**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
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 O₂; 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 (E₂), gonadosomatic index (GSI) and hepatosomatic index (HSI) were used for association analysis. Table 1 presented the mean and standard deviations of four traits.

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

| Names   | Sequences                                      | Length (bp) | Tm (°C) | Regions   |
|---------|------------------------------------------------|-------------|---------|-----------|
| Primer1 | 5-ATGTGCCTGTGGGAACCTGACGC-3  
5-TGGCTGCTGTGGTAATGTGGG-3 | 236         | 60      | Exon1     |
| Primer2 | 5-CACATTCAACCACGCAGCCAAA-3  
5-GCCACTGACTGTGATGTTCCA-3 | 220         | 60      | Exon1     |
| Primer3 | 5-TGGAAACATCATGCTCAGTGGC-3  
5-TCTCCTTTCTGCGCTCGTGAT-3 | 160         | 60      | Exon1     |
| Primer4 | 5-CACAATGTGACCTATGTTTCATC-3  
5-TGGTAGAGATCTCACAGAAG-3 | 177         | 58      | Exon2     |
| Primer5 | 5-GGTGCATTGCGGTGTTCAAAG-3  
5-CCCTTCCGGAAGTGAAGTGGA-3 | 258         | 59      | Exon3     |
| Primer6 | 5-TCACCTACCTTCGCAAAGGG-3  
5-TGGACTGTTTCTGATCTCAGGC-3 | 258         | 56      | 3'-UTR    |

**Cytosolic 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 ¹²⁵I radioimmunoassay (Wen et al., 2006). Four reproductive traits, T, E₂, HSI and GSI, were used for association analysis. The aim of this study was to investigate the significance of the GnRHR gene on reproductive 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|
| E₂ (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 (TaKaLa, 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, E2, 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 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 E2 level (p<0.01) and GSI (p<0.05), respectively (Table 4). And, multiple comparisons analysis showed that E2 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 E2 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 E2 level and GSI (p<0.05). Multiple comparisons are shown in Table 6. The results indicated that E2 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).
Table 3. Frequencies of alleles and genotypes of three SNPs of Japanese flounder GnRHR gene

| Loci | Genotypes frequencies (%) | Alleles frequencies |
|------|---------------------------|---------------------|
|      | AA (21)                   | AB (39)             | BB (15) | A   | B   |
| P1   | 28.0                       | 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  | E2  | HSI  | GSI  |
|------------|----|-----|------|------|
| P1         | NS | **  | NS   | *    |
| P2         | NS | NS  | NS   | *    |
| Diplotype  | NS | **  | NS   | *    |

* p<0.05, ** p<0.01.

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

| P1 locus genotype | E2 (pg/ml) | GSI (%) | P2 locus genotype | GSI (%) |
|-------------------|------------|---------|-------------------|---------|
| AA(21)            | 4.124±0.431b | 0.117±0.040b | AA(42)          | 0.093±0.024b |
| AB(39)            | 5.083±0.235b | 0.107±0.021b | AB(33)          | 0.165±0.027a |
| BB(15)            | 7.378±1.518a | 0.164±0.030a | -                | -       |

* 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\(^b\) |
| D2        | 30            | AB | AA   | 5.405±1.815\(^b\)  | 0.100±0.027\(^a\) |
| 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\(^a\)  |
| D6        | 10            | BB | AB   | 5.921±2.964\(^b\)  | 0.115±0.057\(^a\)  |

\(^1\)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
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 E2 level and GSI. Further, there was significant association between diplotypes D4 based on 3 SNPs with E2 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.

ACKNOWLEDGMENTS

This work was supported by Natural Science Foundation of Shandong Province, China (ZR2009DQ011), Research Fund for the Doctoral Program of Higher Education of China (20090132120006) and the open-fund of Key Laboratory of Fisheries Genetic Resources & Aquaculture, Chinese Academy of Fisheries Sciences (2008B1207).

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