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Mutations in Exons of the *CYP17-Ⅱ* Gene Affect Sex Steroid Concentration in Male Japanese Flounder (*Paralichthys olivaceus*)

MA Ruiqin, HE Feng*, WEN Haishen, LI Jifang, SHI Bao, SHI Dan, LIU Miao, MU Weijie, ZHANG Yuanqing, HU Jian, HAN Weiguo, ZHANG Jianan, WANG Qingqing, YUAN Yuren, and LIU Qun

Fisheries College, *Ocean University of China*, *Qingdao* 266003, *P. R. China*

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Abstract As a specific gene of fish, cytochrome P450c17-Ⅱ (*CYP17-Ⅱ*) gene plays a key role in the growth, development and reproduction level of fish. In this study, the single-stranded conformational polymorphism (SSCP) technique was used to characterize polymorphisms within the coding region of *CYP17-Ⅱ* gene in a population of 75 male Japanese flounder (*Paralichthys olivaceus*). Three single nucleotide polymorphisms (SNPs) were identified in *CYP17-Ⅱ* gene of Japanese flounder. They were c.G594A (p.G188R), c.G939A and c.G1502A (p.G490D). SNP1 (c.G594A), located in exon 4 of *CYP17-Ⅱ* gene, was significantly associated with gonadosomatic index (GSI). Individuals with genotype GG of SNP1 had significantly lower GSI ($P<0.05$) than those with genotype AA or AG. SNP2 (c.G939A) located at the CpG island of *CYP17-Ⅱ* gene. The mutation changed the methylation of exon 6. Individuals with genotype AA of SNP2 had significantly lower serum testosterone (T) level and hepatosomatic index (HSI) compared to those with genotype GG. The results suggested that SNP2 could influence the reproductive endocrine of male Japanese flounder. However, the SNP3 (c.G1502A) located in exon 9 did not affect the four measured reproductive traits. This study showed that *CYP17-Ⅱ* gene could be a potentially useful candidate gene for the research of genetic breeding and physiological aspects of Japanese flounder.

Key words *CYP17-Ⅱ* gene; SNPs; sex steroid; Japanese flounder

1 Introduction

Cytochrome P450c17, an important member of the cytochrome P450 subfamily, is a central enzyme for the synthesis of androgens and estrogens. P450c17 possesses 17α-hydroxylase and 17,20-lyase activities, and occupies the key position in the steroidogenic pathway for the production of C-18, -19 and -21 steroids in the gonads and head kidney of fish (Nakajin *et al*., 1981; Zhou *et al*., 2007). Pregnenolone or progesterone could be catalyzed into 17α-hydroxypregnenolone or 17α-hydroxyprogesterone by the 17α-hydroxylase activity of P450c17, and then the 17, 20-lyase activity breaks 17α-hydroxypregnenolone or 17α-hydroxyprogesterone into dehydroepiandrosterone or androstenedione. These metabolites are the necessary precursors for the production of gonadal androgens or estrogens and adrenal cortisol in vertebrates (Nakajin *et al*., 1981; Payne, 1990; Conley and Bird, 1997). Being different from mammals, fish have two types of p450c17s, P450c17-Ⅰ and P450c17-Ⅱ. They are encoded by *CYP17-Ⅰ* and *CYP17-Ⅱ* genes, respectively. However, P450c17-Ⅱ possesses only 17α-hydroxylase activity (Zhou *et al*., 2007).

A single nucleotide polymorphism (SNP) is a nucleotide variation including deletion, insertion and substitution at a special location in the genome, which can greatly impact gene expressions and protein functions. SNPs are the most common form of genetic variation in the genome (Singhal *et al*., 2011). Many studies have reported *CYP17* genetic variation with the risk of many diseases in mammalian, especially in human. However, to our knowledge, there is no report on the SNPs of *CYP17-Ⅱ* gene in Japanese flounder (*Paralichthys olivaceus*).

In this study, we examined the SNPs of *CYP17-Ⅱ* gene obtained from Japanese flounder (*P. olivaceus*) (FJ613529.2), and then analyzed the associations of the SNPs on the concentrations of serum testosterone (T) and serum 17*β*-estradiol (E_2), hepatosomatic index (HSI) and gonadosomatic index (GSI).

