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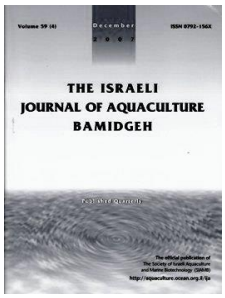

Polymorphism in Growth Hormone Gene and its Association with Growth Traits in *Siniperca chuatsi*

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Key words: *Siniperca chuatsi*, Growth hormone, High-resolution melting, SNP, Growth traits

Abstract

Growth hormone (GH) is a candidate gene for growth traits in fish. In this study, we assessed associations between single nucleotide polymorphisms (SNPs) in GH gene with growth traits in 357 *Siniperca chuatsi* individuals using high-resolution melting. Two SNPs were identified in GH gene, with one mutation in exon 5 (g.5045T>C), and one mutation in intron 5 (g.5234T>G). The corrections analysis of SNPs with the four growth traits was carried out using General Linear Model (GLM) estimation. Results showed that both of them were significantly associated with growth performance in *S. chuatsi*. For g.5234T>G, it was significantly associated with body weight ($P<0.01$), body length ($P<0.05$), body depth ($P<0.01$), and body width ($P<0.01$), and the individuals of genotype GG grew faster than those of genotypes TT and TG ($P<0.05$). A further diplotype-trait association analysis confirmed that in fish with H3H2 (TC-GG) diplotype body weight, body length, and body width was greater than in those with other diplotypes ($P < 0.05$). These results demonstrated GH gene SNPs could be used as potential genetic markers in future marker assisted selection of *S. chuatsi*.

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Introduction

Growth hormone (GH) gene is very closely associated with growth, development, reproduction, food conversion, immune function, and appetite of fish (Quick *et al*., 2010; Clayton *et al*., 2010). Therefore, the identification of GH gene allelic variation may provide useful genetic markers for selection of fish with desirable growth traits. Extensive research has suggested that GH is a very important candidate gene for growth performance in fish. The genetic polymorphism of GH gene have been reported in several fish, such as brown trout *Salmo trutta* (Gross and Nilsson, 1995), rainbow trout *Oncorhynchus mykiss* (Rezaei, 2012),Chinook salmon *Oncorhynchus tshawytscha* (Park *et a*l., 1995), *Salmo truttacaspius* (Rezaei, 2012), Atlantic salmon *Salmo salar* (Rezaei, 2012), bleak *Alburnus alburnus* (Schlee *et al*., 1996), and common bream *abramis brama* (Gross *et al*., 1996). SNPs of GH gene have been reported to be associated with weight in Atlantic salmon (Gross and Nilsson, 1999), growth and immune function in Chinook salmon (Docker and Heath, 2002), and growth traits in large yellow croaker *Larimichthys crocea* (Ni *et al*., 2012).

Chinese perch *Siniperca chuatsi* is the most economically and geographically important freshwater fish in China. However, some desirable characteristics, such as fast-growing and high disease resistance and suitability, have inhibited rapid development of its cultivation. Although the GH gene has been cloned and identified in *S. chuatsi* (Liu *et al*., 2009), there are relatively few reported studies about the relation between genetic markers of GH gene and growth traits in this species. In recent years, wild *S. chuatsi* were collected and some purebred and crossbred strains were produced in our laboratory. Relative growth performances have been assessed (unpublished) and polymorphisms of insulin growth factor (IGF-I) were found to significantly affect the growth traits of cross-sinipercid species (Wang *et al*., 2013). The current study aimed to correlate SNP variation with individual growth traits according to the results of earlier studies. We cloned GH gene sequences, described a high resolution melt (HRM assay) to identify polymorphisms and genotypes of GH, and investigated whether they were associated with growth traits in *S. chuatsi* population. There is potential for the application of GH gene polymorphisms associated with growth traits in future *S. chuatsi* breeding programs and to improve the efficiency of the selection process in this species.

Materials and Methods

Animals and sampling. At the Shunde Lvyuan Fish Farm in Guangdong Province, *S. chuatsi* were hatched and cultured in a pond (1500 fish /667m^2) where competition existed between fish. The 357 individuals were randomly selected at the age of 8 months. Blood (0.2-0.5 μl) was collected from caudal vein of each fish and preserved at -80℃. Total genomic DNA was extracted from the blood using the TIANamp Blood DNA Kit (Tiangen, Beijing, China). Four body measurements, body weight (BWT), body length (BL), body depth (BD), and body width (BWH), were recorded for association analysis with single nucleotide polymorphisms (SNPs) and growth traits.

