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Association between growth hormone gene polymorphisms and growth traits in wild common carp, *Cyprinus carpio* from the Caspian Sea

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Abstract

Growth hormone (GH) is the most important hormone that controls somatic cell growth and syntheses of protein, fat, and carbohydrates. This study was conducted to identify gene polymorphism of GH-1 using PCR-single strand conformation polymorphism (SSCP) technique and its association with growth traits including condition factor (CF), body weight (BW) and total length (TL) in common carp (*Cyprinus carpio*). A total of 150 carps in 4 age classes of 4, 6, 12 and 24 months were randomly selected and DNA was extracted from the caudal fin by modified salting out method. A fragment of 373 bp from exon 4, intron 4, and exon 5 of GH-1 gene was amplified using polymerase chain reaction (PCR). Genotyping of samples by SSCP analysis yielded 8 different banding patterns as A, B, C, D, E, F, G and H with frequencies of 31.33, 10.67, 20.67, 22.67, 4.0, 2.0, 2.67 and 6.0 %, respectively. Analysis of marker-trait correlation by General Linear Model (GLM) showed significant associations between carp GH-1 banding patterns and body weight, but the gene was not significantly associated with TL and CF parameters. The body weights of fish with a banding pattern of D were significantly (p<0.05) higher than the other genotypes. Considering the economic importance of common carp and the positive association between body weight and the banding pattern found in this study, the marker site in GH-1 gene could be used in marker-assisted selection (MAS) in this species. To achieve a reliable conclusion, further experiments employing an appropriate sample size are required.

Keywords: *Cyprinus carpio*, Growth hormone gene, PCR-SSCP.

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Introduction

Common carp (*Cyprinus carpio*) from the family Cyprinidae, is native to Central Asia, and has been grown in different parts of the world over the centuries (Sheikhzadeh *et al*., 2011; Yousefian, 2011). The Caspian Sea is the most important habitat for the native common carp as one of the economic fishes and also an important food source. Hence, there is a large-scale stock releasing program for resource enhancement of common carp in the sea (Yousefian, 2011). Although *C. carpio* naturally exists throughout the Caspian Sea coastal waters and enters the estuaries for reproduction, due to over-fishing and loss of spawning areas, its generation has declined in recent years rendering the need to protect this species in the region (Ghelichpour *et al*., 2010; Fallahbagheri *et al.*, 2013).

Growth hormones (GH) play essential roles in growth regulation, cell division, and cell differentiation and enlargement. It has long been shown that increases in the rate of growth are higher in animals especially in fish as a result of GH manipulation (Sundt-Hansen *et al*., 2009; Zhong *et al*., 2013). GH stimulates growth directly by increasing the synthesis of DNA, protein, and also by lipolysis in muscles. Indirectly, however, the growth process is controlled through the production and release of a mitogen called IGF-I produced by both the liver and most of the peripheral tissues (Zhong *et al*., 2013).

Research has shown that the growth hormone gene in fish is not protected. For instance, GH in carps has four introns and five exons similar to those in mammals (Ho *et al*., 1991) while most other fishes have an additional exon (six exons and five introns) (Agellon *et al*., 1988). Exon size is approximately equal in all fish species except that Exon 5 is divided into two parts during the evolution (Almuly *et al*., 2000). It is assumed that the addition of Intron 5 to Exon 5 as well as division of Exon 5 into two parts have led to a divergence between cypriniformes and other teleosts (Ber and Daniel, 1993).

Various studies on animals have shown that the growth hormone gene accounts for a candidate gene affecting some production traits such as growth and disease resistance, and those candidate genes typically have roles in the regulation of specific metabolic pathways targeted to specific impacts on quantitative traits (Yowe and Epping, 1996; De-Santis and Jerry, 2007). Functional genomic regions known to affect growth in better-studied vertebrates can be explored as candidate genes for growth in teleost fishes (Tao and Boulding, 2003). In candidate gene studies, first the polymorphisms of putative genes are examined, and then the correlation between specific alleles and phenotypic expression of the trait is tested. If certain relationships were found, they evidence a direct effect of the gene on the genetic control of the trait (Yowe and Epping, 1996; De-Santis and Jerry, 2007). Therefore, geneticists are seeking alleles of growth hormone gene axis that are associated with phenotypes of low and/or high growth rates (Tao

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and Boulding, 2003). Mutations at different regions of genes have been regarded by many professionals of eugenics. Gene polymorphism associated with a particular phenotype such as growth has widely been studied in other animals; nonetheless, similar investigations are limited in fish species (Gross and Nilsson, 1999).

