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SIGNIFICANT ASSOCIATION BETWEEN A NON-SYNONYMOUS SNP IN *IGFBP5* GENE AND THE GROWTH OF STRIPED CATFISH (*Pangasianodon hypophthalmus*, Sauvage, 1878)

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SUMMARY

Insulin-like growth factor binding protein 5 (IGFBP5) is the highest conserved member of IGFBP family, and has the broad range of biological activities effecting on the cell growth. This study aims to investigate the association between genetic variation in *IGFBP5* gene and the growth of striped catfish (*Pangasianodon hypophthalmus*). Single nucleotide polymorphisms (SNPs) were discovered and validated in *IGFBP5* gene from two growth-selected populations (fast- and slow- growing fish). For SNP discovery, the fragments of *IGFBP5* from sample sets of 10 fast- growing fish and 10 slow-growing fish were directly sequenced by Sanger sequencing. In this stage, 4 exonic SNPs were discovered, including a non-synonymous SNP 525 T>A (p. Val16Glu) in exon 1, and three synonymous SNPs (8859 G>A, 11713 C>A, 11992 T>C) in exon 4. The non-synonymous SNP 525 T>A (p.Val16Glu) was filtered to the next step of SNP validation. For validation, the SNP was individually genotyped in the test populations of 70 fast- growing fish and 70 slow- growing fish by single base extension method. Data analysis from the total SNPs which were collected from 80 fast-growing fish and 80 slow- growing fish indicated that non-synonymous SNP 525 T>A (p.Val16Glu) was significantly associated to the growth of striped catfish (p-value <0.001). Analysis of genetic diversity parameters (PIC, MAF) suggested that this SNP is a common variant, contributes significantly to the genetic variance. The non-synonymous SNP 525 T>A (p.Val16Glu) in *IGFBP5* gene would become a SNP marker candidate for marker assisted selection (MAS) that can be used in pangasius breeding.

Keywords: Growth, *IGFBP5*, marker assisted selection, non-synonymous SNP, striped catfish

INTRODUCTION

Insulin-like growth factor binding proteins (IGFBPs) regulate the signaling of IGF system of vertebrate, which also includes IGF ligands (IGF1 and IGF2), IGF receptors (IGF1R and IGF2R) and IGFBP- related proteins. In biological fluid, IGFBPs bind to IGF, effecting on the cell growth by both IGF-dependent and IGF-independent mechanism (Hwa *et al.*, 1999). Among members of IGFBP family in teleost (IGFBP1-6), IGFBP5 is the highest conserved

member and has the broadest range of biological activities (Duan, Allard, 2020). Through IGF-independent mechanism, IGFBP5 stimulates/inhibits IGF signaling, concentrating IGFs in certain cells and tissues, and prolonging the half-life IGFs in the circulation (Duan, Allard, 2020). IGFBP5 also has nuclear functions (transcriptional activities), due to the ability to translocate into the nucleus through nuclear localization sequence in the C terminus of this protein (Xu *et al.*, 2004; Sun *et al.*, 2017).

The role of *IGFBP5* gene on the growth of fish was well reported, especially in expression level. The up-regulation of *IGFBP5* was indicated in the switching to fast growth of Atlantic salmon (Bower *et al.*, 2008), in *GH* transgenic coho salmon (Alzaid *et al.*, 2018), upon injection of GH in liver tissue of grass carp (Zheng *et al.*, 2017), and supported anterior muscle growth of gilthead seabream (Vélez *et al.*, 2016). On the other hand, *IGFBP5* was down-regulated in the atrophying muscle of rainbow trout (Salem *et al.*, 2010), and in skeletal muscle of grass carp during fasting (Zheng *et al.*, 2017). Although the available evidences at expression level suggested that *IGFBP5* gene plays conserved functions in the growth of fish, especially in muscle tissue, the association between genetic variations of this gene and the muscle growth has not been surveyed yet.

