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Novel EST-SNPs Polymorphisms and their Association with Growth Traits in *Schizothorax prenanti*

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HY, QL and HL conceived the idea and designed the experiments. TJ, ZL and FL fed the fishes. ZSZ and MZ performed the experiment. YY, MX and BH analysed the data. YY, HY and QL wrote the manuscript and drafted the article.

Key words
***Schizothorax prenanti*, EST-SNP, Growth traits, Correlation analysis, Marker-assisted breeding**

ABSTRACT

Schizothorax prenanti (*S. prenanti*), an economic fish in China, has rich nutritional value and high economical value. In order to acquire some reliable molecular genetic markers for growth traits, correlation analysis between 31 SNP markers and growth traits in *S. prenanti* was analyzed using 164 samples with the same growth conditions. Principal component analysis showed that the body weight accounted for 94.50% of the variance, the eigenvalue was greater than 1, and the accumulative variance was more than 85%. So, the body weight was the first principal component of the growth traits for *S. prenanti*. Association analysis indicated that ug25066-1502 had significantly associated with total length and body weight; and ug22539-1605 and total length, body length, body height, and body weight were significantly associated. In conclusion, ug25066-1502 and ug22539-1605 were significantly associated with the growth traits, which could be used as important candidate molecular markers for selective breeding of *S. prenanti*.

INTRODUCTION

With the development of genomics technology, it is likely that productivity and commercial value could be improved when genomic methods are applied to select superior parents. Marker-assisted selection (MAS) is a powerful method to improve and develop high-quality strains. Compared with traditional methods used in animals, MAS accelerates genetic improvement and the achievement of breeding goals (De-Santis, 2007). It is not affected by the external environment and age stages, which can also use genetic markers for early selection to shorten generational intervals and increase selection intensity (Lu and Wu, 2002).

Single nucleotide polymorphism (SNP) describes polymorphisms caused by point mutations that give rise to different alleles containing alternative bases at a given nucleotide position within a locus (Liu and Cordes, 2004). SNPs have been widely exploited in molecular marker development and genome mapping due to their high abundance, genotyping efficiency, data quality, and genome-wide coverage (Emahazion *et al*., 1999; Liu and Cordes, 2004; Wang *et al*., 1998). Moreover, SNPs developed from ESTs are type I markers, which have the advantages of good versatility, clear bands, and analytical simplicity. SNPs from candidate genes are becoming important and efficient molecular markers for MAS (Spelman *et al*., 1999). In recent years, SNP markers associated with important traits have been reported in animal and plant, especially growth trait. In aquatic animals, SNP markers associated with growth traits have been reported in *Chlamys farreri* (Guo *et al*., 2012), *Pelteobagrus fulvidraco* (Li *et al*., 2016), *Crassostrea gigas* (Cong *et al*., 2013; Cong *et al*., 2014), *Micrpterus salmoides* (Li *et al*., 2012), *Macrobrachium rosenbergii* (Thanh *et al*., 2010), *Siniperca chuatsiand* (Dong *et al*., 2019) and so on.

Schizothorax prenanti, one of the indigenous economic fish species in China, mainly distributed in the upper reaches of the Yangtze River and its tributaries with

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rich nutritional value is of high economic value (Chen and Cao, 2000; Wu and Wu, 1992). With the increase in artificial breeding intensification, the germplasm resources of *S. prenanti* have been degraded, which is revealed in individual miniaturization, slow growth, and decreased disease resistance (Luo *et al*., 2016; Ye *et al*., 2018). Therefore, there is an obvious need for more molecular markers associated with growth traits for intensive study on the molecular marker-assisted breeding. Although the SNP of *S. prenanti* have been developed in recent years (Luo *et al*., 2016), the genetic molecular markers associated with growth traits remain scarce.

In this study, 31 novel EST-SNP markers were analyzed their polymorphism using Mass ARRAY® MALDI-TOF System. To provide valuable information in molecular assisted breeding and genetic improvement for *S. prenanti,* we explored associations between SNP markers and growth traits in the cultured populations of *S. prenanti*.

MATERIALS AND METHODS

Sample collection, genomic DNA preparation and commercial traits measurement

The experimental protocols were approved by the institutional animal care and use committee of Southwest University. A total of 164 individuals were randomly selected from a cultured population in E Meishan, (Sichuan Province, China) in July 2017. The samples were bred in the same batch and pond. Fishes were anesthetized using tricaine meth-anesulfonate (MS222). Then Total length (TL), body length (BL) and body height (BH) were measured by Vernier Caliper (0.1 mm accuracy). Body weight (BW) was weighed using an electronic balance (0.1 g accuracy). After obtaining the measurement data, fins were cut and preserved in 95% alcohol, and genomic DNAs were extracted using Animal genomic DNA extraction kit (Sangon biotech, Shang Hai, China). DNA was examined by performing 1% agarose gel electrophoresis, and concentrations were determined using photometry (Eppendorf, German). The working DNA concentration was 30 ng/uL.

