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Association of a single nucleotide polymorphism of the *Leptin* gene with growth traits in *Takifugu rubripes*

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Abstract

Leptin (LEP) plays a role in animal growth, appetite regulation, energy partition, and body composition. In this study, 296 cultivated *Takifugu rubripes* individuals were screened for LEP gene polymorphisms using PCR-single strand conformation polymorphism (PCR-SSCP) and DNA sequencing. Two single nucleotide polymorphism (SNP) mutations were detected in *T. rubripes* LEP gene: C/G at position 232 nucleotide (nt) and C/T at position 310 nt. One-way ANOVA of the SNPs and growth traits showed that genotype CC of primer L2 had a significantly higher body length (BL), body weight (BW), and absolute growth rate of BL (ABL) and BW (ABW) than genotypes CG and GG ($P<0.05$). The genotype frequencies of CC, CG, and GG were 61.15%, 35.14%, and 3.17%, respectively. Genotype CC at the C232G site was advantageous and significantly associated with growth traits. This site would be an excellent molecular marker to select and cultivate through statistical analysis. Additionally, the second unmutated and mutated mRNA structures were compared and analyzed. These results indicate that the *T. rubripes* LEP gene may be a candidate for these growth traits.

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Introduction

Leptin (LEP) is known as the hormone product of the *obese* gene and plays a role in animal growth, appetite regulation, energy partition, and body composition (Zhang et al.,2017). LEP was originally discovered in a mouse model of obesity (Zhang et al.,1994). These mice manifested hyper phagia and extreme obesity due to a homozygous LEP gene mutation. As a candidate gene, LEP gene can be selected based on its participation in growth and muscle development in animal breeding (Lee,2009). Both human and animal studies have demonstrated the close association between body fat, LEP mRNA, and LEP plasma levels(Levin et al.,1996). An increase in fat storage leads to increased LEP gene expression, which thereby inhibits the satiety center in the hypothalamus (Heymsfield et al.,1999). Furthermore, LEP binds to LEP receptors in the hypothalamus, which are signaled through JAK–STAT pathways, alter transcription, and ultimately affect phenotypes. High LEP attenuates and low LEP stimulates appetite (Zhang et al.,1994). Several studies have shown that LEP affects growth, food breeding, and the body fat content in sheep (Delavaud et al.,2000), poultry (Bruggeman et al.,2000), *Rattusnorvegicus* (Sun et al.,2012), *Equuscaballus* (El-Maaty et al.,2010), *Xenopuslaevis* (Crespi et al.,2006), and *Macacamulatta* (Lujan et al., 2006).

In parts, as compared to studies on LEP gene in farm animals, studies in fish have been limited. Previously, it was proved that LEP affects fish growth, food intake and body fat content in the same way as in mammals (Li et al.,2010; Bakshi et al., 2022). Johnson et al. (2000) were the first to suggest that fish express LEP by documenting an LEP-immunoreactive protein. Kurokawa et al. (2005) first cloned *Takifugu rubripes LEP(TrLEP). TrLEP* cDNA contains a 459 nucleotide (nt) open reading frame (ORF) that encodes a putative polypeptide of 152 amino acids and the primary structure of *TrLEP* has only 13% similarity with human LEP gene (Kurokawa et al.,2005). Threading algorithms that used the carbon backbone of the human-LEP crystal structure predicted that the tertiary structure of fish LEP was very similar to mammalian LEP (Zhang et al.,1997; Kurokawa et al.,2005; Gorissen et al., 2009). In mandarin fish, the leptin family contains two members, lepA and lepB. LepB played an important role in food intake and glucose homeostasis regulation, while LepA showed a limited role in gluconeogenesis and lipid metabolism (Yuan et al., 2020).In grass carp liver, LEP acutely reduced the expression of stearoyl CoA desaturase-1, an enzyme critical for the biosynthesis of monounsaturated fatty acids (Li et al.,2010). The food intake of rainbow trout (*Oncorhynchus mykiss*) was lowered after LEP injection, suggesting that LEP also suppressed food intake mediated by hypothalamic regulation (Murashita et al.,2008). In orange-spotted grouper, six polymorphisms were identified in leptin genes. Three SNPs were associated with growth traits (Wei et al., 2013). Other fish LEPs, including *Danio rerio* (Gorissen et al.,2009), *Oryziaslatipes* (Chisada et al., 2014), and *Moronesaxatilis* (Won et al.,2012), have been identified, and their structures and functions have been investigated.

