisulphate PCR (BS-PCR)

The CpG rich regions of *cyp17-II* were identified by online MethPrimer design software (http://www.urogene.org/methprimer/index1.html). BS-PCR primers were designed on oligo 6 software using the sense strand of the bisulphate-converted DNA. The PCR reactions were carried out in a total of 25 μl volume containing 3 μl of treated DNA, 0.50 mM each primer: forward 5′-TTTTGTTGAAGGGTAAGATGG. ATA-3′ and reverse 5′-CAACAAA-TAAACCAAAATCCACAAC-3′, 0.20 mM each dNTP, 5U TransStart DNA Polymerase. The amplification conditions were 94 °C for 5 min; 40 cycles of 94 °C for 30 s, 54 °C for 30 s, and 70 °C for 1 min; and then a final extension at 70 °C for 5 min. as above described. Three independent PCR amplifications were carried out

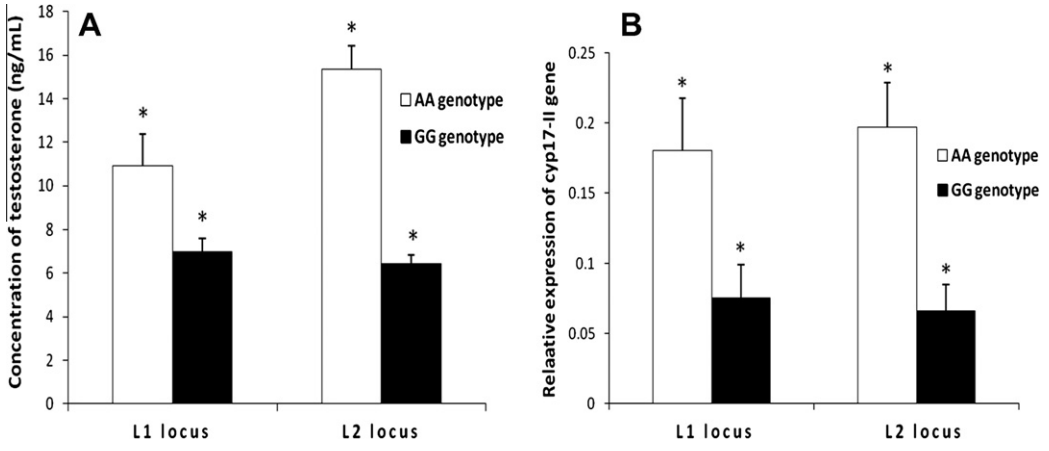

Fig. 2. (A) The serum testosterone (T) level of AA and GG genotype in L1 and L2 locus. (B) Relative abundance of *cyp17-II* gene of AA and GG genotype in L1 and L2 locus was quantified by Quantitative PCR. **P* < 0.05, significantly difference from each other.

for each sample. Genomic region corresponding to the CpG islands (i.e. exon 6) were also amplified from untreated genomic DNA for comparison purpose. All the PCR products were separated on 1.5% agarose gel followed by gel extraction and sequenced, as above described. At least three clones of each product were custom sequenced by ABI 377 sequencer.

In order to evaluate the efficiency of the bisulfite modification step, the rate of conversion of 30 (AA genotype) or 29 (GG genotype) cytosine, which were not in the context of a CpG dinucleotide, were examined.

2.9. Statistical analysis

The associations between genotypes and four reproductive traits (T level, E_2 level, HSI and GSI) or gene expression of Japanese flounder were analyzed by one-way ANOVA using Stat View software version 9.0 (SAS Institute Inc., Cary, NC) as previously described [2]. Differences among means of different genotypes were assessed using Duncan's multiple-range test. Statistical significance was considered as $P < 0.05$.

3. Results

3.1. Polymorphisms within exons of cyp17-II gene

Among the eight sets of primers, two Primer pairs were polymorphic in SSCP pattern. SNP1 (c. G594A) located in exon 4, was a non-synonymous mutation, which caused an amino acid change from Gly^{188} to Arg^{188}, named as L1 locus; another two SNPs were c.A939G and c.C975T which located in exon 6, named as L2 locus (Fig. 1C-F). The SNPs of L2 locus were linked together and were synonymous mutations. Two genotypes were found in each locus and named as AA and GG (Fig. 1A and B).