Polymorphism SNP loci screening and SNP genotyping

EST-SNP sequences were obtained from transcriptome of Spleen in *S. prenanti* in our previous research (Luo *et al*., 2016). Based on EST-SNP sequence information, primers were designed (Table I). PCR was performed in a 10 uL reaction volume, containing 1.25 uL 10×PCR Buffer with 15 mM $MgCl_2$, 0.65 uL 25 mM $MgCl_2$, 2 uL dNTP (2.5 mM), 2 uL forward primer (0.5 uM), 2 uL reverse primer (0.5 uM), 0.2 uL ExTaq DNA polymerase (5 U/ uL) (TaKaRa), using 2 uL genome DNA as template, adding 1.9 uL ddH_2O. PCR profiles included an initial denaturation at 94 °C for 15 min, 94°C denaturation for 20 s, annealing temperature 56 °C for 30 s, 72 °C for 60 s for 45 cycles and a final extension at 72 °C for 3 min. SNP sequence-specific extension primers were added to the PCR-amplified product and a base was extended at the SNP site. The extended product was purified and co-crystallized with a MassARRAY® SpectroCHIP chip with a surface-covered substrate, and the crystals were placed in a vacuum tube of a mass spectrometer to automatically analyze the SNPs site information.

Data analysis

Observed heterozygosity (Ho), expected heterozygosity (He) and Hardy-Weinberg equilibrium (HWE) for SNP loci were calculated using POPGENE version 32 software (Yeh and Boyle, 2000). Polymorphic information content (PIC) was calculated by PIC-CALC software.

Morphological analysis was performed with SPSS 19.0. Multivariate analysis of variance and independent-samples T test in the general linear model (GLM) were used to analyze the association between SNP locus and growth traits. Significant differences were tested using Duncan's multiple range test. Differences were considered to be statistically significant when $p < 0.05$ or $p<0.01$.

RESULTS

Morphological analysis

The descriptive statistics including mean, skewness, kurtosis, minimum and maximum for the growth traits were summarized in Table II. Skewness and kurtosis are parameters to test normal distribution. Skewness test and kurtosis test both indicated the four traits (BW, TL, BL and BH) obeyed normal distribution ($p>0.05$) (West *et al*., 1995).

Pearson correlation analysis showed that there was a significant correlation between the traits ($p<0.01$) (Table III). Maximum correlation coefficient was 0.982 between BL and TL. Minimum correlation coefficient was 0.887 between TL and BH.

Principal component analysis (PCA) indicated that body weight accounting for 94.50% of the variance, the eigenvalue more than 1, the accumulative variance more than 85%, it was the first principal component of the growth traits of *S. prenanti* (Table IV).

The characters of SNP loci

Point mutations of SNP included transitions and transversions. Among the 31 SNP markers, the number of transitions and transversions was respectively 18 and 13, and the ratio between them was approximately 1.38.

23 loci were polymorphic and the proportion was 74.2%. Genetic parameters of 23 SNPs are shown in Table V. The observed heterozygosity (H_o) ranged from 0.0854 to 0.3476, while the expected heterozygosity (H_e) ranged from 0.0820 to 0.3471, with the average values of 0.2094 and 0.2019, respectively. PIC value ranged from 0.0121 to0.3750 with an average of 0.2270. In addition, 13 loci had moderate polymorphism (0.25≤PIC<0.5) (Botstein *et al*., 1980). While the remained 10 loci had low polymorphism (PIC<0.25). 13 of the 23 SNPs were in accordance with HWE (p>0.05).

