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NON-SYNONYMOUS POLYMORPHISM IN *IGFBP-3* GENE ASSOCIATED WITH GROWTH TRAITS IN STRIPED CATFISH (*Pangasianodon hypophthalmus*, Sauvage, 1878)

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SUMMARY

Insulin-like growth factor binding protein-3 (IGFBP-3) has an important role on the growth of teleost fish. The association between genetic variations of *IGFBP-3* gene and the growth of striped catfish, *Pangasianodon hypophthalmus*, was investigated in this study. To discover SNPs, fragments of *IGFBP-3* gene from 10 fast-growing fish and 10 slow-growing fish were directly sequenced. Among 10 putative SNPs, a non-synonymous SNP 704 C>G (p.Leu8Val) located at coding region of exon 1 was filtered and subjected to validate in 70 fast-growing fish and 70 slow–growing fish by individual genotyping. Our statistical analysis showed the significant association between SNP 704 C>G (p.Leu8Val) and growth traits of striped catfish ($p < 0.01$). The genetic diversity of the SNP was evaluated by polymorphism content (PIC) and minor allele frequency (MAF). These values indicated that this SNP was common variant with moderate genetic diversity. The non-synonymous SNP 704 C>G (p.Leu8Val) in *IGFBP-3* gene is a potential candidate for subsequent development of molecular marker for growth traits in breeding of the striped catfish.

Keywords: genetic variations, growth, *IGFBP-3*, MAS, *Pangasianodon hypophthalmus*, SNP, striped catfish

INTRODUCTION

Identification of association between polymorphism in candidate genes and the traits of interest is an effective approach to discover gene markers which can be potential for marker-assisted selection. Genes belong to Insulin-like Growth Factor (IGF) system are reported as interesting candidates because the proteins play a central role in the neuroendocrine regulation of the growth of all vertebrates

(Allard, Duan, 2018; Tran *et al.*, 2021). Insulin-like growth factor binding protein 3 (IGFBP-3) is one of six ancestral subtypes of IGFBPs (IGFBP-1, -2, -3, -4, -5, -6) which binds to insulin-like growth factor (IGF) with high affinity (delaSerrana, Macqueen, 2018). IGFBP-3 carriers IGF in the circulation through ternary complexes with the acid-labile subunit (ALS) (Hossner *et al.*, 1997; Winston *et al.*, 2006). The ternary complex stabilizes mature IGFs and increases IGF half-life, so that regulates IGF bioavailability (Winston *et al.*, 2006; Kannian, Ryan, 2019).

In teleost fish as well as other vertebrates, IGF system plays essential role in muscle growth. IGFBP-3 expression in teleost fish has been examined, that elucidates the function of the protein in muscle growth. As the growth contributing factor, *IGFBP-3* gene in muscle of salmon increased the expression during growth-supporting condition as post-fasting refeeding (Macqueen *et al.*, 2013), and growth hormone (GH) transgenic (Alzaid *et al.*, 2018). Moreover, the down regulation of *IGFBP-3* in skeletal muscle of fine flounder (*Paralichthys adspersus*) as response to crowding chronic stress also indicated the directly effect on the growth of this gene (Valenzuela *et al.*, 2018). In flounder and yellowtail, significant increase of the expression of *IGFBP-3* genes in liver and/or muscle in response to food deprivation elucidated the function nutritional regulation of *IGFBP-3* (Pedroso et al., 2009; Safian et al., 2012). Although the significant association between the expression level of *IGFBP-3* gene and the growth of teleost fish was well illustrated, there has a few studies investigating the association between the genetic variations in this gene and the growth traits.

