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Identification of single nucleotide polymorphism cytochrome P450-c19a and its relation to reproductive traits in Japanese flounder (*Paralichthys olivaceus*)

Feng He, Hai S. Wen *, Shuang L. Dong, Bao Shi, Cai F. Chen, Lian S. Wang, Jun Yao, Xing J. Mu, Yu G. Zhou

Fisheries College, Ocean University of China, 5 Yushan Road, Qingdao 266003, China

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ABSTRACT

CYP19 is considered as an important factor affecting reproductive endocrinology in many fishes, and plays an important role in ovarian development, reproductive function and sexual differentiation. In this study, three single nucleotide polymorphisms (SNPs) within CDS of the *CYP19a* gene were tested and the associations between their genotypes and four reproductive traits were analyzed in 65 Japanese flounder individuals with Polymerase chain reaction and Single-stranded conformational polymorphism (PCR–SSCP). Results indicated that a SNP in the exon7 of *CYP19a* gene, SNP2, was significantly associated with 17β-estradiol (E_2) ($P<0.05$) and gonadosomatic index (GSI) ($P<0.05$). Individuals with genotype AB of SNP2 had significantly higher serum E_2 levels ($P<0.05$) and GSI ($P<0.05$) than those of genotype AA or BB. In addition, there was significant association between one diplotype based on three SNPs and reproductive trait. The genetic effects for both serum E_2 of diplotype D9 and GSI of diplotype D1 were respectively much higher than those of other diplotypes ($P<0.05$). The evidence of the associations between genetic variants with serum E_2 and GSI may help explain effects of *CYP19a* gene in reproductive endocrinology of Japanese flounder.

1. Introduction

Cytochrome P450arom, as a member of the cytochrome P450 superfamily, is a key component of the enzymatic aromatase complex converting androgens to estrogens in vertebrates. The protein that catalyzes the aromatization of steroid hormones is encoded by the *CYP19* gene (Thompson and Siiteri, 1974; Simpson et al., 1994). Estrogens, especially 17β-estradiol (E_2), have been shown to play a key role in ovarian development, reproductive function and sexual differentiation in various species (Miyashita et al., 2000; Miyata and Kubo, 2000; Kuntz et al., 2003a; Kato et al., 2004). There are two isoforms of *CYP19* genes including *CYP19a and CYP19b* present in Japanese flounder (*Paralichthys olivaceus*). They are primarily expressed in the ovary and brain, respectively (Kitano et al., 1999). Both aromatase *CYP19* isoforms are involved in the sexual differentiation, regulation of the reproductive cycle and male reproductive behavior in Japanese flounder (Kitano et al., 1999, 2000). The gene mutation or disruption of either activity or production of this enzyme is likely to result in altered development or reproductive biology of organisms. Due to its key function in estrogen biosynthesis and association with reproductive processes, aromatase has been considered as an important factor to affect reproductive endocrinology in many fishes (Sanderson et al., 2002; Hayes et al., 2002; Rotchell and Ostrander, 2003).

Single nucleotide polymorphisms (SNP), one base variant including deletion, insertion, and substitution, can greatly influence gene expression and the functions of proteins. In agricultural and aquaculture species, SNPs are especially important if they cause differences in economic traits, or are linked to the mutations that do so. For example, in the centromeric region of *Bos taurus autosome* (BTA) 14, the acyl-CoA: diacylglycerol acyltransferasel gene (DGAT1) has been identified as the most likely causative gene underlying a QTL for milk fat yield and content (Grisart et al., 2002; Winter et al., 2002; Thaller et al., 2003). This information can be used to increase the accuracy of selection for these traits, thereby increasing the rate of genetic gain and production efficiency. While a large number of SNPs have been reported in some important livestock species (e.g. Kim et al., 2003; Jungerius et al., 2003), significant numbers of SNPs in the aquaculture species have been few reported by He et al. (2003), Heikki and Craig (2006) and Hayes et al. (2007) .Those SNPs were only distributed in EST sequence. With regard to polymorphism of *CYP19a* gene, many studies focused mainly on diseases in different human populations (Kristensen and Borresen-Dale, 2000; Mitrunen and Hirvonen, 2003; Dunning et al., 1999; Ribeiro et al., 2006). While, polymorphisms in *CYP19* gene of fish were firstly reported by Galay-Burgos et al. (2006).

