# Eight single nucleotide polymorphisms and their association with food habit domestication traits and growth traits in largemouth bass fry (*Micropterus salmoides*) based on PCR-RFLP method

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**Background.** The largemouth bass (*Micropterus salmoides*), an economically important freshwater fish species widely farmed in China, is traditionally cultured using a diet of forage fish. However, given the global decline in forage fish fisheries and increasing rates of waterbody pollution and disease outbreaks during traditional culturing, there is a growing trend of replacing forage fish with formulated feed in the largemouth bass breeding industry. The specific molecular mechanisms associated with such dietary transition in this fish are, nevertheless, poorly understood.

**Methods.** To identify single nucleotide polymorphisms (SNPs) related to food habit domestication traits and growth traits in largemouth bass fry, we initially genotyped fry using eight candidate SNPs based on polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method, with genetic parameters being determined using Popgen32 and Cervus 3.0. Subsequently, we assessed the associations between food habit domestication traits of largemouth bass fry and these SNPs using the Chi-square test or Fisher's exact test. Furthermore, we used a general linear model to assess the relationships between the growth traits of largemouth bass fry and these SNPs. The Pearson correlation coefficient between growth traits and the SNPs was also determined using bivariate correlation analysis in IBM SPSS Statistics 22. Finally, the phenotypic variation explained (PVE) by the SNPs was calculated by regression analysis in Microsoft Excel.

**Results.** The genotyping results obtained based on PCR-RFLP analysis were consistent with those of direct sequencing. Five SNPs (SNP01, SNP02, SNP04, SNP05, and SNP06) were found to be significantly correlated with the food habit domestication traits of fry ($P < 0.05$); SNP01 ($P = 0.0011$) and SNP04 ($P = 0.0055$) particularly, had showed highly significant associations. With respect to growth traits, we detected significant correlations with the two SNPs (SNP01 and SNP07) ($P < 0.05$), with SNP01 being significantly correlated with body length, and height ($P < 0.05$), and SNP07 being significantly correlated with body height only ($P < 0.05$).

**Conclusions.** Our findings indicated that the PCR-RFLP can be used as a low-cost genotyping method to identify SNPs related to food habit domestication and growth traits in largemouth bass, and that these trait-related SNPs might provide a molecular basis for the future breeding of new varieties of largemouth bass.

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## Abstract

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**Keywords:** *Micropterus salmoides*; single nucleotide polymorphism; food habit domestication traits; growth traits; polymerase chain reaction-restriction fragment length polymorphism

## Introduction

Food habit domestication is one of the important aspects of fishery cultivation. For carnivorous fish, switching to formulated feed can effectively reduce the pollution of the aquatic environment and the occurrence of diseases and also contributes to the conservation of marine resources (*Shao et al., 2022*). It is well established that during the fry stage, many fish species require live bait and do not readily switch to formulated feed. However, given that the use of live bait leads to high cultivation costs and may cause water pollution; the comprehensive replacement of forage fish with formulated feed is a foreseeable trend (*Welch et al., 2010*). Although studies on feed domestication have been carried out in mammals to analyze the related mechanism associated with feed transformation, there have been relatively few studies that have examined the molecular regulation mechanisms underlying the regulation of feed domestication in fish (*Wiener & Wilkinson, 2011; Zhao et al., 2010*). Among those studies that have been conducted, some have reported the possibility of "imprinting" fish with alternative components or nutritional levels in early life to improve their utilization in later life, although the specific regulatory mechanisms remain unclear (*Kwasek et al., 2021; Sammons, 2012*). In addition, based on the molecular mechanisms of feeding, some studies have attempted to identify the important regulatory factors associated with the consumption of compound feed, to promote the comprehensive substitution of forage fish with formulated feed. In this regard, the findings of previous studies have indicated that the feeding habits of carnivorous fish are influenced by pathways associated with the regulation of retinal photosensitivity, circadian rhythm, appetite control, learning, and memory (*He et al., 2013*). Given that genetic factors play an important role in switching to formulated feed, some studies have used candidate genes, such as *gh* (*growth hormone*) (*Dou et al., 2020*), *LPL* (*lipoprotein lipase*) (*Ma et al., 2018a; Yang et al., 2011*),

*ghrelin* (*Liu et al., 2016*) and *PEP* (*pepsinogen*) (*Fang et al., 2011*), and association analysis to identify key molecular markers related to the traits involved in switching to formulated feed in carnivorous fish, with the aim of providing references for molecular assisted breeding of such traits.