The striped catfish, *Pangasianodon hypophthalmus* Sauvage, 1878, belonging to the Asian catfish family Pangasiidae (Roberts, Chavalit, 1991), is one of the most important aquaculture product which reached nearly 2,360,000 tons in 2018 (FAO, 2022). This catfish is the major fish species cultured in the Mekong river delta in Vietnam and its production in Vietnam is the biggest in the world (Phan *et al.*, 2009; Kim *et al.*, 2018; Fletcher, 2020). To enhance the pangasius production efficiency, developing selection program for fast growth of this fish is one of the important approaches, which has been conducted based on phenotype for a long time in Vietnam (Nguyen *et al.*, 2012), (Vu *et al.*, 2019), and in Indonesia (Irwan *et al.*, 2019). However, genotype-based selection for growth traits, which towards maker assisted selection (MAS), was carried on recently by studying the association between the growth of striped catfish and the genetic variations such as microsatellites (Marnis, 2018) and SNPs in candidate genes (Tran *et al.*, 2021), (Jiang *et al.*, 2022). Based on available genome sequence of *P. hypophthalmus* (Kim *et al.*, 2018), in this study, we aimed to screen and characterize SNPs in *IGFBP5* gene associated with growth traits of striped catfish, to look for the potential candidate SNP marker for the MAS in pangasius breeding program.

MATERIALS AND METHODS

Collecting sample from growth-selected line of striped catfish

Samples of striped catfish (*P. hypophthalmus*) were collected from populations which had been produced by a breeding program to improve growth traits at Research Institute of Aquaculture No.2 (RIA2), Vietnam. The sampling was described in our own previous study (Tran *et al.*, 2021). Briefly, the growth-selected line of the striped catfish has been selected through three generations (G1, G2, G3) using traditional genetic selection method since 2001 (Nguyen *et al.*, 2012). In this study, the samples were collected from 226 full-sib G3-merged families. After randomly marked 88 individuals per family by Passive Integrated Transponder tags (PIT-tags, Sokymat, Switzerlands), 20,027 fish were tagged, then stocked in separate pond and nursed for 192 days to calculate average body weight, survival rate and feed conversion ratio. Heritability and estimated breeding values (EBV) for body weight were estimated based on the animal linear mixed model and calculated by using ASReml software version 2.0 (VSN International Ltd) (Nguyen *et al.*, 2012). As described in our previous study (Tran *et al.*, 2021), to discover and filter SNPs in *IGFBP5* gene, discovery sample set, including 10 fast- growing fish with the highest EBV individuals from 9 highest EBV families and 10 slow- growing fish with the

lowest EBV individuals from 9 lowest EBV families, was chosen. In the next procedure, the validation sample set, including 70 fast- growing fish with the highest ranking EBV from 24 highest EBV families and 70 slow- growing fish with the lowest ranking EBV from 31 lowest EBV families, were used to validate the discovered SNPs by individually genotyping (Tran *et al.*, 2021). Fin clips cut from these 160 individuals were preserved in 95% ethanol at −20°C until processing for DNA extraction.

Extraction of DNA genome

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. Total DNA extraction was performed using the standard phenol/chloroform method. The quantity and quality of the extracted DNA were checked by NanoDrop One spectrophotometer (Thermo Fisher Scientific) and electrophoresis on 1% agarose gel.

Amplification and Sanger sequencing of *IGFBP5* gene's fragments

Fragments of *IGFBP5* gene in 20 individuals of discovery sample set were amplified and sequenced by Sanger method. Primer pairs were designed by Primer3 (v.0.4.0) software (Untergasser *et al.*, 2012) (Figure 1, Table 1), based on the sequence of *IGFBP5* gene in scaffold VN_pangasius sc0000003 (NW_020824198.1) at position from 9142631 to 9154838 (Kim *et al.*, 2018). Polymer chain reactions (PCR) was performed in total volume of 25 µL, containing 1 µL of diluted DNA genome template extracted from each of 10 fast-growing fish and 10 slow- growing fish, 1 µL of each primer (10 pmol/µL), and 12.5 µL of Taq 2X Master Mix (NEB). The thermal cycle was carried on with the initial denaturation at 95ºC for 5 minutes, 30 cycles at a denaturation temperature of 95ºC for 30 seconds, annealing temperature for 30 seconds (depended on each primer pairs shown in Table 1), extending temperature of 68ºC for 1 minute, and the final elongation at 68ºC 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).