The striped catfish, *Pangasianodon hypophthalmus* Sauvage, 1878, is classified into the Asian catfish family Pangasiidae (Roberts, Vidthayanon, 1991). In total of the world production of air-breathing fish in inland, the production of striped catfish took approximate a half, with 2520.4 thousand tons of live weight (FAO, 2022). The striped catfish is majorly cultured in the Mekong river delta in Vietnam and its production in Vietnam is the biggest over the world (Phan *et al.*, 2009; Kim *et al.*, 2018; Fletcher, 2020). To improve the economically important traits of striped catfish such as growth rate, fillet yield and disease resistance, the breeding program based on traditional genetic selection has been conducted in Vietnam from 2001 (Nguyen *et al.*, 2012; Vu *et al.*, 2019) and in Indonesia from 2009 (Irwan *et al.*, 2019). Recently, maker assisted selection (MAS) breeding program for striped catfish has been paid attention. The associations between genetic variations in candidate genes and the related economically important traits have been investigated to develop molecular markers for MAS (Le *et al.*, 2021; Tran *et al.*, 2021; Jiang *et al.*, 2022). Based on available genome sequence of *P. hypophthalmus* (Kim et al., 2018), this study aimed to discover SNPs in *IGFBP-3* gene and characterize the association between these SNPs and the growth traits of striped catfish, suggesting potential MAS for pangasius growth selection.

MATERIALS AND METHODS

Sampling strategy

Striped catfish (*P. hypophthalmus*) were sampled from the phenotypic selected populations which had been produced by a breeding program to improve growth traits at

Research Institute of Aquaculture No.2 (RIA2), Vietnam since 2001. Individual samples in this study were described in our own previous study (Tran *et al.*, 2021). Briefly, growth-selected line was selected through three generations G1, G2, G3 since 2001, and for this study, 226 full-sib family of G3-merged population were produced in May, 2014. Average 88 individuals per family were randomly marked by Passive Integrated Transponder tags (PIT-tags, Sokymat, Switzerlands) to have total 20,027 fish stocked in separate pond. These fish were nursed for 192 days then were measured the average body weight, survival rate and feed conversion ratio to calculate the heritability and estimated breeding values (EBV) for body weight based on the animal linear mixed model and ASReml software version 2.0 (VSN International Ltd) (Nguyen *et al.*, 2012). Our sampling strategy was performing the discovery sample set with small size to discover SNPs in *IGFBP-3* gene and the validation set with larger size to validate the association of these SNPs with the growth traits (Figure 1). The discovery sample set included 10 fastest-growing fish from 9 highest EBV families and 10 slowest-growing fish with lowest EBV individuals from 9 lowest EBV families. The validation sample set included 70 fast-growing fish having highest ranking EBV from 24 highest EBV families and 70 slow-growing fish having lowest ranking EBV from 31 lowest EBV families (Tran *et al.*, 2021). Fin clips cut from these 160 individuals were preserved in 95% ethanol at −20°C until processing for DNA extraction.