The largemouth bass (*Micropterus salmoides*) is a typical predator fish native to North America, that has become an economically important freshwater fish in China (*Ma et al., 2020*). With gradual progress in the genetic improvement of largemouth bass, important breakthroughs have been made with respect to the breeding for switching to formulated feed. For example, the growth and feed conversion traits of the new variety “Youlu No.3” have been significantly improved compared with those of its predecessor “Youlu No.1” and have made a significant contribution to the rapid development of the largemouth bass breeding industry (*Li et al., 2018*). However, the “Youlu No.3” fry must experience the succession of “live bait-dead bait-formulated feed”. Thus, switching to formulated feed remains a relatively long process, and the success rate of switching to formulated feed still requires further improvement (*Zhao et al., 2019*). In addition, during the process of feeding habit domestication, the time of domestication varies markedly among individuals, and there are still individuals that fail to undergo successful domestication. Given that domestication has great influence on the survival rate and benefit of breeding, it is desirable to identify candidate markers related to switching to formulated feed in largemouth bass to improve the success rate of cultivation and shorten the period of switching to formulated feed.

Previously, we observed that some “Youlu No.3” do not need to transition via the “dead bait” stage prior to being able to consume formulated feed, and can fill the stomach. Accordingly, in the present study, we used "Youlu No. 3" fry as the experimental material, and the process of switching to formulated feed was directly from “live bait-formulated feed” without “dead bait” transition stage. According to the degree of difficulty in receiving artificial formulated feed, we identified two extreme groups designated domesticated and non-domesticated. Subsequently, potential single nucleotide polymorphisms (SNPs) associated with the food habit domestication traits and growth traits were excavated by genotyping by sequencing (GBS). Among the SNPs identified, eight were successfully used to genotype fry based on polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) for identification in large sample sizes, and we used these selected SNPs for further analysis of their association with feeding traits and growth traits. In the present study, we demonstrate that the largemouth bass fry can be directly transferred from the “live bait - the compound feed” without the “dead bait” stage. The purpose of this study was to identify the SNPs related to the food habit domestication traits and growth traits of largemouth bass, and to screen out the largemouth bass that do not experience the “dead bait” stage and directly feed on formulated feed. Thus, our work provides a valuable reference for simplifying switching process of largemouth bass, and provides a theoretical basis for the further genetic improvement to increase the tolerance of largemouth bass to the formulated feed, which will promote the sustainable and healthy development of the largemouth bass breeding industry.

## Materials & Methods

### 2.1 Sample collection

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Academic Committee of Henan Normal University (HNSD-2021-08-06). The "Youlu No.3" largemouth bass fry used in this study, which were the offspring of the random mating and natural spawning of 93 parental fish (51 females and 42 males), were obtained in May 2021 from a population cultured at the Lantian Aquaculture Professional Cooperative (Zhoukou, China). Approximately 150, 000 fertilized eggs were hatched on May 26 in a round tank (diameter = 1.5 m) fitted with a circulating water system (temperature = 25 ± 0.5 °C, DO = 8 – 9 mg/L). The fry were fed with artificially hatched brine shrimps (*Artemia salina*) from May 29 to June 20, during which time, the fry were gradually divided into 10 similar round tanks, corresponding with a reduction in feeding frequency decreased from 6 to 4 times a day. Approximately 1,200 fish (20.34 ± 1.78 mm) were randomly selected from the ten round tanks mentioned above and transferred into another round tank (the same as above) for 24 h of starvation. The powdered formulated feed with which the fry were subsequently provisioned was mixed with water and the bass were fed continuously for 2 hours on June 21. The fry were subsequently anesthetized with MS-222 (3-aminobenzoic acid ethyl ester methanesulfonate; Sigma, Saint Louis, MO, USA), and growth data (body standard length from the front of the mouth to the base of the caudal fin and height) were measured using ImageJ software, expressed as the average of three consecutive readings (*Mishra et al., 2021*). Subsequently, the stomach of the fry were removed under a stereomicroscope and weighed. The fry were accordingly defined as non-domesticated (stomach/body weight < 8%, totaling 236 individuals) and domesticated (stomach/body weight > 24%, totaling 113 individuals) (*Zhao et al., 2019*). Every 96 juveniles were randomly selected from both the domesticated and non-domesticated groups, and their caudal fins were cut and preserved in absolute ethanol. Genomic DNA was then extracted using the Animal Genome Rapid Extraction Kit (Sangon, China). The quality and concentration of DNA were detected using 1% agarose gel electrophoresis and the NanoDrop 2000 (Thermo Fisher Scientific, USA). The DNA was dissolved in sterile water at 20 ng/μL, and stored at -20 °C.

### 2.2 High-throughput sequencing and primer design

30 fry (15 individuals from each group) were randomly selected for the construction of GBS libraries and sequencing. Genomic DNA was digested with the restriction enzyme *Mse*I-*Nla*III-*Msp*I. PCR amplification was performed after digesting the products with adapters, and 215-240 base pair (bp) fragments were recovered to construct the GBS library. After the GBS library preparation, Illumina HiSeq PE150 sequencing was subsequently performed (Novogene, China). The raw sequencing reads (BioProject: PRJNA769836) were filtered using fastp (v0.20.1) software with default parameters (*Chen et al., 2018*), and the resulting clean reads were aligned to the *Micropterus salmoides* reference genome (GenBank assembly accession:

GCA_019677235.1) using BWA (v0.7.17) software (*Li & Durbin, 2010*). SNPs were called using the Genome Analysis Toolkit (GATK 4.1.9.0) software (*McKenna et al., 2010*). A total of 728 SNPs were found to differ significantly between the two groups ($P<0.05$) based on preliminary analysis using a Chi-square test, of which 41 could be digested by *EcoR*V (3), *Hinf*I (20), *Pst*I (7), *Pvu*II (6), *Xba*I (3) and *Xho*I (2). On the basis of the flanking sequences and the results of restriction site analysis using an online website (*http://www.detaibio.com/sms2/rest_summary.html*), we designed 17 primer pairs using Primer Premier 5 software (*Lalitha, 2000*), however, only eight SNPs were successfully genotyped using PCR-RFLP (Table 1).

