Association study between single nucleotide polymorphisms in *leptin* and growth traits in *Cyprinus carpio* var. Jian

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ABSTRACT. Leptin is a hormone that affects the regulation of body weight, energy expenditure, fat metabolism, food intake, and appetite. In this study, we cloned the *jlLEP-A1* and *jlLEP-A2* genes in Jian carp (*Cyprinus carpio* var. Jian) and performed an association analysis between identified polymorphisms and growth traits. Three polymorphisms in exons of *jlLEP-A1* (A1-T113C) and *jlLEP-A2* (A2-G415A and A2-G427A) were identified, and genotyped by the polymerase chain reaction - restriction fragment length polymorphism method in 263 female and 294 male Jian carp. All three SNPs were missense mutations. Association analysis revealed that the three SNPs were significantly associated with growth traits in male Jian carp. Only SNP A1-T113C was significantly associated with growth traits in female Jian carp. Analysis of diplotypes derived from *jlLEP-A2* SNPs revealed an association with growth traits in male but not female Jian

carp. These results demonstrate that polymorphisms in the leptin gene are associated with growth traits and may be used for marker-assisted selection programs in Jian carp breeding and production.

Key words: *Leptin*; Single nucleotide polymorphisms; Growth traits; *Cyprinus carpio* var. Jian

INTRODUCTION

Leptin, a product of the obese (ob) gene, is a cytokine that was first discovered in mouse by Zhang et al. (1994). In mammals, leptin is involved in a diverse range of physiological functions, including appetite and body weight regulation, bone remodeling, fat metabolism, immune responses, and reproduction (Houseknecht et al., 1998; Moschos et al., 2002; Mácajová et al., 2004; Roubos et al., 2012). In fish, the function of leptin remains unclear, although several studies have been conducted on fish *leptin* genes. The first *leptin*-like gene was identified in the pufferfish (*Takifugu rubripes*) using syntenic gene analysis (Kurokawa et al., 2005). *Leptin* genes have subsequently been cloned from other teleost species, including common carp (*Cyprinus carpio*) (Huising et al., 2006), zebrafish (*Danio rerio*) (Gorissen et al., 2009), medaka (*Oryzias latipes*) (Kurokawa and Murashita, 2009), grass carp (*Ctenopharyngodon idella*) (Li et al., 2010), rainbow trout (*Oncorhynchus mykiss*) (Murashita et al., 2008), Atlantic salmon (*Salmo salar*) (Rønnestad et al., 2010; Angotzi et al., 2013), yellow catfish (*Pelteobagrus fulvidraco*) (Gong et al., 2013), orange-spotted grouper (*Epinephelus coioides*) (Zhang et al., 2013), and Jian carp (*Cyprinus carpio* var. Jian) (Tang et al., 2013). Unlike most vertebrates, which possess a single copy of *leptin*, several teleost fish have duplicate *leptin* genes, including zebrafish, Japanese medaka, Atlantic salmon, orange-spotted grouper, and Jian carp. Type A leptin was found to be present in the majority of species studied. In Jian carp, *jlLEP-A1* and *jlLEP-A2* are expressed in a wide range of tissues, which suggests that leptin-A may have pleiotropic physiological effects (Tang et al., 2013).

Advances in molecular genetics have led to the identification of DNA polymorphisms that affect traits of interest in animals. Analysis of single nucleotide polymorphisms (SNPs) have been increasingly utilized in various genetic disciplines, particularly in marker-assisted selection (MAS) programs. Several association studies of SNPs in the *leptin* gene with growth traits in farmed animals have been published, especially in chicken (Sadeghi et al., 2012), swine (Pérez-Montarelo et al., 2012), cattle (da Silva et al., 2012), and fox (Zhang and Bai, 2015). In teleost fish, however, only the studies of Wei et al. (2013) and Huang et al. (2014) have reported polymorphisms in the orange-spotted grouper (*Epinephelus coioides*) *leptin-a* gene and their associations with growth traits.