The Sanger sequencing of the purified PCR products were performed using ABI®3500 Genetic Analyzer (Applied Biosystems). Peak data collected from the ABI sequencer trace files was analyzed using BioEdit software (Hall, 1999) and examined by eyes.

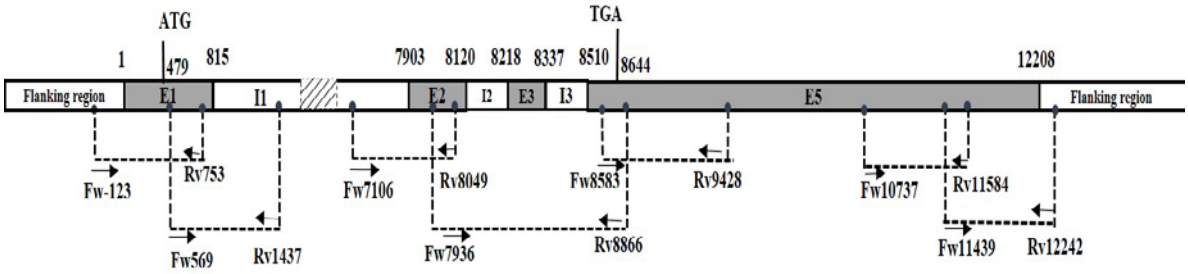

Figure 1. Positions of 7 primer pairs used to amplify fragments of *IGFBP5* gene. The nucleotide of gene was re-numbered based on the annotation for the gene in scaffold, i.e. the first nucleotide of this gene was numbered 1. The gray boxes denoted "E" are exons numbered in ascending order. The starting and ending points of each exon are presented by positive integer above this exon. The white boxes present upstream and downstream flanking regions at the both ends, and introns of this gene. Introns are denoted by "I" with the numbers corresponding to the previous exons.

Table 1. Primer pairs used to amplify fragments of *IGFBP5* gene.

Primers	Sequence	T_a (°C)	Predicted size of fragment (bp)
Fw-123	CGTCATTTATTAGGGCGTCAGG	53	876
Rv753	AGTGCGTGTAGTGGCTTCTC		
Fw569	CAGAAGGCGCTGTCCATGTG	53	869
Rv1437	AGGAATGCAAGTGGGGATGT		
Fw7106	AAGTGTTTTTGGCCTGTTATTCCTC	53	944
Rv8049	GCTGCTTCTTCTTGTCCTTGCG		
Fw7936	AGACTGACACAACGGAGGAG	56	931
Rv8866	GGGACTCAGCTCGTAACACA		
Fw8583	CCGACTACAGTGGAGGGAAC	53	846
Rv9428	AGTGGGCCAGTGAGAGAATG		
Fw10737	AGCCATTCCCTGCCACAG	56	848
Rv11584	CCCACTTGACGGGGTAGTGA		
Fw11439	CACAGCTATTGATCTCAGTCCA	51	804
Rv12242	AACATTTTCACGGTCCTCCTG		

SNP discovery

The corresponding Sanger sequences of 10 fast- growing fish and 10 slow- growing fish in the discovery sample set were aligned to reference sequence by MUSCLE (Edgar, 2004) to identify putative SNPs. These discovered SNP, which were determined in both of 20 individuals, were then filtered by criteria illustrated in the Figure 2. Top priority is non-synonymous SNP because the change of amino acid in protein sequence may has biological significance (Suárez-Salgado *et al.*, 2020), (Diopere *et al.*, 2013). On the other hand, if SNPs are synonymous SNPs or located in non-coding regions, they were continuously evaluated when the ratio of alternative/ reference allele (Alt/Ref) at least in one group ≥0.3. If achieving this strictly criterion, SNPs were further investigated the significant difference between corresponding genotypic composition and/or allelic composition between fast- and slow- growing group. The significant difference 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) (Figure 2).