Design of primers and mutation screening by HRM. 5 pairs of HRM primers were designed to amplify partial regions of GH gene according to *S. chuatsi* DNA sequence (GenBank: EF205280) (Figure 1), and were analyzed for specificity in all target regions. Annealing temperatures were kept around 60 ℃ using Primer Premier 5.0 software (Table 1).

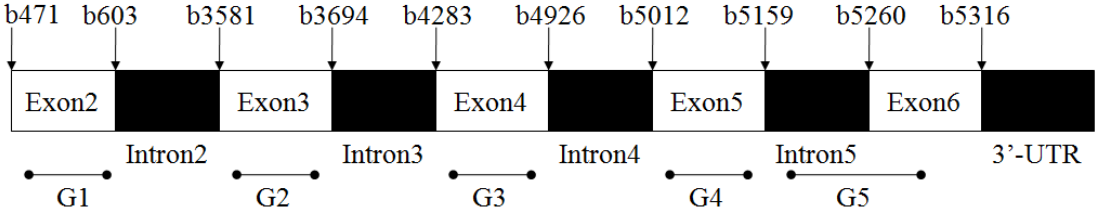

Fig. 1. Schematic structure and amplified fragments of growth hormone gene for this study. White boxes, black boxes and bold lines represent exons, introns and amplified fragments. Numbers represent the start of end positions of exons.

Table 1. Primer sequences and information of sinipercid specie GH genes

Names	*Primer sequences (5'>3')*	*Tm(℃)*	*Length(bp)*	*Amplicons*
G1	F: GTTGTCCTCCTGCTGTCGG R: TCTGAGCGAGCAGGTGGA	59	118	Exon2
G2	F: CGGAGGAGCAGCGTCAACT R: GCGTTGTGTCTCGTGCTTGTC	61	95	Exon3
G3	F: TATCGATTGGTTGAGTCTTGG R: CAGCAGGATTCCCGTCTTC	59	111	Exon4
G4	F: TCAGGACGGAGCCGAGAT R: CCAGCAGTTCGTATGTTCGTC	60	116	Exon5
G5	F: GGAAGAGGAGGGGTATGATGT R: ATTTAGCCACCGTCAGGTAGG	60	118	Intron5 and exon6

F: Forward primer; R: Reverse primer

The 357 DNA samples were assayed for polymerase chain reaction (PCR) amplifications using the LightCycler480 system (Roche, Barcelona, Spain). Each 10μl reaction contained about 25 ng diluted genomic DNA, 1x LightCycler480 HRM Master Reaction Mix (Roche) 5 μl, 2.5 mM $MgCl_2$, and 200nM primers (PAGE purified). Standard samples (known genotypes by sequenced) and non-template control samples were added for each amplicon tested. The same PCR program and melting conditions were used for all amplicons: 95℃ for 10 min; 45 cycles of 95℃ for 10 s, 59-61℃ for 15 s and 72℃ for 10 s; 95℃ for 1 min; 40℃ for 1 min; a melt of 65-95 ℃ (0.02℃/s, 25 acquisitions/℃); and 40℃ for 30 s (1.5℃/s). Melting curves were analyzed using Gene Scanning software (Roche). PCR products of each type were sequenced directly by Beijing Genomic Institute.

Genetic polymorphism analysis. The population genetic indexes including Homozygosity (Ho), Heterozygosity (He), effective number of alleles (Ne), polymorphism information content (*PIC*) were calculated by Nei's method (Weller, 1994).

$$Ho = \sum_{i=1}^{n} (P_i)^2$$

$$He = 1 - \sum_{i=1}^{n} (P_i)^2$$

$$N_e = 1 / \sum_{i=1}^{n} P_i^2$$

P_i: the frequency of the allele i; n: the number of allele.

$$PIC = 1 - \sum_{i=1}^{n} p_i^2 - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2 p_i^2 p_j^2$$

P_i: the frequency of the allele i; P_j: the frequency of the allele j; n: the number of alleles. Generally, *PIC* is classified into the following three types, low polymorphism (*PIC*<0.25), median polymorphism (0.25<*PIC*<0.5) and high polymorphism (*PIC*>0.5).

