Single Nucleotide Polymorphisms of the GnRHR Gene Associated with Reproductive Traits of Japanese Flounder (*Paralichthys olivaceus*)

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ABSTRACT : Gonadotropin-releasing hormone receptor (*GnRHR*) gene is expressed at the anterior pituitary gland and plays a key role in gonad development. This study aimed to investigate molecular genetic characteristics of the *GnRHR* gene and elucidate the effects of single nucleotide polymorphisms (SNPs) of *GnRHR* gene on sex steroid level in Japanese flounder (*Paralichthys olivaceus*). We used polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP) and sequencing of the *GnRHR* gene in 75 individuals. We identified three SNPs in the *GnRHR* gene: P1 locus (C759A and C830T) in the coding region of exon2 which were both linked together and P2 locus (G984T) in the coding region of exon3, which added a new transcript factor (ADR1) and a new methylation site (CG). Only C830T of P1 leads to amino acid changes Thr266Ile. Statistical analysis showed that P1 was significantly associated with 17β-estradiol (E_2) level (p<0.01) and gonadosomatic index (GSI) (p<0.05). Individuals with genotype BB of P1 had significantly higher serum E_2 levels (p<0.01) and GSI (p<0.05) than those of genotype AA or AB. Another SNP, P2, synonymous mutation, was significantly associated with GSI (p<0.05). Individuals with genotype AB of P2 had significantly higher GSI (p<0.05) than that of genotype AA. In addition, there was a significant association between one diplotype based on three SNPs and reproductive traits. The genetic effects for both serum E_2 level and GSI of diplotype D4 were super diplotypes (p<0.05). These results suggest that the SNPs in Japanese Flounder *GnRHR* are associated with E_2 level and GSI. (**Key Words :** Japanese Flounder, GnRHR, SNPs, Diplotype, Reproductive Traits)

INTRODUCTION

Endocrine system plays a major role in the control of reproductive functions which are regulated by the hypothalamus-pituitary-gonad axis and its interactions. However, gonadotropin-releasing hormone (GnRH) plays a pivotal role in the regulation of reproduction in vertebrates through interaction with a specific receptor. The GnRH-stimulated gonadotropin synthesis and release are regulated by the GnRH receptors (GnRHRs). Expression of the *GnRHR* genes vary with sex and reproductive state, due in part to feedback of FSH and LH and steroids including estradiol, testosterone and maturation inducing hormone (MIH) (Mathews et al., 2002; Levavi-Sivan et al., 2006;

Canosa et al., 2007; Moles et al., 2007).

Single nucleotide polymorphisms (SNP), one base variant including deletion, insertion, and substitution, can greatly influence gene expression and the functions of proteins. Several mutations in *GnRHR* have been described. Most mutations in *GnRHR* that either activate or inactivate their functions were reported in humans as responsible for several reproductive genetic disorders (Rosenthal et al., 1996; De Roux et al., 1997; Layman et al., 1998; Kottler et al., 2000; Ines et al., 2005; Quintos et al., 2009). Few numbers of SNPs have been also reported in some important livestock and poultry species (Dunn et al., 2004; Wu et al., 2007; Milazzotto et al., 2008). However, there is no report describing polymorphisms of *GnRHR* gene in teleosts, and few studies about the relationship between mutants and reproductive traits.

Japanese flounder (*Paralichthys olivaceus*) is a teleost fish which has XX (female)/XY (male) sex determination system. Genetic females can be experimentally sex-reversed to phenotypic males when the larvae are reared at high

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Table 1. Primer sequences and information of Japanese flounder *GnRHR* gene

Names	Sequences	Length (bp)	Tm (°C)	Regions
Primer1	5-ATGTGCTGGTGGAACCTGACGC-3 5-TGGCTGCTGTGGTGAATGTGGG-3	236	60	Exon1
Primer2	5-CACATTCACCACAGCAGCCAAA-3 5-GCCACTGGACTGTGATGTTCCA-3	220	60	Exon1
Primer3	5-TGGAACATCACAGTCCAGTGGC-3 5-TCCTCTTTCTGGCCTCGTTGAT-3	160	60	Exon1
Primer4	5-CACAATGTGACCATCGTTCATC-3 5-TCGTTTGGAGATCTCACAGAAG-3	177	58	Exon2
Primer5	5-GGTGCATTTGCGGTGTTCAAAG-3 5-CCCTTTCGGAAGTGAATGGTGA-3	258	59	Exon3
Primer6	5-TCACCATTCACTTCCGAAAGGG-3 5-TGGACTGGTTTCGATCTCGAGC-3	258	56	3'-UTR

water temperature or treated with sex steroid hormones (Kitano et al., 1999). So this fish species has been regarded as a useful model for developmental and reproductive biology (Norifumi et al., 2004; Toshiya et al., 2007). *GnRHR* of Japanese flounder encodes a protein of 415 amino acid residues exhibiting the typical arrangement of the G protein-coupled receptors with seven transmembrane domains. Reverse transcription polymerase chain reaction amplification products were found in the brain and the ovary (Fang et al., 2006).

