Fish Physiol Biochem (2009) 35:333–340
DOI 10.1007/s10695-008-9237-y

Polymorphisms within promoter of Japanese flounder (*Paralichthys olivaceus*) ovary cytochrome P450-c19 (CYP19a) gene associated with reproductive traits

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Received: 2 January 2008 / Accepted: 17 May 2008 / Published online: 7 June 2008

Abstract Cytochrome P450 aromatase, which is encoded by the *CYP19a* gene, converts androgens to estradiol. Considerable evidence suggests that estrogens play an important role in fish reproductive process. Therefore *CYP19a* is an excellent candidate gene for reproductive traits. Variants in the promoter of the *CYP19a* gene might also be involved in the control of aromatase expression and affect regulatory mechanism linking cholesterol metabolism to the synthesis of sex steroids. In this study, nine single-nucleotide polymorphisms (SNPs) were detected with polymerase chain reaction-single stranded conformational polymorphism (PCR-SSCP), namely A-680G, G-672A, AGTAGT-649 inserting or deleting, T-623C, C-410A, T7-454A, T-402C, TTTCCAGACTGA-345 inserting or deleting, and G-297C. Nine SNPs within the promoter of the *CYP19a* gene were tested for association with four reproductive traits [serum testosterone (T), serum 17β-estradiol (E_2), hepatosomatic index (HSI), and gonadosomatic index (GSI)] in a population of 50 female Japanese flounder individuals. A locus, P3 (TTTCCAGACTGA-345 inserting or deleting, G-297C), was significantly associated with 17β-estradiol (E_2) level ($P < 0.05$) in female Japanese flounder. In addition, there was significant association between one diplotype based on nine SNPs and reproductive trait. The genetic effect for E_2 level of diplotype D3 was significantly higher than those of other diplotypes ($P < 0.05$). Results indicate that these genetic effects of those variants on E_2 level may help to explain *CYP19a* gene status in the reproductive endocrinology of Japanese flounder.

Keywords Japanese flounder · *CYP19a* gene promoter · SNPs · Diplotype · Reproductive traits

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Introduction

Estrogen biosynthesis is catalyzed by the cytochrome P450 aromatase encoded by the *CYP19a* gene. *CYP19a* expression in the gonads has been proposed to be a key step of estrogen synthesis that is crucial for ovarian maturation and female reproduction in teleosts (Guiguen et al. 1999). Aromatase activity progressively increases parallel with the gonadosomatic index (GSI) (Jeng et al. 2005). The GSI is a gross quantitative indicator of gonad condition and represents the simplest way to measure changes in size and weight of this organ in relation to the total weight of the organism (Hervey et al. 2006). Testosterone (T) was shown to be translated into 17β-estradiol (E_2) by the *CYP19a* gene, and the functions of T and E_2 transited by estrogen receptor (ER) and androgen receptor (AR) subtypes into regulation of

reproductive and metabolic homeostasis (Goksoyr 2006; Tabb and Blumberg 2006). It is known that the liver is the site of production of vitellogenin, a glycoprotein precursor to yolk. The size of the liver may increase with the number of eggs maturing or spawned in a single event. The HSI has been shown to increase with measures of vitellogenin production (Pereira et al. 1993) and to decrease with ovarian development (Yoneda et al. 2001). So we hypothesized that the *CYP19a* gene promoter variants or disruption of either activity or production of this enzyme is likely to result in altered development or reproductive biology of organisms.

Single-nucleotide polymorphism (SNP), one-base variation including deletion, insertion, and substitution, plays an important role in the transcription and translation of genes and affects function of protein. For example, in the centromeric region of *Bos taurus autosome* (BTA) 14, the acyl-CoA: diacylglycerol acyltransferase gene (*DGAT1*) has been identified as the most likely causative gene underlying a quantitative trait nucleotide (QTN) for milk fat yield and content (Grisart et al. 2002; Winter et al. 2002; Thaller et al. 2003). Recently, polymorphisms in the promoter of the *CYP19a* gene have been reported in teleosts. Three gonadal aromatase promoter sequences have been described in goldfish that differ in sequence by 9–15%, with the high sequence divergence suggesting that they represent separate genes (Tchoudakova et al. 2001). Galay-Burgos et al. (2006) described that analysis of promoter sequences from sea bass suggested the presence of three promoter alleles that arose due to three single-nucleotide polymorphisms (SNPs) in linkage disequilibrium. Some transcription factors, such as SF-1, FoxL2, Sox, and so on, are combined to aromatase genes promoter. Variants in the promoter of *CYP19a* gene might also be involved in the control of aromatase expression, suggesting the possible existence of a regulatory mechanism linking cholesterol metabolism to the synthesis of sex steroids. However, in those studies, there is no analysis of simple correlation between gonadal aromatase alleles and phenotype traits.

