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Association of reproductive performance with SNPs of *FOXL2* gene by SSCP in Japanese flounder (*Paralichthys olivaceus*)

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ABSTRACT

FOXL2 which is a putative winged helix/forkhead transcription factor gene and a sexually dimorphic marker of ovarian differentiation plays an important role in ovarian development, granulosa cell differentiation, and thus the proper maintenance of ovarian function. The aims of this study were to characterize polymorphisms within the *FOXL2* gene in a population of 52 female Japanese flounder and analyze the association of *FOXL2* polymorphisms with reproductive performance by polymerase chain reaction-single stranded conformational polymorphism (PCR-SSCP). Results indicated that five single nucleotide polymorphisms (SNPs), which were SNP1 [c.540A>C (p.Asn102His) and c.591A>G (p.Asn119Asp)], SNP2 [c.864G>A (p.Lys210Glu)and c.875G>A] and SNP3 (c.1169C>A), were identified in the *FOXL2* gene. General Linear Model (GLM) analysis showed that SNP1 in the forkhead domain was significantly associated with gonadosomatic index (GSI) ($P<0.05$). SNP2 in the downstream of forkhead domain was significantly associated with serum 17β-estradiol (E_2) level ($P<0.05$). And SNP3 in the 3′-UTR was significantly associated with hepatosomatic index (HSI) ($P<0.05$). Moreover, the evaluation of the genetic effects for both Testosterone (T) level of diplotype D3 and GSI of diplotype D5 suggested they were significantly higher than those of other four diplotypes ($P<0.05$), respectively. These results implied that these SNPs could influence reproductive endocrinology of female Japanese flounder and be also used in marker-assisted selection (MAS) program to reproductive performance in female Japanese flounder in the future.

1. Introduction

FOXL2 is one of the earliest ovarian markers and it offers, along with its transcriptional target genes, an excellent model to study ovarian development and function in normal and pathological conditions (Moumné et al., 2008). Recent reports have described the cloning of *FOXL2* from six fish species, rainbow trout (*Oncorhynchus mykiss*), Nile tilapia (*Oreochromis niloticus*), Japanese medaka (*Oryzias latipes*), Japanese flounder (*Paralichthys olivaceus*), honeycomb grouper (*Epinephelus merra*) and Southern catfish (*Silurus meridionalis*) (Baron et al., 2004; Wang et al., 2004; Nakamoto et al., 2006; Liu et al., 2007; Yamaguchi et al., 2007; Alam et al., 2008), and shown expression in the ovary. The role of FOXL2 in cholesterol metabolism and steroidogenesis in the ovary was further strengthened by the fact that it can up-regulate the expression of ovary-type P450 aromatase (*CYP19a1*), the steroidogenic enzyme responsible for the transformation of androgens to estrogens in granulosa cells (Galay-Burgos et al., 2006; Yamaguchi et al., 2007; Wang et al., 2007a). Hence, FOXL2 is a candidate regulator of the gonadal aromatase gene (Galay-Burgos et al., 2006).

SNPs are becoming a focal point in molecular marker development since they are the most abundant polymorphisms in any organism and adaptable to reveal polymorphism not detected with other genetic markers and methods (Liu and Cordes, 2004). SNP describes polymorphisms caused by point mutations that give rise to different alleles containing alternative bases at a given nucleotide position within a locus, and plays an important role in the transcription and translation of genes and affects function of protein. For instance, Allelic variation due to a single nucleotide transition (cytosine [C] to thymine [T] transition that results in a Arg25Cys) has been demonstrated to be associated with higher leptin mRNA levels in adipose tissue and increased fat deposition in mature beef (Kononoff et al., 2005).

