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Single nucleotide polymorphisms of Δ6-desaturase and Elovl5 segments and their associations with common carp (*Cyprinus carpio*) growth traits

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ABSTRACT. Highly unsaturated fatty acids (HUFAs) are crucial for the nutritional health, physiology, and reproduction of vertebrates. The Δ6-desaturase and Elovl5 elongase genes produce essential enzymes in the biosynthetic pathway of HUFAs. Single nucleotide polymorphism (SNP) analysis of genes functionally related to the growth traits of the common carp (*Cyprinus carpio* var. Jian) can provide useful information for common carp molecular breeding. In this study, we isolated two Δ6 Fad genes and two Elovl5 genes from the common carp. Polymerase chain reaction-restriction fragment length polymorphism was performed, and the genotypes of three SNPs (Δ6Fad-a intron 10_C73T, Δ6Fad-b intron 10_A56G, and Elovl5-a intron 5_C64A) in 712 individuals (383 females and 329 males) were detected. Correlation analysis between the genotypes and weight gain revealed that intron

10_C73T of Δ6Fad-a, intron 10_A56G of Δ6Fad-b, and intron 5_C64A of Elovl5-a were significantly associated with common carp weight gain. Weight gain increased with the enrichment of molecular SNP markers, consistent with the characteristics of quantitative traits. Our results indicate that Δ6Fad and Elovl5 elongase genes could be candidate genes for the molecular breeding of the common carp. This study provides useful information for the improvement of this species.

Key words: Δ6-desaturase; Elovl5; *Cyprinus carpio* var. Jian; Single nucleotide polymorphism; Growth trait

INTRODUCTION

Fish are an important source of n-3 highly unsaturated fatty acids (HUFAs), which have beneficial health effects in humans (Eilander et al., 2007; Ruxton et al., 2007). The biosynthesis of HUFAs in fish involves the sequential desaturation and elongation of precursor C_{18} polyunsaturated fatty acids (PUFAs), Δ6-desaturase, and Elovl5 elongase, which are crucial enzymes in the biosynthetic pathway of HUFAs (Tocher et al., 2004; Zheng et al., 2004). Δ6-desaturase catalyzes the insertion of double bonds at the Δ6 position in the carbon backbone, and Elovl5 elongase is responsible for the pre-existing chain (Jakobsson et al., 2006).

The identification of molecular genetic markers for growth rate is attracting increasingly more attention in the selective breeding of fish. Once an association between a DNA polymorphism and a trait is found, this polymorphism is considered a potential genetic marker for marker-assisted selection (MAS) programs. Genetic markers that are either linked to loci that affect economically important traits, or have a direct effect themselves, can be used to genetically improve animals.

The common carp (*Cyprinus carpio* var. Jian) is farmed throughout China. As part of a MAS program to improve growth rate in the species, growth-related genes, such as myostatin and growth hormone receptor, have been studied in our laboratory, and we have identified growth-rate markers (Yu et al., 2010, 2011). However, no studies on the common carp Δ6-desaturase and Elovl5 elongase genes have been conducted, despite it having been extensively studied.

Although association studies cannot determine whether gene markers are responsible for variations in a trait or variations are due to closely linked loci that affect the trait, there is evidence to suggest that the gene affects the trait. Therefore, the aim of the present study was to identify polymorphisms in the Δ6-desaturase and Elovl5 elongase genes, and to analyze associations between the polymorphisms and growth rate in the common carp. The results of this study may provide a better understanding of the effects of Δ6-desaturase and Elovl5 elongase genes on common carp growth traits.

MATERIAL AND METHODS

Fish sampling

A total of 712 individuals, including 383 females and 329 males, were passive integrated transponder-tagged and used in the experiment after four months of breeding. All of the fish were fed in the same pond at the Yi Xing site of the Freshwater Fisheries Research Center, Chinese Academy of Fishery Science. Body weight was recorded at two and 15 months after tagging, and venous blood was collected for genomic DNA extraction.

DNA extraction

Genomic DNA was extracted using the classic phenol-chloroform method (Sambrook et al., 1989) and preserved in TE buffer. DNA was examined by performing 1% agarose gel electrophoresis, and concentrations were determined using photometry (Eppendorf, German). The working DNA concentration was 50 ng/μL.