T. rubripes is an economical fish species farmed on its excellent taste, rich nutrition, and high medicinal value in the coastal provinces of Northern China. Recently, *T. rubripes* cultivation has continuously expanded throughout China. Its culturing methods are evolving rapidly. The present study was planned for correlation analysis on the growth traits of *T. rubripes* using single nucleotide polymorphisms (SNPs) analysis of the LEP gene to advance the culture of *T. rubripes* through molecular marker-assisted breeding.

Materials and Methods

Animals and traits

The healthy individuals of *T. rubripes* (n=296) were selected having age of 102 days (d) from Zhuanghe Dalian in China. Their body length (BL), body height (BH) and body weight (BW) were scored according to the methods described by Wang et al. (2014). The muscles of each individual were sampled and preserved in 95% ethanol for further analysis.

DNA extraction, PCR amplification and sequence annotation

The genomic DNA was extracted by the phenol/chloroform method and dissolved in ultrapure water with a concentration of 100 ng/mL and stored at -20 °C.

Five pairs of primers were designed according to *TrLEP* (GenBank accession no.: NM_001032725). Primers' information was shown in **Table 1**. PCR amplification was performed in a reaction volume of 25 μL including 1 μL genomic DNA, 1μLeach primer, 2.5μL10×PCR Buffer, and 0.2 μL Taq DNA polymerase. The PCR thermal cycling condition was initial denaturation for 5 min at 94 °C; 30 cycles of the 30s at 94 °C, 52-64 °C for 30s, 30s at 72 °C; and a final 7 min extension at 72 °C.

Table 1 The information of new primers designed for PCR amplification of Leptin gene from *T.rubripes*

Locus	Primer sequence(5′ to 3′)	Length(bp)	Tm(℃)	Location[a]
L1	F :TGCCGACATGGATCACATTC R :GGTCCGAAATCAGGTTGTTG	254	56	58-311
L2	F:GATGAAGTCAAAGGTGACCTGG R: ACAGAGGTGGATCCCTCCAC	114	52	157-270
L3	F: TTCGACACCGACAAGGTG R:GAAGATATTCCTCCTCGGCC	198	57	236-433
L4	F: ACAACCTGAT TTCGGACCGC R: CAGCCATGAC TCCCAAACAC	261	57	294-554
L5	F: GGATCTTCT GGAGAGATG R: CCATTTTTCT CTGTCGGCGC	284	60	502-785

[a]Location represents the position of the *T. rubripes Leptin* sequence (Genbank accession no: NM_001032725)

PCR-single strand conformation polymorphism (PCR-SSCP) and DNA sequencing were used to perform genotyping. One microliters of the PCR product was diluted with 5 μL of loading buffer (98% formamide, 10 mM EDTA pH8.0, 0.025% xylene cyanol FF, 0.025% bromophenol blue, 2% glycerol). After denaturing at 98 °C for 10 min, the mixture was immediately placed on ice for 10 min. Next it was loaded on a 10% acrylamide/bisacrylamide gels (acr:bis = 29:1). The PCR product was electrophoretically resolved (10 mV/cm for 14–16 hours). The gel was stained using the silver staining method. Each homozygote PCR product was purified, recovered, and sequenced using the Sanger sequencing method in TaKaRa Biotechnology (Dalian) Co. Ltd. (TaKaRa Dalian).

The second structure of TrLEP mRNA

All the second structures of *TrLEP* mRNA were predicted by the software (Reuter and Mathews, 2010).

Statistical analysis

Absolute growth rate was calculated according to the equation described by Wang et al. (2010):

$ABL/ABW = y/t$

where y was BL or BW, and t was the age in days (t=102 d).

The body length, body height, body weight, ABL, and ABW of the *T. rubripes* were scored, and the data were analyzed by one-way ANOVA through software SPSS 14.0. The correlation between the SNPs and growth traits were analyzed through post hoc multiple comparisons (Duncan method, P-value < 0.05).

A fixed model was adopted according to the factors that affect growth traits using the following equation (Wang et al., 2014):

$y_{ij} = \mu + G_i + e_{ij}$

where

y_{ij} is the observed value of j^{th} individual of genotype i,

μ is the mean of observed values,

G_i is the practical value of the genotype i and

e_{ij} is the random residual effect corresponding to the observed values.

Note: The effect of the surrounding environment was included in the unexpected error.