The efficacy of PCR-based single strand conformation polymorphism (PCR-SSCP) method is quick and easy to do, and in addition, because even a single-base change in a sequence is likely to result in several copies, very close species can be carefully separated even with very short pieces (Teletchea, 2009). SSCP point mutation detection rate is 80%-90% at different systems, hence, it is an efficient, convenient, and economical detection of mutations (Xin and Jin-Gou, 2011). Examples of SSCP application include polymorphism detection in intron 2 of GH-I gene in crucian carp, *Carassius carassius* (Mo *et al*., 2004), determination of seven different haplotypes (a, b, c, d, e, f, g) in nine breeding lines (Polish, Hungarian, German, Ukraine, and Lithuanian) of *C. carpio* and their associations with resistance to different pathogens (Rakus, 2008), and very recently, PCR-SSCP identification of polymorphic GH genes associations with fish growth traits in the Chinese perch, *Siniperca chuatsi*, with three genotypes significantly associated with growth performance (Tian *et al*., 2014). This technique, to the best of our knowledge, has not ever been examined in *C. carpio* from the Caspian Sea. Additionally, the genetic mechanism underlying different traits has not yet been elucidated in this species (Xu *et al*., 2012). Therefore, this research aimed at the identification of polymorphism in GH-1 gene and estimation of gene and genotype abundances using PCR-SSCP technique through observation of different allelic forms in this locus and their relations to growth traits in the common carp.

Materials and methods

A total of 150 common carp specimens produced by the breeders at the southern Caspian Sea were obtained from Nasr Fish Culture Co., Sari, in the north of Iran. The fish were at the ages of 4 (n=84), 6 (n=5), 12 (n=54), and 24 months (n=7) with unknown maturity ages and genders. Caudal fin samples (2-3 g) were collected and fixed in 96% ethanol and preserved at −20 °C until DNA extraction. The collected samples were transferred to the Laboratory for Molecular Genetics and Animal Biotechnology at Sari Agricultural Science and Natural Resources University and genomic DNA was extracted using a modified salting out method. The quality and quantity of DNA were determined using spectrophotometry and agarose gel electrophoresis. To identify gene polymorphisms in GH-1 of common carp, specific primers of:

F-5'-GGAAGCTTAACCCCAACCAGCTCACTGAGAA-3' and R-5'-CTACAGGGTGCAGTTGGAATCCAG-3' were used to amplify a 373-bp fragment from exon 4, intron 4, and exon 5. In addition, a pair of oligonucleotide

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primers for PCR amplification and sequencing was designed to match the highly conserved sequences of the *GH* gene from common carp *C. carpio* L. (Gross and Nilsson, 1996) (Accession No. U21920). The reaction mixture (25 µL) contained 200 ng of template DNA, 2.5 mL of a buffer, 1.5 mM of $MgCl_2$, 10 pmol of each of forward and reverse primers, 200 mol dNTP, one unit of *Taq*- polymerase enzyme (Cinnagen Co., Tehran, Iran), and di-ionized water. The desired locus was amplified in a thermocycler apparatus (BioRad, USA) at 95 °C for 3 min including these steps: primary denaturation, 30 cycles at 95 °C for 30 s, primer annealing at 65.4°C for 30 s, primer extension at 72°C for 60 s, and a final extension at 72°C for 5 min. After PCR denaturation, the amplified products were electrophoresed on 10% polyacrylamide overnight at 4°C (Teglab, 41-2025, Germany). The fragments were stained with silver nitrate for counting the bands and genotyping the fish samples. Genotype frequencies were calculated using the software Popgene, version 1.32 (Quinn and Keough, 2002). The effects of each observed genotype on the condition factor (CF), body weight (BW) and total length (TL) were estimated by the following statistical model (GLM: General Linear Model) with SAS software (Version 9.1) (Quinn and Keough, 2002):