Therefore, *GnRHR* was chosen as a candidate gene to investigate their effects on reproductive performance of Japanese flounder. SNP based on PCR-SSCP in coding region of the Japanese flounder *GnRHR* gene was identified. The associations of the *GnRHR* genotypes and the reproductive traits were analyzed. The aim of this study was to investigate the significance of the *GnRHR* gene on reproductive traits.

MATERIALS AND METHODS

DNA samples

The population was obtained as described by Shi et al. (2009). Japanese flounder (*Paralichthys olivaceus*) were fed a commercially prepared diet at 2-5% of body weight (bw)/d and reared in natural sea water under 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 reached 6 months of age. Seventy-five females were selected from the population in the light of peri-nucleolus oocytes by histological examination. Four reproductive traits of female Japanese flounder including testosterone (T), 17β-estradiol (E$_2$), gonadosomatic index (GSI) and hepatosomatic index (HSI) were used for association analysis. Table 1 presented the mean and standard deviations of four traits.

Genomic DNA was isolated from blood sample by the phenol-chloroform method (He et al., 2008). Six pairs of

primers were designed to amplify three exons of Japanese flounder *GnRHR* based on its cDNA sequence (GenBank Accession No. DQ011872) using the Oligo6.0 software (Table 1).

HSI and 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. GSI or HSI = (Gonad or liver weight/(body weight-viscera weight))× 100

Steroid assays by radioimmunoassay

The fish were anesthetized with 100 ppm 3-aminobenzoic acid ethyl ester (MS222, Sigma) and blood samples were taken from the caudal vessels by using heparinized disposable syringes. After centrifugation, the serum was stored at -40°C for steroid analysis. The serum testosterone and estradiol-17β were measured by ^{125}I radioimmunoassay (Wen et al., 2006). Four reproductive traits, T, E$_2$, HSI and GSI, were used for association analysis. Table 2 presented the mean and standard deviations of four traits.

PCR-SSCP and DNA sequencing

PCR reactions were carried out according to He et al. (2006). The PCR products of GnRHR were genotyped by 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),

Table 2. Means and standard deviations of reproductive traits

Performances	Mean±SD[1]
T (ng/dl)	17.64±7.36
E$_2$ (pg/ml)	6.31±3.74
HSI	1.55±0.567
GSI	0.155±0.122

[1] Standard deviation.

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 (He et al., 2006). Individual genotypes were defined according to band patterns.

PCR products of each type of homozygotes were purified with DNA Fragment Quick Purification/Recover Kit (TaKaLRa, Japan). 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.

Statistical analysis

The genotype frequencies of each polymorphism were calculated by Excel. The diplotypes were constructed on the base of three SNPs with Excel. Associations between genotypes or diplotypes of Japanese flounder *GnRHR* gene and four reproductive traits (T, E_2, HSI and GSI) and genetic effects were respectively analyzed by one-way Anova using Stat View software version 9.0 (SAS Institute Inc., Cary, NC). Significant differences among means of different genotypes or diplotypes were calculated using Duncan's multiple-range test, and p values <0.05 were considered statistically significant.

**Analysis of DNA and protein sequences**

The sequencing results of PCR products of different SSCP patterns in this study were compared with that from the GenBank, respectively. In addition, the amino acid sequences of different genotypes were compared with DNA star software (version 7.1).

RESULTS

Sequence variation and PCR-SSCP analysis

Among the six sets of primers used to amplify the gene fragments by PCR-SSCP analysis, the PCR products of primer4 and primer5 were polymorphic, respectively (Figure 1). Three SNPs, namely SNP1, SNP2 and SNP3, were located at positions of C759A and C830T in exon2, which were linked together, and G984T in exon3 of Japanese flounder *GnRHR* gene (Figure 2). So SNP1 (C759A) and SNP2 (C830T) were regarded as P1 locus, and SNP3 (G984T) as P2 locus. Three genotypes were observed at P1 locus and named as AA, AB, and BB (Figure 1). P2 locus has only two SSCP pattern: AA and AB (seen in two peaks of sequencing picture) (Figure 2).