Results

PCR results and analysis

Three TrLEP fragments with the same genotype through amplification by the same primer were sequenced three times. The PCR products of primers L2 and L3 presented polymorphism (**Figure 1**). Comparison of the sequences with the known *TrLEP* sequence (GenBank accession no.: NM_001032725) showed two polymorphic sites. Both C232G mutation and C310T mutation are located in an open reading frame (ORF). For the SNP C232G, the C and G are at the third position of the codons CT(U)C and CT(U)G. Both the codons encode leucine (Leu). For the SNP C310T, the C and T are at the third position of the codons GAC and GAT (U), respectively. Both the codons encode Aspartic acid (Asp), representing synonymous mutation.

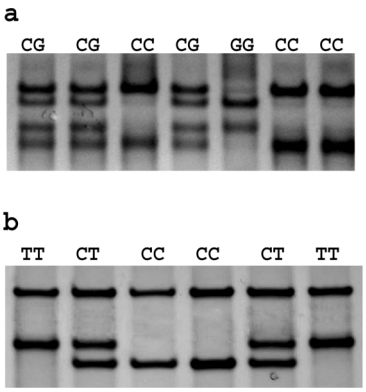


Figure 1 Band patterns for the two SNPs **(a)** Genotypes of primer L2; **(b)** Genotypes of primer L3

To identify the three genotypes generated by primer L2, the PCR fragments' sequence, which has the base 'C' at 232nt, was defined as allele 'C.' While the sequence which has the base 'G' at 232nt was defined as allele 'G'. For primer L3, the PCR fragments' sequence, which has the base 'C' at 310nt, was defined as allele 'C'. At the same time, the sequence which has the base 'T' at 310nt was defined as allele 'T' (**Table 2**).

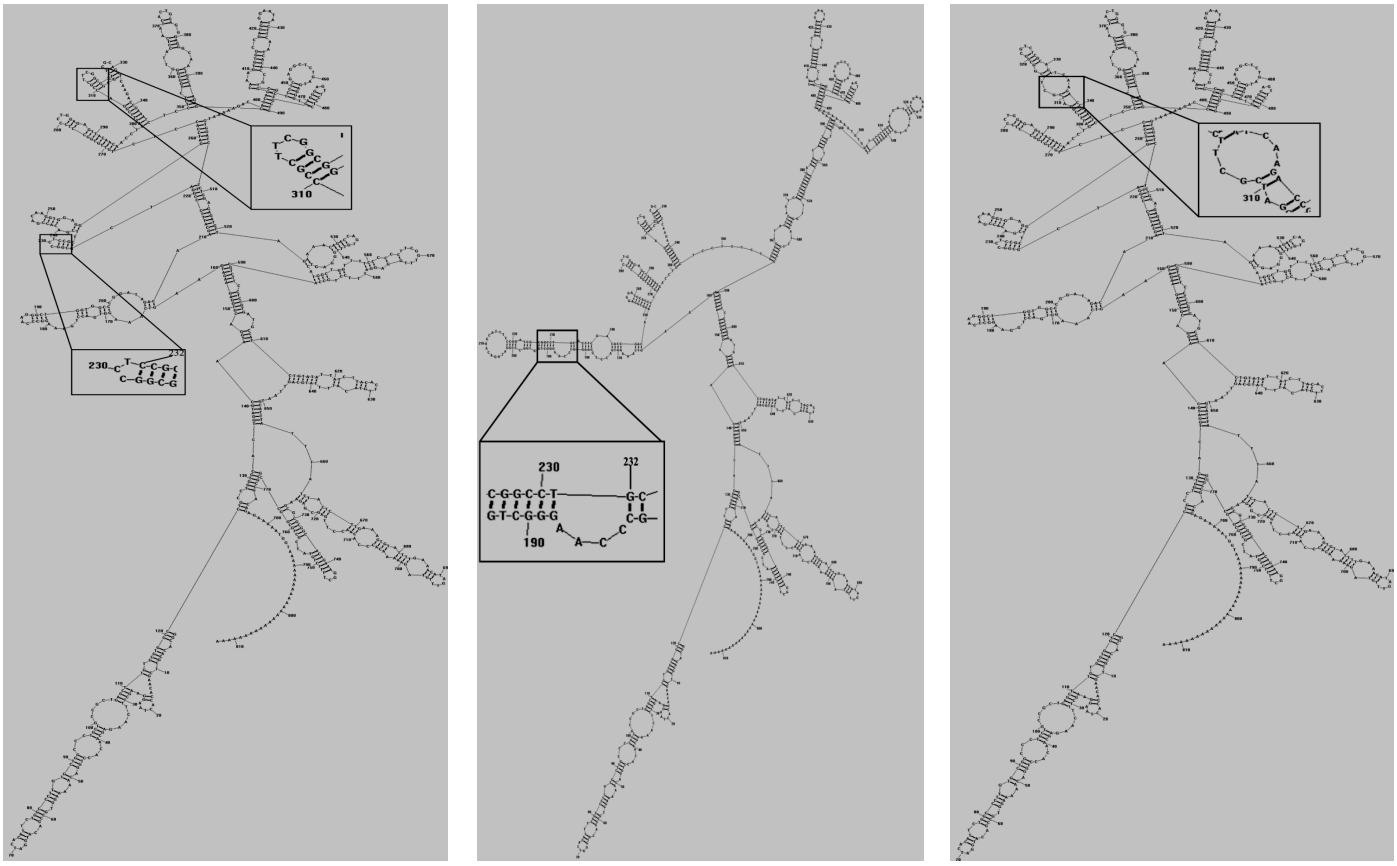
Frequencies of genotypes and alleles of L2, and L3 were also calculated (**Table 2**). In L2 loci, genotype CC, CG, and GG frequencies were 61.15%, 35.14%, and 3.71%, respectively. Allele C and G frequencies were 78.72% and 21.28%, respectively. In L3 loci, genotype CC, CT, and TT frequencies were 47.64%, 43.58%, and 8.81%, respectively. Allele C, and G frequencies were 69.43% and 30.57%, respectively.