Genotyping filtered SNPs by single base extension (SBE) reaction

Fragments containing the filtered SNPs of 70 fast-growing fish and 70 slow- growing fish in validation sample set were amplified then purified to be used as template for individual genotyping by SBE reaction (Syvanen, 1999). In this study, the filtered SNP in *IGFBP5* was genotyped at the same time with other filtered SNPs of other genes (data not shown) in a multiplex SBE reaction. Therefore, SBE primers were designed with 25 bases in core, which bind specifically to the adjacent regions of filtered SNPs, and 5' non-homologous tails with different length to distinguish SBE products in multiplex reaction. The sequence of SBE primer for SNP 525 T>A was 5'- TTTTTGTGATGGTGCCGTTTCTGT CGGCTG-3'. SBE reactions were performed using an ABI SNaPshot Multiplex PCR Kit (Applied Biosystems) according to the manufacturer's instructions. The purification for SBE product by 1 U of Shrimp Alkaline Phosphatase - SAP (Thermo Fisher) at 37°C for 30 minutes was required to prevent high background signal in mini-sequencing generated by co-migrating of unincorporated terminators

with extension products, then SAP was inactivated by incubation at 65°C for 15 minutes. The fluorescence and size of the extended products were determined by capillary electrophoresis on an ABI®3500 Genetic Analyzer (Applied Biosystems). Total volume of 10 µL containing 0.5 µL purified SBE products, 0.5µL GeneScan-120 LIZ Size Standard (Applied Biosystems) and 9 µL formamide was denatured at 95°C for 5 minutes before loading onto the genetic analyzer. The SNP data was collected using GeneMapper 4.1 software.