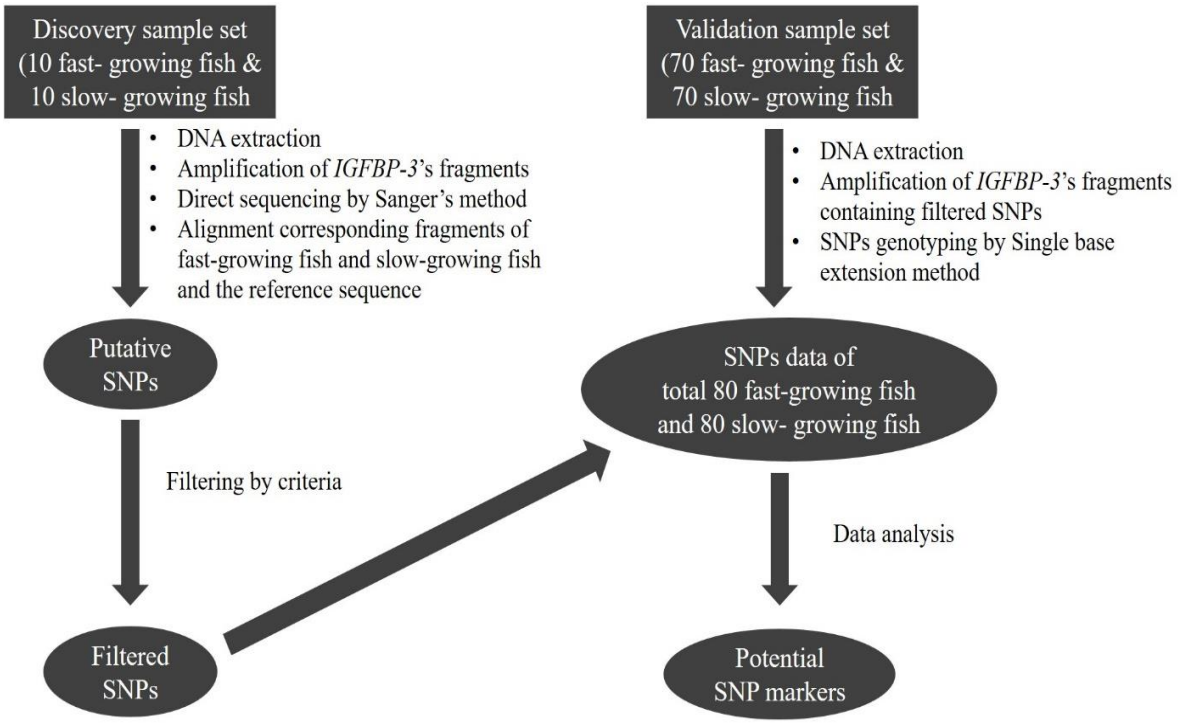

Figure 1. Workflow to discover and validate SNPs in *IGFBP-3* gene.

DNA extraction

Fin clips were powdered in liquid nitrogen and homogenized in lysis solution (0.01 M EDTA, 0.01 M Tris-HCl pH 7.5, 0.1 M NaCl, 2.1% SDS and 100 µL/mL proteinase K) for 3 hours at 56ºC. DNA extraction was performed using the standard phenol/chloroform method (Sambrook, Russell, 2001). The quantity and quality of the extracted DNA were checked by NanoDrop One spectrophotometer (Thermo Fisher Scientific) and electrophoresis on 1% agarose gel.

Fragments of *IGFBP-3* gene amplification

Based on the sequence of *IGFBP-3* gene in scaffold VN_pangasius sc0000042 (NW_020824237.1) at position from 1921250 to 1939687 (Kim *et al.*, 2018), 5 primer pairs were designed by using Primer3 (v.0.4.0) software (Untergasser *et al.*, 2012) to amplify exons and their adjacent intronic regions (Figure 2), (Table 1).

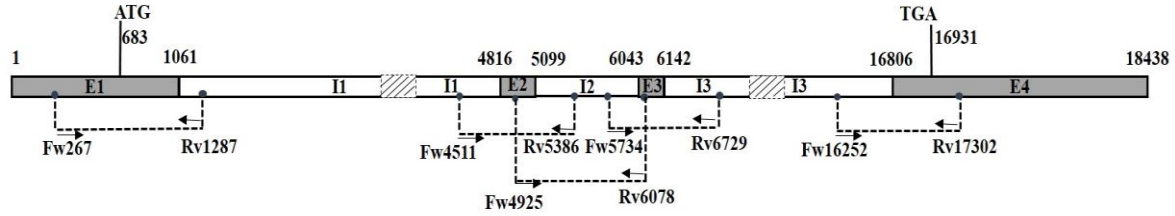

Figure 2. Positions of 5 primer pairs used to amplify fragments of *IGFBP-3* gene. The nucleotide of gene was numbered based on the annotation for the gene in NCBI (Gene ID 1939687). Exons and introns were denoted by E and I, respectively and numbered in ascending order.

Table 1. Primer pairs used to amplify fragments of *IGFBP-3* gene.