**2.3 PCR-RFLP genotyping and identification**

The PCR-RFLP method was used for SNPs genotyping. PCR was performed in a 20 μL volume mixture containing 0.5 μmol/L primer, 10 μL of 2 × Master Mix (Vazyme, China), 60 ng template DNA, and deionized water added to 20 μL. The PCR conditions were as follows: pre-denaturation at 95 °C for 3 min; denaturation at 95 °C for 30 s, annealing time for 30 s (the annealing temperature (Ta) is shown in Table 1), extension at 72 °C for 45 s for a total of 34 cycles, and a final extension at 72 °C for 5 min. The PCR products were detected using 1% agarose gel electrophoresis, and the qualified PCR products were digested. The restriction enzymes (Sangon, China) corresponding to the eight SNPs are listed in Table 1. The enzyme restriction system was performed in a 10 μL volume containing 5 μL of PCR product, 0.5 μL restriction enzyme, 1 μL of 10 × Speedy One Buffer, and deionized water supplemented to 10 μL. The reaction was then performed at 37 °C for 45 min. The fragment size of the digested product was detected using 2% agarose gel electrophoresis. PCR products corresponding to the different genotypes of each SNP were randomly selected for direct sequencing.

**2.4. Statistical analysis**

Microsoft Excel (Microsoft Corp., Redmond, WA, USA) was used for statistical analysis of the morphological data and genotyping results. Analyses of the observed heterozygosity (Ho), expected heterozygosity (He), and the polymorphic information content (PIC) were performed using Cervus 3.0 software (*Botstein et al., 1980; Kalinowski, Taper & Marshall, 2007*). Popgen32 software was used to analyze the Hardy-Weinberg equilibrium (*Yeh & Boyle, 1996*). The correlation between genotypes at each locus and food habit domestication traits of the fry was analyzed using the Chi-square or Fisher exact test in R software. The general linear model in IBM SPSS Statistics 22 (IBM Corp., Armonk, NY, USA) was used to analyze the correlation between the genotypes at each locus and body height, and length of largemouth bass fry (*Ma et al. 2018b*). The bivariate correlation analysis was used to analyze the correlation between the genotypes of each locus and growth traits in IBM SPSS Statistics 22 (*Weaver & Wuensch, 2013*), and the phenotypic variation explained (PVE) was calculated using regression analysis in Microsoft Excel.

198 # Results

199 **3.1. Comparative analysis of direct sequencing peak and PCR-RFLP**

200 The genotyping results revealed that all eight selected SNPs were successfully genotyped using
201 the PCR-RFLP method. Each locus had a homozygous wild genotype, heterozygous mutant
202 genotype, and homozygous mutant genotype. The PCR products of different genotypes at each
203 locus were randomly selected and subjected to direct sequencing. Comparison of a sequencing
204 peak map with the banding patterns of the PCR-RFLP products on agarose (Figure. 1),
205 confirmed the direct sequencing results were consistent with the PCR-RFLP genotyping results,
206 thereby indicating the applicability of the PCR-RFLP approach for SNP genotyping.
207

208 **3.2. Polymorphism analysis of eight SNPs**

209 The genotype frequencies of eight SNPs were analyzed using Microsoft Excel. The results
210 revealed that the Ho of eight SNPs ranged from 0.3490 to 0.5417, He ranged from 0.3514 to
211 0.5013, and PIC ranged from 0.2891 to 0.3750. All eight SNPs were moderately polymorphic
212 ($0.25 \leq PIC < 0.5$), and SNP03 deviated significantly from the Hardy-Weinberg equilibrium
213 (Table 2).
214

215 **3.3. Correlation analysis between eight SNPs and food habit domestication traits**

216 The Chi-square test or Fisher's exact test were used to analyze the correlation between the eight
217 SNPs and the food habit domestication traits of the fish fry (Table 3). The results showed that
218 three SNPs (SNP02, SNP05, and SNP06) were significantly correlated with food habit
219 domestication traits ($P < 0.05$), with PVE values of 3.29, 0.02 and 3.11, respectively. In addition,
220 two SNPs (SNP01 and SNP04) were highly significantly correlated with food habit
221 domestication traits ($P < 0.01$), with PVE values of 7.08 and 5.39, respectively.
222