Moreover, there is no report describing polymorphisms of *CYP19a* gene in Japanese flounder, and few studies about the relationship between mutants and reproductive traits. In this study, SNPs in Japanese flounder *CYP19a* and its effect on the synthesis of the E_2 or sex steroids were studied. Single-stranded conformational polymorphism (SSCP) analysis is one of the simplest, most reliable, and most sensitive methods for detecting mutations based on PCR (Orita

* Corresponding author. Tel./fax: +86 532 82031825.
E-mail address: wenhaishen@ouc.edu.cn (H.S. Wen).

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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 of *CYP19a* and their associations with reproductive traits.

2. Materials and methods

2.1. Animals

Japanese flounder were reared in sea water at room temperature. Fish were decapitated and the gonads were removed and weighed. Gonads were fixed in 10% neutral buffered formalin, dehydrated, embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin for histological examination. Animal populations were 65 female individuals of Japanese flounders, individual weight approximately 239.23 ± 74.93 (g). Four reproductive traits, serum testosterone (T), serum 17β-estradiol (E_2), Hepatosomatic index (HSI) and gonadosomatic index (GSI), were used for association analysis. Table 1 presented the mean and standard deviations of four traits.

2.2. Hepatosomatic index (HSI) and gonadosomatic index (GSI)

The Hepatosomatic index or gonadosomatic index 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.

2.3. T and E_2 assays by radioimmunoassay

The blood was sampled by puncturing the caudal vasculature with a 25-guage 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 separated the serum by centrifugation (15,000 rpm) for 5 to 7 min and stored at −40 °C. The serum testosterone and estradiol-17β were quantified by ^{125}I radioimmunoassay basing on double antibody assay, using diagnostic kits from 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).

2.4. PCR–SSCP analysis

Genomic DNA was isolated from blood sample by the phenol-chloroform method. Nine pairs of primers were designed to amplify eight exons of Japanese flounder *CYP9a* based on its cDNA sequence (GenBank Accession No. AB017182) using the Oligo6.0 software (Table 2). PCR reactions were carried out in a total of 25 μl volume containing 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. Amplification condition was 94 °C for 5 min followed by 35 cycles of 94 °C for 45 s, 57 °C for 45 s, 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) method. Two μl 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 on 12% polyacrylamide gel for 14 h at 4 V/cm. 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 DNA Fragment Quick Purification/Recover Kit. The purified PCR products were ligated to the PMD 18-T vector and transformed into DH5-α *Escherichia coli*. Positive recombinant colonies were sequenced on the ABI 377 sequencer.

Table 1
Means and standard deviations of reproductive traits

Traits	Mean	SD[1]
T (pg/ml)	17.638	7.36
E_2 (pg/ml)	6.306	3.739
HIS	1.548	0.567
GSI	0.155	0.122

[1] Standard deviation.

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

Names	Sequences	Length (bp)	Tm (°C)	Amplicons
Primer1	5-GTCGTCCAGTTTGTGCAG-3 5-TCTCCTGTCTGTGTGGCT-3	240	57	5′-UTR-exon1
Primer2	5-GTCCACCTTTCTGTTTGG-3 5-TGCTGAGGATGAGTGTCT-3	150	57	Exon2
Primer3	5-CATGTACTGAAGAATGGA-3 5-CTTTGGAGAAATAGTTGC-3	139	49	Exon3
Primer4	5-TCTGACAGGTCCAGGTTTG-3 5-GGGCACATCAAGGAAGAGT-3	159	60	Exon4
Primer5	5-GAGCTGCTGCTGAAGATT-3 5-TGCTGTCTTATGCCTCTG -3	108	56	Exon5
Primer6	5-CTGTCTGCGGAGAACGTGGT-3 5-CAGTGTCAATCTCTCGCAGC-3	145	57	Exon7
Primer7	5-CTGGAGAGCTTCATCAAC-3 5-TCTCAAAGTTGTCCAGGC-3	193	57	Exon8
Primer8	5-GACGTTACTTCCAGCCAT-3 5-TCAGAGTGTTTGCCAGCT-3	250	55	Exon9
Primer9	5-TGATCCACACTGCTTCAT-3 5-TTCCTACTTGGAAAGTGC-3	237	53	3′-UTR