$Yij = \mu + G_i + Ag_j + e_{ij}$, where Y_{ij}: observations of the studied traits, µ: population mean for the trait in question, G_i: the i^{th} effect of banding patterns, Ag_j is the effect of age and e_{ij} is the residual random error. The means of different band patterns were compared with Duncan's multiple range tests. It should be noted here that the fish age was considered as a covariate in the analyses. Furthermore, because of high differences in body size, the 24-month age group was not included in the statistical analyzes. As noted above, the carp population studied were gender-mixed so the sexes were not separately examined.

Results

In this study, a 373-bp fragment from exon 4, intron 4, and exon 5 of GH-1 gene was amplified in common carp (Fig. 1). This fragment is registered with a similar size in NCBI. The SSCP analysis showed eight different banding patterns as A, B, C, D, E, F, G, and H with frequencies of 31.33, 10.67, 20.67, 22.67, 4.0, 2.0, 2.67, and 6.0 %, respectively, in the studied fish population (Table 1).

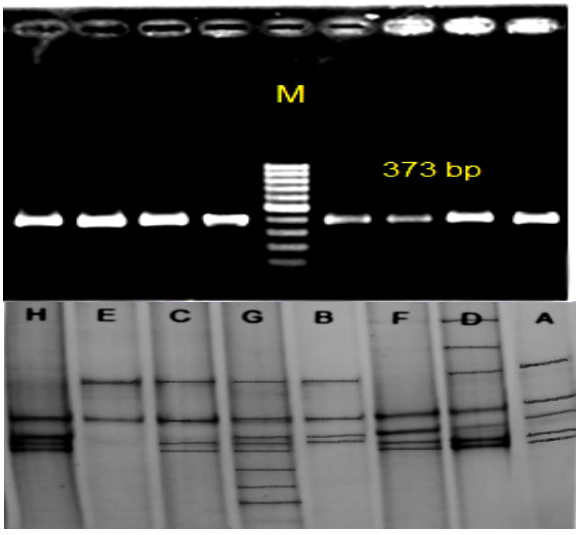


Figure 1: PCR product of Exon 4, Intron 4, and Exon 5 of GH-I gene (left) and banding patterns observed for GH-I gene (right) in common carp; M: molecular weight marker (mi-E8200s, 50 µg).

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The association studies on marker-trait showed significant association between GH-1 gene polymorphisms in carp and BW but the gene was not associated with both TL and CF parameters (Table 2). Fish with a banding pattern of D genotype were significantly (p<0.05) heavier than the other genotypes (Table 2).

Table 1: Genotypic frequencies observed in GH-1 loci of *C. carpio* samples (n= 150).

Banding pattern	Frequency (%)
A	31.33
B	10.67
C	20.67
D	22.67
E	4.0
F	2.0
G	2.67
H	6.0

Table 2: Results of GLM analysis and comparison of the least square means of different banding patterns.

locus	genotype	BW	TL	CF	Age (month)
GH-1	A	23.75^{d}	11.76^{a}	1.23^{a}	4,6,12
	B	27.33^{b}	12.06^{a}	1.27^{a}	4,6,12
	C	25.71^{d}	11.97^{a}	1.26^{a}	4,6,12
	D	30.52^{a}	12.50^{a}	1.26^{a}	4,6,12
	E	23.39^{c}	12.17^{a}	1.16^{a}	4,6,12
	F	26.67^{d}	12.57^{a}	1.15^{a}	4,6,12
	G	22.31^{d}	11.57^{a}	1.26^{a}	4,6,12
	H	24.03^{c}	11.68^{a}	1.29^{a}	4,6,12
P- value		0.04^{*}	0.09^{ns}	0.7^{ns}	
SEM		1.65	0.22	0.016	

* Significant, ns: Non significant, Means with same superscript in each column do not have significant differences (p<0.05).