Primer name	Sequence (5'-3')	T_a (ºC)	Predicted size of fragment (bp)
Fw267	GGGCAGTTGTATAGCTCGTG	54	1021
Rv1287	TCCTGTAGAAGTTTCGTTCGATT		
Fw4511	ACTGTTGAGTGTTGATCGGT	51	876
Rv5386	TCCTTCTCTTTCCTGACTCCTAATA		
Fw4925	CGGTATGCAGAAAACAGAGCTG	51	1154
Rv6078	CTTCAAAACGCTTTCCATCTCTC		
Fw5734	AGAGAGGTGTGTTCAAGTGGTAT	53	996
Rv6729	TGAGTGAGAGCATTTACAAGCAG		
Fw16252	GCTTCTCCAACTGTCATATTTCCT	51	1051
Rv17302	GCCAACCACATACTTTCAGTCA		

PCR mixture contained 1 µL of diluted DNA template extracted from each individual, 1 µL of each primer (10 pmol/µL), 12.5 µL of Taq 2X Master Mix (NEB) and H_2O up to final volume of 25 µL. The reaction was conducted including the

initial denaturation at 95^{o}C for 5 minutes, 30 cycles of denaturation at 95^{o}C for 30 seconds, annealing at the temperature shown in Table 1 for 30 seconds, extending at 68^{o}C for 1 minute, and the final elongation at 68^{o}C for 7 minutes. The PCR products were checked the quality by electrophoresis on 1% agarose gel then purified by Thermo Scientific GeneJET PCR Purification Kit (Thermo Fisher).

SNPs discovery

The purified PCR products then were directly sequenced by Sanger method using ABI®3500 Genetic Analyzer (Applied Biosystems). The sequencing data was analyzed by BioEdit software (Hall, 1999) and the peaks were checked by eyes. To identify putative SNPs, the corresponding fragment from 20 individuals of discovery sample set were aligned to reference sequence using MUSCLE (Edgar, 2004).

The putative SNPs were filtered to the next step of SNP validation if they met at least one of two criteria. The first criterion was non-synonymous SNPs because the substitution of amino acid sequence may lead to the changing of structure or function of protein (Diopere *et al.*, 2013), (Suárez-Salgado *et al.*, 2020). The second criterion was that the ratio between alternative nucleotide and reference nucleotide (Alt/Ref) in at least one group was higher than 0.3 and the corresponding genotypic and/or allelic composition between fast- and slow-growing group significantly differed. In case the Ref is minor allele, the ratio would be calculated by Ref/Alt (minor allele/ major allele). The significant difference of genotypic composition and/or allelic composition between two groups was assessed by using p-value from Fisher's exact test (p-value <0.05), which was calculated by SHEsis software (Shi, He, 2005).

SNP validation by genotyping in multi-individuals

The filtered SNP in *IGFBP-3* was then individually genotyped in the validation sample set by Single Base Extension (SBE) method (Syvanen, 1999). To be used as templates for SBE reaction, fragments with average size of 500 bp containing the filtered SNP in 70 fast- growing fish and 70 slow-growing fish were amplified by PCR using primer pair Fw446 and Rv998 (Table 2). Then, the PCR products were purified by Thermo Scientific GeneJET PCR Purification Kit (Thermo Fisher). SBE primer was designed with 25 bases in core, which bound specifically to the adjacent regions of the filtered SNP (Table 2).

Table 2. Primers used for SNP genotyping.

Primer name	Sequence (5'-3')	Purpose	Predicted size of fragments (bp)
Fw446	CTTGTTGCCCGTGCCTTTTC	Amplifying fragment containing the filtered SNP	553
Rv998	GCAGCGGTTTGCTCTCAC		
SBE	GAACATGAAGCCCATATTCCGC TCT	Genotyping the filtered SNP by SBE reaction	26

SBE: Single base extension.

SBE reactions were performed using an ABI SNaPshot Multiplex PCR Kit (Applied Biosystems) according to the manufacturer's instructions. SBE products were purified by 1 U of Shrimp Alkaline Phosphatase - SAP (Thermo Fisher) at 37°C for 30 minutes to prevent the co-migrating of unincorporated terminators, which could generate the high background signal in mini-sequencing, then SAP was inactivated by incubation at 65°C for 15 minutes. Total volume of 10 µL containing 0.5 µL purified SBE product, 0.5 µL GeneScan-120 LIZ Size Standard (Applied Biosystems) and 9 µL formamide was denatured at 95°C for 5 minutes before loading onto the ABI®3500 Genetic Analyzer (Applied Biosystems) to determine the fluorescence and size of the extended products. The SNP data was analyzed using GeneMapper 4.1 software.