2.5. Statistical models and analysis

The genotype frequencies of each polymorphism were calculated by Excel. The diplotypes were constructed on the base of 3 SNPs with phase 2.0. Associations between genotypes and diplotypes of 3 SNPs of Japanese flounder *CYP9a* gene and four reproductive traits (T, E_2, HSI and GSI) and genetic effects were respectively analyzed using GLM procedure of SAS 8.02 software. The following models were used.

$$Y = \mu + G \text{ (or } H) + e$$

where Y is value measured of four reproductive traits; μ is mean value of four reproductive traits, G or H is fixed effects of genotypes of each SNP or diplotype, e is random error effect. Considering the all experimental fish were female from the same site and slaughtered at the same age, so other effects were not taken in this model such as sex, generation and site. Significant differences among least-square means of different genotypes or diplotypes were calculated using Duncan's multiple-range test, and P values of 0.05 were considered statistically significant.

3. Results

3.1. Polymorphisms within exons of CYP19a gene

Among the nine sets of primers used to amplify the gene fragments by PCR–SSCP analysis, the PCR products of primer1, primer6, and primer7 were polymorphic, respectively (Fig. 1). Three SNPs, namely SNP1, SNP2 and SNP3, were located at positions of A193C, T993G and A1297G of Japanese flounder *CYP19a* gene (Fig. 2). Three genotypes were found for each SNP and named as AA, AB, and BB, respectively (Fig. 1).

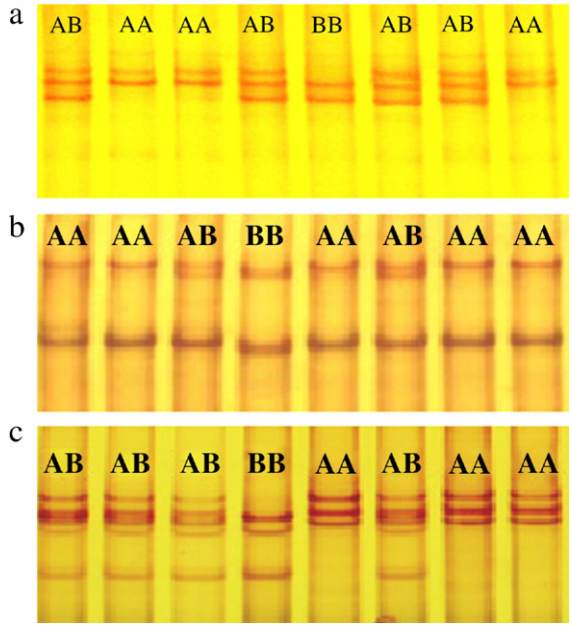

Fig. 1. Band patterns for the 3 SNPs a: Genotype of SNP1; b: Genotype of SNP2 and c: Genotype of SNP3.

At the SNP1 locus, a nucleotide transition, A193C (GenBank accession no. AB017182), was present in the exon1 of *CYP19a*. The single nucleotide polymorphism (SNP) is synonymous mutation because the mutation does not lead to amino acid variation. In addition, at the SNP3 locus, A1297G identified in exon8 was also a synonymous mutation. At the SNP2 locus, T993G was detected in the exon7 of *CYP19a*, which caused an amino acid change from Val^{308} to Gly^{308} (GenBank accession no. AB017182).