223 **3.4. Associations between eight SNPs and growth traits**

224 The analysis of the correlation between eight SNPs and growth traits of the fry using the general
225 linear model revealed that two SNPs (SNP01 and SNP07) were significantly associated with the
226 growth traits of largemouth bass fry (Table 4). The body height differed significantly with
227 respect to the three genotypes of SNP01 ($P < 0.05$), the Pearson correlation coefficient between
228 SNP01 and body height was 0.291 and PVE value was 8.45. Whereas body length showed a
229 significant difference only between the GG and GA genotypes ($P < 0.05$), the Pearson
230 correlation coefficient between SNP01 and body length was 0.172 and PVE value was 2.95.
231 Additionally, the body height of the SNP07 AA genotype had a significantly high correlation
232 with that of the CC genotype ($P < 0.05$). The Pearson correlation coefficient between SNP07 and
233 body height was 0.156, and PVE value was 2.43.
234

235 # Discussion

236 **4.1. Application of PCR-RFLP for SNPs genotyping of largemouth bass fry**

As the third generation of molecular genetic markers, SNPs have broad application prospects in animal and plant breeding because of their large number, wide distribution, and considerable effects on phenotypes (*Lambert et al., 2016; Siccha-Ramirez et al., 2018; Zhang et al., 2019*). However, SNPs have their own application limitations, such as its relatively high genotyping costs. Fortunately, several methods have been developed and improved to decrease SNP genotyping cost, including allele-specific PCR (AS-PCR), SNaPshot, and PCR-RFLP (*Zhao et al., 2017*). The PCR-RFLP is a cost-effective method and has been successfully applied in SNP genotyping of many species (*Forche, Steinbach & Berman, 2009; Jiang et al., 2021*). In the 1980s, *Botstein et al.* (*1980*) used DNA RFLP to construct a genetic linkage map of human genes, which pioneered the use of DNA polymorphic genetic markers. However, the procedures associated with this technique are notably complex, and there are certain complications, such as the increase, decrease and movement of enzyme digestion sites, which limits the widespread application of RFLP markers. PCR-RFLP combines the advantages of PCR and RFLP, and this combined technique is frequently used as the method of choice in analyses of the genetic variation of genomic DNA to reveal SNPs loci or in the use of known SNPs loci for genotyping is increasingly favored. For example, *Viana et al.* (*2007*) designed a PCR-RFLP strategy for the G/A mutation site at base pair 1440 bp of the human *CXCR2* gene and successfully genotyped this, whereas Ma et al. (*2011*) also used this technique to identify a SNP site on the polymorphism of the partial sequence of the antimicrobial peptide gene $SCY^2$ in *Scyllapar amamosain*, which was not found by direct sequencing, thereby confirming that PCR-RFLP has considerable applicability in detecting molecular genetic variations.

In terms of SNP genotyping, PCR-RFLP technology has clear advantages compared to direct sequencing, including low cost, rapidity, and reliable analytical results. Nevertheless, it has certain limitations. If SNPs cannot form restriction sites, PCR-RFLP cannot be used directly for genotyping. Even if SNPs can form restriction sites, the genotyping cost of each site may vary greatly due to the different costs of restriction enzymes (*Xu & Shen, 2003*). For example, in a 20 μL enzyme restriction system, the genotyping cost of SpeedyCut *Eco*RI was 0.5 CNY/site, while the genotyping cost of SpeedyCut *Fsp*I was 6 CNY/site. In addition, the PCR-RFLP method is susceptible to the type and number of restriction enzyme sites in flanking sequences (*Yan et al., 2011*). For example, in the present study, there was a G/C mutation at SNP05 in this study, which *Pvu*II could restrict, and the PCR product fragment containing this SNP site was 648 bp. However, another restriction enzyme site in the PCR product was recognized by *Pvu*II, which led to the 101 and 547 bp bands. Therefore, there were two bands of 101 and 547 bp in the digested products of the homozygous wild-type GG, and *Pvu*II could completely digest the 547 bp PCR product of the homozygous mutant CC to produce 169 and 378 bp, giving three bands of 101, 169, and 378 bp. In the PCR products of heterozygous mutant type GC, only part of the 547 bp could be digested by *Pvu*II to produce 169 and 378 bp, giving four bands of 101, 169, 378, and 547 bp. Furthermore, the PCR-RFLP method usually requires enzyme restriction after PCR amplification, which can easily cause pollution and affect the genotyping results due to the open

operation. In addition, each locus must be digested after PCR amplification, which is more suitable for SNP genotyping with a small number of loci and a medium/large sample size.