Several approaches have been used for SNP discovery, and one of them is the single-strand conformation polymorphism (SSCP) procedure. It is a simple, fast, reliable and inexpensive technique used to detect sequence variants (Sheffield et al., 1993; Vidal-Puig and Moller, 1994). Concerning genetic variants of *FOXL2* gene, positive associations have been observed that *FOXL2* mutation causes blepharophimosis/ptosis/epicantus inversus syndrome (BPES), a genetic disorder characterized by eyelid abnormalities and premature ovarian failure (POF) in human. Furthermore, the sequence variant of *FOXL2* is responsible for polled/intersex synodrome in goats, which is associated with the transformation of genetically female to sterile males (Crisponi et al., 2001; Pailhoux et al.,

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Table 1
Means and standard deviations of reproductive index.

Physiological index	Mean	SD[a]
T (ng/dL)	17.64	7.36
E_2(pg/mL)	6.31	3.74
HSI	1.55	0.57
GSI	0.16	0.12

T = Testosterone.
E_2 = 17β-estradiol.
HSI = Hepatosomatic index.
GSI = Gonadosomatic index
[a] Standard deviation.

2001). In those studies, genetic variants may influence the expression of transcriptional target genes of *FOXL2* and the synthesis of sex steroids, and in turn affect disease. In teleost, Polymorphisms of the *FOXL2* gene have been firstly reported by Shirak et al. (2006). To our knowledge, to date, there are no reports describing polymorphisms of the Japanese flounder *FOXL2* gene, and association studies between genetic diversities and reproductive performances are also scarce.

The steroid hormones, testosterone (T) and 17β-estradiol (E_2), play an important role in the gonad development and reproduction of teleosts (Struessmann and Nakamura, 2002). Testosterone (T) can be transformed into 17β-estradiol (E_2) by the *CYP19a1* gene. Estrogens, especially 17β-estradiol (E_2), have been shown to play an important role in fish sex differentiation, sexual maturation, final maturation of oocytes and ovulation (Struessmann and Nakamura, 2002; Lee and Yang, 2002). It is generally accepted that the liver is site of production of vitellogenin, which is an estrogen-induced egg-yolk protein synthesized in the liver of oviparous female fish. 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). For aquaculture operations, the GSI is a useful index for monitoring the progression of gametogenesis and estimating reproductive activity in teleost (Marcano et al., 2007).

In this study, we selected four measured physiological indexes, namely testosterone (T), 17β-estradiol (E_2), hepatosomatic index (HSI) and gonadosomatic index (GSI), tested polymorphisms within the Japanese flounder *FOXL2* gene and determined whether mutation detected in the flounder *FOXL2* gene could be relative to reproductive performance. The results would provide the conceptual basis for better understanding molecular mechanism of functional candidate gene mutation affecting reproductive endocrinology in the female Japanese flounder, and would help us carry out a more efficient selection program related to reproductive performance.

2. Materials and methods

2.1. The experimental fish

The 300 Japanese flounder (*P. olivaceus*) individual mass approximately 20.38 ± 3.71 g, were reared in a pond of a commercial fish farm (Rushan, Shandong, PR China). They were fed with a commercially prepared diet at 2–5% of body weight (b.w.)/day and reared in natural sea water under the controlled conditions (20 ± 0.5 °C; ≥4 mg L^{-1} O_2; 14:10 h light:dark cycle). We randomly chose 150 Japanese flounder (242.17 ± 30.76 g) from this pond when fish were reared at six months. Their wet weights were first measured, and then their gonads and livers were dissected and weighed. A piece from the central part of the gonad was fixed in Bouin's solution and embedded in paraffin, followed by sectioning at 5 μm. Developmental stages of germs cells were examined using transverse sections stained with hematoxylin and eosin. Histological examination indicated that developing ovaries were predominated by the peri-nucleolus oocytes. Four reproductive indexes including testosterone (T), 17β-estradiol (E_2), gonadosomatic index (GSI) and hepatosomatic index (HSI) were represented as means ± standard deviation (presented in the Table 1), and subjected to one-way analysis of variance followed by Duncan's multiple range test. Differences were considered significant if $P<0.05$. We combined those data of analysis with histological examination, and then chose 52 female fish in subsequent experiments.