At the P1 locus, C759A and C830T in exon2, C759A is synonymous mutation because the mutation does not lead to amino acid variation and C830T caused an amino acid change from Thr to Ile at the position of 266 amino acid (GenBank accession no. DQ011872). In addition, at the P2
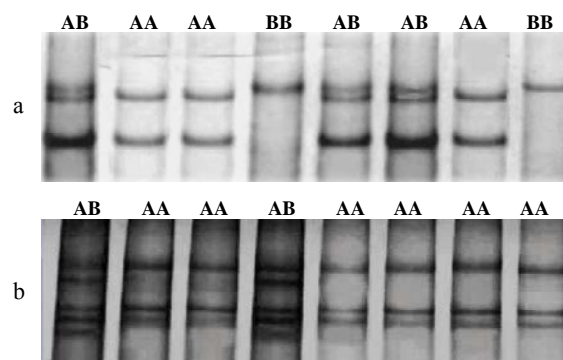
Figure 1. Band patterns for the 3 SNPs. a: Genotypes of SNP1 and SNP2 at P1 locus; b: Genotypes of SNP3 at P2 locus.

locus, G984T identified in exon3 was also a synonymous mutation.
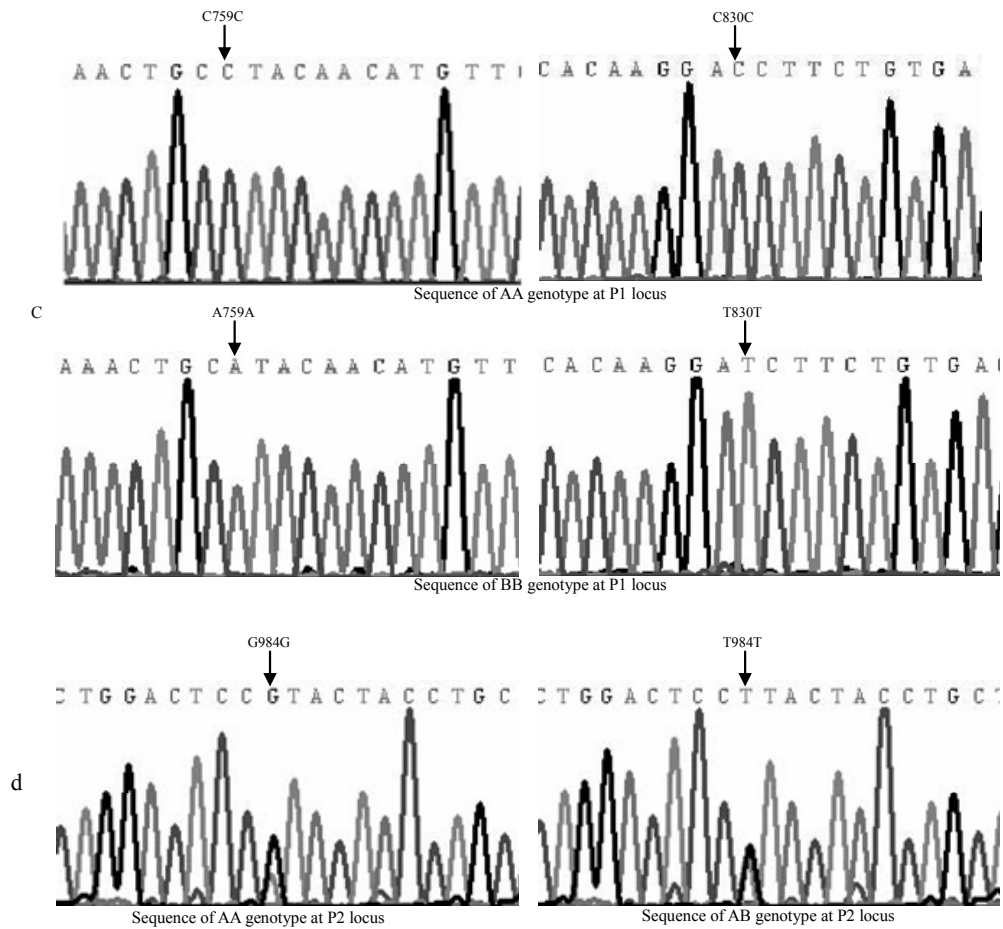
Frequencies of genotypes and alleles

Gene and genotypic frequencies were listed in Table 3. The frequencies of AA and BB for P1 locus were respectively 28.0% and 20.0% which were very low. A relatively high frequency of the genotype AA for P2 locus was 56%. However, the frequency of AB for P1 was relatively high (52.0%) and the frequency of AB for P2 was low (44.0%).