### 4.2. Association analysis of food habit domestication traits and growth traits in largemouth bass fry

The largemouth bass is an economically important freshwater fish in China, wherein it has been widely cultured in recent years. During the culturing process, the cost of rearing can be effectively reduced by directly transfer from "live bait" to "formulated feed" without transitioning through the "dead bait" stage. Although largemouth bass fry do not readily switch to formulated feed, while some studies have shown that an early transfer to formulated feed can increase food intake and improve the later growth performance (*Skudlarek, Coyle & Tidwell, 2013*). Therefore, improving the success rate and shortening the period of switching to formulated feed of largemouth bass would be advantageous (*Ehrlich et al., 1989*). In this regard, the use of molecular markers to screen largemouth bass that can be easily switched to formulated feed without "dead bait" stage can effectively solve the problems of breeding environment contamination and disease associated with "dead bait" stage in the cultivation process of largemouth bass. With the publication of the largemouth bass genome and the decrease in high-throughput sequencing costs (*Sun et al., 2021*), it is possible to use high-throughput sequencing technology to identify molecular markers related to the economic traits of largemouth bass. In this study "Youlu No.3", which did not experience the "dead bait" stage, was used as the experimental material to screen SNPs related to food habit domestication. We identified five SNPs related to food habit domestication traits and two SNPs related to growth traits were successfully verified. The results showed that there were significant differences in the body height traits among the three genotypes of SNP01 (GG > GA > AA, $P < 0.05$), with respect to body height, and between the two genotypes of SNP01 (GA > AA, $P < 0.05$), with respect to body length, whereas the two genotypes of SNP07 were found to be associated with body length, which may be related to growth rate differences at different stages (*Gong et al., 2022; Jiang et al., 2020*). Further research on the other potential food habit domestication-related SNPs based on GBS data is required to verify the potential application of marker-assisted selection for largemouth bass in the future.

## Conclusions

In summary, the PCR-RFLP method was successfully and accurately applied in the genotyping of eight randomly selected SNPs. Five food habit domestication-related SNPs and two growth-related SNPs were identified in largemouth bass fry. The results of the present study suggest that the PCR-RFLP might be a low-cost and effective method for excavating trait-related SNPs, especially using "small sample/big data" to excavate and then the correlation is verified by "slight amount markers/big sample". Overall, our findings provide candidate markers for further genetic improvement of the related traits of largemouth bass.

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## References

**Botstein D, White RL, Skolnick M, Davis RW. 1980.** Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *American Journal of Human Genetics* **32(3)**: 314-331.

**Chen SF, Zhou YQ, Chen YR, Gu J. 2018.** fastp: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics* **34:** 884–890.

**Dou YQ, Liang XF, Gao JJ, He S, Shi LJ, Shi DY. 2020.** Single nucleotide polymorphisms in pepsinogen gene, growth hormone gene and their association with food habit domestication traits in *Siniperca chuatsi*. *Journal of Fisheries of China* **27(5)**: 485-493.

**Ehrlich KF, Cantin MC, Rust MB, Grant B. 1989.** Growth and survival of larval and postlarval smallmouth bass fed a commercially prepared dry feed and/or *Artemia nauplii*. *Journal of the World Aquaculture Society* **20**:1-6.

**Fang R, Liang XF, Yang YH, Du JH, Cao L, Ye W, Fu Y. 2011.** Association of polymorphism detection of SNPs in exon 7 of pepsinogen (*PEP*) and feeding behavior domestication in *Siniperca chuatsi*. *Journal of Fishery Sciences of China* **18(5):** 992-999.

**Forche A, Steinbach M, Berman J. 2009.** Efficient and Rapid identification of *Candida albicans* allelic status using SNP-RFLP. *FEMS Yeast Research* **9(7)**: 1061-1069.

**Gong YT, Fu JJ, Zhu WB, Zhang LB, Wang, LM, Qiang J, Gao C, Hu GF, Hong B, Dong ZJ. 2022.** Growth traits and path analysis comparison of two populations of largemouth bass (*Micropterus salmoides*) and their reciprocal hybrids. *Journal of Shanghai Ocean University* **33(1)**: 131-139.

**He S, Liang XF, Sun J, Li L, Yu Y, Huang W, Qu CM, Cao L, Bai XL, Tao YX. 2013.** Insights into food preference in hybrid F1 of *Siniperca chuatsi* (♀) ×*Siniperca scherzeri* (♂) mandarin fish through transcriptome analysis. *BMC Genomics* **14:** 601.

**Jiang X, Lin XQ, Ma H, Chen F, Xiong Y, Wang XH. 2020.** Correlation and path analysis on body weight and morphometric traits of 5-mouth-old *Micropterus salmoides*. *Shandong Agricultural Sciences* **52(5)**: 124-129.

**Jiang Z, Cui J, Shao JQ, Dong CJ, Du JX, Shen YB, Li SJ, Zhang M, Li XJ. 2021.** Development and characterization of 37 SNP markers for the largemouth bass (*Micropterus salmoides*) by using PCR-RFLP method. *Conservation Genetics Resources* **13(4)**: 429-433.

**Kalinowski ST, Taper ML, Marshall TC. 2007.** Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Molecular Ecology* **16(5)**: 1099-1106.