Statistical models and analysis. The alleles and genotype frequencies of each SNP were calculated by Microsoft Excel. Haylotypes of these SNPs were analyzed using Arlequin 3.0 software. The two haplotypes were merged into a diplotype. Associations between genotypes and diplotypes of SNPs of GH gene and growth traits were analyzed using GLM procedure of SPSS17.0 software. The following models were used.

$$Y = \mu + G \text{ or } D + e$$

Where *Y* is the measured value of the growth trait, μ is the mean value of the growth trait, *G or D* refers to the fixed effects of genotypes of each SNP or diplotype, and *e* is the random error effect. Considering that all of the experimental fish were selected from the same site and weighed and measured at the same age, the statistical model only included the fixed effect of the genotype. Significant differences among means of different genotypes were calculated using Tukey's HSD method in the GLM program and *P* values of 0.05 were considered statistically significant.

Results

Polymorphisms of GH gene in Chinese perch population. The HRM method was applied to investigate the potential sequence variations of genomic DNA of the GH gene from 357 *S. chuatsi* individuals. PCR productions of P4 and P5 were found to be polymorphic. Two SNPs with one mutation in exon 5 (g.5045T>C, synonymous mutation), other mutations in intron 5 (g.5234T>G) were identified in GH gene.

Frequencies of genotypes and genetic polymorphic paraments. Table 2 shows the genotype and allele frequencies, Ho, He, Ne and *PIC*. The CC genotype was not found at locus g.5045T>C. Allele T was the dominant allele for the two SNPs g.5045T>C and g.5234T>G. A relatively low frequency of the genotype GG was 16% for the SNP g.5234T>G. The frequency of heterozygous genotype was 48% and 41% respectively which was high at the two SNPs loci. The Ne of g.5234T>G was rather high (1.855). According to the classification of *PIC*, the population from five *S. chuatsi* families belonged to the median polymorphism level (0.25 < *PIC* < 0.5).

Table 2. Frequencies of genotypes and alleles, genetic polymorphic parameters of two SNPs in Siniperca chuatsi GH gene

Loci	*g.5045T>C*		*g.5234T>G*	
Genotypes frequencies	TT	0.52	TT	0.43
	TC	0.48	TG	0.41
	CC	0	GG	0.16
Allele frequency	T	0.76	T	0.64
	C	0.24	G	0.36
Homozygosity	0.635		0.539	
Heterozygosity	0.365		0.461	
Ne	1.575		1.855	
PIC	0.299		0.355	

Ne, effective number of alleles; PIC, polymorphism information content.

Association between SNPs with growth traits. Association analysis of the two SNPs of GH gene with growth traits in *S. chuatsi* individuals was carried out using least square estimation (Table 3). Statistical results suggest that the association between the two SNPs and growth performance was significant. g.5045T>C exhibited significant effect on BWH ($P<0.05$). The relationship between g.5234T>G and all four growth traits in *S. chuatsi* population was highly significant ($P<0.01$).

Multiple comparisons of growth traits with highly significant associations in different genotype were also presented in Table 3. Statistical results showed that individuals with the GG genotype at locus g.5234T>G had a significantly faster growth rate than those of genotypes TT and TG on BWT ($P<0.01$), BL ($P<0.05$), BD ($P<0.01$), BWH ($P<0.01$) in *S. chuatsi* population. At the position of g.5045T>C, *S. chuatsi* with the TT genotype had significantly greater values for BWH than those with the TC genotype ($P<0.05$).

Table 3. Association of genotypes of two loci (least square means±SD) with growth traits in *Siniperca chuatsi* GH gene

Loci	*Genotype*	*Traits*			
		BWT (g)	*BL (cm)*	*BD (cm)*	*BWH (cm)*
g.5045T>C	TT(185)	370.48±9.39	23.05±0.48	9.34±0.20	$3.79±0.10^{b}$
	TC(172)	346.87±10.47	22.51±0.82	9.07±0.13	$3.49±0.07^{a}$
g.5234T>G	TT(154)	$261.13±26.45^{a}$	$20.77±0.67^{a}$	$8.11±0.26^{a}$	$3.44±0.13^{a}$
	TG(147)	$315.29±14.14^{b}$	$21.84±0.36^{ab}$	$8.75±0.14^{b}$	$3.68±0.07^{b}$
	GG(56)	$381.32±24.94^{c}$	$23.12±0.63^{b}$	$9.41±0.25^{c}$	$4.42±0.13^{c}$

The number of individuals of each genotype is in brackets.
^{a,b,c} Different superscript letters of mean within a column means significant difference at $P<0.05$.
BWT, body weight; BL, body length; BD, body depth; BWH, body width.