Table 2 SNPs in T. rubripes leptin gene: genotype and allele frequencies

SNP	Position	Mutation type	Genotype frequencies	Allele frequencies
C310T	exon	Synonymous (GAC→CAT)	CC (47.64%);CT (43.58%);TT (8.8%)	C (69.43%);T (30.57%)
C232G	exon	Synonymous (CTC→CTG)	CC (61.15%);CG (35.14%);GG (3.71%)	C (78.72%);G (21.28%)

The second structure of TrLEP mRNA

All the second structures of *TrLEP* mRNA, C232G mutation (G-mRNA), and C310T mutation (T-mRNA) were predicted by RNA structure (**Figure 2**). After C mutated T at 310nt, the second structure of T-mRNA had a minor change that only one stem-loop structure was different (**Figure 2a** and **Figure 2c**). But when 232 nt of *TrLEP* mRNA was G, G-mRNA second structure changed significantly and the original stem-loop structures were broken basically (**Figure 2a** and **Figure 2b**).



(a) (b) (c)

Figure 2 Comparison of the second structure of three mRNAs, including *leptin* mRNA, C232G mutation mRNA and C310T mutation mRNA of *T. rubripes* LEP gene. (a) The second structure of *leptin* mRNA;(b) The second structure of C232G mutation mRNA; (c) The second structure of C310T mutation mRNA.

Analysis of the association between different genotypes of the TrLEP and growth traits

The association analysis of the two loci within the *TrLEP*, with the growth traits, was carried out using one-way ANOVA. Three loci (L1, L4, and L5) did not find SNPs. Other loci (L3) did not show any significant effects on the examined traits in *T. rubripes*. L2 was significantly associated with body length, body weight, ABL, and ABW. Furthermore, multiple comparison analysis was performed in three genotypes of L2. Results showed that the *T. rubripes* with genotype CC of L2 had significantly higher body length, body weight, ABL, and ABW than those of genotype GG and CG (**Table 3**).

Table 3 Analysis of the SNP polymorphism of primer L2 and growth traits (ANOVA)

Genotype	N[#]	Body weight(g)	Body length(mm)	Body height(mm)	ABL (mm/d)	ABW (g/d)
CC	181	20.216±7.881[a]	96.221±12.122[a]	17.403±2.936[a]	0.943±0.119 [a]	0.198±0.0773 [a]
CG	104	16.680±6.855[ab]	90.894±11.044[ab]	16.125±2.635[a]	0.891±0.108 [ab]	0.164±0.0672 [ab]
GG	11	14.687±3.046[b]	89.000±5.329[b]	16.272±2.370[a]	0.873±0.0522 [b]	0.144±0.0299 [b]

[#]N is the numbers of *T. rubripes* individuals. The data are presented as the mean ± SE of the three independent Genotype populations. [a,b, ab] Within the same column of each Genotype with different letters are significantly different (P-value < 0.05).