2 Materials and Methods

2.1 The Experimental Fish

The adult experimental fish were bought from Shan-

* Corresponding Author. Tel: 0086-532-82031953
E-mail: hefengouc@ouc.edu.cn

dong Kehe High Technology Co., Ltd. They were then reared in a commercial fish pond for six months, provided with natural seawater under controlled conditions (20℃ ± 0.5℃; ≥4 mg L^{-1} O_2; 14:10 h light:dark cycle), and fed with a commercially prepared diet. Their average wet weight was 452.89 g. Out of these individuals in the pond, 100 male fish were randomly chosen and then anesthetized. After weighting wet weights and checking body lengths, blood was extracted from the tail of fish. Fish were dissected with the gonads and livers taken out and weighed. Muscle samples were stored at −80℃.

2.2 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. Formulas used are as follows:

$$HSI = \frac{\text{liver weight}}{\text{body weight} - \text{viscera weight}} \times 100$$

$$GSI = \frac{\text{gonad weight}}{\text{body weight} - \text{viscera weight}} \times 100$$

2.3 Steroid Radioimmunoassay (RIA)

Blood samples were stored at 4℃ for 8 h before centrifugation at 12 000 g for 10 min. The plasma was divided into aliquots in 1.5 mL plastic centrifuging tubes and then stored at −40℃. The steroid RIA was carried out with the method of Wen *et al*. (2006) to determine the serum concentrations of T and E_2.

2.4 DNA Extraction and Polymerase Chain Reaction (PCR)

Nine exons form the cDNA sequence of Japanese flounder *CYP17-Ⅱ* gene. Oligo 6.0 software was used to design eight pairs of primers to amplify the single exon of *CYP17-Ⅱ* gene (Table 1). The primers of exon 5 were not designed because their sequences were less than 100 base pairs (bp). Genomic DNA was extracted from muscle samples using the phenol chloroform method. PCR reactions were carried out in a total of 25 μL volume containing 50 ng of genomic DNA, 0.20 mmol L^{-1} dNTP (each), 2.5 mmol L^{-1} $MgCl_2$, 0.20 mmol L^{-1} primers and 0.5 U *Taq* DNA polymerase. Amplification condition was 94℃ for 5 min followed by 35 cycles of 94℃ for 30 s, 60℃–66℃ (Table 1) for 30 s, 72℃ for 30 s and a final extension at 72℃ for 10 min. A 5 μL aliquot of each PCR product was electrophorized in a 2% agarose gel containing ethidium bromide and then visualized under a UV transilluminator.

Table 1 Primer sequences and information of Japanese flounder *CYP17-Ⅱ* gene

Name	Sequence	Length (bp)	Temp (℃)	Amplicon
Primer1	ACATGGGATTTCATTTTCTCTTTGTTCCGGGTGAAGAGCAGGT	279	60	Exon 1
Primer2	GGTTAACAGGTACGGCTCTCTG CTCGGTCGTCCAGCAAAGT	125	60	Exon 2
Primer3	TGACCTGTTGACCAGAGGAGGTGATGTCCTGCAGACGACTGGTT	130	61	Exon 3
Primer4	GTCTCTGTGTCGAGTTGTTGTCCAGCCTTCATCCAGGGATAAATGTCC	213	62	Exon 4
Primer5	CTTCTGGACGCCTTGCTGAGCAGGTAGGCCAGGATC	158	60	Exon 6
Primer6	AGTGCAGAAGGAGCTGGACGAAATAGCGGTGTGTGGGATCAGGA	147	62.5	Exon 7
Primer7	TATTGGAGGTCACTCTGTCGGTCTGGGTTGAGGAGGTCCGGT	104	62.5	Exon 8
Primer8	ATGACCAGGGTCAGCGGGTCACTGGTTGCAGGACCACGCCAAGG	197	66	Exon 9