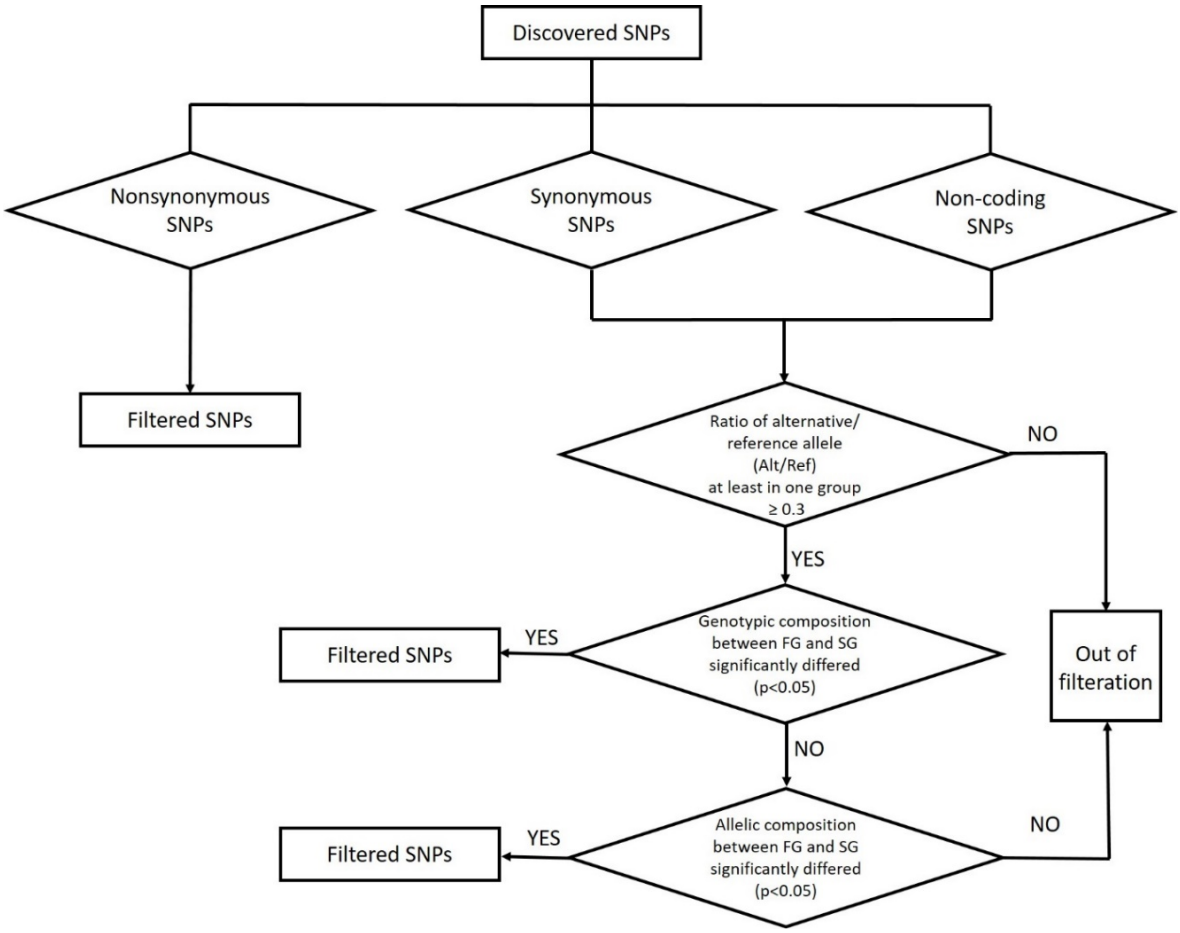

Figure 2. Schema used to filter discovered SNPs in *IGFBP5* gene. Frequency of Alt/Ref in each group was calculated by the number of genotype carrying alternative allele (Alt) divided by the number of genotype carrying reference allele (Ref), and must be at least 0.3 in one group. FG and SG denoted for fast-growing group and slow-growing group, respectively.

Data analysis

All SNP data collected from discovery and validation sample sets (totally, 80 inviduals of fast-growing fish and 80 inviduals of slow- growing fish) was analyzed to identify candidate SNP markers for growth trait in *P. hypophthalmus*. These SNPs were assessed if the corresponding genotypic composition and/or allelic composition between fast- and slow-growing groups differred significantly. The difference was confirmed by Fisher's exact test determined by SHEsis software (Shi, He, 2005), with p-value <0.05. Genetic diversity of 160 individuals including polymorphism information content (PIC), minor allele frequency (MAF) was analyzed by Gene-Calc software (https://www.gene-calc.pl).

RESULTS AND DISCUSSION

SNPs discovery and filteration

There were 4 SNPs identified in *IGFBP5* gene of individuals in discovery sample set, including 525 T>A, 8599 G>A, 11713 C>A and 11992 T>C (Figure 3). Based on the structure diagram of *IGFBP5* gene (Figure 1), these 4 discovered SNPs were determined locating in exons with SNP 525 T>A in exon 1, and 3 other SNPs in exon 4. In details, two SNPs 525 T>A and 8859 G>A were in coding region (CDS), while two SNPs 11713 C>A and 11992 T>C were located in 3'-untranslated region (3'-UTR) (Table 2).