Table 3
Frequencies of alleles and genotypes of three SNPs of Japanese flounder *CYP19a* gene (%)

	Genotypes Frequencies			Alleles frequencies	
SNPs	AA	AB	BB	A	B
SNP1	41.5	46.2	12.3	64.6	35.4
SNP2	10.2	38.5	51.3	29.45	70.55
SNP3	79.8	17.6	2.6	88.60	11.40

3.2. Frequencies of genotypes and alleles

Gene and genotypic frequencies were listed in Table 3. The frequency of BB for each of SNP1 and SNP3 was respectively 12.3% and 2.6% which were very low. A relatively high frequency of the genotype AA was 41.5% and 79.8%, respectively. However, at the locus SNP2, the frequency of BB was relatively high (51.3%) and the frequency of AA was low (10.2%).

3.3. Associations between SNPs with reproductive traits

The association analysis of the 3 SNPs within Japanese flounder *CYP19a* gene with the reproductive traits was carried out using least square estimation. Statistical results indicated that, among the three

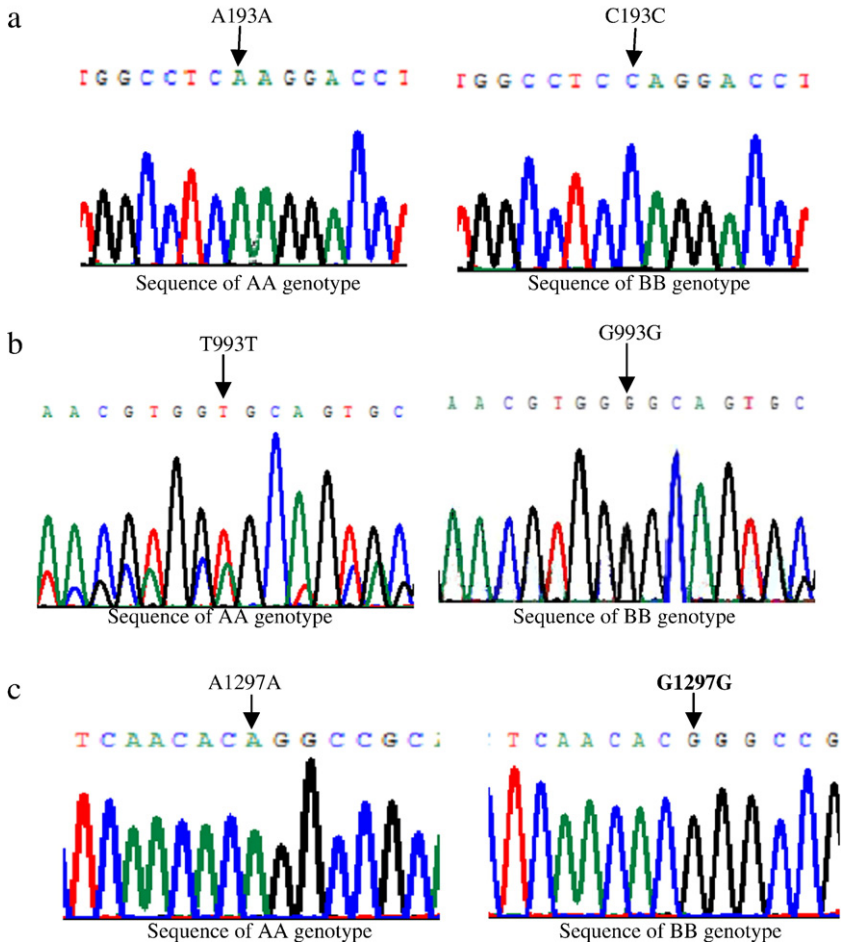

Fig. 2. Sequences of the 3 SNPs a: Sequences of genotypes at SNP1 locus; b: Sequences of genotypes at SNP2 locus; c: Sequences of genotypes at SNP3 locus.

Table 4
Associations between each of three SNPs and reproductive traits by a factor analysis

SNPs	T	E_2	HSI	GSI
SNP1	NS	NS	NS	NS
SNP2	NS	*	NS	*
SNP3	NS	NS	NS	NS

* $P \leq 0.05$. NS: Not significant.