2.2. Gonadosomatic index (GSI) and hepatosomatic index (HSI)

The ratio of the gonad or liver wet weight to the whole body net weight was calculated as following:

$$GSI = [\text{gonad} / (\text{body weight} - \text{viscera weight})] \times 100 \quad (1)$$

$$HSI = [\text{liver weight} / (\text{body weight} - \text{viscera weight})] \times 100 \quad (2)$$

2.3. Steroid radioimmunoassay (RIA)

The fish were anesthetized with 3-aminobenzoic acid ethyl ester (MS222, Sigma) and blood was taken from the caudal vasculature by heparinized disposable syringes. Blood samples were held on ice before centrifugation at 12,000 g for 10 min at 4 °C. The plasma was aliquoted into 1.5-mL plastic microfuge tubes and stored at −40 °C until analysis. The steroid RIA was carried out using the method of Wen et al. (2006).

2.4. DNA extraction and PCR amplification

Genomic DNA was extracted from blood samples using the phenol–chloroform method. Six pairs of primers were designed to amplify the single exon of Japanese flounder *FOXL2* gene, based on its cDNA sequence (GenBank Accession No. AB303854) using the Oligo6.0 software (Table 2). PCR reactions were performed in a Tpersonal thermocycler (Biometra) according to the following conditions: 50 ng DNA template, 2.5 mM $MgCl_2$, 0.20 mM dNTP, 0.20 mM primers and 0.5 U Taq DNA polymerase, for a final volume of 25 μL. Thermal cycling comprised 94 °C for 5 min followed by 35 cycles 94 °C for 30 s, annealing at 60–65 °C(Table 2) for 30 s, and an extension temperature of 72 °C for 30 s, followed by a final

Table 2
Primer sequences and information of Japanese flounder *FOXL2* gene.

Names	Sequences	Length (bp)	Tm (°C)	Amplicons
Primer1	5-CTGGTGCATGACACTGAGCT-3 5-CCATTGCGTCATCCTCTGAGT-3	241	65	5′-UTR-Exon (35 bp–276 bp)
Primer2	5-ACTCAGAGGATGACGCAATG-3 5-GAAGGGGAACTTGCTGATGA-3	230	60	Exon (255 bp–485 bp)
Primer3	5-TCATCAGCAAGTTCCCCTTC-3 5-TGTAGTTCCCCTTCTCGAAC-3	180	65	Exon (465 bp–645 bp)
Primer4	5-CATGAACAACTCATGGTCGC-3 5-AAGAGCAGTGCATCATGGAC-3	335	64	Exon (770 bp–1105 bp)
Primer5	5-GTCCATGATGCACTGCTCTT-3 5-GAACAGCTCGTCAGAATGGT-3	200	65	Exon-3′-UTR(1084 bp–1284 bp)
Primer6	5-ACCATTCTGACGAGCTGTTC-3 5-CCCCAACATGAAGGAGACAA-3	232	62	3′-UTR(1264 bp–1496 bp)