2.5 Single-stranded Conformation Polymorphism (SSCP) Analysis

The single-stranded conformation polymorphism (SSCP) method was used for genotype *CYP17-Ⅱ* gene (He *et al*., 2010). Only 75 samples were used for analysis by PCR-SSCP. Four μL amplification products of each individual were mixed with 5 μL denaturing buffer (98% formamide, 0.09% xylene cyanole FF and 0.09% bromophenol blue). The samples were heat-denatured at 98℃ for 5 min and then placed on ice for 10 min. The denatured DNA samples were electrophorized in 12% non-denaturing polyacrylamide gel and at 110 V for 12 h–14 h; SSCP patterns on the gels were visualized by silver staining (Qu *et al*., 2005). Each genotype was defined according to the band patterns. The location and chemical nature of each mutation were confirmed by the sequencing of the reamplified product. PCR products of heterozygous and homozygous samples were purified with DNA Fragment Quick Purification/Recover Kit (TIANGEN), then inserted into the PGM-T vector (TIANGEN) and transferred into Trans-5α Chemically Competent Cell (Beijing TransGen Biotech Co., Ltd). Positive recombinant colonies were sequenced by the ABI 377 sequencer.

2.6 Statistical Analysis

The gene frequencies for each polymorphism were calculated by Microsoft Excel. Associations between genotypes and four reproductive traits (T, E_2, HIS, and GSI) of Japanese flounder were respectively analyzed by one-way ANOV using Stat View software version 9.0 (SAS Institute Inc., Cary, NC). Differences among means of different genotypes were calculated using Duncan's multiple-range test and $P<0.05$ was considered statistically significant.

3 Results

3.1 Polymorphisms within Exons of *CYP17-Ⅱ* Gene

The PCR products of Primer pair 4, Primer pair 5 and Primer pair 8 were polymorphic in SSCP pattern among the eight sets of primers (Fig.1). The three SNPs, namely SNP1, SNP2 and SNP3, were respectively located at positions of c.G594A, c.G939A and c.G1502A of Japanese flounder *CYP17-Ⅱ* gene (Fig.2). Three genotypes were found for the SNP1 and SNP3 and named as AA, AG and

GG. Two genotypes were found for the SNP2 and named as AA and GG.

At the SNP1 locus, c.G594A identified in exon 4 was a non-synonymous mutation, which caused an amino acid change from Gly^{188} to Arg^{188}. At the SNP2 locus, the nucleotide transition c.G939A was synonymous mutations. At the SNP3 locus, the non-synonymous mutation G1502A caused an amino acid change from Gly^{490} to Asp^{490}.

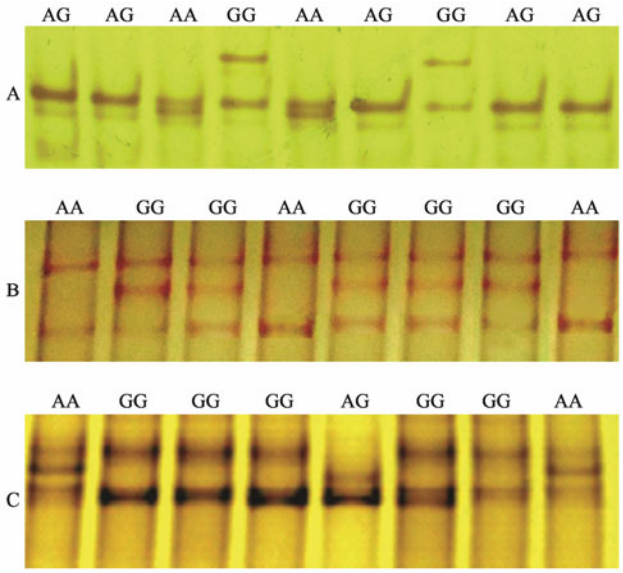

Fig.1 Band patterns for the three SNPs of Japanese flounder *CYP17-Ⅱ* gene. A: Genotype of SNP1; B: Genotype of SNP2; C: Genotype of SNP3.