SNPs, only SNP2 in the exon7 was significantly associated with E_2 ($P<0.05$) and GSI ($P<0.05$) (Table 4). And, multiple comparisons analysis showed that E_2 ($P<0.05$) and GSI ($P<0.05$) were higher in Japanese flounder with genotype AB than in those of genotype AA and BB. Other two SNPs showed no association with reproductive traits ($P>0.05$). Multiple comparisons of E_2 and GSI in different genotype were presented in Table 5.

3.4. Association between diplotypes and reproductive traits

Diplotypes were constructed based on three SNPs in the experiment population by use of the Phase program. Nine diplotypes with the minor allelic frequencies of above 2% were identified (Table 6). Association analysis indicated that there was significant association between diplotype and E_2 and GSI ($P<0.05$). Multiple comparisons are shown in Table 6. The results indicated that E_2 level was not significantly different among diplotype D1, D3, D4, D5, D6, D7 and D9 or among diplotype D1, D2, D3, D4, D5, D7 and D8. Similarly, GSI did not differ significantly among diplotype D1, D3, D4, D5, D6, D7, D8, and D9 or among diplotype D2, D, D3, D4, D5, D6 and D8. However, we found that E_2 level in diplotype D9 was much higher than in diplotype D2 and D8 ($P<0.05$) and GSI in diplotype D1 was significantly higher than in diplotype D2 ($P<0.05$).

4. Discussion

Aromatase regulation and activity play a pivotal role in sexual development and in communicating reproductive processes in vertebrates. Accurate transcriptional regulation of the genes encoding steroidogenic enzymes such as aromatase is critical to the regulation of sex steroid homeostasis, essential to ordinary sexual development processes in animals (Yamada et al., 1995). Thus, improper and untimely changes in *CYP19* gene expression may affect reproductive success in animals (Trant et al., 2001; Kuntz et al., 2003b). Previous studies on the human genome have shown that SNPs are associated with diseases (Kristensen and Borresen-Dale, 2000; Mitrunen and Hirvonen, 2003; Dunning et al., 1999; Ribeiro et al., 2006). Although those SNPs are associated with diseases, SNPs in human *CYP19a* gene could modify *CYP19a* catalysis activity and lead to reduced or increased estrogen levels. An analysis of promoter sequences from individual European sea bass suggested the presence of three promoter alleles that had arisen due to three single nucleotide polymorphisms (SNPs) in linkage disequilibrium (Galay-Burgos et al., 2006). Some transcription factors, such as SF-1, FoxL2, Sox, are known to bind to the promoter regions of aromatase genes. Mutations in the promoter of *CYP19a* gene might also be involved in the control of aromatase expression, suggesting the possible existence of regulatory mechanism linking cholesterol metabolism to the synthesis of sex steroids.

Table 5
Multiple comparisons of reproductive traits among three genotypes of SNP2 locus

Traits	AA	AB	BB
E_2 (pg/ml)	$8.432 \pm 1.970^{a,b}$	9.340 ± 1.721^{a}	3.987 ± 1.792^{b}
GSI	$0.133 \pm 0.058^{a,b}$	0.159 ± 0.051^{a}	0.053 ± 0.046^{b}

[a,b]Different superscript letters of mean within a row mean significant difference at $P<0.05$.

Table 6
Associations between diplotypes of *CYP19a* gene and reproductive traits[1] in Japanese flounder

Diplotype	Frequency(%)	SNP1	SNP2	SNP3	E_2 (pg/ml)	GSI
D1	18.46	AA	AB	AA	$4.473 \pm 1.093^{a,b,c}$	0.211 ± 0.030^{a}
D2	7.69	AA	BB	AA	1.996 ± 1.761^{c}	0.100 ± 0.048^{b}
D3	3.07	AA	BB	AB	$2.795 \pm 2.698^{a,b,c}$	$0.199 \pm 0.074^{a,b}$
D4	9.23	AA	AA	AA	$5.697 \pm 1.535^{a,b}$	$0.127 \pm 0.042^{a,b}$
D5	24.62	AB	AB	AA	$4.202 \pm 0.956^{a,b,c}$	$0.184 \pm 0.026^{a,b}$
D6	10.77	AB	AB	AB	$4.936 \pm 1.519^{a,b}$	$0.207 \pm 0.042^{a,b}$
D7	10.77	AB	AA	AA	$4.433 \pm 1.412^{a,b,c}$	0.207 ± 0.039^{a}
D8	6.15	BB	AA	AA	$1.887 \pm 1.268^{b,c}$	$0.106 \pm 0.052^{a,b}$
D9	6.15	BB	AB	AA	8.053 ± 1.865^{a}	0.203 ± 0.059^{a}