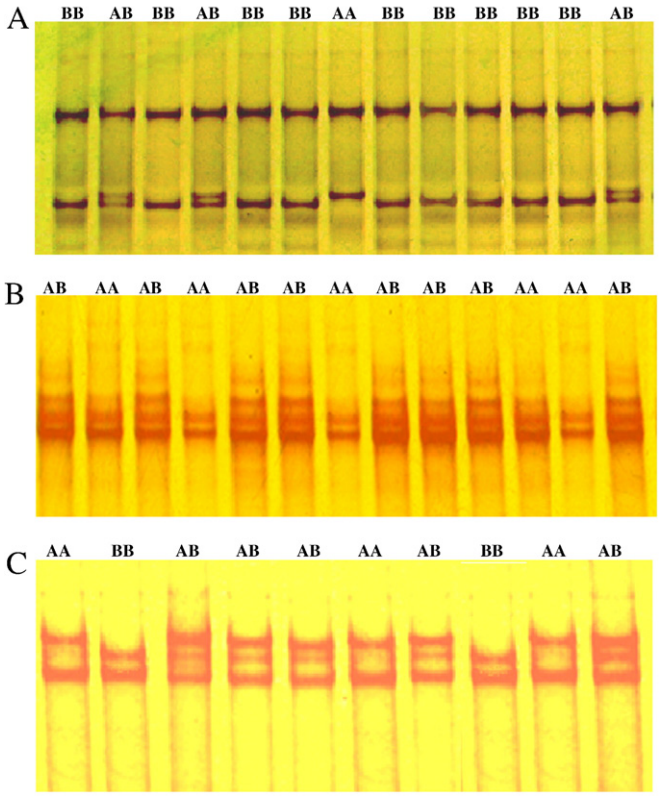

Fig. 1. Band patterns for the five SNPs. A: Genotypes of SNP1 locus (primer3); B: Genotypes of SNP2 locus (primer4); C: Genotypes of SNP3 locus (primer5). AA: the homozygote, consistent to sequence of GenBank Accession Number AB303854. BB: the homozygote, inconsistent to sequence of AA. AB: heterozygote.

extension of 72 °C for 10 min. Each amplification product was verified by electrophoresis on a 2% agarose gel (5 V/cm) in 1×TAE buffer. Gels were stained with ethidium bromide.

2.5. Single-stranded conformation polymorphism (SSCP) analysis

For SSCP analysis, 2 μL of each amplification product was added to, respectively, 5 μL denaturing buffer (98% formamide, 0.09% xylene cyanole FF, and 0.09% bromophenol blue). The samples were heat-denatured at 94 °C for 5 min and then placed on ice for 10 min. Electrophoresis of the denatured DNA was conducted in 12% non-denaturing polyacrylamide gel; 120 V for 12–14 h; 4 °C. SSCP patterns on the gels were visualized 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 (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 by an ABI 377 sequencer.

2.6. Statistical models and analysis

The genotype frequencies of each polymorphism were calculated by Excel. The five diplotypes were constructed on the base of five SNPs with Excel. The combination, having same genotype at each corresponding locus, was counted as a kind of diplotype. Associations between genotypes and diplotypes of five SNPs of Japanese flounder *FOXL2* gene and four physiological indexes (T, E_2, GSI and HSI) and genetic effects were analyzed using General Linear Model (GLM) procedure of Statistical Analysis System (SAS) 8.02 software, respectively. The following models were used.

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

where Y is value measured of four physiological indexes; μ is mean value of four physiological index, G or H is fixed effects of genotypes of each SNP or diplotype, e is random error effect. Considering that 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. PCR-SSCP analysis and base mutation

The PCR-SSCP method was used for the detection of nucleotide sequence polymorphism of the *FOXL2* gene. The target fragment of gene was amplified and denatured, and the polymorphism was found using polyacrylamide gel electrophoresis (Fig. 1). No polymorphisms were detected in the PCR products of primer1, primer2 and primer6 under various electrophoretic conditions (data not shown). However, analysis of the PCR products of primer3, primer4 and primer5 revealed polymorphisms. Those variants were confirmed by

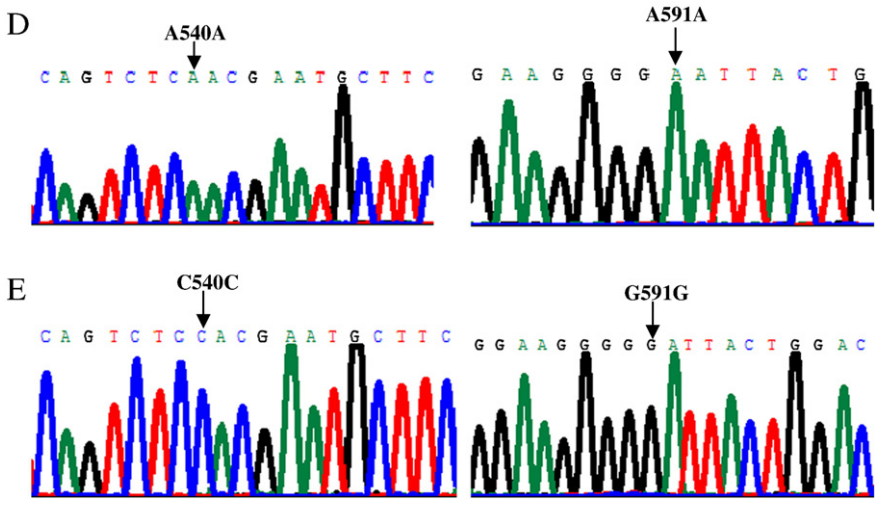

Fig. 2. Sequences of three genotypes of SNP1 locus (primer3). D: Sequence of AA genotype of SNP1 locus (primer3); E: Sequence of BB genotype of SNP1 locus (primer3).

