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Single nucleotide polymorphisms within the estrogen receptor beta gene are linked with reproductive indices in Japanese flounder, *Paralichthys olivaceus*

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ABSTRACT

The objectives of this study were to characterize polymorphisms within the coding region of estrogen receptor β (*ERβ*) gene in a population of 57 female Japanese flounder (*Paralichthys olivaceus*) and to analyze the association of *ERβ* polymorphisms with reproductive indices by polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP). Two single nucleotide polymorphisms (SNPs), SNP1 (c.577delC) and SNP2 [c.A891T (p.Gln114Leu)], were identified in the *ERβ* gene. A one-way ANOVA revealed that SNP1 was significantly associated with the gonadosomatic index (GSI) in female Japanese flounder ($P<0.05$). And SNP2 was significantly associated with the serum 17β-estradiol (E_2) level and GSI ($P<0.05$). Individuals with genotype AB of SNP2 had significantly higher serum E_2 level and GSI than those of genotype AA ($P<0.05$). Moreover, the hepatosomatic index (HSI), a marker for genetic effects, was significantly higher for diplotype D2 compared with the other three diplotypes ($P<0.05$). These results obtained in this study suggested that SNP2 could influence reproductive endocrinology of female Japanese flounder and be useful as a potential candidate genetic marker for the selection of reproductive indices in female Japanese flounder.

1. Introduction

Estrogens play important roles in the growth and differentiation of reproductive tissues and in the maintenance of fertility by binding to a nuclear receptor protein, the estrogen receptors (ERs) (Drummond et al., 1999). The receptor-mediated estrogenic actions in the female reproductive tract are mainly regulated by ERα, while ERβ is the key mediator of estrogen action in the ovary (Couse and Korach, 1998; Krege et al., 1998; Muramatsu and Inoue, 2000). Moreover, *ERβ* gene has been shown to be expressed in the ovary of several teleost species, including goldfish, *Carassius auratus* (Tchoudakova et al., 1999), channel catfish, *Ictalurus punctatus* (Xia et al., 2000), European sea bass, *Dicentrarchus labrax* (Halm et al., 2004), Nile tilapia, *Oreochromis niloticus* (Wang et al., 2005), sea bream, *Sparus auratus* (Pinto et al., 2006), Common sole, *Solea solea* L (Caviola et al., 2007) and killifish, *Fundulus heteroclitus* (Greytak and Callard, 2007). A number of reports have demonstrated that ERs are involved in embryonic development, gonadal differentiation and gonad development in fish (Cavaco et al., 1998; Lassiter et al., 2002). In addition, they also play a pivotal role in regulating vitellogenesis (Filby and Tyler, 2005).

In aquaculture species, Very few SNP markers have been developed (He et al., 2003; Ryynänen and Primmer, 2006; Hayes et al., 2007; Moen et al., 2008) and those SNPs were distributed in an expressed sequence tag. Furthermore, there were few correlation analyses between allelic variants and phenotype traits (Tao and Boulding, 2003; He et al., 2008a,b). With regard to ER polymorphisms, previous studies have documented a significant effect of ER polymorphism on reproduction traits in pigs (Noguera et al., 2003; Muñoz et al., 2007). Those observations led us to consider that the ER gene may be a candidate gene maker associated with reproductive indices in fish. To our knowledge, to date, nucleotide sequence polymorphisms of the teleosten *ERβ* gene have been reported (Tchoudakova et al., 1999; Pinto et al., 2006; Greytak and Callard, 2007). However, no data are available about the association between genetic diversity of the *ERβ* gene and reproductive indices in any fish species.

The Japanese flounder (*Paralichthys olivaceus*), a member of the flatfish species, has become an economically important cultivated marine species in China. Japanese flounder, which is a multiple spawner with oocytes showing asynchronous development, spawns almost daily during the spawning season (Matuyama et al., 1995; Ozawa et al., 1996). In addition, the Japanese flounder is a rare marine teleost that can be reared with a closed water recirculation system, and gonadal maturation is easily induced by long photoperiod and low-water-temperature treatment (Honda et al., 1993; Mizuta et al., 1996). Therefore, the Japanese flounder is unique and a good model for the study of fish reproduction.