Isolation of Δ6-desaturase and Elovl5 segments

Primers were designed based on Δ6-desaturase and Elovl5 genomic sequences (data not shown) (Table 1). JLFAD-E9F and JLFAD-I11R-a were used to amplify partial exon 9, intron 9, exon 10, intron 10, and partial exon 11 of Δ6Fad-a, and JLFAD-E9F and JLFAD-I11R-b were used to amplify partial exon 9, intron 9, exon 10, intron 10, and partial exon 11 of Δ6Fad-b. JLELO-E5F-A and JLELO-E6R-A were used to amplify partial exon 5, intron 5, and partial exon 6. The primer sequences are shown in Table 1. Polymerase chain reaction (PCR) amplifications using the primers and *Taq* DNA polymerase (Takara, Japan) were performed with an initial denaturation at 94°C for 2 min, 30 cycles of denaturation at 94°C for 30 s, annealing at Tm (annealing temperature according to different primers) for 30 s, and extension at 72°C for 1 min, followed by a final extension at 72°C for 8 min. The gel-purified PCR products were cloned into a pMD18-T vector (Takara，Japan), and positive clones were sequenced by Biosune Inc., Shanghai, China.

Table 1. Characteristics of the primers used in the study.

Primer	Sequence (5'→3')	Tm (°C)	Product
JLFAD-E9F	CAGATGAGCCACATCCCCATG	57.3	Partial E9, I9, E10, I10, partial E11
JLFAD-I11R-A	CACTGGCGCCTTCTGCAAGAA	60.0	
JLFAD-E9F	CAGATGAGCCACATCCCCATG	57.3	Partial E9, I9, E10, I10, partial E11
JLFAD-I11R-B	ATAGGAATAGTTCGGAGCAAGAGCAC	57.5	
JLELO-E5F-A	ATCTTCTGATTGTGTGGATGGGACC	59	Partial E5, I5, partial E6
JLELO-E6R-A	GTGAAATGTGACATGCTCTTGACAGG	58.7	

I, intron; E, exon.

Screening for polymorphic sites

Primers designed according to the above sequences were used to amplify DNA from 10 randomly chosen fish (Table 1). The amplified fragments were sequenced and aligned using Clustal W (Thompson et al.,1994). A locus was considered to be an SNP site if it had an alternative base in four or more animals of the 10 samples. JLFAD-E9F-a and JLFAD-I11R-a were used to screen for SNP sites in intron 9, exon 10, and intron 10 of Δ6-Fad-a; JLFAD-E9F and JLFAD-I11R-b were used to screen for SNP sites in intron 9, exon 10, and intron 10 of Δ6-Fad-b; and JLELO-E5F-A and JLELO-E6R-A were used to screen for SNP sites in partial exon 5, intron 5, and partial exon 6 of Elovl5-a. We designed the primers to examine three SNP sites by PCR-restriction fragment length polymorphism (PCR-RFLP) (Table 2). The 5-μL PCR product was mixed with 0.15 μL endonuclease, 1.0μL buffer, and 3.85μL ddH_2O, and digested at a temperature that was dependent on the enzyme used for between 4 and 6h. The digestion product was then separated by 2% agarose gel electrophoresis to determine the allele type.

Table 2. Primers, restriction enzymes, and restriction fragments of single- nucleotideloci.

Gene region	Primer and sequence (5'→3')	Restriction enzyme and temperature	Length of restriction fragment
Δ6-Fad-a	F: ACGCATCACAGAACTGCGTATGTG	*Hpy*F3I, 37°C	CC: 809
I-10_C73T	R: TGCTTAAACCCAGTGCGGCTGT		TT: 221,588
Δ6-Fad-b	F: CAGTCGCATGACTGTGGTGTGAATG	*Hha*I, 37°C	GG: 352,67
I-10_A56G	R: CGCCAGTAATTGTGCCGAGGC		AA: 419
Elovl5-a	F: ACTGTGTAAGTGGAAAATCAGAAGTGGG	*Tas*I, 65°C	AA: 373,208,239
I-5_C64A	R: CACCATTCGATTCGATTCACATTC		CC: 581,239

Sequence analysis and data processing

Sequences were assembled using DNASTAR (Lasergene, San Francisco, CA, USA), and aligned with Clustal W (Thompson et al., 1994). A general linear model in SPSS17.0 (SPSS Inc., USA) was used to analyze relationships between the alleles and growth traits.

RESULTS

Analysis of Δ6-desaturase and Elovl5 and screening for polymorphic sites

Based on the results of a preliminary experiment, two Δ6-desaturase (Δ6Fad-a and Δ6Fad-b) and two Elovl5 (Elovl5-a and Elovl5-b) genomic DNA fragments were cloned from the common carp. Segments of the Δ6Fad-a, Δ6Fad-b, and Elovl5-a genomic sequences were amplified from 10 randomly selected carp and aligned with Clustal W. Sites with an alternative base in four or more samples were counted as valid SNP sites. We identified nineteen SNPs in the Δ6Fad-a segment, seven in the Δ6Fad-b segment, and six in the Elovl5-a segment. Three SNPs were examined using the PCR-RFLP technique, one in intron 10_C73T of Δ6Fad-a, two in intron 10_ A56G of Δ6Fad-b, and one in intron 5_C64A of Elovl5-a (Figure 1).