Jian carp (*Cyprinus carpio* var. Jian) was first bred artificially at the Freshwater Fisheries Research Center of the Chinese Academy of Fishery Sciences (FFRC, CAFS) and is now widely cultured in China. *Leptin* is a potential candidate gene that influences growth traits in Jian carp; therefore, the objectives of this study were to investigate polymorphisms in the *leptin* gene and to explore the relationship between genotypes and growth traits. The results of this study may provide a basis for MAS programs aiming to improve growth traits and preserve important genetic resources.

MATERIAL AND METHODS

Animals and phenotypic data

A total of 557 (female 263; male 294) samples comprising 12 full-sibling families were used, which were created by crossing 12 sires and 12 dams from Yixing Breeding Base at the Freshwater Fisheries Research Center of the Chinese Academy of Fishery Sciences (Wuxi, China). The offspring were cultured in different cages for the first month. About 50 individuals per family were randomly selected, passive integrated transponder (PIT) tagged, and mixed in the same pond when the fish reached ~25 g in weight. Considering that the growth rate differs between the juvenile and adult stages, the body weight gain (BWG) was measured during the two developmental stages respectively. The BWG of the juvenile and adult stages in Jian carp was defined by subtracting the initial BW from juvenile BW, and juvenile BW from adult BW, respectively. All animal experiments complied with the Guidelines for the Care and Use of Laboratory Animals of Jiangsu Province, China.

PCR amplification and SNP discovery

Blood samples (0.5 mL each) were collected from the tail vein of each animal using disposable syringes containing the anticoagulant citrate-dextrose. Genomic DNA was extracted using a Blood Genome DNA extraction Kit (Takara, Dalian, China), following the manufacturer recommendation. The concentration of genomic DNA was determined using a UV spectrophotometer.

To discover putative SNPs in *jlLEP-A1* and *jlLEP-A2*, DNA samples from eight unrelated Jian carp parents were selected. Nucleotide sequences of the primers and restriction enzymes used are shown in Table 1. PCR was performed in a 12.5-μL reaction volume containing 100-200 ng genomic DNA using HotStar Taq (Takara, Dalian, China). The thermal cycle consisted of 3 min initial denaturation at 94°C, 30 cycles of 30 s at 94°C, 30 s at 56-58°C, and 1 min at 72°C, followed by a 5 min final extension at 72°C. The *jlLEP-A1* and *jlLEP-A2* genes were cloned as previously described (Tang et al., 2013). The sequences were aligned by the Clustal X program to identify potential SNPs. Nucleotide sites that contained an alternative base in two or more individuals were considered putative SNP loci.

Table 1. Primers, restriction enzymes, and restriction fragments of *leptin* SNPs.

Locus	Primers	Restriction enzyme (T)	Fragments (bp)
A1-T113C	*jlLEP-A1*-1F: CTGCACTGGTGCCAAGTTTAA	*Bse*GI (55°C)	TT: 333
	jlLEP-A1-1R: ACCTCAGGGTAAAGTTCTGGATC		CC: 109, 224
A2-G415A	*jlLEP-A2*-1F:CCACTTCAAAAGGGACAAATATGTACC	*Xag*I (37°C)	AA: 792
	jlLEP-A2-1R:CTTGAAAATCCTCTTGGCCTCGACT		GG: 382, 410
A2-G427A	*jlLEP-A2*-2F:CCACTTCAAAAGGGACAAATATGTACC	*Alu*I (37°C)	GG: 181
	jlLEP-A2-2R:CTTGAAAATCCTCTTGGCCTCGACT		AA: 129, 52

SNP genotyping

The SNPs in *jlLEP-A1* and *jlLEP-A2* were genotyped using the polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) method. Pairs of primers were designed using Primer Premier 5 software. Restriction enzymes (Thermo, USA),

including *Bse*G I, *Xag* I, and *Alu* I, were selected based on the mutation sites. The amplification products were completely digested in a 10-μL reaction solution containing 6 μL PCR products and 0.1 μL enzyme at the appropriate temperature for 1-2 h. The genotypes of samples were identified by 2% agarose gel electrophoresis.