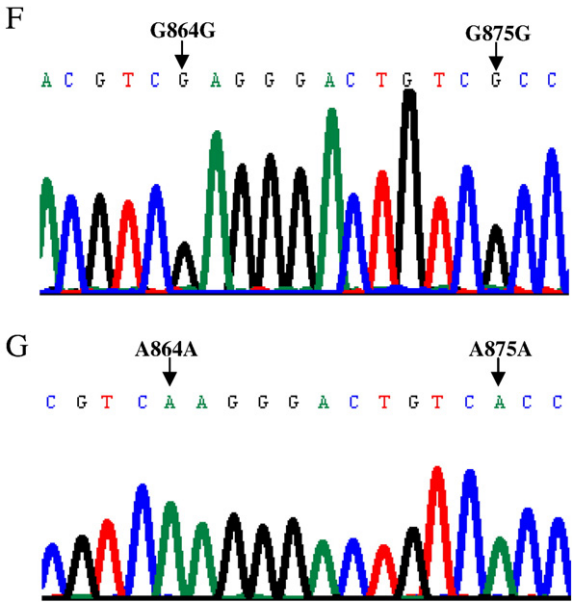

Fig. 3. Sequences of two genotypes of SNP2 locus (primer4). F: Sequence of AA genotype of SNP2 locus (primer4); G: Sequence of BB genotype of SNP2 locus (primer4).

subcloning the PCR products from each genotype of fish into pGEM-T and sequencing multiple subclones from chosen individual. The homozygote, consistent to sequence of GenBank Accession No. AB303854, was named AA genotype, another homozygote was named BB genotype and heterozygote was named AB genotype. Each experimental fish was classified as either genotypes AA, AB or BB with respect to the *FOXL2* genotype.

At primer3, we detected two mutations which were linked together (Fig. 2). One mutation was c.540A>C (p.Asn102His), and the other mutation was c.591A>G (p.Asn119Asp). At primer4, we found two mutations which were also linked together (Fig. 3). One of mutations was c.864G>A (p.Lys210Glu) and another c.875G>A mutation was synonymous mutation. At primer5, there was c.1169C>A mutation (Fig. 4), which was located in 3′-UTR of the *FOXL2* gene. And, the five SNPs were named SNP1 locus (c.540A>C and c.591A>G), SNP2 locus (c.864G>A and c.875G>A) and SNP3 locus (c.1169C>A), respectively. Three genotypes were found in SNP1and SNP3 locus, but only two kinds of genotypes were observed in SNP2 locus.

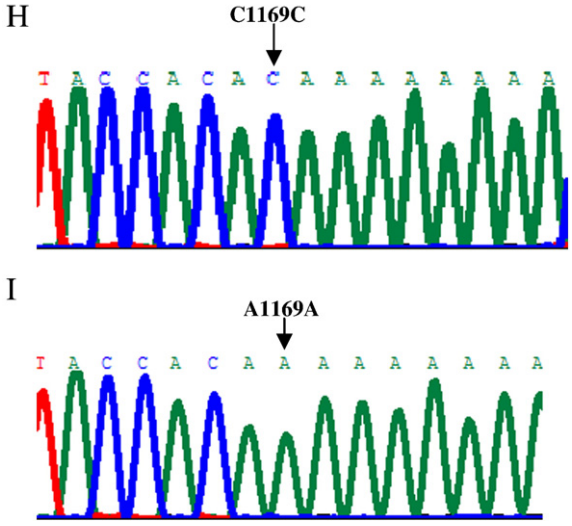

Fig. 4. Sequences of three genotypes of SNP3 locus (primer5). H: Sequence of AA genotype of SNP3 locus (primer5); I: Sequence of BB genotype of SNP3 locus (primer5).