Table I. The primer information of 31 EST- SNPs in *S. prenanti.*

Locus	Mutation	Sequence (5' to 3')
ug25066-1464	C-T	F:CCTCAGCTTTCAGGGTAAAC
		R:TCCCATCCTAACCAGTACTC
ug23056-2291	C-T	F:TCTGTAACACCCCAAATGCC
		R:AAGGTGTCATATCCTTGCAG
ug25066-2545	T-C	F:AACCCTTTGTGTGGTGTCTG
		R:TGTGCCAGACTGAAGGTTTC
ug22539-1613	A-G	F:TCATCGTCTTAAAGAACTG
		R:ATGGGCATGTTGTTCCTCAC
ug23056-1182	T-C	F:ATCCCTGGAGCATAACTCAC
		R:GCAGGTTAAGCTGTCTAAGG
ug23056-1969	G-A	F:GATTACCACGGAGACTTAGC
		R:CTGGATCTGGATTACAATGG
ug22539-1718	C-T	F:TTTCTGACTCTGAAATGCAC
		R:GAAATGGAATATATCCCATGC
ug25066-1003	G-T	F:TTTGATGCAATCACCGGCAC
		R:ACCAAAAGAACTGCCCATGC
ug22539-1428	T-G	F:CCCTATCTCTGAGTTTCGAC
		R:TTTGAAGGACTGCTGTTCCC
ug23056-2461	T-A	F:TGTCTTCAGTACTGTGGATG
		R:ATGGTAAACCGTATGCTGGG
ug22539-1359	G-A	F:CTATAGGACCATTTGATGGAC
		R:GTATCCCATACCTACAACCC
ug23056-1317	C-A	F:AATCCAGTGTCTTCAGGTGC
		R:GAGCATCTATGCATGGCAAA
ug23056-2066	T-G	F:TGTGACACGTTGGACAGATG
		R:GCTGCATGCCTCTGTTTTTG
ug23056-2976	T-C	F:CTTCTACCAACAGGGTCAAC
		R:CGGACATACCAAATAAGGAC
ug25066-703	G-A	F:GGTGTCTCTAGAGATGCTTG
		R:GATCCAGCACATGGAATCTC
ug25066-1502	G-C	F:CGGACATGTTCTTAAATGGG
		R:TTTCACGCTTCTGACCCTTG
ug23056-2381	T-C	F:TGACACCTTTTAAGGCACTG
		R:GGATTCTCCATCCTTGGAAG
ug23056-1955	A-G	F:ATGTAACTCAGAGAAGCCGC
		R:CCTCAAGGATGCCACAAAAC
ug25066-2515	T-A	F:CCAACCATCCTTCAGGAAAC
		R:GACCATCTTACCAACATGCG
ug25066-779	C-T	F:AGGGTCAGAAGCGTGAAATG
		R:TTCCATGTGCTGGATCTCTC
ug22539-1605	C-A	F:GAACTGTATTTAGGCCCCAC
		R:ATGGGCATGTTGTTCCTCAC
ug23056-1165	A-T	F:ATCCCTGGAGCATAACTCAC
		R:GCAGGTTAAGCTGTCTAAGG
ug25066-2587	A-C	F:GTATGTTCCCTACAACCAGC
		R:AGAAACCTTCAGTCTGGCAC
ug225391-831	C-T	F:ATCAGAGCTTGGTGAGAAGG
		R:TTGCTCTTGCTCCCAGACAG
ug22539-400	T-C	F:CAAACTCAAAGCGACACCAG
		R:GAAGGAATAATGGGCAGTCG
ug23056-2938	G-T	F:CGGACATACCAAATAAGGAC
		R:CTTCTACCAACAGGGTCAAC
ug22539-817	C-T	F:TTGCTCTTGCTCCCAGACAG
		R:ATCAGAGCTTGGTGAGAAGG
ug22539-387	T-G	F:GAAGGAATAATGGGCAGTCG
		R:CAAACTCAAAGCGACACCAG
ug22539-551	C-A	F:CGTTCTCCAGATTCTCCATC
		R:CTGGTCGACAAGAATGTTCC
ug25066-2659	G-A	F:TATAAGTCCTGGCTGTCGTC
		R:CATACCGCAGTACAGGTTAC
ug25066-1540	C-T	F:AGTACTGGTTAGGATGGGAC
		R:AGCTGTTTCCAGATAGGTCC

Table II. Descriptive statistics for the growth traits.

Growth traits	Mean ±standard deviation	Skewness	Kurtosis	Minimum	Maximum	P
Body weight /g	72.74±30.43	0.62	-0.17	18	158	0.168
Total length/cm	19.76±2.72	-0.05	-0.47	12.4	26	0.697
Body length/cm	16.40±2.4	0.1	-0.27	10.1	23.1	0.615
Body height/cm	3.53±0.55	0.42	0.06	2.1	5.2	0.054

Table III. Correlation coefficients of body weight, total length, body length, body heigh.