Discussion

The GH-1 polymorphism of *C. carpio* sampled from the Nasr Fish Culture Co., Sari, north of Iran, offered eight different genotypes with significant relationships between GH-1 and body weight at three age groups of 4, 6 and 12 months. The results suggest that some allelic forms of GH-1 are capable of stimulating the growth of common carp, but the functional performances are somewhat different with different genotypes detected. Genotype D presented the highest frequencies followed by genotypes C and B. Likewise, in seven wild crucian carp colonies with a sample number almost corresponding to that of our carp samples, seven length types (A, B, C, D, E, F, and G) were determined in intron 2 of GH-1 by PCR-SSCP method, for which the observed numbers ranged from 3 to 6, 6 to 10, and 2 to 6, respectively, with base pair lengths of 189, 196, 204, 205, 206, 207, and 209 bp (Mo *et al*., 2004).

These findings suggest various genetic diversities of GH-1 in different carp populations. Also, in hybrid and mirror carps, six SNPs were detected in

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intron 3 and exon 3 of growth hormone gene by PCR-SSCP method (Hu and Ying, 2010). In another similar research, results of PCR-SSCP in a population of yellow croaker fish, *Larimichthys crocea*, yielded two haplotypes of intron 1, i.e. genotypes AA and AB (Ni *et al*., 2012). In AB genotype, a single nucleotide polymorphism (SNP) was noticed, which was negatively associated with the body weight (Ni *et al*., 2012). This is not in agreement with what was found in the present study for the carp at all the ages of 4, 6, and 12 months as the carp samples with D-banding pattern gained higher weights than the other genotypes; one of the main causes for this discrepancy might be the difference in the number of samples.

Most polymorphisms detected in fish GHs have been reported to be established in introns (Forbes *et al*., 1994; Park *et al*., 1995; Gross *et al*., 1996; Almuly *et al*., 2000; Mo *et al*., 2004). Consequently, the mutations detected here should also have occurred in the introns, though, the exact determination requires DNA sequencing.

Whereas introns are non-coding regions of genes, mutations in GH introns could nonetheless effectively contribute to the gene's transcription, translation, expression, and other processes hence influencing the growth and developmental stages of the fish (Ni *et al*., 2012). It was also suggested that the GH gene association of growth traits with polymorphisms at the DNA level may be due to the self-regulation of the growth hormone gene (Kang *et al*., 2002).

According to the marker-trait association study, the studied region of GH-1 gene signified statistical association with weight gain in our samples of common carp whereas it revealed no associations with both TL and CF parameters. This finding is in concordance with Tian *et al*., (2014) in a *S. chuatsi* population, for which four novel SNPs were detected in the GH gene from 282 individuals; all of the SNPs presented median polymorphisms and significant associations with growth traits indicating that these SNPs had large genetic variations and selection potentials. Similar to our study, a significant association between *g*.4940A>C SNP and body weight has been observed in *S. chuatsi* population.

There was no significant association between GH gene polymorphisms and length of body in the present study, whereas a genetic correlation of 0.98 was reported between weight and length in 8-week-aged *C. carpio* determined by eight microsatellite markers (Vandeputte *et al*., 2004). According to this high correlation, it is expected that with a larger sample size significant association between GH gene and body length can be detected in *C. carpio* population. The growth traits are also under the effects of other genes such as second copy of growth hormone (GH-2), GH receptor (GHR), insulin-like growth factor 1 and 2 (IGF-I and II), GH-inhibiting hormone (GHIH), somatostatin (SRFI14), and GH-releasing hormone (GHRH) (Murkaeva, 2008). Further research is needed on the

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simultaneous impacts of all these genes in larger populations. It is also necessary to examine more individual fish as well as more stocks to establish highly significant correlations between determined polymorphisms and growth traits.

The association between GH-1 genotypes and growth traits including body weight, body length and condition factor of *C. carpio* was assessed by SSCP technique in the present study. This technique proved to be appropriate in polymorphism screening for the carp samples. The polymorphic genotypes observed here may be useful markers for growth traits in the selection of common carp. Due to a limited sample size, however, the findings of this study should further be evaluated through a sufficient sample size.

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