**Kwasek K, Wojno M, Patula S, Oliaro F, Cabay C, Pinnell LJ. 2021.** The effect of first feeding exposure of larval largemouth bass to a formulated soybean meal-based or Soy Saponin-

supplemented diet on fish growth performance and gut microbiome. *North American Journal of Aquaculture* **83**: 312-326.
**Lalitha S. 2000.** Primer Premier 5. *Biotech Software & Internet Report* **1(6)**: 270-272.
**Lambert P, Campoy JA, Pacheco I, Mauroux JB, Cassia DSL, Micheletti D, Bassi D, Rossini L, Dirlewanger E, Pascal T, Troggio M, Aranzana MJ, Patocchi A, Arús P. 2016.** Identifying SNP markers tightly associated with six major genes in peach [*Prunus persica* (L.) Batsch] using a high-density SNP array with an objective of marker-assisted selection (MAS). *Tree Genetics & Genoms* **12(6)**: 121.
**Li H. Durbin R. 2010.** Fast and accurate long-read alignment with Burrows–Wheeler transform. *Bioinformatics* **26(5)**: 589–595.
**Li SJ, Bai JJ, Han LQ, Sun JG. 2018.** Breeding example of largemouth bass "Youlu No.3". *Scientific Fish Farming* **9**: 44-45.
**Liu H, Bai JJ, Li SJ, Fan JJ, Quan YC. 2016.** SNPs detection of *ghrelin* gene and its association with growth traits in largemouth bass (*Micropterus salmoides*). *Journal of Fisheries of China* **40(4)**: 521-527.
**Ma HY, Ma CY, Zhang D, Cui HY, Jiang KJ, Ma LB. (2011).** Analysis of SNP of antimicrobial peptide *SCY*$^2$ gene in *Scylla paramamoain* by by PCR-RFLP assay. *Journal of Wuhan University (Natural Science Edition)* **57(4)**: 343-349.
**Ma DM, Zhu ZM, Bai JJ, Quan YC, Fan JJ, Tian YY, Liao GL, Liang XF. 2018a**. SNPs in *LPL* Genes and study of their association relationship with the adaptability to formulated feed in *Micropterus salmoides*. *Journal of Agricultural Biotechnology* **26(1)**: 140-149.
**Ma DM, Quan YC, Fan JJ, Hu J, Bai JJ, Liu H. 2018b.** Development of SNPs related to bait domestication based on largemouth bass (*Micropterus salmoides*) transcriptome and association analysis with growth traits. *Journal of Fisheries of China* **42(11)**: 1684-1692.
**Ma DM, Fan JJ, Zhu HP, Su HH, Jiang P, Yu LY, Liao GL, Bai JJ. 2020.** Histologic examination and transcriptome analysis uncovered liver damage in largemouth bass from formulated diets. *Aquaculture* **526**: 735329.
**McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, Garimella K, Altshuler D, Gabriel S, Daly M, DePristo MA. 2010.** The genome analysis toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Research* **20:** 1297–1303.
**Mishra N, Agsar D, Deepthi S, Kattegouda UM, Shetty PR, Pandey V. 2021.** Novel application of Nerium leaf and Image J software in drop collapse assay for rapid screening of biosurfactant producing microorganisms. Indian Journal of Experimental Biology **59(7)**: 484-492.
**Sammons SM. 2012.** Diets of juvenile and sub-adult size classes of three Micropterus spp. in the Flint River, Georgia: Potential for trophic competition. *Southeastern Naturalist* **11**: 387-404.
**Shao JQ, Du JX, Lei CX, Li SJ, Dong CJ, Zhang M, Li XJ. 2022.** Development of genes and SNP markers related to food domestication based on largemouth bass transcriptome. *Journal of Fishery Sciences of China* **29(3)**: 421-434.

**Siccha-Ramirez ZR, Maroso F, Pardo BG, Fernández C, Martínez P, Oliverira C. 2018.** SNP identification and validation on genomic DNA for studying genetic diversity in *Thunnus albacares* and *Scomberomorus brasiliensis* by combining RADseq and long read high throughput sequencing. *Fisheries Ressearch* **198**: 189-194.

**Skudlarek N, Coyle SD, Tidwell JH. 2013.** Development of first-feeding protocols for indoor larviculture of largemouth bass (*Micropterus salmoides*). *Journal of Applied Aquaculture* **25**: 9-23.

**Sun CF, Li J, Dong JJ, Niu YC, Hu J, Lian JM, Li WH, Li J, Tian YY, Shi Q, Ye X. 2021.** Chromosome-level genome assembly for the largemouth bass *Micropterus salmoides* provides insights into adaptation to fresh and brackish water. *Molecular Ecology Resources* **21(1)**: 301-315.

**Viana AC, Kim YJ, Cirelli JA, Orrico SRP, Curtis KC, Cano VSP, Valentini SR, Scarel-Caminaga RM. 2007.** A novel PCR-RFLP assay for the detection of the single nucleotide polymorphism at position +1440 in the human *CXCR2* gene. *Biochemical Genetics* **45**: 737-741.

**Weaver B, Wuensch KL. 2013.** SPSS and SAS programs for comparing Pearson correlations and OLS regression coefficients. *Behavior Research Methods* **45:** 880–895.