Growth traits	Body weight	Total length	Body length	Body height
Body weight	1	0.939**	0.951**	0.910**
Total length		1	0.982**	0.887**
Body length			1	0.889**
Body height				1

Table IV. Principle component analysis on growth traits of *S.prenanti.*

Component	Initial of component			Sum of squares of extracted component		
	Total	Variance ratio	Accumulation variance ratio	Total	Variance ratio	Accumulation variance ratio
Body weight	3.78	94.50	94.50	3.78	94.50	94.50
Total length	0.14	4.67	98.06			
Body length	0.06	1.51	99.57			
Body height	0.02	0.43	100.00			

Table V. Genetic parameters of 23 SNP loci in *S. Prenanti.*

Locus	Mutation	H_o	H_e	P_{HWE}	PIC
ug22539-1613	A-G	0.2744	0.3188	0.0727	0.2673
ug22539-1718	C-T	0.0183	0.0182	0.9232	0.0179
ug23056-1182	T-C	0.1646	0.1912	0.0712	0.1725
ug23056-1317	C-A	0.1768	0.1617	0.2229	0.1482
ug23056-2066	T-G	0.3963	0.5015	0.0071	0.3750
ug23056-2976	T-C	0.1951	0.1766	0.1736	0.1606
ug25066-2545	T-C	0.2805	0.3151	0.1572	0.2648
ug22539-1359	G-A	0.3049	0.4102	0.001	0.3253
ug22539-1428	T-G	0.6951	0.4997	0.0000	0.3741
ug23056-1969	G-A	0.6103	0.4256	0.0000	0.3341
ug23056-2291	C-T	0.4146	0.3297	0.0009	0.2746
ug23056-2461	T-A	0.6829	0.4511	0.0000	0.3486
ug25066-1003	G-T	0.5915	0.4178	0.0000	0.3298
ug25066-1464	C-T	0.9207	0.4984	0.0000	0.3734
ug22539-1605	C-A	0.0854	0.0820	0.5827	0.0784
ug23056-1165	A-T	0.1951	0.2240	0.0953	0.1983
ug23056-1955	A-G	0.1890	0.2285	0.0253	0.2019
ug23056-2381	T-C	0.3476	0.3471	0.9868	0.2862
ug25066-2515	T-A	0.0122	0.0122	0.9558	0.0121
ug25066-2587	A-C	0.0305	0.0301	0.8595	0.029
ug25066-703	G-A	0.2317	0.3700	0.0000	0.3008
ug25066-779	C-T	0.0675	0.0654	0.6712	0.063
ug25066-1502	G-C	0.3476	0.3471	0.9868	0.2862
Mean		0.3145	0.2792		0.2270

observed heterozygosity= (H_o), expected heterozygosity= (H_e), polymorphic information content = (PIC), Hardy-Weinberg equilibrium= (HWE).

Associations between EST-SNPs and growth traits

The results of multivariate analysis of variance in the general linear model indicated that CC genotype at ug25066-1502 had significantly higher values for BW and TL than did individuals with the GG genotype ($p<0.05$), and CC genotype had significantly greater values for BW than CG genotype ($p<0.05$). For ug23056-2976, TT genotype was significantly lower than CT genotype for BH ($P<0.05$). Moreover, compared with CC genotype, individuals with the AC genotype at ug22539-1605 had significantly higher value in TL, BL, BH, and BW ($p<0.05$) (Table VI). Further analysed, the allele C was significantly higher than allele A in TL, BL and BH (Table VII) ($p<0.05$).

DISCUSSION

Principal component analysis is a dimension reduction technique that used to describe the relations between several response variables and explain the total variation in the data (Abbas and Wasin., 2019). To date, PCA has been widely used in aquatic area, such as aquatic ecosystem (Uddameri *et al*., 2014), aquatic nutrition (Casu *et al*., 2017; Gammanpila *et al*., 2017), morphological analysis (Jiang *et al*., 2012; Li *et al*., 2015). It is useful when the variables under study are highly correlated. In our study, the correlation analysis showed that four traits (BW, TL, BL and BH) have an extremely significant relationship. What is more, the result of PCA showed that BW is the first principal component of the growth traits of *S. prenanti*. Therefore, body weight is a main indicator in selective breeding of *S. prenanti*. Furthermore, TL, BH and BL could be indirect indicators to reflect the growth.

Table VI. Correlation analysis between genotypes of SNPs and growth traits in *S. prenanti*.