Statistical analysis

Allele and genotype frequencies were calculated using a simple allele counting method. Hardy-Weinberg equilibrium was tested for goodness-of-fit by comparing expected and observed genotype frequencies using a Chi-square test (Rodriguez et al., 2009). Association analysis between genotypes of SNPs in *leptin* and growth traits was performed using the general linear model (GLM) procedure with SPSS 15.0 software. We used the following statistical model: $Y = u + G + e$ where, Y is the phenotypic value of a growth trait; u is population mean value of growth traits, G is the fixed genotype effect of each SNP, and e is the random error effect. Multiple comparisons between different genotypes were tested using the least significant difference (LSD) method. $P < 0.05$ was considered as statistically significant and $P < 0.01$ was considered as extremely significant.

RESULTS

Identification and genotyping of SNPs

The DNA sequence of *jlLEP-A1* (GenBank accession No. KC496017) and *jlLEP-A2* (GenBank accession No. KC496018) were 741- and 792-bp long, respectively. *jlLEP-A1* and *jlLEP-A2* contained an intron of 93 and 102 bp, respectively, and both contained an open reading frame (ORF) of 516 bp, which encoded a 171-amino acid protein. Five SNPs were identified by the alignment of *jlLEP-A1* and *jlLEP-A2* fragments from eight individuals. Two SNPs (*jlLEP-A1*-G533A, *jlLEP-A2*-G360A) out of five were not analyzed because there was no available restriction enzyme for RFLP genotyping. Therefore, the three SNPs in exons of *jlLEP-A1* (A1-T113C) and *jlLEP-A2* (A2-G415A and A2-G427A) were examined. All three SNPs were missense mutations. There was a T/C substitution at position 113 bp of the *jlLEP-A1* gene, which replaced serine with proline, and a G/A substitution at 415 and 427 bp of the *jlLEP-A2* gene, which each replaced arginine with lysine. Three SNPs were genotyped by PCR-RFLP in 263 female and 294 male Jian carp. Enzyme digested products of A1-T113C, A2-G415A, and A2-G427A SNPs in partial samples are shown in Figure 1A, B, and C, respectively.

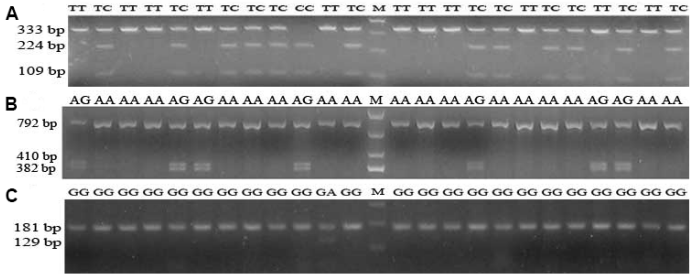

Figure 1. Digestion production of three SNPs. **A.** A1-T113C by *Bse*GI; **B.** A2-G415A by *Xag*I; **C.** A2-G427A by *Alu*I.

At locus A2-G427A, there were only two genotypes (GG and GA). Exact tests showed that one locus (A1-T113C) deviated significantly from Hardy-Weinberg equilibrium ($P < 0.05$) in the test population of Jian carp. At locus A1-T113C, the frequency of the TT genotype (53.6%) was higher than that of the TC genotype (43.3%) in female Jian carp, while the opposite was true in male Jian carp. At locus A2-G415A, the frequency of the AA genotype (61.660.9%) was higher than that of the GG genotype (3.0/3.4%) in female and male Jian carp. At locus A2-G427A, the G allele occurred significantly more frequently (96.697.8%) than allele A, and the frequency of the GG genotype (93.2/95.6%) was higher than that of the GA genotype (6.8/4.4%) in female and male Jian carp (Table 2).

Table 2. Frequencies of genotypes and alleles of three SNPs of *leptin* in female and male Jian carp.

Locus	Sample size (female/male)	Genotype frequencies [female/male (%)]			Allele frequencies [female/male (%)]		P (female/male)
A1-T113C	263/294	TT	TC	CC	T	C	0.007*/4.2E-07*
		53.6/46.6	43.3/51.7	3.1/1.7	75.3/72.4	24.7/27.6	
A2-G415A	263/294	AA	AG	GG	A	G	0.216/0.252
		61.6/60.9	35.4/35.7	3.0/3.4	79.3/78.7	20.7/21.3	
A2-G427A	263/294	GG	GA	AA	G	A	0.566/0.698
		93.2/95.6	6.8/4.4	0/0	96.6/97.8	3.4/2.2	

*Significant at the $P < 0.05$ level.