Table 3
Frequencies of alleles and genotypes of five SNPs of Japanese flounder *FOXL2* gene (%).

Locus	Genotype frequencies (%)			Allele frequencies (%)	
	AA	AB	BB	A	B
SNP1	65.38	17.31	17.31	74.04	25.96
SNP2	11.54	88.46	–	55.77	44.23
SNP3	63.46	28.85	7.69	77.88	22.12

3.2. Genotypic and allelic frequencies

The genotypic and allelic frequencies of two loci were presented in Table 3. The value of AB genotypic frequency was highest in SNP2 locus, AA genotypic frequency was highest in SNP1and SNP3 locus, but BB genotypic frequency was lowest in SNP3 locus. Generally, the frequency of allele A was relatively higher than the frequency of allele B in this population.

3.3. Correlation between FOXL2 gene polymorphisms and reproductive performance

The association analysis of the three loci within Japanese flounder *FOXL2* gene with the reproductive indexes was carried out using GLM estimation (Table 4). The results showed that SNP1, SNP2, and SNP3, were significantly associated with GSI, E_2 level and HSI in female Japanese flounder ($P<0.05$), respectively. However, the three loci showed no significant effects on T level in this population.

Further, multiple comparisons analysis were performed in different genotypes of three loci (Table 5). The results showed that GSI and HSI of the individuals with genotype AB were significantly higher than that of those with genotype AA and BB in SNP1 and SNP3 ($P<0.05$), respectively. And E_2 level of the individuals with genotype AA was significantly higher than that of those with genotype AB in SNP2 ($P<0.05$).

3.4. Construction of diplotypes and the correlation with diplotypes and the reproductive performance

Five diplotypes, with the minor allelic frequencies above 6%, were constructed on the basis of five SNPs in the experiment population (Table 6). Association analysis indicated that there was significant association between diplotypes and T level and GSI ($P<0.05$). By multiple comparisons, we found that the genetic effects for both T level of diplotype D3 and GSI of diplotype D5 were respectively much higher than those of other four diplotypes ($P<0.05$).

4. Discussion

Genetic variants within the *FOXL2* gene have been previously investigated as candidates for diseases (Pailhoux et al., 2001; De Baere et al., 2002; Harris et al., 2002; Kumar et al., 2004; Wang et al., 2007b). Those studies indicated that the forkhead protein is known to be involved in different developmental and metabolic processes (Kaufmann and Knöchel, 1996; Carlsson and Mahlapuu, 2002) and mutations in some of them are responsible for genetic developmental diseases (Carlsson and Mahlapuu, 2002). However, how they influence diseases is not exactly known. It is possible that the mutations influence sex hormone levels and this, in turn, leads to

Table 4
Associations between each of SNPs within Japanese flounder *FOXL2* gene and reproductive index.

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

*$P\leq0.05$, NS: No Significance.

Table 5
Association of genotypes of three loci (mean ± SD) with reproductive index.

Locus	Physiological index	AA	AB	BB
SNP1	GSI (%)	0.0413 ± 0.0287^{ab}	0.1371 ± 0.0402^{a}	0.0561 ± 0.0341^{b}
SNP2	E_2 (pg/mL)	6.4321 ± 1.8416^{a}	5.3513 ± 0.9601^{b}	–
SNP3	HSI (%)	1.2173 ± 0.1617^{b}	1.6885 ± 0.1604^{a}	1.5195 ± 0.4555^{ab}

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

disease. In this study, we adopted a PCR-SSCP method to detect polymorphisms within the Japanese flounder *FOXL2* gene. Five SNPs that could be relative to reproductive performance were first identified within the Japanese flounder.