The steroid hormones, testosterone (T) and 17β-estradiol (E_2), play an important role in the gonad development and reproduction of teleost (Struessmann and Nakamura, 2002). Testosterone (T) can be

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translated 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 measurable physiological indices, namely testosterone (T), 17β-estradiol (E_2), hepatosomatic index (HSI) and gonadosomatic index (GSI), and screened polymorphisms within the Japanese flounder (*P. olivaceus*) *ERβ* gene using an optimized PCR-SSCP procedure and determined whether mutations detected in the Japanese flounder *ERβ* gene were related to reproductive indices.

2. Materials and methods

2.1. The experimental fish

Three-hundred Japanese flounder (*P. olivaceus*, Paralichthyidae, Pleuronectiformes), weighing approximately 20.38 ± 3.71 g each, were reared in a pond at a commercial fish farm (Rushan, Shandong, PR China). They were fed a commercially prepared diet at 2–5% of body weight (b.w.)/day 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. Their wet weights were measured, and their gonads and livers were then dissected and weighed. Three parts (anterior, middle, posterior) of either the right or the left gonad were fixed with Bouin's solution, embedded in paraffin, sectioned at a thickness of 5 μm. Developmental stages of germs cells were examined using transverse sections, which were stained with hematoxylin and eosin. This experimental result showed that there were 78 female and 72 male in this population of 150 Japanese flounder. Histological examination indicated that the developing ovaries were predominantly contained peri-nucleolus oocytes. Four reproductive indices of female Japanese flounder including testosterone (T), 17β-estradiol (E_2), gonadosomatic index (GSI) and hepatosomatic index (HSI) were subjected to one-way analysis of variance followed by Duncan's multiple range tests. *P* values of 0.05 were considered statistically significant. We analysed the data based on the results for the 78 female Japanese flounder and then chose 57 female Japanese flounder for subsequent experiments. Four reproductive indices of 57 female, T, E_2, GSI and HSI, were used for association analysis. Table 1 presented the mean and standard deviations of four indices.

Table 1
Means and standard deviations of reproductive indices.

Physiological index	Mean	SD[1]
T (ng/mL)	0.1764	0.0736
E_2 (pg/mL)	6.31	3.74
HSI	1.55	0.57
GSI	0.16	0.12

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

Table 2
Primer sequences for the Japanese flounder *ERβ* gene.

Names	Sequences	Length(bp)	Tm (°C)	Amplicons
Primer1	5-CTCACGGAAGCTGAAGCCAC-3 5-TGACAGAGCAGGGACACGGT-3	461	61	Exon1
Primer2	5-CCGATACTGAGACTGTCTGT-3 5-CAGCGTAGCTGAAGATGGT-3	250	63	Exon2
Primer3	5-CCCAGACTGTCCCTCTGTCC-3 5-CCACGGACTCTGGATGGGCT-3	135	64	Exon2
Primer4	5-AGGAGGCGTTCCCAGGAGAG-3 5-GGCCTTACACCCCTCACACG-3	150	63	Exon3
Primer5	5-ACACAACGACTACATCTGCC-3 5-TTGGTCATGCCGACTTCGTA-3	110	55	Exon4
Primer6	5-AGCACTGACCCCAGAGCAGC-3 5-CCAGGTGATCATGTGAACCAGC-3	150	65	Exon5
Primer7	5-GTTTGTGGAGCTGGGCCT-3 5-GAGGTCCGGGGAGAAGAT-3	127	65	Exon6
Primer8	5-AAGAGGGGAGCTGCGTCC-3 5-AGGCAGACGTACTCCTCCC-3	106	64	Exon7

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 using the following formulae:

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

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

2.3. Steroid radioimmunoassay (RIA)

The fish were anesthetized with 3-aminobenzoic acid ethyl ester (MS222, Sigma, St. Louis, MO) and blood was taken from the caudal vasculature using heparinized disposable syringes. Blood samples were kept on ice before centrifugation at 12,000 *g* for 10 min at 4 °C. The plasma was transferred into 1.5-mL plastic microfuge tubes and stored at −40 °C. 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. Eight pairs of primers were designed to amplify the seven exons of Japanese flounder *ERβ* gene, based on its cDNA sequence (GenBank accession no. AB070630) using the Oligo6.0 software (Table 2). PCR reactions were performed using a Tpersonal thermocycler (Biometra, Goettingen, Germany) with 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 reaction volume of 25 μL. Thermal cycling comprised 94 °C for 5 min followed by 35 cycles 94 °C for 30 s, annealing at 55–65 °C (Table 2) for 30 s, and extension at 72 °C for 30 s, followed by a final 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-strand conformation polymorphism (SSCP) analysis

For SSCP analysis, 2 μL of each amplification product was added to 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 using 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.

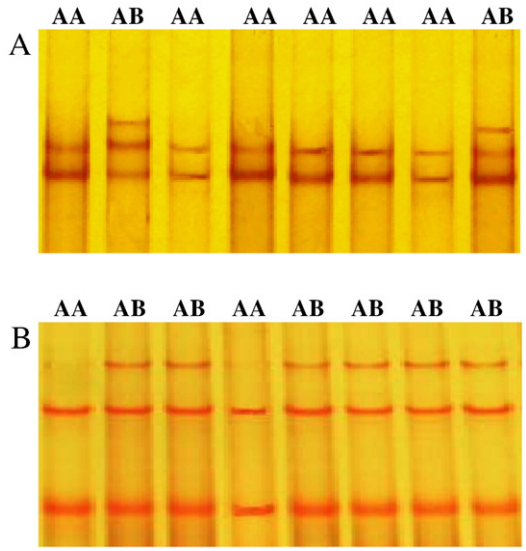

Fig. 1. Band patterns for the two SNPs. A: Genotypes of SNP1 locus (primer 2); B: Genotypes of SNP2 locus (primer 3). AA: the homozygote, which is consistent with sequence of GenBank accession no. AB070630. AB: heterozygote.

PCR products of homozygous individuals of different genotype were purified with DNA Fragment Quick Purification/Recover Kit (Invitrogen, San Diego, CA, USA). 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 using an ABI 377 automatic sequencer (Applied Biosystems, Foster City, CA, USA).

2.6. Statistical models and analysis

The genotype frequencies of each polymorphism were calculated using Excel. The four diplotypes were constructed on the base of two SNPs detected in this study with Excel (Microsoft, Redmond, WA, USA). Combinations with the same genotype at each corresponding locus were considered to be types of diplotypes. Considering that all experimental fish were females, from the same site and were killed at the same age, other effects were not taken into consideration such as sex, generation and site. Associations between genotypes and diplotypes of two SNPs of Japanese flounder *ERβ* gene and four physiological indices (T, E_2, GSI and HSI) and genetic effects were analyzed using one-way analysis of variances (ANOVA) with Duncan's Multiple Range Test (DMRT). A value of $P<0.05$ was considered as statistically significant. These statistical analyses were carried out using the Statistical Analysis System (SAS) 8.02 software (SAS Inst. Inc., Cary, NC, USA).

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

Locus	Genotype Frequencies (%)		Allele Frequencies (%)	
	AA	AB	A	B
SNP1	64.91 (37)	35.09 (20)	82.46	17.54
SNP2	26.32 (15)	73.68 (42)	63.16	36.84

3. Results

3.1. PCR-SSCP analysis and base mutation

The PCR-SSCP method was used to detect nucleotide sequence polymorphisms of the *ERβ* gene. The target gene fragment was amplified and denatured, and polymorphisms identified using polyacrylamide gel electrophoresis. No polymorphisms were detected in the PCR products of primers 1, 4, 5, 6, 7 or 8 under various electrophoretic conditions (data not shown). However, analysis of the PCR products of primers 2 and 3 revealed polymorphisms (Fig. 1). These variants were confirmed by subcloning the PCR products from each genotype of fish into pGEM-T and sequencing at least three subclones from the chosen individual. The homozygote, which was consistent with the sequence of GenBank accession number AB070630, was named AA genotype, another homozygote was named BB genotype and heterozygote was named AB genotype.