Haplotype and diplotype analysis

Based on genotype data for *jlLEP-A2* (A2-G415A and A2-G427A) SNPs, four haplotypes were found in the Jian carp population, the frequencies of which are summarized in Table 3. Haplotype 1, which is the most common haplotype, had an estimated frequency of 0.761/0.778 in female and male Jian carp. Another common haplotype, haplotype 3, occurred only at a frequency of 0.203/0.200 in female and male Jian carp. The two common diplotypes, H1H1 and H1H3, composed of haplotype 1 and 3, respectively, accounted for 89.7 and 92.5% of all diplotypes in female and male Jian carp (Table 4). Only one male Jian carp with the diplotype H3H4 was deleted in the following association analysis.

Table 3. Pattern and frequency of each reconstructed haplotype in the genotyped female and male population. The individual alleles for each haplotype are given in the order A2-G415A and A2-G427A.

	H1	H2	H3	H4
Haplotype	AG	AA	GG	GA
Frequency (female/male)	0.761/0.778	0.032/0.010	0.203/0.200	0.004/0.012

Table 4. Frequency of each reconstructed diplotype in the haplotyped female and male population.

Diplotype	H1H1	H1H3	H1H2	H3H3	H1H4	H3H4
Frequency (female/male)	0.570/0.595	0.327/0.330	0.046/0.01	0.030/0.031	0.027/0.027	0/0.003

Association of SNPs and diplotypes of SNPs with growth traits

Associations of *leptin* gene polymorphisms with growth traits were analyzed by one-way ANOVA in SPSS 15.0 software. The results are shown in Tables 5 and 6. All three single SNPs (A1-T113C, A2-G415A, and A2-G427A) showed significant or extremely significant

associations with growth traits in male Jian carp. Only one SNP (A1-T113C) was significantly or extremely significantly associated with growth traits in female Jian carp. Diplotype-based analysis indicated that five diplotypes were significantly associated with growth traits in male Jian carp.

Table 5. Association of SNPs with body weight gain in male and female Jian carp.

Locus	Genotype	Juvenile stage	Adult stage	Sample size
Male Jian carp				
A1-T113C	TC	100.38 ±1.70	683.80 ± 10.63^{a}	152
	TT	96.61 ± 2.39	652.28 ± 11.18^{b}	137
	CC	98.84 ± 10.28	690.12 ± 46.54^{ab}	5
A2-G415A	AA	99.60 ± 1.75^{a}	670.27 ± 9.18^{A}	179
	AG	98.64 ± 5.60^{a}	679.34 ± 13.86^{A}	105
	GG	80.26 ± 3.36^{b}	544.11± 31.12^{B}	10
A2-G427A	GG	99.30 ± 1.47^{a}	671.52 ± 7.94^{a}	281
	GA	83.46 ± 4.02^{b}	619.41 ± 16.51 ^{b}	13
Female Jian carp				
A1-T113C	TC	110.41 ± 2.24^{A}	828.89 ± 14.27^{a}	114
	TT	103.30 ± 2.33^{AB}	787.82 ± 12.98^{b}	141
	CC	83.44 ± 6.02^{B}	757.66 ± 50.12^{ab}	8

Data are reported as means ± SE. Different capital letters in the same column show extremely significant differences ($P < 0.01$); different small letters in the same column show significant differences ($P < 0.05$).

Table 6. Association of diplotypes with body weight gain in male Jian carp.

Diplotype	Juvenile stage	Adult stage	Frequency
H1H1	99.93 ± 1.77^{a}	670.20 ± 9.37^{ab}	175
H1H3	99.98 ± 2.77^{ab}	686.54 ± 14.72^{a}	97
H3H3	79.69 ± 3.70^{c}	535.34 ± 33.39^{c}	9
H1H4	82.40 ± 2.41^{bc}	591.96 ± 15.35^{bc}	8
H1H2	85.10 ± 13.45^{abc}	673.40 ± 31.13^{abc}	4

Different lowercase letters in the same column represent a significant difference ($P < 0.05$).