Data analysis

The total SNP data from discovery and validation sample sets (80 individuals of fast-growing fish and 80 individuals of slow-growing fish) was analyzed to characterize the association with growth traits and validate SNP marker candidates for growth trait in *P. hypophthalmus*. The significant difference of genotypic composition/ allelic composition between fast- and slow-growing groups was assessed by p-value from Fisher's exact test (p-value <0.05), which was calculated by SHEsis software (Shi, He, 2005). Parameters of genetic diversity of 160 individuals including polymorphism information content (PIC) and minor allele frequency (MAF) were calculated by Gene-Calc software (https://www.gene-calc.pl).

RESULTS AND DISCUSSION

SNPs discovery

Fragments of *IGFBP-3* gene from 10 fast-growing fish and 10 slow-growing fish were amplified, then sequenced by Sanger sequencing, and aligned to reference sequence to discover SNPs (Figure 3).

A total of 10 putative SNPs were discovered in *IGFBP-3* gene, including a non-synonymous SNP and 9 SNPs located in non-coding sequence (Table 3). Non-synonymous SNP was rarely occurred in the examined fragments of *IGFBP3* gene, which was consistent with previous SNP discoveries in teleost fish, such as salmon (Ferchaud *et al.*, 2018), channel catfish (Suárez-Salgado *et al.*, 2020), sole (Diopere *et al.*, 2013), and turbot (Vera *et al.*, 2013; Robledo *et al.*, 2017). The explanation for this phenomenon is that SNPs in intron regions may accumulate more easily than in exon regions because of the differences in selective pressures (Mu *et al.*, 2011; Zhang *et al.*, 2019). Non-synonymous variations are usually associated with deleterious mutations, hence, they should be preferentially eliminated by evolutionary constraints (Hubert *et al.*, 2010).

Based on the criteria of SNP filtration, non-synonymous SNP 704 C>G causing the substitution of amino acid Leucine to Valine at position 8 of protein (p.Leu8Val) was filtered for the next step of SNP validation. Among 9 SNPs located in non-coding region, two SNPs, 1123 G>T in intron 1 and 17119 A>G in 3'-UTR, had the ratio Alt/Ref > 0.3 in at least one group (Table 3), but they did not show the significant difference in genotypic as well as allelic composition between fast- and slow-growing groups (p > 0.05). Thus, these two SNPs were not filtered to be validated in the next procedure.