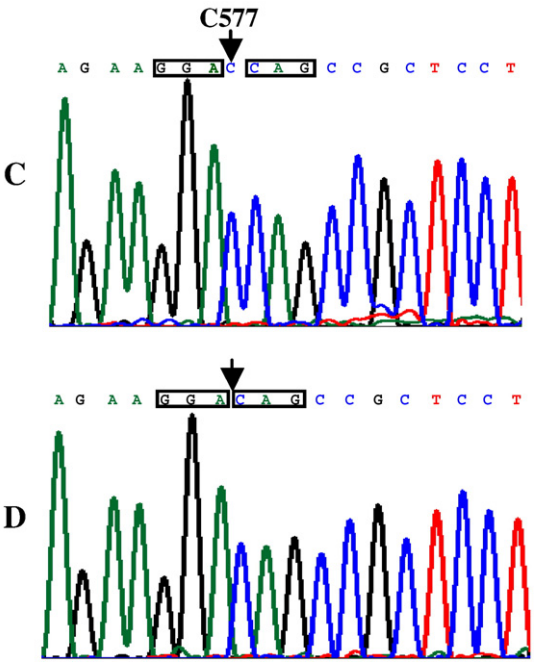

Fig. 2. Sequences of two genotypes of SNP1 locus (primer2). C: Sequence of AA genotype of SNP1 locus (primer2); D: Sequence of AB genotype of SNP1 locus (primer2).

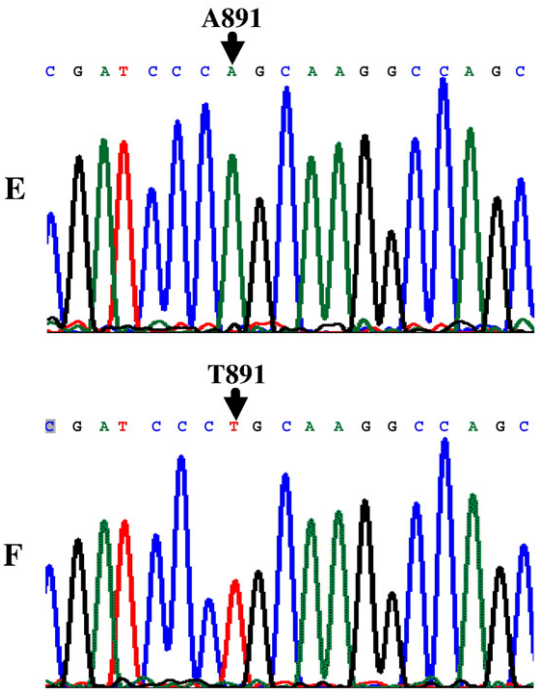

Fig. 3. Sequences of two genotypes of SNP2 locus (primer3). E: Sequence of AA genotype of SNP2 locus (primer3); F: Sequence of AB genotype of SNP2 locus (primer3).

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

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

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

Two SNPs, namely SNP1 and SNP2, were located at c.577delC and c. A891T of the *ERβ* gene in Japanese flounder (Figs. 2 and 3). Two genotypes were found for each SNP and named AA and AB (Fig. 1). At the SNP1 locus, a nucleotide deletion, c.577delC, was present in exon2 of *ERβ* gene. This mutation leaded to a truncated predicted protein. At the SNP2 locus, the c.A891 T caused an amino acid change from Gln^{114} to Leu^{114} (GenBank accession no. AB070630).

3.2. Genotypic and allelic frequencies

The genotypic and allelic frequencies of two loci were presented in Table 3. At the SNP1 locus, the frequency of AA was higher than the frequency of AB. In contrast, the frequency of AB was relatively high and the frequency of AA was low at the SNP2 locus. The frequency of allele A was relatively higher than the frequency of allele B in this population.