When specimens were assigned to three groups based on their genotype at the A1-T113C locus, the group with the TC genotype performed significantly better for BWG than did the other groups (CC and TT) at the juvenile and adult stages. However, this difference was not observed in the male juvenile stage.

At the A2-G415A locus, males who inherited the GG genotype were significantly inferior for BWG compared to those who inherited the AA or AG genotypes at both the juvenile and adult stages; however, there was no significant difference among genotypes in females. At the A2-G427A locus, BWG was significant only in males, for which the GG group showed significantly better performance than the GA group. No difference was observed for this trait in females at this locus.

Male Jian carps possessing H1H3 had the highest values for growth traits, while those with H3H3 had the lowest values for growth traits. At the juvenile stage, the growth traits of Jian carp with H1H1 had significantly higher values than those of Jian carp with H3H4 and H1H4. However, the growth traits of Jian carp with H1H3 were significantly higher than those with H3H4 and H1H4.

DISCUSSION

In the present study, one SNP and two SNPs were found in *jlLEP-A1*and *jlLEP-A2*,

respectively. The three SNPs could be successfully genotyped by the PCR-RFLP method. There are always two paralogues genes with high sequence similarity within the Jian carp genome; therefore, it is difficult to find and identity SNPs between the two paralogous genes. When SNPs exists in AT rich regions, it is clear that appropriate primers cannot be designed by the PCR-RFLP method. SNPs should be widely used in fish breeding with other methods of detection such as tetra-primer PCR method and the amplification refractory mutation system (tetra-primers ARMS) (Ye et al., 2001) and intron-primed exon-crossing (IPEC) (Ryynänen and Primmer, 2006).

Several studies have showed that introns span a much larger portion of the genome than exons (Venter et al., 2001), and introns are usually more highly diversified than adjacent exons (Özlem and Dursum, 2011). As a result, introns include more polymorphisms than exons. However, all three SNPs identified in the present study existed in *leptin* exons and were missense mutations. There were no significant differences in phenotypic characterization, which implies that the mutated amino acids were not essential and did not change the function of *leptin*.

Leptin polymorphisms may have an effect on growth traits in animal husbandry. Yang et al. (2007) reported that *leptin* may be a candidate gene for growth traits such as height at hip cross and was applied to MAS in Chinese cattle breeds. Kulig and Kmieć (2009) showed that the average daily weight gain between 3 and 210 days of age was significantly higher in Limousin cattle possessing the CT/CT haplotype compared with those possessing the CC/CC and CC/CT haplotypes for the A59V polymorphism in exon 3 of the *leptin* gene. Clempson et al. (2011) discovered that four *leptin* SNPs (A1457G, A59V, UASMS1, UASMS2) were associated with different types of growth performance in Holstein cows. Recently, Wei et al. (2013) reported that several polymorphisms in the *leptin-a* gene are associated with growth traits and can be used for MAS in orange-spotted grouper populations. In conclusion, these reports have revealed that the *leptin* gene may be an important candidate gene associated with growth traits.

Single SNP analysis is a traditional tool in MAS. Recently, haplotypes and/or diplotype analysis has gradually been used to elucidate the relationships between candidate gene polymorphisms and economical traits (Zuo et al., 2014). The results of our study clearly demonstrate that one and three SNPs were significantly and extremely significantly associated with growth traits in female and male Jian carp, respectively. Diplotype analysis derived from two SNPs showed that these were only associated with growth traits in male Jian carp. Jian carp with H1H1 and H1H3 had higher values for growth traits than those with other diplotypes. However, Jian carp with H3H3 had the lowest values for growth traits in five diplotypes. These results suggest that the H1 and H3 haplotypes may have positive and negative effects on growth traits, respectively. Growth traits could be ultimately affected by the interaction of SNPs. *Leptin* SNPs could be applied as useful genetic markers in future Jian carp breeding programs.

Growth is a typical quantitative trait controlled by multiple genes. It is necessary to detect more polymorphisms in other growth-related genes and to evaluate major effects of single and combined gene markers. The results of these kinds of studies will be of importance to MAS in fish.

Conflicts of interest

The authors declare no conflict of interest.

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