FOXL2 of human has four different homopolymeric runs of amino acids including polyglycine (35–43), polyalanine (221–234), polyproline (284–292) and polyalanine (301–304), except a characteristic 100 amino acid DNA-binding forkhead domain (52–152) (Crisponi et al., 2001; De Baere et al., 2003). According to multiple sequence alignment of FOXL2 protein sequences among different species, we have analyzed the structure domains of the FOXL2 protein in Japanese flounder. The results suggested that FOXL2 of Japanese flounder has a conserved forkhead domain (45–145), but has not an alanine-rich region. Our analysis was consistent with the hypothesis that the conservation of the *FOXL2* open reading frame (ORF) and protein sequences, especially at the level of the forkhead domain and the C-terminal region, while the homopolymeric runs of amino acids, such as the polyalanine tract, are less conserved (Cocquet et al.,2003).

FOXL2 is a forkhead transcription factor essential for proper reproductive function in females (Uhlenhaut and Treier, 2006). Investigators have described the mutations within the forkhead domain. These mutations are at the following positions: 404delC, 486C→G, 568–569insT; 553C→T, 564C→A (De Baere et al., 2001, 2003). In our study, SNP1 [c.540A>C (p.Asn102His) and c.591A>G (p.Asn119Asp)] was detected in the forkhead domain. Only two SNPs were found in this domain of female Japanese flounder for the possibility that the forkhead domain maybe was highly conserved between species.

Missense mutation 564C→A(N109K) within the human *FOXL2* leaded to Blepharophimosis–Ptosis–Epicanthus-Inversus syndrome (BPES) (De Baere et al., 2003). In the current research, statistical results indicated that SNP1 was significantly associated with GSI ($P<0.05$). Since the discovery of FOXL2 in relation to BPES, genetically engineered knock-out mice models have shown that *FOXL2*$^{-/-}$ mice cannot form the functional granulosa cells which lead ultimately to progressive follicular deletion and ovary atresia (Uda et al., 2004; Schmidt et al., 2004). At present, we deduced that these polymorphisms associated with the GSI of female Japanese flounder would have effect on the structure and function of the FOXL2 protein. It is reasonable to suppose those mutations would influence the oocyte development and final gonadal maturation in Japanese flounder. However, all these possibilities remain to be investigated in the future.

The mutations in the downstream of forkhead domain have been described in human *FOXL2* gene. They are at the following positions: 848G→A, 892C→T; 887C→T, 945–946insC, 1041–1042insC ,1164–1165insA, 1335delG; c.881A–>G; c.650C>G (Crisponi et al., 2001; De Baere et al., 2001; Kumar et al., 2004;Wang et al., 2007b). In our study, SNP2 [c.864G>A (p.Lys210Glu)and c.875G>A] was detected in the downstream of forkhead domain. We speculated that the mutation of *FOXL2* gene, G864A (K210E) could have functional significance.

Interaction of FOXL2 with the aromatase promoter, with a subsequent activation of the gene expression in various species, strongly suggest that FOXL2 could play a direct regulatory role on *CYP19a1* transcription (Pailhoux et al., 2001; Baron et al., 2004; Govoroun et al., 2004; Pannetier et al., 2005, 2006; Galay-Burgos et al., 2006; Wang et al., 2007a). In this study, Statistical results indicated that SNP2 was significantly associated with E_2 ($P<0.05$). Moreover, recent report has described that the FOXL2 was involved in the transcriptional regulation of CYP19a1 in Japanese flounder (Yamaguchi et al., 2007). It was possible that SNP2 could modify *CYP19a1* catalysis activity and also affect E_2 synthesis. However, the real molecular mechanisms underlying the association of the SNP2 with reproductive performance was not clear. As shown above, it was conceivable that this mutation might alter hormone synthesis and influence gonadal development and reproduction in the female Japanese flounder.