Associations between SNPs with reproductive traits

The association analysis of the three SNPs within Japanese flounder *GnRHR* gene with the reproductive traits was carried out. Statistical results indicated that P1 and P2 were significantly associated with E_2 level (p<0.01) and GSI (p<0.05), respectively (Table 4). And, multiple comparisons analysis showed that E_2 level (p<0.01) and GSI (p<0.05) for P1 were higher in Japanese flounder with genotype BB than in individual of genotypes AA and AB. GSI (p<0.05) for P2 was higher in individual with genotype AB than in individual with genotype AA. Other reproductive traits (T level and HSI) showed no association with three SNPs. Multiple comparisons of E_2 level and GSI in different genotype were presented in Table 5.

Association between diplotypes and reproductive traits

Diplotypes were constructed based on three SNPs in the experiment population by use of the Excel program. Six diplotypes with the minor allelic frequencies of above 9% were identified (Table 6). Association analysis indicated that there was significant association between diplotype and E_2 level and GSI (p<0.05). Multiple comparisons are shown in Table 6. The results indicated that E_2 level with diplotype D4 was significantly highest among D2, D3 and D6 (p<0.05). Similarly, GSI in diplotype D4 was much higher than that in diplotype D3 (p<0.05).

Figure 2. Sequences of the 3 SNPs. c: Sequences of genotypes at P1 locus; d: Sequences of genotypes at P2 locus.

Table 3. Frequencies of alleles and genotypes of three SNPs of Japanese flounder *GnRHR* gene

Loci	Genotypes frequencies (%)			Alleles frequencies	
	AA	AB	BB	A	B
P1	28.0 (21)	52.0(39)	20.0(15)	0.54	0.46
P2	56.0(42)	44.0(33)	-	0.78	0.22

Table 4. Associations between each of three SNPs and reproductive traits

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

* $p \leq 0.05$, ** $p \leq 0.01$.

Table 5. Multiple comparisons of reproductive traits among genotypes of P1 and P2 loci

P1 locus genotype	E_2 (pg/ml)	GSI (%)	P2 locus genotype	GSI (%)
AA(21)	4.124±0.431[b]	0.117±0.040[b]	AA(42)	0.093±0.024[b]
AB(39)	5.083±0.235[b]	0.107±0.021[b]	AB(33)	0.165±0.027[a]
BB(15)	7.378±1.518[a]	0.164±0.030[a]	-	-

[a,b] Different superscript letters of mean within a same column mean significant difference at p<0.05.

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

Diplotype	Frequency (%)	P1	P2	E_2 (pg/ml)	GSI (%)
D1	22	AB	AB	9.457±2.567[a]	0.114±0.033[a,b]
D2	30	AB	AA	5.405±1.815[b]	0.100±0.027[a,b]
D3	16	AA	AA	5.069±3.121[b]	0.060±0.035[b]
D4	12	AA	AB	9.606±1.711[a]	0.267±0.050[a]
D5	10	BB	AA	7.217±2.964[ab]	0.118±0.057[a,b]
D6	10	BB	AB	5.921±2.964[b]	0.115±0.057[a,b]

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

DISCUSSION

Gonadotropin-releasing hormone plays a critical role in the control of reproductive functions in both mammals and teleosts from the pituitary (Kumar and Trant, 2001; Kah et al., 2007). In fish, GnRH is synthesized in the hypothalamus and the hypothalamic GnRH nerve fibers directly innervate in the anterior pituitary, where the GnRH binds to a specific high affinity receptor (Yaron et al., 2003). Therefore, GnRH and its receptor play a pivotal role in the start of the cascade that produces the appropriate growth, maturation and maintenance of the gonads.

GnRHR is a key molecule in the hypothalamic-pituitary-gonadal axis that controls sex steroid status and reproductive processes. Several mutations in the receptor that either activate or inactivate their functions were reported in humans as responsible for several reproductive genetic disorders (Rosenthal et al., 1996; De Roux et al., 1997; Layman et al., 1998; Kottler et al., 2000; Wilkinson et al., 2008; Quintos et al., 2009). Many SNPs have also been reported in some important livestock and poultry species (Dunn et al., 2004; Wu et al., 2007; An et al., 2009). Although those SNPs are associated with ovary disease or reproductive production, SNPs in *GnRHR* gene could modify *GnRHR* activity and lead to regulate steroid secretion levels and further affect gonad development. In this study, P1 (C759A and C830T) had significant effects on E_2 level and GSI. E_2 is one of the most prominent hormones in females and impact GSI (Tian et al., 2010). 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). In our study, there is a good reason to believe that the gene would influence the traits in Japanese flounder. The *GnRHR* is expressed in the pituitary, the gonads and the hypothalamus and has the pharmacological profile of an avian GnRH receptor (Sun et al., 2001; Fang et al., 2006). Since the *GnRHR* occurs in the gonads as well as the pituitary, its effect might occur at the level of the ovary, possibly by affecting cell proliferation

and apoptosis as suggested in mammals (Takekida et al., 2000).