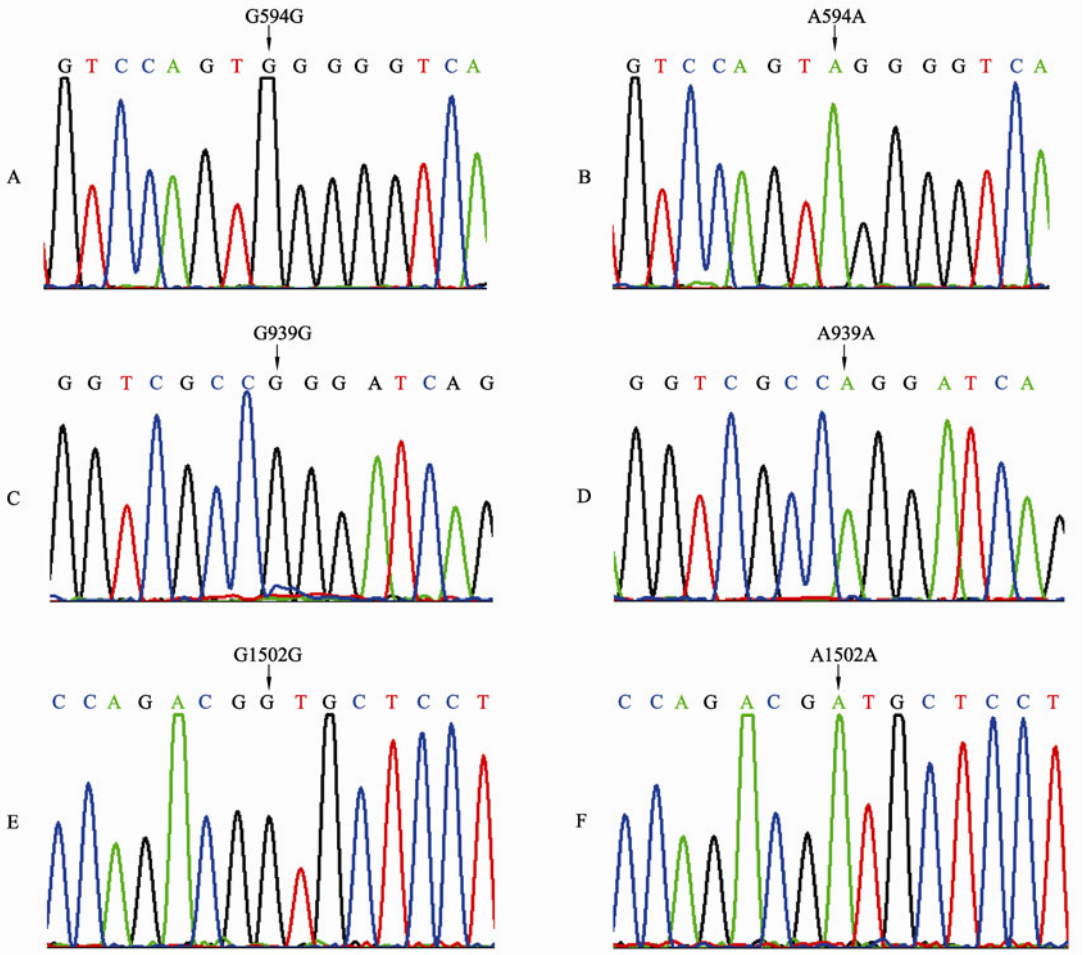

Fig.2 Sequences of the SNPs of Japanese flounder *CYP17-Ⅱ* gene. A: Sequence of GG genotype at SNP1 locus; B: Sequence of AA genotype at SNP1 locus; C: Sequence of GG genotype at SNP2 locus; D: Sequence of AA genotype at SNP2 locus; E: Sequence of GG genotype at SNP3 locus; F: Sequence of AA genotype at SNP3 locus.

3.2 Frequencies of Genotypes and Alleles

Table 2 shows the frequencies of genotypes and alleles of the three SNPs of Japanese flounder *CYP17-Ⅱ* gene. The AG genotype of SNP1 and the GG genotype of SNP2 and SNP3 were obviously richer than the other genotypes. At the loci SNP2 and SNP3, the frequency of allele G was relatively higher than the frequency of allele A in the experimental population. But the frequencies of allele G and allele A were approximately equal at the loci SNP1.