Assessment of polymorphic loci and associations with body weight

Relationships between the genotypes and body weight were investigated in 712 samples (Table 3). Significant differences were found in intron 10_C73T of Δ6Fad-a between the genotypes and body weight. Multiple comparison results indicated that individuals with the TT genotype were significantly heavier than those with the CC genotype ($P < 0.05$). Significant differences were also found in intron 10 _A56G of Δ6Fad-b between the genotypes and body weight. Multiple comparison results indicated that individuals with the GG genotype were significantly heavier than those with the AA genotype ($P < 0.01$). Significant differences were found in intron 5_C64A of Elovl5-a between the genotypes and body weight. Multiple comparison results indicated that individuals with the CC genotype were significantly heavier than those with the AA genotype ($P < 0.05$).

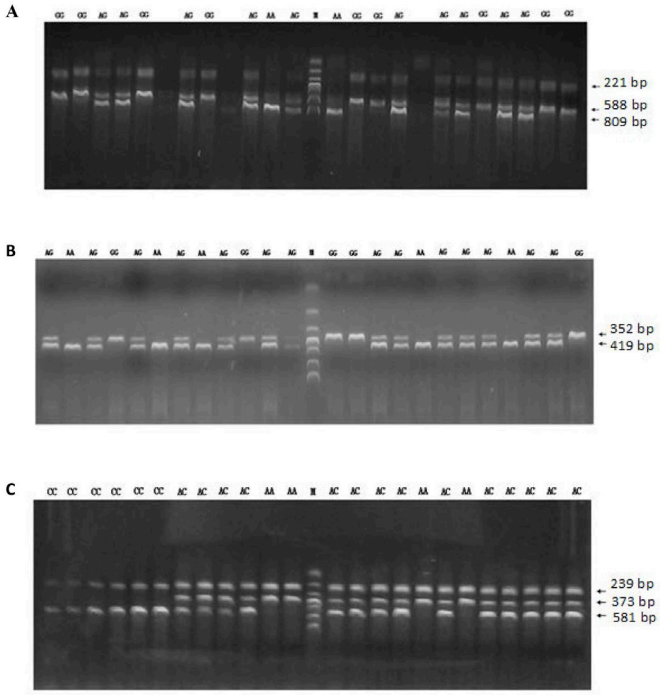

Figure 1. Electrophoresis patterns of different genotypes in three single nucleotide polymorphic loci. **A.** Δ6-Fad-a, I-10_C73T; **B.** Δ6-Fad-b, I-10_A56G; **C.** Elovl5-a, I-5_C64A.

Table 3. Correlations between Δ6-desaturase and Elovl5 genotypes and weight gain in *Cyprinus carpio* (mean ± SE).

SNP locus	Genotype	Juvenile		Adult	
		Male	Female	Male	Female
Δ6-Fad-a I-10_C73T	CC	98.48 ± 8.64 (n + 113)	91.57 ± 3.19^{b} (n + 101)	648.77 ± 10.68^{b} (n + 113)	769.65 ± 12.49^{b} (n + 101)
	CT	96.56 ± 3.03 (n + 88)	106.60 ± 3.36^{a} (n + 76)	671.05 ± 15.34^{ab} (n + 88)	815.83 ± 20.69^{ab} (n + 76)
	TT	101.20 ± 3.59 (n + 81)	110.52 ± 4.49^{a} (n + 79)	696.60 ± 15.58^{a} (n + 81)	853.49 ± 18.29^{a} (n + 79)
P		0.891	<0.001	0.045	0.002
Δ6-Fad-b I-10_A56G	AA	92.87 ± 7.81 (n + 128)	92.02 ± 3.49^{b} (n + 95)	634.88 ± 9.52^{b} (n + 128)	769.75 ± 13.89^{b} (n + 95)
	AG	98.31 ± 2.75 (n + 108)	100.71 ± 3.54^{b} (n + 97)	650.55 ± 11.87^{b} (n + 108)	799.64 ± 17.17^{b} (n + 97)
	GG	102.91 ± 3.32 (n + 76)	112.87 ± 3.54^{a} (n + 79)	736.13 ± 16.63^{a} (n + 76)	868.26 ± 17.45^{a} (n + 79)
P		0.059	<0.001	<0.001	<0.001
Elovl5-a I-5_C64A	AA	93.21 ± 2.71^{b} (n + 126)	99.44 ± 4.12 (n + 103)	643.23 ± 9.59 (n + 126)	763.48 ± 25.74^{b} (n + 103)
	CA	98.26 ± 2.38^{b} (n + 152)	98.68 ± 3.18 (n + 127)	666.55 ± 10.42 (n + 152)	822.15 ± 12.83^{ab} (n + 127)
	CC	109.26 ± 8.16^{a} (n + 32)	106.32 ± 4.95 (n + 41)	679.48 ± 27.52 (n + 32)	833.69 ± 24.13^{a} (n + 41)
P		0.036	0.515	0.177	0.046