We have previously shown, by means of single-stranded conformational polymorphism (SSCP) analysis, that three SNPs detected in the coding regions of the *CYP19a* gene were significantly associated with reproductive traits in Japanese flounder (*Paralichthys olivaceus*) (He et al. 2008a, b). Although previous study has focused on association between polymorphisms in the coding regions of the *CYP19a* gene and economically important traits, studies in humans and other species have shown that polymorphisms in the *CYP19a* gene promoter may be of major importance. This is because such polymorphisms are generally associated with the sequence elements and factors regulating gene expression (Shozu et al. 2001; Enjuanes et al. 2003; Tong et al. 2003; Toshiya et al. 2007). The Japanese flounder *CYP19a* gene promoter has been sequenced (accession no. AB303853) (Toshiya et al. 2007). In the present study, we searched for polymorphisms within these regulatory regions and studied their association with four reproductive traits: serum testosterone (T), serum 17β-estradiol (E_2), hepatosomatic index (HSI), and gonadosomatic index (GSI).

SSCP analysis is one of the simplest, most reliable, and most sensitive methods for detecting variants s based on PCR (Orita et al. 1989; Sheffield et al. 1993). We have optimized the SSCP procedure to detect single-nucleotide polymorphisms (SNPs) and used this method to evaluate polymorphisms in promoter of *CYP19a* and their association with reproductive traits.

Materials and methods

Animal populations

Fifty female Japanese flounder (*Paralichthys olivaceus*) (239.23 ± 74.93 g) were reared at natural seawater temperature (20 ± 0.5°C) in our laboratory under the same rearing and management conditions. Fish were decapitated and the gonads were removed and weighed. Four reproductive traits, namely, testosterone (T), 17β-estradiol (E_2), hepatosomatic index (HSI), and gonadosomatic index (GSI), were used for association analysis. Table 1 presents the means and standard deviations of these four traits.

Table 1 Means and standard deviations of reproductive traits

Traits	Mean	SD
T (ng/dl)	17.638	7.36
E_2 (pg/ml)	6.306	3.739
HSI	1.548	0.567
GSI	0.155	0.122

SD, standard deviation; T, testosterone; E_2, 17β-estradiol; HSI, hepatosomatic index; GSI, gonadosomatic index

Hepatosomatic index (HSI) and gonadosomatic index (GSI)

The hepatosomatic index or gonadosomatic index (percentage) of each animal was calculated as the ratio of the gonad or liver wet weight to the whole net body weight according to: gonadosomatic or hepatosomatic index = [gonad or liver weight/(body weight — viscera weight)] × 100.

T and E_2 radioimmunoassay assays

The blood was sampled by puncturing the caudal vasculature with a 25-gauge 1.3-cm needle attached to a 1.0-ml disposable syringe. Blood samples were allowed to clot on ice for several hours, and then the serum was separated by centrifugation (15,000 rpm) for 5–7 min and stored at −40°C. The serum testosterone and estradiol-17β were quantified by ^{125}I radioimmunoassay, using kits from the Diagnostic Products Corporation (Tianjin Nine Tripods Medical & Bioengineering Co., Ltd., Sino-US joint-venture enterprise). Steroids were assayed directly on the serum, the antisera are highly specific with an extremely low crossreactivity to other naturally occurring steroids, the crossreactivity was less than 0.1% to most circulating steroids. Intraassay variability was 7.4% for the estradiol-17β assay and 8.0% for the testosterone assay. Any sample with coefficient of variation higher than 10% was not included in the analyses. The assay sensitivity reached to 2 ng/dl for T and 4 pg/ml for E_2 in a modified protocol provided by Wen et al. (2006).