Table 2 Frequencies (%) of genotypes and alleles of three SNPs of Japanese flounder *CYP17-Ⅱ* gene

Locus	Genotype frequency			Allele frequency	
	AA	AG	GG	A	G
SNP1	14.67	77.33	8	53.33	46.67
SNP2	5.33	–	94.67	5.33	94.67
SNP3	8	5.33	86.67	10.6	89.34

3.3 Correlation Between *CYP17-Ⅱ* Gene Mutations and Sex Steroid and Reproductive Traits

The association of SNPs in *CYP17-Ⅱ* gene with reproductive traits was analyzed (Table 3). Statistical results indicated that, among the three SNPs, SNP1 was significantly associated with GSI ($P<0.05$) in male Japanese flounder. SNP2 was found to have significant associations with serum T level and HSI ($P<0.05$).

Moreover, multiple comparisons were performed for different genotypes of two loci (Table 4). The results showed that GSI for individuals with genotype GG was significantly lower than individuals with genotypes AA and AB in SNP1 ($P<0.05$), and T level and HSI were lower for individuals with genotype AA than genotype GG in SNP2 ($P<0.05$).

3.4 Construction of Diplotypes and the Correlation with Diplotypes and Reproductive Traits

Diplotype was constructed based on the three SNPs in the experimental population by use of the Phase program. Six diplotypes were identified (Table 5). Association analysis indicated that there was significant association between diplotype and T level and GSI ($P<0.05$). Multiple comparisons indicated that the T level of diplotype D1 and the GSI of diplotype D6 were lower than the other five diplotypes.

Table 3 Associations between SNPs of Japanese flounder *CYP17-Ⅱ* gene and reproductive traits by ANOVA

Locus	T (ng mL^{-1})		E_2 (pg mL^{-1})		HSI		GSI	
	F value	P value	F value	P value	F value	P value	F value	P value
SNP1	1.89	0.158	0.50	0.6110	2.10	0.1305	4.65	0.0126†
SNP2	4.29	0.0419†	0.20	0.6525	6.56	0.0125†	3.10	0.0825
SNP3	0.25	0.7798	0.48	0.6184	2.69	0.0745	0.10	0.9043

Note: $^{\dagger}P\leq0.05$.

Table 4 Multiple comparisons of reproductive traits† among different genotypes of SNP1and SNP2 of Japanese flounder *CYP17-Ⅱ* gene

Locus	Trait	AA genotype	AG genotype	GG genotype
SNP1	GSI	0.552 ± 0.079^{a}	0.598 ± 0.0347^{a}	0.252 ± 0.108^{b}
SNP2	T (ng mL^{-1})	18.905 ± 4.953^{b}	–	40.144 ± 7.518^{a}
	HSI	1.462 ± 0.150^{a}	–	1.065 ± 0.035^{b}

Notes: †Means ± standard deviation; within the same row, different superscript letters mean significant differences at $P<0.05$.

Table 5 Associations between diplotypes of *CYP17-Ⅱ* gene and reproductive traits† in Japanese flounder

Diplotype	Frequency (%)	SNP1	SNP2	SNP3	T (ng mL^{-1})	GSI
D1	4	AG	AA	GG	17.66 ± 7.649^{d}	0.305 ± 0.147^{ab}
D2	13.33	AA	GG	GG	98.526 ± 4.788^{bc}	0.507 ± 0.080^{ab}
D3	5.33	AG	GG	AA	158.925 ± 6.543^{ab}	0.593 ± 0.127^{ab}
D4	56	AG	GG	GG	105.370 ± 2.191^{abc}	0.639 ± 0.039^{a}
D5	5.33	AG	GG	AG	170.953 ± 4.542^{a}	0.640 ± 0.127^{a}
D6	8	GG	GG	GG	55.582 ± 5.672^{cd}	0.252 ± 0.104^{b}

Notes: †Means ± standard deviation; within the same column, different superscript letters mean significant differences at $P<0.05$.

4 Discussion

Many previous studies indicated that the gonads and the adrenal glands were the main established sites for P450c17 expression (Sakai *et al*., 1992; Trant, 1995; Freking *et al*., 2000; Kazeto *et al*., 2000; Wen *et al*., 2009), and in other non-steroidal tissues including the brain, the expression of P450c17 was also found (Matsunaga *et al*., 2001; Yu *et al*., 2002; Halm *et al*., 2003; Wang and Ge, 2004). In this study, we first detected three SNPs c.G594A, c.G939A and c.G1502A. They were located at the exon4, exon6 and exon9 of Japanese flounder *CYP17-Ⅱ* gene, respectively. SNP1, a non-synonymous mutation, was significantly associated with GSI ($P<0.05$). It is reasonable to suppose that this mutation could alter the function of *CYP17-Ⅱ* gene and subsequently

influence gonadal development. However, these possibilities remain to be investigated in the future.