**Welch A, Hoenig R, Stieglitz J, Benetti D, Tacon A, Sims N, O'Hanlon B. 2010.** From fishing to the sustainable farming of carnivorous marine finfish. *Reviews in Fisheries Science* **18(3)**: 235-247.

**Xu YH, Shen J. 2003.** DNA molecular markers techniques and their principles. *Journal of Lianyungang Normal College* **3:**78-82.

**Yan MY, Song PS, Lai J, Guo YH, Ni L, Zhu XC. 2022.** Identification of *Aucklandia Costus*，*Dolomiaea Souliei* and *Inula Racemosa* by PCR-RFLP based on ITS sequence. *Journal of Nanjing University of Traditional Chinese Medicine* **38(4):** 339-346.

**Yang YH, Liang XF, Fang R, Peng MY, Huang ZY. 2011.** Polymorphism of SNPs in the lipoprotein lipase (*LPL*) in *Siniperca chuatsi* and their association with feed habit domestication. *Hereditas* **33(9)**: 996-1002.

**Yeh FC, Boyle TJB. 1996.** Population genetic analysis of co-dominant and dominant markers and quantitative traits. *Belgian Journal of Botany* **129:** 157.

**Wiener P, Wilkinson S. 2011.** Deciphering the genetic basis of animal domestication. *Proceedings Biological Sciences* **278(1722)**: 3161-3170.

**Zhang SY, Li X, Chen XH, Pan JL, Wang MH, Zhong LQ, Qin Q, Bian WJ. 2019.** Significant associations between prolactin gene polymorphisms and growth traits in the channel catfish (*Ictalurus punctatus* Rafinesque, 1818) core breeding population. *Meta Gene* **19**: 32-36.

**Zhao HB, Yang JR, Xu HL, Zhang JZ. 2010.** Pseudogenization of the umami taste receptor gene *Tas1r1* in the giant panda coincided with its dietary switch to bamboo. *Molecular Biology & Evolution* **27(12)**: 2669-2673.

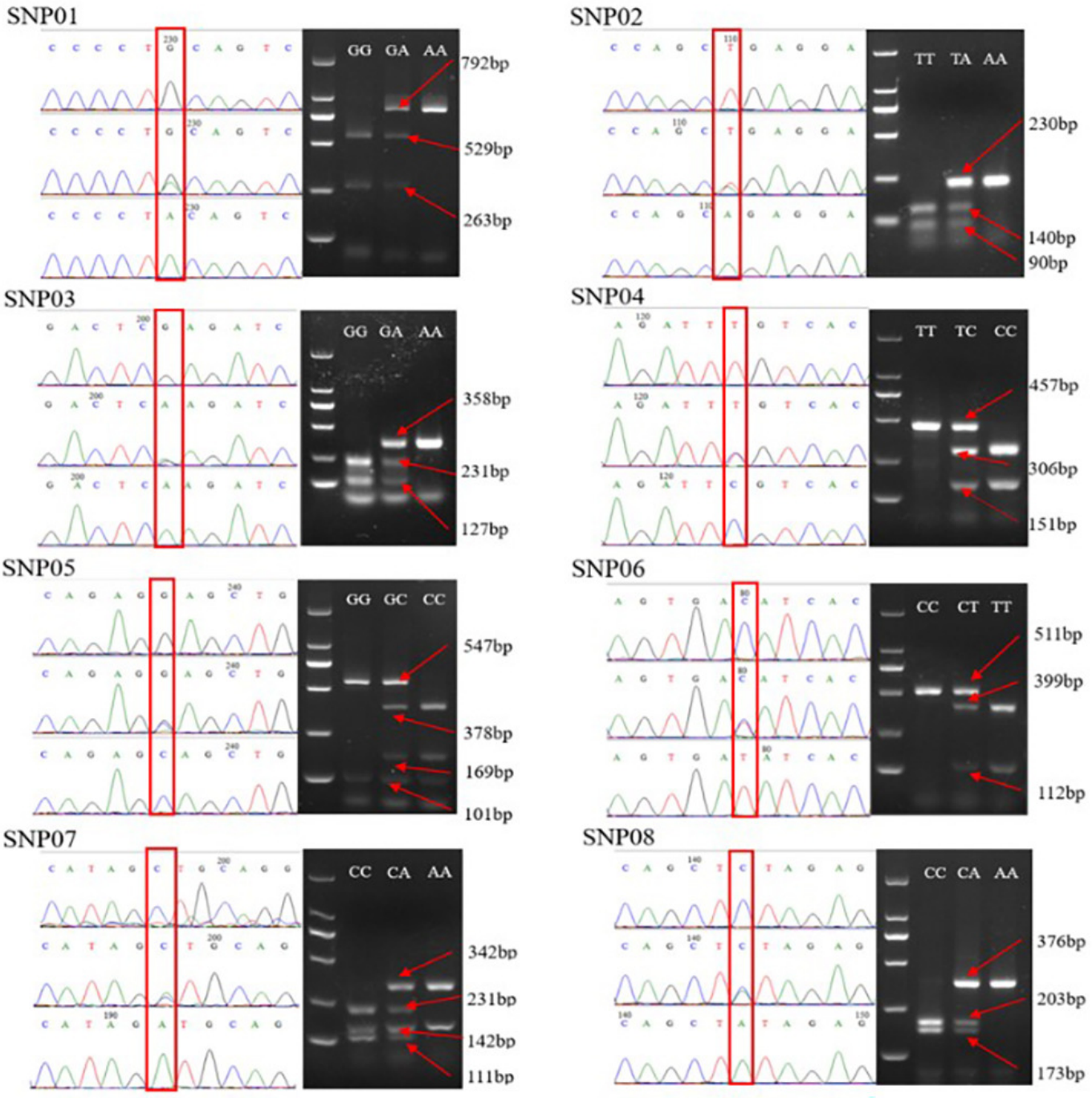
**Zhao L, Xue B, Gao H, Yan BL. 2017.** Progress on the SNP molecular markers in economic crustaceans. *Marine Fisheries* **39(2)**: 233-240.