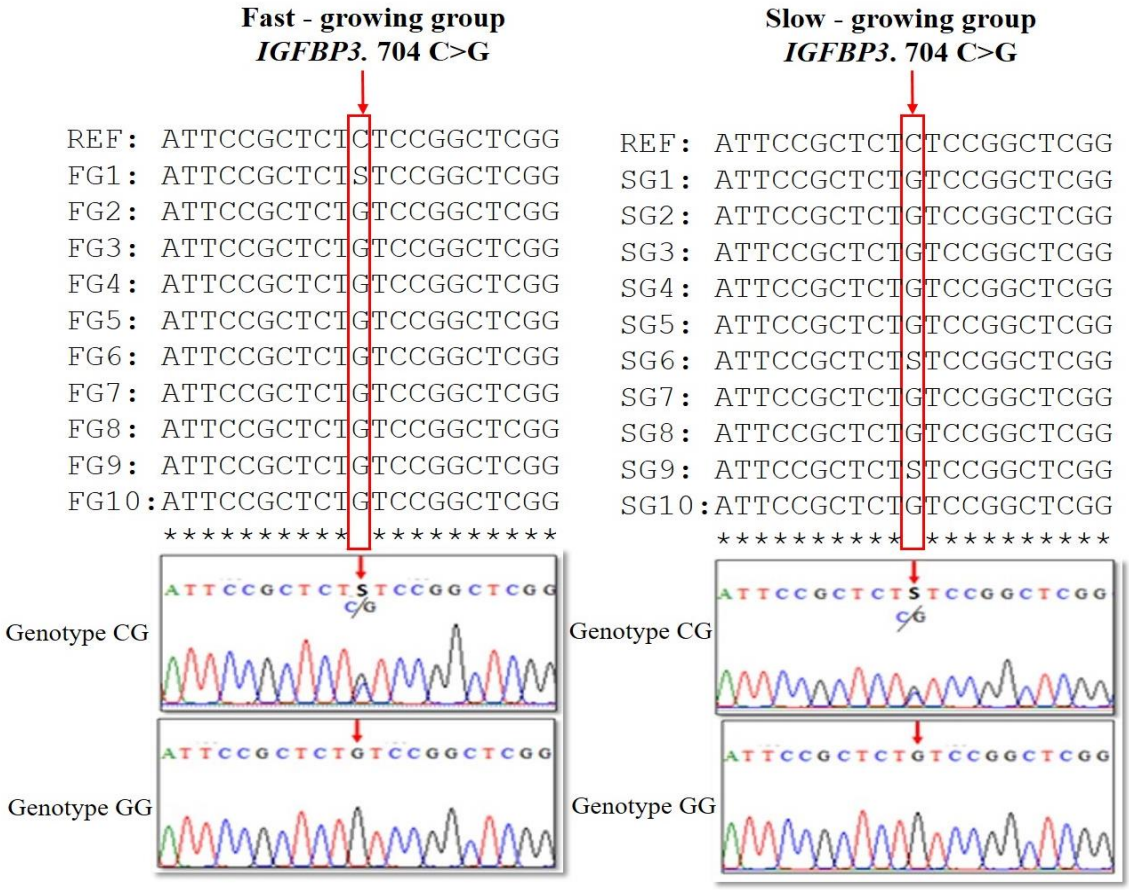

Figure 3. Illustration of a discovered SNP 704 C>G in *IGFBP-3* gene by Sanger sequencing in 10 fast- growing fish and 10 slow- growing fish. REF, FG and SG denotes sequence of reference, fast-growing fish and slow-growing fish, respectively. Red borders and red arrows denote positions of this SNP. Heterozygous genotype is illustrated by degenerate nucleotides, S for C and G.

SNPs validation in bigger population and association analysis with growth traits of the striped catfish

The filtered non-synonymous SNP 704 C>G (p.Leu8Val) was individually genotyped in 70 fast- growing fish and 70 slow-growing fish by SBE method. SNP data collected in both discovery and genotyping panels, totally 80 fast- growing fish and 80 slow- growing fish, were used to validate the association between the SNPs and growth traits of striped catfish (Table 4). The non-identified (NN) genotyped individual was caused by technical errors of SBE method. In our study, the error rate was only 1.25% (Table 4) which was acceptable for statistical analysis (Bonin *et al.*, 2004). Two genotypes, CG and GG, presented in both fast- and slow-growing groups. However, genotype CG appeared more frequently in fast-growing group while genotype GG made up the majority in slow-growing group. The difference between the genotypic compositions of these two groups was statistically significant with p-value <

0.01. Moreover, the allelic composition at this locus showed significant difference between the fast- and slow-growing groups ($p<0.05$) (Table 4). The result proved that the non-synonymous SNP 704 C>G (p.Leu8Val) is significantly associated with the growth traits. In previous studies, the associations between SNPs in *IGFBP-3* gene and the growth of human (van der Kaay *et al.*, 2009), swine (Wu *et al.*, 2016), common carp (Mehrabi *et al.*, 2015) and chicken (Ou *et al.*, 2009) were found at loci in non-coding region such as promoter, intron and 5'-UTR. In this study, we firstly reported a non-synonymous SNP of *IGFBP-3* gene in *P. hypophthalmus*, which was associated with growth traits of this fish. By *in silico* analysis, the position of substituted amino acid (Leu8Val) in IGFBP-3, which was caused by non-synonymous SNP 704 C>G, was located in N-terminal signal peptide of proteins (data not shown). Because the signal peptide is crucial for the transport and secretion of IGFBP-3 (Varma Shrivastav *et al.*, 2020), this non-synonymous SNP might effect on downstream response. The function of the SNP needs to be further clarified.