According to Gardiner-Garden sequence criteria, a genomic region has to fulfill three conditions to be classified as a CpG island: (1) GC content above 50%, (2) ratio of observed to expected numbers of CpG dinucleotides above 0.6, and (3) length greater than 200 bp. The potential CpG island within *CYP17-Ⅱ* gene of Japanese flounder was assessed by the CpG island searcher software (http://www.urogene.org/methprimer/). Interestingly, SNP2 located just at the CpG island and it was observed to be significantly associated with serum T level ($P<0.05$) and HSI ($P<0.05$). DNA methylation is the most important epigenetic mechanism. The most widely accepted mechanisms for active DNA demethylation involve DNA repairment and replacement of the methylated cytosine nucleotide by an unmethylated cytosine nucleotide (Szyf, 2010). Klose and Bird (2006) indicated that demethylation of CpG sites in intra- and/or extra-genic positions could increase the transcription rate of a specific gene. It has been proven that in many species DNA demethylation could regulate gene expression (Zhong *et al*., 2009). From these results, we assume that the mutation of G939A might change the methylation of CpG island and subsequently improve the translation activity of *CYP17-Ⅱ* gene.

The cortisol, essential for the osmoregulation and energymetabolism in fish, is produced by the hydroxylase activity of P450c17-Ⅱ in the interrenal cells of the head kidney (Gallo and Civinini, 2003; Zhou *et al*., 2007). Many studies suggested that the deficiency of 17α-hydroxylase in human could reduce the content of cortisol and then induce the loss of adrenal's function and the abnormality of sexual differentiation (Yanase *et al.,* 1991; Costa-Santos *et al*., 2004; Zhang *et al.*, 2008). Due to the important function of 17α-hydroxylase catalyzing androstenedione to synthesize adrenal cortisol, we propose that the improved translation activity of *CYP17-Ⅱ* gene resulted in raising cortisol levels, and therefore regulating the synthesis of T.

Liver is the biggest and most important digestive gland for fish. Hepatosomatic index (HSI) is a good indicator of liver energy content (Lambert and Dutil, 1997). At the SNP2 locus, it is interesting to note that the HSI of individuals with AA genotype was lower than that with the other genotypes. However, the underlying mechanism of HSI with T and cortisol was not clear.

In addition, we detected a non-synonymous mutation SNP3 with Primer pair 8. However, no association was observed between the mutation and the four reproductive traits examined in this study. Further studies with larger population are necessary to confirm the result.

The diplotypes constructed by united SNPs would supply more information than by a single SNP (He *et al*., 2006). In this study, six diplotypes were constructed and their associations with reproductive traits were analyzed. We found that diplotype D1 was significantly correlated with T level and diplotype D6 was associated with GSI. We speculated that D1 and D6 may be promising molecular markers for reproductive indices.

5 Conclusions

We have shown the association between the SNPs of *CYP17-Ⅱ* gene in coding region and the reproductive traits in Japanese flounder. Three SNPs were detected in *CYP17-Ⅱ* gene. SNP1, located in exon 4, was significantly associated with GSI. The GSI of GG genotype was much lower than that of the other two genotypes. The SNP2 was significantly associated with serum T level ($P<0.05$) and HSI ($P<0.05$). Individuals with AA genotype of SNP2 had lower serum T level and HIS compared with GG genotype. The SNP3 did not show association with the reproductive traits examined in this study. Diplotypes composed of the three SNPs may provide more powerful tool for analyzing associations between *CYP17-Ⅱ* gene and reproductive traits. The SSCP method does not identify all SNPs potentially present in the samples tested. Therefore, our study was designed as the first step in detecting genetic markers for *CYP17-Ⅱ* gene and showed that *CYP17-Ⅱ* gene was a valuable candidate gene for further investigation into mutations and breeding programs of Japanese flounder.

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