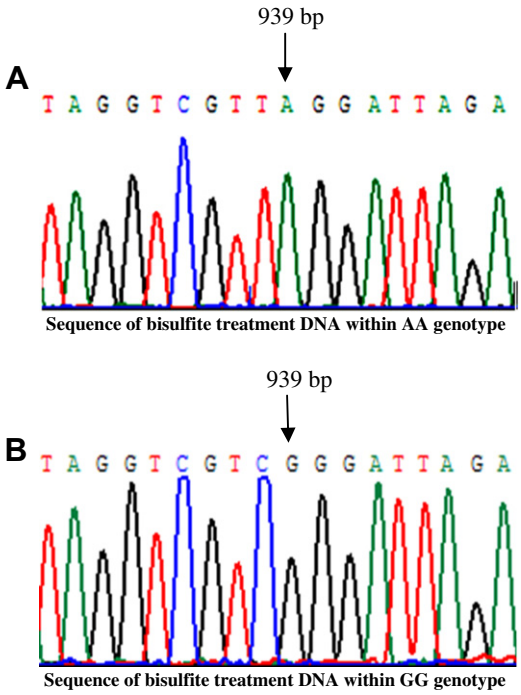

Fig. 3. A part of exon 6 sequence of *cyp17-II* gene was amplified from bisulfate treatment DNA. The number indicidate the position of mutation (A/G) site: (A) The cytosine close to adenine was converted to thymine in AA genotype; (B) there was a novel CpG dinucleotides due to guanine substitute for adenine at 939 bp position. The cytosine was methylated in GG genotype.

3.2. Correlation between two loci and reproductive traits

The association between the two loci (L1 and L2) within *cyp17-II* gene and the reproductive traits of Japanese flounder was showed in Table 2. Statistical results indicated that, both L1 locus ($P < 0.05$) and L2 locus ($P < 0.01$) were significantly associated with serum testosterone level in female Japanese flounder. No significantly association was found between two loci and other traits. Furthermore, multiple comparison analysis demonstrated that T level for individuals with AA genotype were higher than those with GG in L1 locus and L2 locus ($P < 0.05$) (Fig. 2A).

3.3. Relationship between two loci and gene expression of cyp17- II in ovary of female Japanese flounder

The correlation analysis demonstrated that there were significant associations between the two loci and *cyp17-II* gene expression of female Japanese flounder ($P < 0.05$) (Table 3). Additionally, multiple comparisons showed that fish with AA genotype of both two loci have significantly higher gene expresses level ($P < 0.05$) than GG genotype (Fig. 2B).

3.4. DNA methylation level in CpG rich region of cyp17-II gene

Evaluation of the efficiency of bisulfite treatment showed that only three non-converted C were found in all copies of three different samples of CpG dinucleotide sequence analyzed, and almost above 99% of all C had been chemically converted by the bisulfite treatment. This demonstrates that the DNA modification procedure was very efficient and should not produce considerable artifacts due to incomplete conversion.

Eight CpG dinucleotide sites were found in the sequence of exon 6 of *cyp17-II* of Japanese flounder with GG genotype, which has one more CpG dinucleotides than AA genotype due to the substitution from A to G at nucleotide 939 bp in exon 6 of *cyp17-II* gene (Fig. 3). The cytosine base of the CpG site at 938 bp position was found to be highly methylated ($\geqslant$ 77.8%) in the individuals with GG genotype. Moreover, except for the CpG site at 938 bp position, the other seven CpG dinucleotides were also highly methylated, but no significant difference was found between AA and GG genotype (Fig. 4).

4. Discussion

Cytochrome P450c17 (17-hydroxylase/C17-20 lyase, *cyp17*) is a central steroidogenic enzyme in the production of androgens and estrogens. Besides, it is also indispensable for the production of oestrogens which are produced from the aromatisation of androgens [44]. However, gene mutation can alter the transcription activity, such as the substitution (T → C) located 34 base pairs upstream from the translation initiation site; This polymorphism makes a potential Sp-1-like (CCACC box) binding site, which is thought to increase *cyp17* gene expression and androgen biosynthesis and is expected to be associated with higher estrogen and androgen levels [3,35]. It is reported that *P450c17*, during preparatory and prespawning/spawning phases, might exert an influence on the shift in steroidogenesis during ovarian and testicular recrudescence [40]. Therefore, improper and untimely changes in their production could have detrimental consequences for normal sexual development [44]. These studies demonstrated that polymorphisms of *cyp17-II* gene would decrease or increase its mRNA expression and T level, and finally affect gonad development. In this study, we have detected single nucleotide polymorphisms (SNPs) and investigated the associations of *cyp17-II* polymorphisms with E2 and T concentration, HSI and GSI, based on SSCP method.