PCR and SSCP conditions

Genomic DNA was isolated from blood samples by the phenol-chloroform method. According to its DNA sequence (GenBank accession no. AB303853) using the Oligo6.0 software, three pairs of primers were designed to amplify the promoter region of Japanese flounder *CYP19a* (Table 2).

PCR (Thermocycler, American) reactions were carried out in a total volume of 25 μl including 50 ng of genomic DNA, 0.20 mM each dNTP, 2.5 mM $MgCl_2$, 0.20 mM primers, and 0.5 U Taq DNA polymerase (Promega). Amplification conditions were 94°C for 5 min followed by 35 cycles of 94°C for 45 s, 56–58°C for 45 s, and 72°C for 45 s, and a final extension at 72°C for 10 min. The PCR products of *CYP19a* were genotyped by single-stranded conformation polymorphism (SSCP). Two-microliter of the PCR products of each individual were mixed with 5 μl denaturing buffer (98% formamide, 0.09% xylene cyanole FF, and 0.09% bromophenol blue), and then denatured at 94°C for 5 min followed by a rapid chill on ice for 10 min. The denatured PCR products were separated for 14 h at 4 V/cm on 12% polyacrylamide gel. The DNA bands were stained by silver staining (Qu et al. 2005). Individual genotypes were defined according to band patterns.

PCR products of each type of homozygotes were purified with the DNA Fragment Quick Purification/Recover Kit (Invitrogen). The purified PCR products were ligated to the PMD 18-T vector (Promega, Southampton, UK) and transformed into DH5-α *Escherichia coli*. Positive recombinant colonies were sequenced on an ABI 377 sequencer.

Statistical models and analysis

The genotype frequencies of each polymorphism were calculated by Excel. The six diplotypes were constructed on the base of nine SNPs with Excel. Combinations having same genotype at each corresponding locus were counted as a kind of diplotype

Table 2 Primer sequences and information of the Japanese flounder *CYP19a* gene promoter

Names	Sequences	Length (bp)	T_m (°C)	Amplicons
Primer1	5-CTGGAGAGCTTCATCAAC-3	361	57	−720 to −359
	5-TCTCAAAGTTGTCCAGGC-3			
Primer2	5-GACGTTACTTCCAGCCAT-3	154	56	−378 to −224
	5-TCAGAGTGTTTGCCAGCT-3			
Primer3	5-TGATCCACACTGCTTCAT-3	271	58	−305 to −34
	5-TTCCTACTTGGAAAGTGC-3			

according to He et al. (2006, 2008a, b). Associations between genotypes and diplotypes of nine SNPs in the promoter of the Japanese flounder *CYP19a* gene and four reproductive traits (T, E_2, HSI, and GSI) and genetic effects were analyzed using the general linear model (GLM) procedure of Statistical Analysis System (SAS) 8.02 software, respectively. The following models were used.

$$Y = \mu + G \text{ or } D + e$$

where Y is the value measured for the four reproductive traits; μ is mean value of the four reproductive traits, G or D is the fixed effect of the genotypes of each SNP or diplotype, and e is the random error effect. Considering that all experimental fish were selected from the same sex and site and slaughtered at the same age, no other effects (such as sex, generation, and site) were introduced into the model. Significant differences among the means of different genotypes or diplotypes were calculated using Duncan's multiple-range test, and P values of 0.05 were considered statistically significant.

Results

Polymorphisms within the promoter of the *CYP19a* gene in Japanese flounder

To investigate the potential sequence variations of the promoter of the *CYP19a* gene, a PCR–SSCP strategy was applied to genomic DNA from 50 female Japanese flounder. These PCR productions of primer1 and primer2 were polymorphic (Fig. 1).