Locus	Genotype	Number	Body weight (g)	Body height (cm)	Total length (cm)	Body length (cm)
ug25066-1502	GG	99	75.26±30.37^{a}	3.59±0.54^{a}	20.00±2.69^{a}	16.62±2.39^{a}
	CG	57	67.07±30.12^{a}	3.40±0.56^{a}	19.16±2.73^{ab}	15.87±2.35^{a}
	CC	8	81.94±30.85^{b}	3.64±0.57^{a}	20.99±2.37^{b}	17.39±2.23^{a}
ug23056-2976	TT	132	70.73±29.22^{a}	3.48±0.53^{a}	19.59±2.66^{a}	16.24±2.34^{a}
	CT	32	81.00±34.26^{a}	3.71±0.62^{b}	20.45±2.87^{a}	17.03±2.56^{a}
ug22539-1605	CC	150	74.13±30.97^{aA}	3.54±0.57^{a}	19.88±2.77^{aA}	16.52±2.44^{aA}
	AC	14	57.8±18.86^{bB}	3.32±0.31^{b}	18.43±1.50^{bB}	15.08±1.34^{bB}

Note: The different superscript lowercase letters within the same column mean significantly difference at 0.05 level, and the different capital letters mean significantly difference at 0.01 level.

Table VII. Correlation analysis between alleles of SNP and growth traits.

Locus	Allele	Number	Body weight (g)	Body height (cm)	Total length (cm)	Body length (cm)
ug22539-1605	C	164	72.74±30.43	3.53±0.55^{a}	19.76±2.72^{Aa}	16.40±2.40^{Aa}
	A	14	57.8±18.86	3.32±0.31^{b}	18.43±1.50^{Bb}	15.08±1.34^{Bb}

Note: The different superscript lowercase letters within the same column mean significantly difference at 0.05 level, and the different capital letters mean significantly difference at 0.01 level.

Abundant SNPs discovered by the next generation sequencing technologies have allowed us to better understand the association between genomic variation and production traits in aquatic species (Yáñez *et al*., 2014). In this study, we identified 31 genomic SNP loci from the unigene data of the transcriptome. The ratio between conversion and transversion was 1.38, similar to turbot (1.35) (Vera *et al*., 2013), lower than lake sturgeon (1.65) (Hale *et al*., 2009), which is related to the differences of the species' own genome and living environment. The ratio between conversion and transversion in point mutations has a great influence on the degree of gene selection pressure and reflected deviation of mutation. Besides, the data of genotyping of SNP in 164 *S. prenanti* showed 23 loci were polymorphic accounting for 74.2%, which was lower than previous research in *S. prenanti* (Luo *et al*., 2016) and higher than *Mauremys mutica* (Zhao *et al*., 2016) and large yellow croaker (Jiang *et al*., 2015).

Heterozygosity could measure the genetic variability; He>5 indicated that the population experiences low selection and maintains higher genetic diversity (Jiang *et al*., 2015; Zhang *et al*., 2019). Our data suggested that the mean value of observed heterozygosity and expected heterozygosity were respectively 0.2094 and 0.2019, which indicated the population has low genetic diversity. After calculating the PIC in the population, 13 loci were found to have moderate polymorphism (0.25≤PIC<0.5), whereas others were low polymorphism (PIC<0.25). PIC demonstrates the degree of DNA variation, the value of which is dependent on the number of alleles and their frequency distribution. If the number of alleles was greater and the allelic frequencies of all alleles were more balanced, the PIC will be greater (nearly 1) (Li *et al*., 2012). Low genetic parameters could be attributed to the fact that the SNP only has two alleles, allelic imbalance, and the samples from cultured population and the population's genetic diversity were low.

If there is a significant association between markers and specific trait in a population, correlation analysis can figure out that which marker is associated with that trait (Doerge, 2002; Lynch and Walsh, 1997). This association has already reached the significant level, which may suggest the relationships between the marker and the trait. Consequently, the selective breeding inferred from phenotype can operate to genotype-assisted selection. In this study, we found that CC genotype at ug25066-1502 was significantly superior than GC genotype and GG genotype with higher BH, which demonstrates that ug25066-1502 was correlated with BH. However, only BL in the CC genotype was significantly superior than the GG genotype, while no significant difference was observed between GG and GC genotype. Therefore, these data imply that ug23056-2976 may have some effects on TL, but the effects are not major. Apart from these, at ug23056-2976 and ug22539-1605, we only detected two genotypes. The results imply that the CC at ug23056-2976 and AA at ug22539-1605 are rare genotypes. Or we need additional work to utilize larger samples for further confirmation. For ug22539-1605, the CC genotype was significantly correlated with the four traits. In our analysis, we found

that the allelic genotype C was significantly higher than allele A in TL, BL and BH. So, the allelic genotype C may be had a major impact on growth traits.

CONCLUSIONS

In our study, principal component analysis indicated that body weight was the first principal component of the growth traits of *S. prenanti*. And we found that ug25066-1502 was correlated with BH and ug22539-1605 was significantly correlated with the four traits. The two loci could be used as important candidate molecular markers for selective breeding of *S. prenanti*.

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Conflict of interest declaration

The authors have no conflict of interest to declare.

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