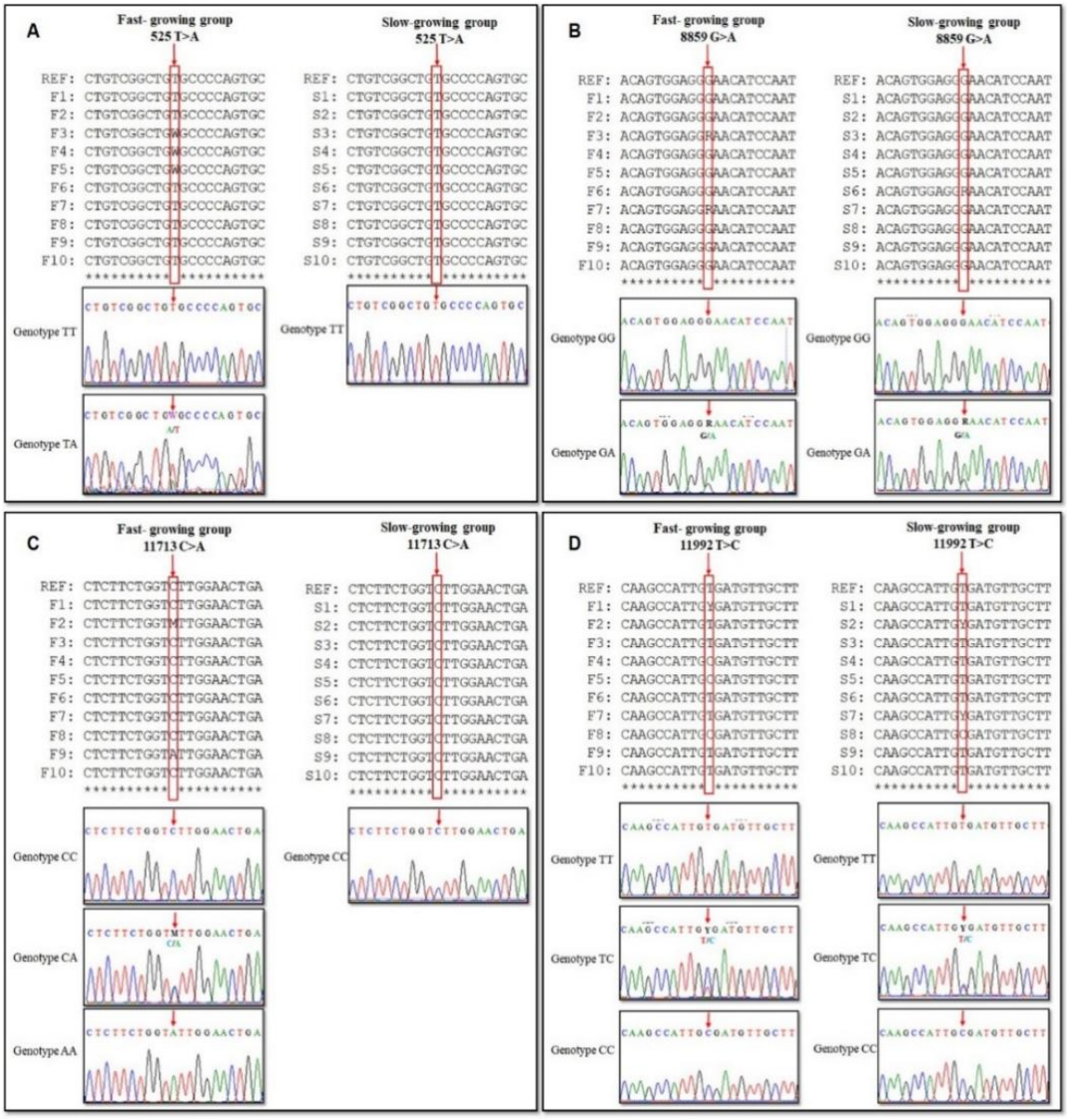

Figure 3. Discovered SNPs in *IGFBP5* gene by Sanger sequencing in 10 fast- growing fish and 10 slow- growing fish of discovery sample set. A, B, C, D: Alignment results and illustration of Sanger peaks of SNP 525 T>A, 8859 G>A, 11713 C>A and 11992 T>C, respectively. Red borders and red arrows denoted positions of SNPs. Heterozygous genotypes were illustrated by degenerate nucleotides, W for T and A (in part A), R for G and A (in part B), M for C and A (in part C), and Y for T and C (in part D).

Table 2. Location and genotypic/allelic composition of discovered SNPs in *IGFBP5* gene.

Nº	Positions of SNPs in gene	Ref	Alt	Genotypic composition		Allelic composition	
				Fast- growing group (Alt/Ref)	Slow- growing group (Alt/Ref)	Fast-growing group	Slow-growing group
1	**Exon 1_ CDS_525**	**T**	**A**	7TT:3TA (0.3)	10TT (0.0)	17T:3A	20T
2	Exon 4_ CDS_8599	G	A	8GG:2AG (0.20)	9GG:1GA (0.10)	18G:2A	19G:1A
3	Exon 4_ 3'-UTR_11713	C	A	8CC:1CA:1AA (0.20)	10CC (0.00)	17C:3A	20C
4	Exon 4_ 3'-UTR_11992	T	C	6TT:1TC:3CC (0.57)	7TT:2TC:1CC (0.3)	13T:7C	16T:4C

Although this study scanned SNP in the through out of target gene including both introns and exons, no intronic SNP was detected. This result was somehow different from other researches in which the appearance of the SNPs in non-coding regions were more frequently than that in coding regions (Wang *et al.*, 2010), (Feng *et al.*, 2014), (Cuevas-Rodríguez *et al.*, 2016), (Zhang *et al.*, 2019).