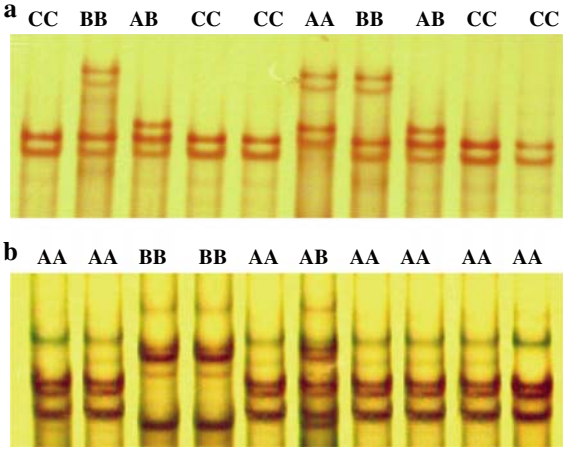

Fig. 1 Band patterns for the nine SNPs. (**a**) Genotypes of P1 and P2 (primer1); P1 locus: AA, AB and BB; P2 locus: AA and CC. (**b**) Genotypes of P3 (primer2)

Those variants were confirmed by subcloning the PCR products from each genotype of fishes into pGEM-T and sequencing multiple subclones from each individual. Three genotypes were found for each locus. The homozygote, consistent with the sequence of GenBank accession number AB303853, was named AA genotype; another homozygote was named BB or CC genotype and the heterozygote was named the AB genotype.

At primer1, four genotypes, seven SNPs were detected, which were linked in a group of five (A-680G, G-672A, AGTAGT-649 inserting or deleting, T-623C, C-410A) and four (A-680G, G-672A, T7-454A, T-402C), respectively. The AB genotype of primer1 is heterozygote between genotypes AA and BB. No heterozygote was found between genotype CC and AA or BB. Three genotypes, two SNPs, TTTCCAGACTGA-345 inserting or deleting and G-297C, were found at primer2. The AB heterozygote was also detected in primer2 between genotypes AA and BB. So the nine SNPs were named loci P1 (A-680G, G-672A, AGTAGT-649 inserting or deleting, T-623C, C-410A), P2 (A-680G, G-672A, T7-454A, T-402C), and P3 (TTTCCAGACTGA-345 inserting or deleting, G-297C), respectively.

Table 3 shows gene and genotypic frequencies. The frequency of CC of P2 (62.0%) was very high. The frequencies of the genotypes BB (P1) and BB (P3) were relatively low: 12.0% and 18.0%, respectively. On the whole, the A and C alleles were predominant over B.

Associations between SNPs and reproductive traits

The association analysis of the three loci within the Japanese flounder *CYP19a* promoter with the

Table 3 Frequencies of alleles and genotypes of SNPs within the promoter of the *CYP19a* gene in Japanese flounder (%)

Alleles	Genotypes frequencies					Alleles frequencies		
	AA	AB	BB	AC	CC	A	B	C
P1	40.0	48.0	12.0	–	–	64.0	36.0	–
P2	38.0	–	–	–	62.0	38.0	–	62.0
P3	46.0	36.0	18.0	–	–	64.0	36.0	–

Table 4 Associations between each of the SNPs within the promoter of the *CYP19a* gene and reproductive traits of Japanese flounder

	T	E_2	HSI	GSI
P1	NS	NS	NS	NS
P2	NS	NS	NS	NS
P3	NS	*	NS	NS
Diplotype	NS	*	NS	NS

* $P \leq 0.05$; NS, not significant

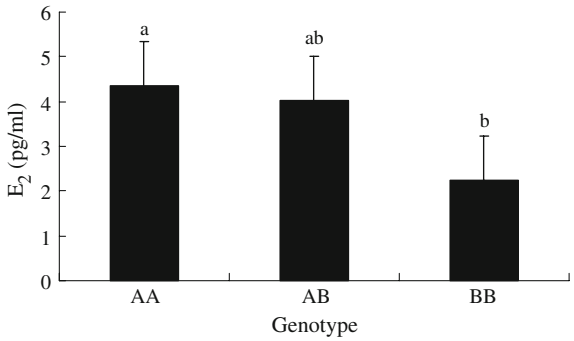

Fig. 2 Multiple comparisons of the effects of E_2 level among three genotypes of P3 (primer2). Different letters indicate statistically significant differences among three genotypes

reproductive traits was carried out using GLM estimation (Table 4). P3 was significantly associated with E_2 level ($P < 0.05$) in female Japanese flounder. P1 and P2 did not show any significant effects on any of the examined traits in female Japanese flounder.