3.3. Compared between ERβ gene polymorphisms and reproductive indices

The association analysis of the two loci within Japanese flounder *ERβ* gene with the reproductive indices was carried out using one way ANOVA (Table 4). SNP1 was significantly associated with the GSI in female Japanese flounder ($P<0.05$). And SNP2 was significantly associated with the E_2 level and GSI in female Japanese flounder ($P<0.05$).

Furthermore, multiple comparisons analysis was performed for different genotypes of two loci (Table 5). The GSI for individuals with genotype AB was significantly higher than for individuals with genotype AA in SNP2 ($P<0.05$). And the E_2 level was significantly higher in individuals with genotype AB compared with individuals with genotype AA in SNP2 ($P<0.05$). However, the genotypes of SNP1 showed no significant differences on any of the reproductive indices in this population ($P>0.05$).

3.4. Construction of diplotypes and the Compared between diplotypes and reproductive indices

Four diplotypes, with minor allelic frequencies above 7%, were constructed on the basis of two SNPs in the experiment population (Table 6). Association analysis indicated that there was a significant association between diplotypes and HSI ($P<0.05$). Multiple comparisons revealed that the genetic effects on HSI were higher with diplotype D2 compared with the other three diplotypes ($P<0.05$).

4. Discussion

Sequence variations in the human *ERβ* gene are associated with some endocrine related disorders, such as ovulatory dysfunction (Sundarrajan et al., 2001), high serum androgen level of premenopausal woman (Westberg et al., 2001) and breast cancer (Tsezou et al., 2008). However, how they influence diseases is not exactly known. It is possible that mutations may influence ERβ protein function and lead to the changes in the androgen-estrogen balance, and eventually result in disease. In the present study, we identified two novel polymorphisms within the Japanese flounder *ERβ* gene by SSCP technique. These polymorphisms were expected to change the corresponding amino acid of the encoded ERβ protein. Our results substantiated several earlier reports that PCR-SSCP analysis is a fast, reliable and inexpensive technique used to detect sequence variants (Sheffield et al., 1993; Vidal-Puig and Moller, 1994).

Genetic variants in the coding region of human *ERβ* gene have been described. They are at the following positions: c.G1082A in exon5 (Westberg et al., 2004), rs1256049 in exon5 (Ichikawa et al., 2005) and rs1256064 in exon6 (Kung et al., 2006). In our study, only two SNPs were found in exon2 of the seven (of eight) exons gene investigated in this study. Because SNPs were not found in other exons, there remains the possibility that the experimental population was not large enough. Therefore, we could not exclude the possibility that other sequence variants might exist within the non-coding region or coding regions of the *ERβ* gene that we have not studied so far. Of the two SNPs in exon2, SNP1 (c.577delC) resulted in a shorter protein, and SNP2 (c.A891T) caused an amino acid change from Gln to Leu at position 114 of the predicted amino acid sequence. Moreover, SNP1 was significantly associated with the GSI in female Japanese flounder ($P<0.05$). And SNP2 was significantly associated with the E_2 level and GSI in these female Japanese flounder ($P<0.05$). ERβ has six functionally independent domains, termed A to F from the amino to carboxyl terminus (Krust et al., 1986). The DNA-binding domain (C domain) and the ligand-binding domain (E domain) are highly conserved between species. There are also variable regions at the N and C termini and between the DNA-binding and ligand binding domains (A/B, F and D domains, respectively). The SNP1 was responsible for the synthesis of a truncated predicted protein, containing an incomplete A/B domain. And SNP2 was detected in the A/B domains. The N-terminal A/B domains show high interspecific variability and transactivate target gene transcription directly through an AF-1 domain or after interaction with co-activators. Because there is no conservation of A/B domains among species, it was unlikely that these polymorphisms have a strong effect on reproductive indices. However, our finding of significant association between these polymorphisms and two reproductive indices suggests theirs functional relevance.