Within the same column, different letters indicate a significant difference at $P < 0.05$. SNP, single nucleotide polymorphism.

Marker enrichment and body weight

The present study identified three markers that were associated with weight gain (Table 4). Individuals with the TT genotype in intron 10_C73T of Δ6Fad-a, the GG genotype in intron 10_ A56G of Δ6Fad-b, and the CC genotype in intron 5_C64A of Elovl5-a gained more weight than individuals with other genotypes. We also found significant differences between individuals with different numbers of markers: individuals with three markers were significantly heavier than those with one or no markers ($P < 0.01$).

Table 4. Effect of marker enrichment on weight gain.

Number of markers	Juvenile weight gain	Adult weight gain	Sample size
0	92.18 ± 2.65^{b}	722.38 ± 13.33^{b}	130
1	100.15 ± 1.43^{b}	749.45 ± 7.42^{b}	504
2	104.62 ± 3.93^{ab}	802.73 ± 21.44^{ab}	68
3	118.96 ± 5.43^{a}	838.08 ± 34.30^{a}	9
P	0.007	0.004	

Within the same column, different letters indicate a significant difference at $P < 0.05$.

DISCUSSION

The Δ6-desaturase and Elovl5 elongase gene DNA fragment sequences were spliced and isolated, while the Δ6Fad-a and Δ6Fad-b DNA sequences were cloned, both being comprised of 13 exons and 12 introns, and the Elovl5-a and Elovl5-b DNA sequences were also cloned, both being comprised of eight coding exons and seven introns. Segments of Δ6Fad-a, Δ6Fad-b, and Elovl5-a were isolated, and SNP loci were detected using three primer pairs (Table 1). The exon sequences were very similar, but the introns differed in size; however, the 5' and 3' ends of the introns contained similar sequences. Intron termini are highly conserved, because they are important for RNA splicing (Fedorova and Fedorov, 2003). A total of 32 polymorphic loci were found in Δ6Fad-a, Δ6Fad-b, and Elovl5-a; 26 were found in the introns, whereas only six were found in the exons, which indicates that there was greater variation in the introns than in the exons.

Three loci were genotyped using PCR-RFLP, and the positive results confirmed that this method could accurately identify genotypes. However, this method requires specific primers, and RFLP also requires a restriction site. Therefore, it may not be suitable for all loci, particularly for loci in introns, as they may contain many repetitive sequences and have a higher AT content. Although many other methods exist, including gene chips and probes for high-throughput SNP analysis, they are expensive and require special equipment. Therefore, it is necessary to develop other detection methods.

Dietary long-chain PUFAs are very important to the nutritional health, physiology, and reproduction of vertebrates (Burr, 1981; Simopoulos, 2000), and are crucially important during early development because of the profound morphological and physiological changes that occur (Tocher et al., 2003). HUFAs have long been recognized as essential components of fish larval diets (Sargent et al., 1999), and play both energetic and structural roles, depending on the class of lipid molecule that they are incorporated in. Δ6-desaturase and Elovl5 elongase are critical enzymes in the biosynthetic pathway of HUFAs (Tocher et al., 2004; Zheng et al., 2004), and may affect growth and development in fish. In the present study, three loci that

were closely related to juvenile and adult carp growth rates were examined. Individuals with mutated genotypes grew faster than those with other genotypes, and those with three markers were heavier than those with one or no markers. Therefore, Δ6Fad-a, Δ6Fad-b, and Elovl5-a play an important role in the common carp. Our results suggest that it is feasible to select molecular makers from candidate genes. In addition, because growth is a quantitative trait that is controlled by multiple genes, markers in other genes that control growth, appetite, muscle development, and cell proliferation should also be selected. To stabilize and develop a high-quality strain, it is necessary to adhere to pedigree selection, and to develop more markers for growth.

In conclusion, this is the first study to report an association between Δ6-desaturase and Elovl5 elongase genes and growth traits in the common carp, and will provide useful information for the improvement of this species.

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