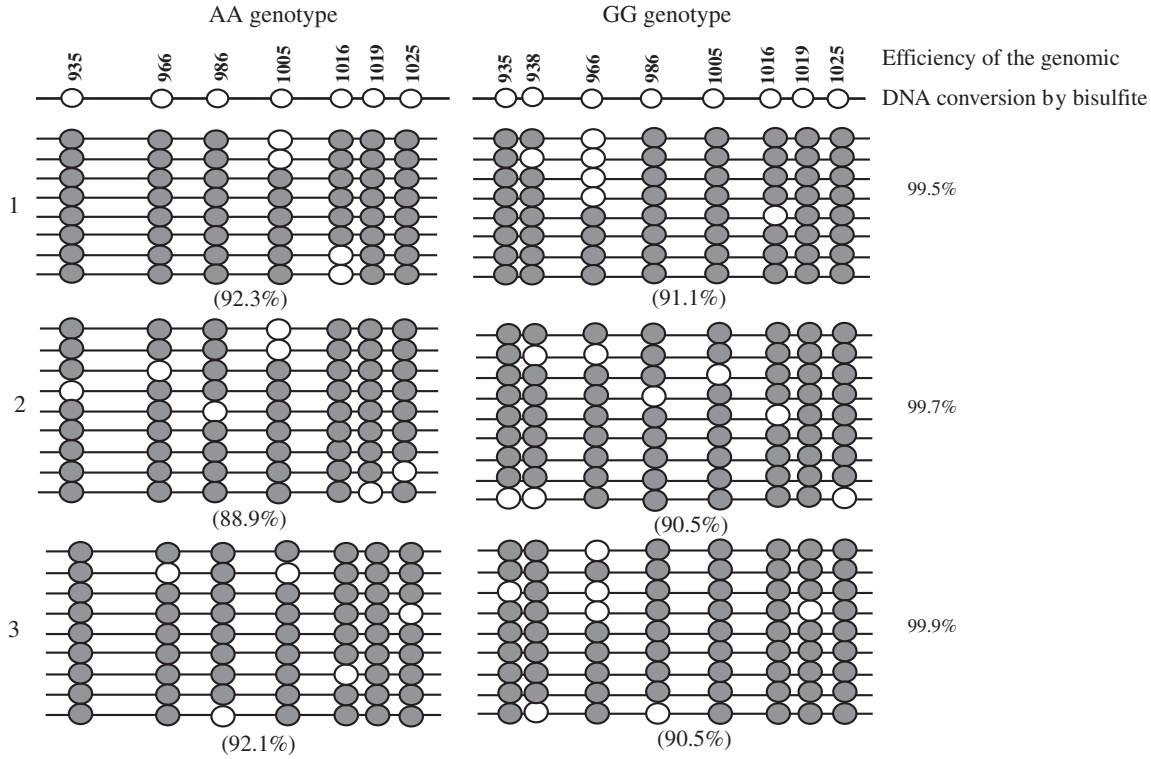

Fig. 4. DNA methylation patterns of exon 6 in *cyp17-II*. Filled and open circles denote methylated and unmethylated sites, respectively. Each line represents one sequenced clone. The first line indicates the localizations of studied CpG sites related to the sequence of *cyp17-II* coding region. No. 1–3 refer to individual fish from each genotype. The percentage indicates the methylation level (under the line), calculated as the number of methylated CpG per total number of CpG sequences except for the second CpG in GG genotype. The average efficiency of the genomic DNA conversion by bisulfite is given on the right for each fish.

Three SNPs of coding region of *cyp17-II* gene in female Japanese flounder were identified, SNP1 (c. G594A) located in exon 4, SNP2 (c.A939G) and SNP3 (c.C975T) were linked together in exon 6. A SNP located at L1 locus was a non-synonymous mutation and two SNPs located at L2 locus did not lead to amino acid changes. Statistic analysis showed that these mutations were significantly associated with T level.

There is a non-synonymous mutation leading to an amino acid change from Gly^{188} to Arg^{188} in exon 4, which was significantly relative to T level. The expression of *cyp17-II* between two genotypes of L1 locus by qPCR showed individuals with AA genotype had significantly higher expression level than that with GG genotype ($P < 0.05$). We proposed that the non-synonymous mutation observed might impede the gene transcription or alter the activity of protein, and then change the level of androgen. However, the activity of protein needs to be evaluated in the future, for well elucidate the relationships between gene polymorphisms and reproductive traits.