Discussion

Single nucleotide polymorphisms (SNPs) is the most frequently occurring form of variation in the animal genome. Many genes have many SNPs, which are mainly used in the studies of disease, even in very small samples (Constantine et al., 2008). By choosing "tag" SNPs, the SNPs can also be used to study the association between them and the production traits. This is an increasingly common approach to genetic association studies. This approach assumes that the variant and its haplotype are relatively in the general population and will be ascertained (Constantine et al., 2008). The candidate gene approach is a powerful method for investigating associations of gene polymorphisms with economically essential traits in farm animals (Rothschild and Soller, 1997). Many studies have examined growth, development, and immune function traits using the candidate gene approach in animals (Wang et al.,2010; Li et al.,2010; Gorissen et al., 2009). In our study, the LEP gene was selected as a candidate gene to investigate associations of gene polymorphisms with growth in cultured populations of *T. rubripes.*

Here we examined the presence of two *TrLEP* SNPs (C232G and C310T) in *T. rubripes*. It is, to our knowledge, the first report of significant association of these polymorphisms with growth traits in *T. rubripes*. In C232G mutation, *T. rubripes* with genotype CC of L2 had significantly higher body length, body weight, ABL, and ABW than genotype GG and CG, though this SNP was a synonymous mutation. Some other studies have shown the critical role of the LEP in body growth using SNPs. Kennes et al. (2001) found that two SNPs (A2845T and T3469C) in the LEP gene had a significant association with intake and growth rate in landrace pigs.

Buchanan et al. (2002 and 2003) found more excellent carcass fat and LEP mRNA expression in cattle T-homozygous for C305T (LEP gene exon 2) that encodes an Arg-Cys change at aa-4 in the mature LEP gene. This suggested higher feed intake by TT genotypes. The LEP gene exon-3 SNP (A59V) associated with weight was identified by Kulig and Kmiec (2009) in cattle. Boucher et al. (2006) reported three LEP gene SNPs in Dorset and Suffolk lambs. A103G associated with thickness and shear force and metabolic activity of *M. longissimus* in Suffolks Zhou et al. (2009) found three SNPs in exon 3 that result in amino acid changes and could affect LEP gene structure and function. These studies showed that SNPs in the LEP gene could affect body growth. Correlation analysis between markers and traits reaching a significant level may indicate the relationship between the characteristics and certain traits. But some SNPs of the LEP gene were not significantly associated with growth traits. Amills et al. (2008) genotyped Landrace for exon-3 C3469T and found no associations between TT or TC and plasma leptin level, growth rate, or carcass fatness. Promoter region SNP does not associate with leptin expression or fatness in Durocs (Stachowiak et al., 2007). In this study, three genotypes of C310T mutation were not significantly associated with growth traits.

Synonymous mutations do not change the protein-coding genes, but they still have a role that can't be ignored (Li and Zhou, 2007). C232G mutations were synonymous mutations in *TrLEP* in this study. The same phenomenon was found in previous studies. Kimchi-Sarfaty et al. (2007) found a "Silent" polymorphism in the MDR1 gene changed substrate specificity, whereas Montera et al. (2001) also found a silent mutation in exon 14 of the APC gene is associated with exon skipping in a FAP family. The synonymous mutations found in our studies might lead to protein with the same amino acid sequence but different structural and functional properties (Komar,2007).

The change of the senior structure of mRNA may affect its translation efficiency. All the second structures of *TrLEP* mRNA, C232G mutation(G-mRNA), and C310T mutation (T-mRNA) were predicted by RNA structure. The second structure of G-mRNA changed significantly, and the original stem-loop structures were broken basically. This might affect the function of the translated protein. In summary, the change of G-mRNA second structure would be a possible cause that genotype CC had significantly higher body length, body weight, ABL, and ABW than genotype GG and CG.

Correlation analysis between markers and traits reaching a significant level may indicate the relationship between the markers and specific traits. Thus, selective breeding based on phenotype can go to genotype-assisted selection (Wang et al.,2006). In this study, 296 cultivated *T. rubripes* individuals were screened for polymorphisms in the *TrLEP* using PCR-SSCP and DNA sequences. The genotype CC of primer L2 had a significantly higher body length, body weight, and absolute growth rate than genotype CG and GG ($P<0.05$). It showed that the CC genotype in the C232G site was an advantage genotype, significantly associated with growth traits. It can be endorsed as a molecular marker for the selection of *T. rubripes* individuals for their culture. The results present evidence that the *TrLEP* may be selected as a candidate gene for various growth traits to be implemented in the breeding program of *T. rubripes*.

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