Because discovered SNPs would be exceptional chosen if they were non-synonymous variations (Figure 2), two SNPs 525 T>A and 8859 G>A locating in CDS were checked whether they induced the substitution of amino acid in protein sequence. The result illustrated in Figure 4 indicated that SNP 525 T>A caused the change of codon gTg to gAg, leading to the substitution of Valine at the position 16 of protein sequence to Glutamic acid (p.Val16Glu), while SNP 8859 G>A is a synonymous SNP at the position Gly255 in protein sequence (Figure 4).

The synonymous SNP 8859 G>A and two non-coding SNPs (11713 C>A and 11992 T>C) were evaluated by criteria to filter SNP listed in Figure 2. Although the ratios Alt/Ref of SNP 11992 T>C in both fast- and slow- groups were bigger than 0.3, there was no significant difference in genotypic as well as allelic composition between two groups (p-values were 0.55 and 0.48, respectively). Therefore, these 3 SNPs were not filtered to the next step of validation in bigger population.

SNP validation, characterization of the non-synonymous SNP 525 T>A and association analysis with growth traits

Among 4 discovered SNPs in *IGFBP5*, only non-synonymous SNP 525 T>A (p.Val16Glu) was filtered and validated in the larger sample size with 70 fast- growing fish and 70 slow-growing fish by single base extension method. Data collected from total 80 fast-growing fish and 80 slow-growing fish of SNP 525T>A was presented as corresponding genotypic and allelic composition in Table 3.

In fast-growing group, there were 3 genotypes TT, TA, AA in which the genotype TA was the most frequently observed. In slow-growing group, there were only 2 genotypes TT and TA in which the genotype TT was predominant (Table 3). The result clearly showed that in the fast- growing fish, the alternative A allele was predominant over the T allele at the locus 525T>A. The genotypic composition of fast- growing group significantly differed from that of slow- growing group, with p-value = 1.13e-8 (Table 3). Allelic composition between fast- growing group and slow- growing group also differed significantly, with p-value = 1.14e-5 (Table 3). Based on the significant

difference of genotypic as well as allelic composition between fast- and slow-growing groups, non-synonymous SNP 525T>A was significantly associated to the growth of striped catfish, thus might become potential SNP marker for growth traits in the striped catfish. This result was consolidated by the observation that non-synonymous SNPs screened in other candidate genes were proven relating to the growth of aquatic species such as razor clam (Xie *et al.*, 2018), hybrid of *Culter alburnus* (♀) x *Ancherythroculter nigrocauda* (♂) individuals (Cheng, Sun, 2015), and channel catfish (Suárez-Salgado *et al.*, 2020).