Haplotype and diplotypes analysis. Based on genotype data for the two SNPs, 3 haplotypes were found in *S. chuatsi* population. Table 4 suggested that the three haplotypes had an estimated frequency 49.17%, 30% and 20.83% respectively. Further, the 5 diplotypes were observed in *S. chuatsi* GH gene in Table 4.

Table 4. Frequency of haplotype and diplotype in *Siniperca chuatsi* GH gene

Haplotype	*g.5045T>C*	*g.5234T>G*	*Frequency%*	*Diplotype*	*g.5045T>C*	*g.5234T>G*	*Frequency %*
H1	T	T	49.17	H1H1	TT	TT	20.00
H2	C	G	30.00	H1H2	TC	TG	45.00
H3	T	G	20.83	H3H2	TC	GG	15.00
				H1H3	TT	TG	13.33
				H3H3	TT	GG	6.67

The diplotype H1H2 accounted for 45% of all diplotype frequency. Association analysis showed that there was significant correlation between diplotypes and growth traits ($P < 0.05$). Multiple comparison analysis was performed in the five diplotypes groups. H2H4 had significantly higher values ($P<0.05$) than those diplotypes on BWT, BL and BWH (Table 5).

Table 5. Association between diplotypes of GH gene and growth traits in *Siniperca chuatsi*

Diplotye	*Traits*			
	BWT(g)	*BL(cm)*	*BD(cm)*	*BWH(cm)*
H1H1	306.08±24.45^{a}	21.53±0.57^{a}	8.70±0.25	3.44±0.13^{a}
H1H2	318.60±16.30^{a}	21.97±0.38^{ab}	8.82±0.17	3.71±0.07^{ab}
H3H2	383.07±28.23^{b}	23.34±0.65^{b}	9.33±0.29	4.05±0.13^{b}
H1H3	337.23±29.94^{ab}	22.27±0.69^{ab}	8.88±0.31	3.44±0.18^{a}
H3H3	361.78±42.34^{ab}	22.93±0.98^{ab}	9.30±0.43	3.76±0.26^{ab}

^{a,b} Different superscript letters of mean within a column means significant difference at $P<0.05$.
BWT, body weight; BL, body length; BD, body depth; BWH, body width.

Discussion

The correlation of DNA markers and traditional selection breeding programs has increased in popularity in aquaculture. DNA markers can accelerate genetic improvement by marker-assisted selection that increases the rate of the selection of genetic improvements by 25 to 50% compared to classical selective breeding programs (Moav *et al*., 1960). Application of DNA markers to breeding programs may be important for the aquaculture industry. In addition to microsatellite markers, SNPs could be important for aquaculture, as they are a relatively new 'tool' in breeding programs. To date, there have been a few screened candidate genes for growth via SNP markers in Arctic charr *Salvelinus alpinus* (Tao and Boulding, 2003), *Penaeus monodon* (Glenn *et al*., 2005), Asian seabass *Lates calcarifer* (Xu *et al*., 2006) and freshwater prawn *Macrobrachium rosenbergii* (Thanh *et al*., 2010). In this study, GH gene was chosen as a candidate gene for growth in *S. chuatsi*, and two SNPs (g.5045T>C, g.5234T>G) were detected from 357 individuals. The high heterozygosity of two SNPs (0.365and 0.461, respectively), showed median polymorphisms (0.299 and 0.355, respectively), indicating large selection potential and genetic diversity in the two SNPs.

For the GH gene, only two genotypes were detected at locus g.5045T>C in *S. chuatsi* in this study. It is possible that alternative homozygotes involve a lethal allele causing mortality. Similar results have been reported involving the SS genotype at locus UNH231 in some tilapia *Oreochromis aueus* families (Palti *et al*., 2002) and the TT genotype at crustacean hyperglycemic hormone gene g.2407 and g.2409 in freshwater prawn (Thanh *et al*., 2010). The motifs that are important splicing signals often were conserved and found near exon-intron boundaries. This suggests that intronic sequences, including enhancer or other cis-acting elements could control genetic transcription (Bachl *et al*., 1998). The enhancers and silencers as regulatory elements may be located near 50-100 bp from the splice sites to regulate normal splicing of exonic sequences (Pagani and Baralle, 2004).The SNP g.5234T>G present in GH gene fifth intron showed a strong association with growth performance of *S. chuatsi*. It was located from 75 bp distant from the exon 5-intron 5 boundary and 19 bp from the intron 5- exon 6 boundary (Figure 2). Maybe the SNP g.5234T>G alter the splicing process or influence recognition of

splicing sites that lead to increased or decreased synthesis of GH gene. Therefore, it could affect fish growth traits. Similar research results have been reported in Arctic charr (Tao and Boulding, 2003), dwarf chicken (Huang *et al*., 1993), and freshwater prawn (Thanh *et al*., 2010).