Furthermore, multiple comparisons analysis were performed in three genotypes of P3. Results showed that fish with genotype AA of P3 had significantly higher E_2 level ($P < 0.05$) than those of genotype AB and BB. Figure 2 presents the result of the multiple comparisons.

Construction of diplotypes and their associations with diplotypes and reproductive traits

Six diplotypes with minor allelic frequencies of above 5% were constructed based on nine SNPs in the experiment population (Table 5). Association analysis indicated that there was significant association between diplotypes and E_2 level ($P < 0.05$) (Table 4). By multiple comparisons, we found that the mean of E_2 level for diplotype D3 was significantly higher than those of other diplotypes ($P < 0.05$), respectively (Fig. 3).

Table 5 Frequencies of six diplotypes within the promoter of the *CYP19a* gene in Japanese flounder

Diplotype	P1	P2	P3	Frequency (%)
D1	AA	AA	BB	12.0
D2	AA	AA	AA	20.0
D3	AA	AA	AB	6.0
D4	AB	CC	AA	22.0
D5	AB	CC	AB	24.0
D6	BB	CC	AB	6.0

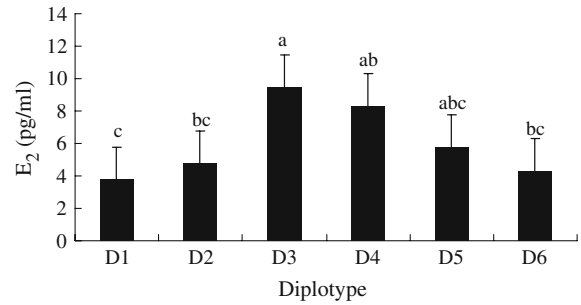

Fig. 3 Multiple comparisons of the effects of E_2 level among six diplotypes. Different letters indicate statistically significant differences among diplotypes

Discussion

In this study, PCR products of three primers span approximately 720 bp, which contains multiple putative binding sites for TATA box, two Ad4BP or SF1, an ERE-half, a forkhead transcription factor FoxL2, and a cAMP-responsive element (CRE). Though the sequences we obtained were different from the Genebank sequence (accession no. AB303853), our results are consistent with putative binding sites reported by Toshiya et al. (2007).

To our knowledge, to date, several polymorphisms have been reported in the promoter of fish *CYP19a* genes, and there was no link between polymorphisms and correlated phenotype trait (Tchoudakova et al. 2001; Galay-Burgos et al. 2006). However, genetic variants in the promoter of the *CYP19a* gene have been previously investigated as candidates for human disease. The polymorphism Aro1 (rs4775936) lays within a regulatory region of the human *CYP19a* gene

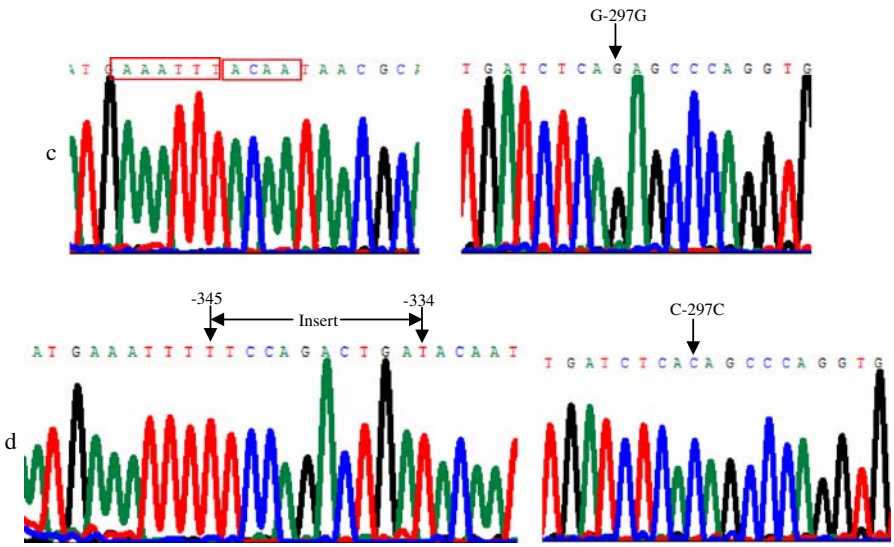

Fig. 4 Sequences of two genotypes of P3 locus (primer2) c: Sequence of AA genotype of P3 locus (primer2); d: Sequence of BB genotype of P3 locus (primer2)