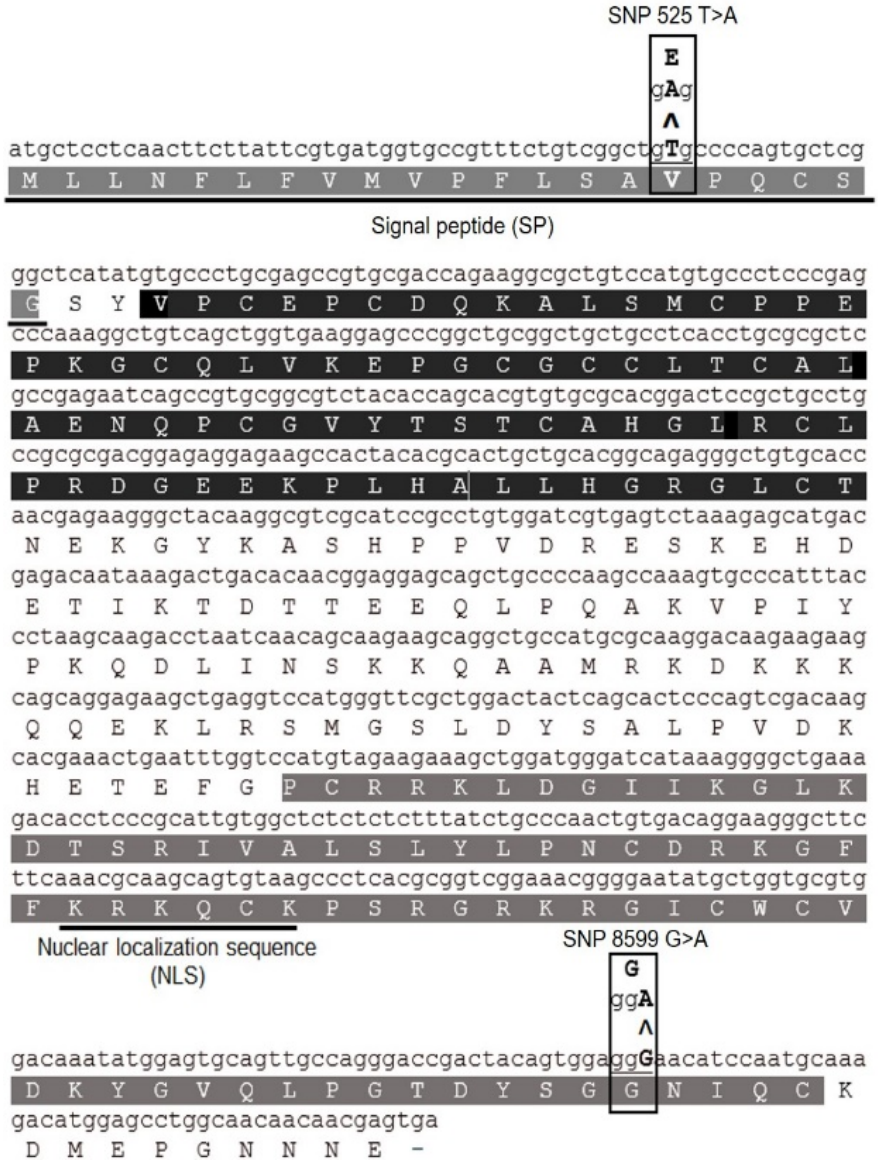

Figure 4. The effect of SNPs 525 T>A and 8859 G>A on the sequence of IGFBP5 protein. Black borders denoted the position of amino acids in protein substituted by these 2 SNPs. Functional region and domains of IGFBP5 protein were highlighted with white letters, in order from top to bottom including signal peptide (SP) (determined by SignalP-5.0 software (data not shown), IGFBP domain, and Thyroglobulin type 1 (determined by blastP to Protein Data Bank (PDB) (data not shown)). Signal peptide (SP) and nuclear localization sequence (NLS), which were necessary to transport IGFBP5 to nuclear, were underlined.

Table 3. Characterization of filtered SNP g.525 T>A (p.Val16Glu) and association analysis with growth trait.

	Fast-growing group	**Slow-growing group**	**p**
Genotypic composition	TT (07) TA (69) AA (03) NN (01)	TT (41) TA (37) AA (00) NN (02)	1.13e-8***
Allelic composition	T (83) A (75)	T (119) A (37)	1.14e-5***
PIC	0.459		
MAF	0.357		

Note: The number of individuals carrying genotype and the number of each allele in each group is shown in parentheses. NN: Non-identified genotype individuals. 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 < 0.001.

Moreover, the genetic diversity of SNP 525 T>A in 160 individuals were evaluated by PIC and MAF values. Table 3 showed that PIC was 0.459 and MAF was 0.357. In the range of 0.25 and 0.5, this PIC value indicated the moderate genetic diversity of this SNP, suggesting the reasonable potential for breeding selection (Vaiman *et al.*, 1994; Chesnokov, Artem'eva, 2015). With high value of MAF (30-50%), SNP 525 T>A was determined as common variant, contributing significantly to the genetic variance (Park *et al.*, 2011).

The effect of non-synonymous SNP 525 T>A on the structure and function of IGFBP5 protein, was *in- silico* analyzed. As described in Figure 4, this non-synonymous SNP caused the substitution of amino acid p.Val16Glu, which was predicted locating in N-terminal signal peptide of IGFBP5 protein by SignalP-5.0 software. Because the signal peptide was crucial for the transport and secretion of IGFBP5 (Luther *et al.*, 2013), the effect of substituted amino acid Val16Glu on the function of IGFBP5 protein needed further clarified.

CONCLUSION

The genetic variation of *IGFBP5* gene of striped catfish was investigated in this study. A novel non-synonymous SNP 525 T>A (p. Val16Glu) was significantly associated with the growth traits of striped catfish. This non-synonymous SNP might be a potential molecular marker for MAS program in pangasius aquaculture.

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