Many studies reported that exons of gene may contain methylation sites and transcript regulator factors (Stephenson et al., 1993; Li et al., 2001). In this study, the consensus *GnRHR* sequence was analyzed for the presence of a CpG Island using Soft berry CpG Finder (http://www.softberry.com/berry.phtml?topic=cpgfinder&group=programs&subgroup=promoter). It is interesting that, the third exon of the GnRHR gene contains nine CpG sites and regulatory factors. P2 (G984T) analyzed in *GnRHR* gene was found in exon3, which was synonymous mutation. The polymorphism "G" in exon3 was at CG site. Putative transcription binding domains were identified by TFSEARCH predictions above 90 scores (Figure 3), which demonstrated that the mutation (CG site) added a new transcript factor: ADR1, which encodes a transcriptional activator involved in the expression of genes (Young et al., 2003). In our study, individual with AB genotype (T/G) had higher GSI than that of animal with AA genotype (G/G). As methylation is tissue specific and inhibits the binding of transcription factors to the respective DNA elements by occupying the same regions with methyl binding proteins and therefore, distorts the DNA orientation for transcriptional factors binding, the transcription of *GnRHR* gene may be inhibited. Therefore, GSI may be low in individual with AA genotype at GC site. However, the exact mechanism is not clear and requires further investigation.

SNPs are frequently used as candidates in the search for causative variation (Cargill et al., 2000). But 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 (Rosenkrans Jr. et al., 2010). In this study, we tried to construct 6 diplotypes on the basis of the three SNPs and analyzed for the associations of diplotypes with reproductive traits. Results showed that diplotype D4 was super for E_2 level and GSI, implying that diplotypes might be used as a marker for improving the Japanese flounder reproductive traits.

In conclusion, three SNPs were first identified in the

e：Exon3 of AB genotype

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 51 CTCTCAAAAT GAGTATAGTT ATTGTCTTGT CTTTTATCAT CTGCTGGACT entry        score
     >                                                     M00029 HSF    100.0
                     <----------                          M00079 Evi-1  93.9
                            ----------->                  M00230 Skn-1  91.0
101 CCTTACTACC TGCTGGGCCT GTGGTACTGG TTCTTCCCAG ACGACCTTGA entry        score
            ------>                                       M00271 AML-1a 100.0
                 <-----                                   M00029 HSF    100.0
                                     --- M00154 STRE   91.5
                                     --- M00083 MZF1   90.4
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f：Exon3 of AA genotype

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 51 CTCTCAAAAT GAGTATAGTT ATTGTCTTGT CTTTTATCAT CTGCTGGACT entry        score
     >                                                     M00029 HSF    100.0
                     <----------                          M00079 Evi-1  93.9
                        <-- M00048 ADR1  92.3
                            ----------->                  M00230 Skn-1  91.0
101 CCGTACTACC TGCTGGGCCT GTGGTACTGG TTCTTCCCAG ACGACCTTGA entry        score
            ------>                                       M00271 AML-1a 100.0
                 <-----                                   M00029 HSF    100.0
        ---                                               M00048 ADR1   92.3
                                     --- M00154 STRE   91.5
                                     --- M00083 MZF1   90.4
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Figure 3. Insilico prediction of transcription factor binding sites in putative proximal region in GnRHR exon3. The transcription factor (TF) binding sites were predicted by TFSEARCH (Ver 1.3) software in the sequence of *GnRHR* gene exon3. The sequence from animals with AB genotype (e) did not have ADR1 transcription factor binding site whereas the sequence of animals with AA genotype (f) had ADR1 transcription factor binding site because of the presence of 'T' rather than 'G 'at position 984 of exon3.

exons of the *GnRHR* gene and associated with Japanese flounder reproductive traits in this study. The SNPs located in exon2 and exon3, P1 and P2, were significantly associated with E_2 level and GSI. Further, there was significant association between diplotypes D4 based on 3 SNPs with E_2 level and GSI. It implied that mutations of *GnRHR* gene could affect sex-steroid biosynthesis and reproductive processes in Japanese flounder. Our findings indicate that the sex-steroid 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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