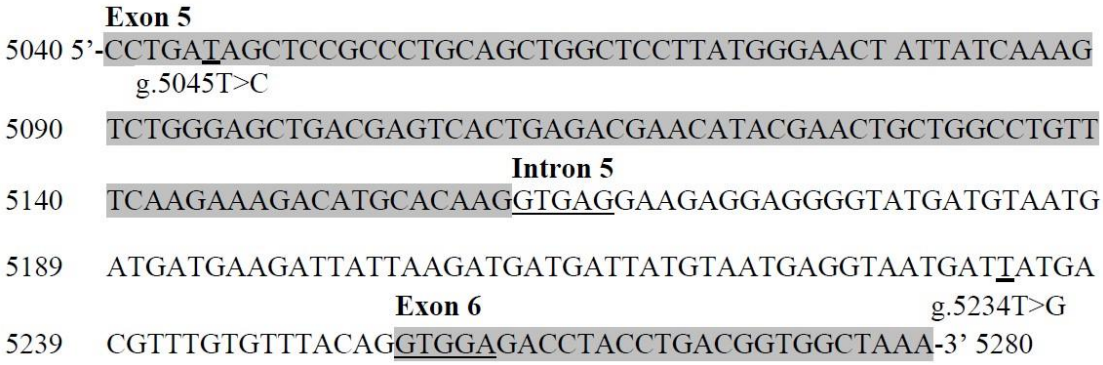

Fig. 2. The positions of two SNPs (g.5045T>C, g.5234T>G) in growth hormone gene. Shaded sequences are exon 5 and exon 6, unshaded sequence is intron 5.

In this study, two SNPs were detected in the GH gene from 357 *S. chuatsi* individuals. The SNP g.5045T>C was synonymous mutation in exon 5. Synonymous mutation can change any single base except amino acids. However, the codon of the same amino acids has a different frequency of use, which can affect translation efficiency, translation speed, and the content of intracellular tRNA (Nickerson *et al*., 1998). This can explain how the locus g.5045T>C could be significantly associated with body width of *S. chuatsi*, the TT genotype having a wider body than the TC genotype. The SNP g.5234T>G in the GH gene intron 5 had a very significant effect on growth traits in *S. chuatsi*, with GG genotype demonstrating faster growth speed than other genotypes ($P < 0.01$). It is hypothesized that the polymorphism of GH introns plays important roles in the genetic transcription, translation, and expression, thereby influencing growth and development of fish. Significant association between SNPs and other traits have been reported in some aquatic species. For example SNPs in amylase gene are associated with growth rate in oyster *Crassostrea gigas* (Prudence *et al*., 2006), parvalbumin gene correlated with growth traits in Asian seabass (Xu *et al*., 2006), cytochome P450-c19a and estrogen receptor α gene associated with reproductive traits in Japanese flounder *Paralichthys olivaceus* (He *et al*., 2008; He *et al*., 2008).

In certain genes, adjacent SNPs were found to be interconnected. The SNPs analyzed in association with some traits may be more accurate than a single SNP. (Weiss and Terwilliger, 2000). Haplotype which is a physical arrangement of SNP alleles on a single chromosome may provide some insight into factors influencing the dependency among SNPs. In this study, there were 3 haplotyes and 5 diplotypes. Associated studies indicated that the diplotype H3H2 (TC-GG genetype) had greater values on BWT, BL and BWH ($P < 0.05$) than the other four diplotypes. In Japanese flounder, although the single SNP of estrogen receptor beta gene has no significant association with reproductive indices, the diplotypes significantly affected reproductive indices (Shi *et al*., 2009). Similar results were found in SNPs of IGF-II gene in largemouth bass (Li *et al*., 2012). The outcome of our study is preliminary and further study is necessary to identify more SNPs in other areas of GH gene to confirm the SNP-trait association in larger independent populations.

In conclusion, our study found significant correlation between polymorphism in the GH gene and individual growth traits in *S. chuatsi*. However, further investigation is needed in a larger population to validate the correlation. Ultimately, SNPs can contribute to the marker-assisted selection (MAS) program for genetic improvement in *S. chuatsi.* From an aquaculture point of view, it seems important that once faster growing populations are selected, they must be isolated to avoid loss of genetic material due to occasional hybridization with wild populations.

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