The mutation c.A566T in exon2 of the human *ERβ* gene was not correlated with endocrine-related diseases in an African population (Zhao et al., 2004). In this study, SNP2 located in exon2 was significantly associated with GSI ($P<0.05$). Several studies have shown highly specific expression of ERβ in the granulosa cell layer of small and developing follicles (Britt et al., 2004; Emmen et al., 2005). Furthermore, the ovary of mice lacking ERβ contained more atretic follicles and fewer corpora lutea (Krege et al., 1998). Those results suggest that ERβ plays an important role in ovarian follicular growth and maturation during the female reproductive cycle. We deduced that the polymorphism associated with the GSI of female Japanese flounder would alter the structure and function of the ERβ.

Table 5
Multiple comparisons of reproductive index among two genotypes of SNP2 locus.

Physiological index	AA	AB
GSI (%)	0.1384 ± 0.036	0.1763 ± 0.073*
E_2 (pg/mL)	7.638 ± 0.9051	8.256 ± 1.407*

* $P<0.05$.

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

Diplotype	Frequency (%)	SNP1	SNP2	HSI
D1	15.79 (9)	AB	AB	1.539 ± 0.1770^{a}
D2	19.30 (11)	AB	AA	1.680 ± 0.0819^{a}
D3	7.02 (4)	AA	AA	$1.326 \pm 0.2375^{a,b}$
D4	57.89 (33)	AA	AB	1.051 ± 0.2108^{b}

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

Therefore, it is reasonable to hypothesize that this mutation might influence ovarian development and function in the Japanese flounder.

Growth and function of female reproductive organs, especially uterus and ovary, are partly regulated by estrogen (Fujimoto et al., 2003). Therefore, estrogen levels might reflect the expression and function of ER in target cells. Moreover, the ERα-deficient mice exhibit follicular growth and development, which together with other evidence (Kuiper et al., 1996; Couse et al., 1997; Tremblay et al., 1997), strongly suggests that responsiveness of the ovary to estrogen occurs predominantly through ERβ. The statistical results of our study indicated that SNP2 was significantly associated with E_2 ($P<0.05$). This experimental result was consistent with a recent report showing that polymorphisms of the human *ERβ* gene were correlated to estrogen variation in women of four races/ethnicities (Sowers et al., 2006). It seemed probable that SNP2 modified the binding affinity or dissociation equilibrium of ERβ with estrogen and consequently affects physiological functions of estrogen. Although the molecular mechanisms underlying the association between SNP2 and reproductive indices were unclear, we speculated that this variation might disturb estrogenic effects as well as reproduction in female Japanese flounder. However, the biological significance of all these hypotheses remains to be investigated in future studies.

In our study, the SNP1 led to a truncated predicted protein. Moreover, SNP1 was significantly related to the GSI ($P<0.05$). We assumed this mutation could alter the function of ERβ and also affect gonadal development and reproduction in female Japanese flounder. Furthermore, the SNP1 may be in linkage disequilibrium with other SNPs that have functional significance. However, the exact molecular mechanisms underling the association of SNP1 with reproductive indices were not clear. And additional functional studies and larger sample association studies are needed to elucidate aforementioned hypotheses.

According to recent research about diplotypes (He et al., 2008a,b), we tried to construct a diplotype in the current study. Diplotypes were constructed with two SNPs and we analyzed their associations with reproductive indices. We found that diplotype D2 was significantly correlated with HSI ($P<0.05$). We speculate that D2 may be a promising molecular marker for reproductive indices.

5. Conclusions

To our knowledge, this is the first report on the relationship between polymorphisms of the *ERβ* gene with reproductive indices in teleostean fish. The obtained results should improve the knowledge about the molecular mechanism by which functional candidate gene mutations affect reproductive endocrinology in Japanese flounder. The results might be beneficial for the development of new tools for DNA-based marker-assisted selection program related to reproductive indices in fish. However, the data generated in current study have to be interpreted with caution since further studies using sufficient number of sample size as well as identifying the possible causative sequence alteration and its function at the genomic level are still needed to extend and confirm the role of *ERβ* gene in the reproduction of Japanese flounder. Nonetheless, the results of this study support the role of SNP2 as a candidate genetic marker for Japanese flounder breeding programs.

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