[1]Means ± standard deviation
[a,b,c]Different superscript letters of mean within an upright means significant difference at $P<0.05$.

In this study, we first identified three SNPs including A193C, T993G, A1297G, which were respectively located in the exon1, exon7 and exon8 of the Japanese flounder *CYP19a* gene. Two SNPs located at SNP1 and SNP3 locus did not lead to amino acid changes. This may be the reason that the mutations were not associated with reproductive traits. The SNP2, T993G, in exon7 missense mutation, leads to Val308Gly in Japanese flounder *CYP19a* gene, which had significant effects on E_2 level and GSI.

In the gonad, estrogen is converted from androgen by catalysis of *CYP19* gene. If the encoding region of *CYP19a* gene mutates, it would modify catalysis activity of *CYP19a*. Recent data identified that an aromatase gene polymorphism (tetranucleotide repeat polymorphism in intron4) was associated with increased androgen levels and relative abdominal adiposity in women who were premenopausal (Baghaei et al., 2003). Joan et al. (2006) found that *CYP19* rs936306 was associated with both insulin sensitivity and a significant difference in the ratio of testosterone to estradiol (lower testosterone and higher estradiol levels) in African American women. In our study, SNP2 affected significantly E_2 level ($P<0.05$) and GSI ($P<0.05$). This indicates that this polymorphism may have functional significance. This amino acid variation may influence the functional structure and, therefore influenced reproductive traits. However, the exact molecular mechanisms underling the association of the SNP of SNP2 with reproductive traits are not clear, and the possible functionality of the exon7 variants of the Japanese flounder *CYP19a* gene, Val308Gly, can only be appreciated from *in vivo* and *in vitro* experiments in the future.

In this study, no association was observed between the *CYP19a* genetic polymorphisms and T level and HSI. It was previously shown that serum T level in females was higher than in males (Prat et al., 1990). Our results were consistent to Prat et al. (1990). Mean of T level was higher than that of E_2 in female Japanese flounder (Seen Table 1). A good possibility is that the female Japanese flounder was not mature and lower aromatase activity in immature females affected efficiency of converting androgens to estrogens. It is known that the liver is 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. 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 this physiological process maybe explains that mutation of SNP2 was associated with GSI, instead of HSI.

A single SNP often provides a little information. If the diplotypes are constructed by united SNPs, they would supply more information and make up for short-coming of single SNP (Daly et al., 2001; Lei et al., 2005; He et al., 2006). In this study, we tried to construct 9 diplotypes on the basis of the three SNPs and analyzed for the associations of diplotypes with reproductive traits. Results showed that two diplotypes were super for E_2 (D9) or GSI (D1).

5. Conclusion

Three SNPs were first identified in the exons of the *CYP19a* gene and associated with Japanese flounder reproductive traits in this study. The SNP located in exon7, SNP2, was significantly associated with E_2 and GSI. Further, there was significant association between diplotypes D1 and D9 based on 3 SNPs with E_2 and GSI. It implied that mutation of *CYP19a* gene could affect estrogen biosynthesis and reproductive processes in Japanese flounder. Our findings indicate that the estrogen biosynthesis and associated reproductive processes have a high genetic variability. Also our data show that PCR–SSCP is a simple and efficient technique for the detection of single base substitutions and can be employed for evaluating genetic variability in large populations. The identified gene variants, however, need large population studies in order to establish a breeding program for marker assisted selection, improvement in productivity of the Japanese flounder resources of China.

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