and may be a functional polymorphism associated with the bone phenotype (Enjuanes et al. 2006). Several polymorphisms in the coding region and intron of *CYP19a* have been reported to be associated with bone mineral density or fragility fractures in postmenopausal women: a four-nucleotide repeat (TTTA) in intron 4 (Masi et al. 2001; Dick et al. 2005; Gennari et al. 2004); a common Arg264Cys change (Zmuda et al. 2003); and another silent SNP in exon X (Tofteng et al. 2004). However, how they influence diseases is not exactly known. It is possible that the polymorphisms influence sex hormone levels and this, in turn, affects disease. In this study, we adopted a PCR-SSCP method to detect polymorphisms in the promoter of the *CYP19a* gene. Nine SNPs were firstly identified in the promoter of the Japanese flounder *CYP19a* gene, which could be related to reproductive traits.

Putative transcription factor binding sites were predicted for nine SNPs by TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html). Results showed that two new transcription factors were added: HSF and E47 (P3 locus, Fig. 4). The binding of heat shock factors (HSF) initiates the transcriptional activation of heat shock protein (HSP) genes to heat shock elements located at the promoter regions (Yeh et al. 2006). E47 is a basic helix-loop-helix (bHLH) transcription factor that plays an essential role in regulatory cascades such as neuronal differentiation (Guillemot et al. 1999). In addition, our statistical analysis showed that there was significant association between P3 and E_2 level ($P < 0.05$). It can be postulated from our experiments that the *CYP19a* gene promoter contains the regulatory elements necessary to direct basal transcription with a high strength. We deduced that P3 could be a potential quantitative trait nucleotide (QTN) affecting reproductive traits in Japanese flounder (Fig. 4). Further studies in a larger population are needed to confirm this result. However, if the exact molecular mechanisms underling the association of the SNP of P3 with E_2 level reported in the present study is related to the regulation of HSF and E47 sites are unknown, the possible functionality of the promoter variants of the Japanese flounder *CYP19a* gene, HSF and E47, can only be appreciated from in vivo and in vitro experiments in the future.

Temperature influences sex differentiation in particular Japanese flounder populations (Kitano et al. 1999, 2000; Toshiya et al. 2007). These results strongly suggest that suppression of *CYP19a* mRNA expression and the resultant inhibition of estrogen biosynthesis trigger the sex reversal of genetic females by high water temperature. Since temperature changes are known to regulate aromatase gene expression in some fish species, we hypothesised that there could be genetic differences in the responsiveness of the gonadal aromatase promoter to temperature shifts. In this study, variants in the promoter did not alter sex under the same temperature (<27°C). It would be of great interest to determine whether there is any difference in

expression between the different alleles and if there is a correlation between the effects of temperature on sex differentiation and gonadal aromatase promoter genotype.

Diplotypes have been described by He et al. (2006, 2008a, b). In this study, we constructed six diplotypes on the basis of nine SNPs and analyzed for the associations of diplotypes with reproductive traits. Results showed that diplotype D3 significantly affected E_2 level (Table 2).

In conclusion, nine SNPs were first identified in the promoter of the *CYP19a* gene and associated reproductive straits of Japanese flounder in this study. The SNPs located in P3, TTTCCAGACTGA-345 inserting or deleting and G-297C, were significantly associated with E_2 level ($P < 0.05$) in female Japanese flounder. Further, there was significant association between diplotypes D3 based on nine SNPs with E_2 level. This implied that variants of the *CYP19a* gene promoter could affect reproductive processes in Japanese flounder.

Acknowledgments This work was supported by the National Key Technologies R & D Program (2006BAD09A01).

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