In contrast, Steroidogenic Acute Regulatory Protein (StAR) is expressed in multiple steroidogenic tissues and is responsible for a rate-limiting step in steroid hormone synthesis. Previous works have revealed that FOXL2 represses transcription of *StAR* (Pisarska et al., 2004). So there was another possibility that those mutations influenced expression of *STAR* gene and, in turn, affected E_2 synthesis. However, it was most likely that the mutation influenced both *CYP19a1*and *STAR* gene, and both of them could affect E_2 synthesis. With regard to polymorphism of *FOXL2*, the genetic variants within the *FOXL2* gene influenced the role of sex steroid hormones during the sex determination of tilapia species (Shirak et al., 2006). It is well known that estrogen plays key roles in ovarian differentiation in fish (Lee et al., 2000). We hypothesized that SNP2 disturbed the physiological function of E_2 in the present research. Thus, this may raise a possibility that the c.864G>A (p.Lys210Glu) is important for FOXL2 function. It is possible that SNP2 could be potential QTN (quantitative trait nucleotide) affecting reproductive performance in female Japanese flounder. However, further studies in larger population are needed to confirm the aforementioned hypothesis.

Recent data identified that a novel insertion mutation (2293–2294insT) in the 3′-UTR of *FOXL2* has been found in a big Chinese family, which was the first report about a close correlation between the 3′-UTR mutation and BPES (Qian et al., 2004). The 3′-UTR of genes has been recognized to be important for the control of gene expression at different levels such as nuclear export, polyadenylation, translation efficiency, and mRNA degradation (Conne et al., 2000; Guhaniyogi and Brewer, 2001; Macdonald, 2001). In our study, one SNP, c.1169C>A, located in the 3′-UTR, was synonymous mutation. Although SNP3 is sited in the 3′-UTR, it could affect the expression and the activity of *FOXL2* gene. Statistical results indicated that SNP3 were significantly associated with HSI ($P<0.05$). In oviparous vertebrates, the ovarian form of P450 aromatase (*CYP19a1*) plays a crucial role in vitellogenesis (Nagahama, 1994). The association of SNP3 with HSI could be that the mutation might influence expression of *CYP19a1* gene and also affect vitellogenin synthesis, and further have an impact on HSI. However, the exact molecular mechanism underlying the association was not clear. Further studies are necessary in this regard.

According to the recent research about diplotype (He et al., 2008a,b), we tried to construct diplotype in current study. Diplotypes were constructed with the five SNPs and analyzed for the associations with reproductive index by multiple comparisons. The current datum showed that diplotype D3 and diplotype D5 was significantly correlated with T level and GSI ($P<0.05$), respectively. We speculated that D3 and D5 may be promising molecular markers for reproductive performance.

Table 6
Frequencies of five diplotypes and associations between diplotypes of the *FOXL2* gene and reproductive index[1] in Japanese flounder.

Diplotype	SNP1	SNP2	SNP3	Frequency (%)	T (ng/dL)	GSI (%)
D1	AA	AB	AB	16.27	4.141 ± 1.285^{b}	0.140 ± 0.038^{b}
D2	AA	AB	AA	53.49	5.872 ± 0.709^{b}	0.115 ± 0.021^{b}
D3	BB	AB	AB	13.95	6.649 ± 0.388^{a}	0.079 ± 0.041^{b}
D4	BB	AB	AA	6.97	6.351 ± 1.964^{b}	0.058 ± 0.046^{b}
D5	AB	AB	AA	9.302	5.831 ± 1.701^{b}	0.337 ± 0.050^{a}

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

5. Conclusions

To the best of our knowledge, this is the first report on the relationship between the polymorphism of *FOXL2* gene with reproductive performance. Five SNPs were identified in the coding sequence and 3′-UTR of the *FOXL2* gene and associated with reproductive performance of female Japanese flounder in this study. Both GSI and E_2 level appear to be under the influence of polymorphisms detected in the single exon and HSI is apparently under the influence of polymorphisms detected in the non-coding region of *FOXL2*. Diplotypes composed of five SNPs may provide a more powerful tool for analyzing associations between *FOXL2* and reproductive performance. The data presented in current study revealed that *FOXL2* is a reproduction related transcription factor gene. Moreover, the results suggested the *FOXL2* gene is useful as a potential marker in the MAS programs. And its single exon is a preferential target for further investigation on mutations that influence reproductive performance in Japanese flounder.

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