Though polymorphisms (c.A939G and c.C975T) in exon 6 were synonymous mutations, the two SNPs happened in CpG rich region of *cyp17-II* gene. This change seemed to be very important because a new methylated CpG site was added at the 938 bp position of *cyp17-II* gene. Many reports indicated that transcription rate was altered by methylated CpG sites in coding region. For instance, Methylation of a specific CpG site within the *phyA'* coding region is critical for maintaining *phyA'* silencing, further, erasure of methylation of this specific site leads to irreversible epigenetic activation of *phyA'* [8]. Hohn et al. reported that activity of a weak promoter (derivative of 35S promoter) in tobacco was suppressed when coding region contained methylated CpG sites [13]. There is a crosstalk between DNA methylation and chromatin structure, showing that chromatin structure is condensed by DNA methylation and consequently leads to transcriptional repression [1,5,6]. DNA methylation in the coding region is an important silencer [14,15]. Moreover, recent evidences demonstrated that methylation in the coding region silenced the human gene expression [21,50]. However, demethylation of CpG sites in intra- and/or extra-genic positions has been shown to increase the transcription rate for a specific gene [18]. In the present study, except for the CpG site at 938 bp position of *cyp17-II* gene, the other seven CpG dinucleotides were also highly methylated, but no significant difference was found between AA and GG genotype in exon 6. The high methylation level of whole CpG sites of exon 6 may be involved in the gene expression rate, but further study is needed to conform it. Furthermore, statistic results showed that T level and mRNA expression of GG genotype (containing eight CpG dinucleotide) were significantly lower than that of AA genotype (containing seven CpG dinucleotide). So, we speculated that epigenetic modification of L2 locus could regulate the *cyp17-II* gene transcription and L2 locus could be regarded as an important genetic or epigenetic marker.

Interestingly, in silico analysis of exon 6 sequence for putative transcription factor binding sites by TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html) showed GATA-1 and GATA-2 near the 938 bp position of *cyp17-II* gene of Japanese flounder. Six members (GATA-1 to GATA-6) of the GATA family transcription factors share a highly homologous DNA-binding domain, exhibiting similar DNA-binding properties [19,25]. GATA-4 and GATA-6 are responsible for activation of *P450c17* gene expression at early stage of mouse embryonic development [38]. Thus, it is possible that methylation status of CpG site at 938 bp position alters the chromatin structure, and subsequently prevents or enhances GATA transcription factors from binding to their consensus sequence. This may reduce the transcriptional activity of *cyp17-II* gene and decrease the concentration of testosterone, finally. However, there were no reports describing that GATA transcription

factors involved in the regulation of *cyp17-II* gene in teleosts. Therefore, further experiments are needed to prove that the GATA1/2 transcription factor is on the *cyp17-II* gene sequence and involved in the regulation of *cyp17-II* gene. Importantly, the influence of epigenetic modifications (especially DNA methylation) on reproductive traits in fish is worth drawing more attention with the purpose of understanding the molecular mechanism of fish reproduction and endocrine physiology.

The GSI is a gross quantitative indicator of gonad condition [12] and HSI is a good indicator of liver energy content [20]. The previous study illustrated that the mutation in coding region of *cyp17-II* gene notably altered the GSI and HSI in male Japanese flounder [22]. But this study in female Japanese flounder showed that GSI and HSI were not associated with polymorphisms of the *cyp17-II* gene. It should be noticed that the ovary tissues studied were all at stage *II* (perinucleolus stage) identified by histological analysis. But some ovary tissues were at early perinucleolus stage and others at late perinucleolus stage. Due to the unclear differences of the two perinucleolus stage, we did not separate them for analyzing. So we do not deny the potential influence of the SNPs on the GSI and HSI, and further studies with larger population of different mature state ovary are required.

5. Conclusions

In this study, we identified 3 SNPs in *cyp17-II* gene of female Japanese flounder which were associated with T level and mRNA expression. Interestingly, the mutation (c.A939G) at L2 locus was located in CpG rich region of *cyp17-II* gene. The individuals with GG genotype of L2 locus has one more CpG site which has been highly methylated, lower gene expression level and lower content of serum T level than those with AA genotype. These results provided vital evidences for elucidating the molecular mechanism of endocrine physiology in fish, and could also support the role of L2 locus as a candidate genetic or epigenetic marker for Japanese flounder breeding programs.

Acknowledgments

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