Table 3. Characteristics of discovered SNPs in *IGFBP-3*.

No	Positions of SNPs in gene	Ref	Alt	Genotypic composition		Allelic composition	
				Fast- growing group (Alt/Ref)[3]	Slow- growing group (Alt/Ref)[3]	Fast-growing group	Slow-growing group
1	Exon 1_ 5'-UTR_647	T	A*	8TT:2AA (0.25)	8TT:1TA:1AA (0.22)	16T:4A	17T:3A
2	**Exon 1_ CDS_704(1)**	**C***	**G**	1CG:9GG	2CG:8GG	1C:19G	2C:18G
3	Intron 1_1115	G	T*	9GG:1TT (0.11)	9GG:1TT (0.11)	18G:2T	18G:2T
4	Intron 1_1123(2)	G*	T	2GG:8TT (0.20)	3GG:7TT (0.43)	4G:16T	6G:14T
5	Intron 1_4638	C	G*	8CC:2CG (0.20)	10CC (0.00)	18C:2G	20C
6	Intron 1_4695	C	A*	9CC:1CA (0.01)	8CC:2CA (0.20)	19C:1A	18C:2A
7	Intron 2_5187	G	A*	10GG (0.00)	9GG:1GA (0.10)	20G	19G:1A
8	Intron 2_5375	G	A*	6GG:2GA:2NN	8GG:1GA:1NN	14G:4A	17G:1A
9	Exon 4_ 3'-UTR_17119(2)	A*	G	3AA:7GG (0.43)	2AA:8GG (0.25)	6A:14G	4A:16G
10	Exon 4_ 3'-UTR_17128	C	G*	8CC:2GG (0.25)	10CC (0.00)	16C:4G	20C

NN presents non-identified individuals. (1) denotes for nonsynonymous SNP. (2) denotes for SNP with the ratio Alt/Ref > 0.3 in at least one group. [3]the ratio was defined as minor allele/ major allele. * denotes for minor allele, which took smaller proportion in total individuals of both fast- growing group and slow- growing group.

Table 4. Characterization of the SNP 704 C>G (p.Leu8Val) and association analysis with growth trait.

	Fast-growing group	**Slow-growing group**	**p**
Genotypic composition	CG (45) GG (35)	CG (26) GG (53) NN (01)	0.003**
Allelic composition	C (45) G (115)	C (26) G (132)	0.013*
PIC		0.347	
MAF		0.223	

Note: The number of individuals carrying genotype and the number of each allele in each group is shown in parentheses. NN: Non-identified genotype individual. p-values for the difference between genotypic composition/allelic composition of the fast- growing group and that of slow- growing group are from Fisher's exact test: *p<0.05, **p < 0.01.

Furthermore, the genetic diversity of SNP 704 C>G in 160 individuals were evaluated by PIC and MAF values shown in Table 4. With PIC value was 0.347, this non-synonymous SNP presented the moderate genetic diversity (0.25 < PIC < 0.5), suggesting the reasonable potential for breeding selection (Vaiman *et al.*, 1994; Chesnokov, Artem'eva, 2015). The moderate MAF value 0.223 (>20%) determined this SNP as a common variant, contributing to the genetic variance (Park *et al.*, 2011).

CONCLUSION

Putative SNPs were discovered in *IGFBP-3* gene of striped catfish, among which a non-synonymous SNP 704 C>G (p.Leu8Val) showed the significant association with the growth of this fish. Therefore, this non-synonymous SNP in *IGFBP-3* might be a potential molecular marker for growth traits in breeding of the striped catfish.

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