**Zhao L, Li SJ, Bai JJ, Liang JH, Han LQ, Liang XF. 2019.** Transfer food from zooplankton to formulated feed in juvenile selectively bred largemouth bass *Micropterus salmoides*. *Fisheries Science* **38(6)**: 846-850.

# Figure 1

Comparison of the sequencing peaks of different genotypes of SNP01-SNP08 and PCR-RFLP.

The red box indicates the mutation site, and different sizes of enzyme fragments are on the right. The GG of SNP05 and the AA of SNP07 have two bands after digestion due to the existence of enzyme sites in the selected flank sequence, which is independent of specific SNP.


SNP01
GG GA AA
792bp
529bp
263bp
SNP02
TT TA AA
230bp
140bp
90bp
SNP03
GG GA AA
358bp
231bp
127bp
SNP04
TT TC CC
457bp
306bp
151bp
SNP05
GG GC CC
547bp
378bp
169bp
101bp
SNP06
CC CT TT
511bp
399bp
112bp
SNP07
CC CA AA
342bp
231bp
142bp
111bp
SNP08
CC CA AA
376bp
203bp
173bp

## Table 1(on next page)

Primer information of eight SNPs in juvenile *M. salmoides*

1 **Table 1.** Primer information of eight SNPs in juvenile *M. salmoides*

| Locus | Flanking Sequencing | Restriction Enzyme | Primer Information | | |
|---|---|---|---|---|---|
| | | | Name | Sequence (5'-3') | Ta(℃) |
| SNP01 | CCCCT[G/A]CAGTC | *Pst*I | F1 | CAGTGGGAATGGTTATACATG | 60 |
| | | | R1 | CTGAGAATGTAGCAGTAAAGTCC | |
| SNP02 | CCAGC[T/A]GAGGA | *Pvu*II | F2 | CAACTAAAGGAAAGGCTGATT | 54 |
| | | | R2 | GGAAGACGAGAAACACGAAA | |
| SNP03 | GACTC[G/A]AGATC | *Xho*I | F3 | TCGGGAAATCCGTGTTGA | 58 |
| | | | R3 | CAGGTTAAACTTTGTTCCTGGTC | |
| SNP04 | AGATT[T/C]GTCAC | *Hinf*I | F4 | GCCCAAGAGGACACTTAGAT | 56 |
| | | | R4 | TTTTTACGAGATAGGGTAGACAT | |
| SNP05 | CAGAG[G/C]AGCTG | *Pvu*II | F5 | CTTCATGCAGTTGGGTATT | 48 |
| | | | R5 | AATACTTATGTTTGCCCTTG | |
| SNP06 | AGTGA[C/T]ATCAC | *EcoR*V | F6 | TTGTGTTATTTGAAGAGTAACTTAAC | 62 |
| | | | R6 | AATCTACCCATCTACAGTCCC | |
| SNP07 | CATAG[C/A]TGCAG | *Pst*I | F7 | ATCCTACAATTGGATGTAACTT | 60 |
| | | | R7 | AAAGCCCAACTATTACCC | |
| SNP08 | CAGCT[C/A]TAGAG | *Xba*I | F8 | ATCTCCCACGCCAAGTCA | 63 |
| | | | R8 | AAAATCCAAGTGCGGTCTG | |

2

3

## Table 2(on next page)

Genotype frequency and genetic parameters of eight SNPs in juvenile *M. salmoides*

* indicates significant differences ($P < 0.05$).

1 **Table 2.** Genotype frequency and genetic parameters of eight SNPs in juvenile *M. salmoides*

| Locus | Genotype Frequency | | | Ho | He | PIC | PHWE |
|---|---|---|---|---|---|---|---|
| SNP01 | GG(0.4896) | GA(0.4219) | AA(0.0885) | 0.4219 | 0.4207 | 0.3316 | 0.9684 |
| SNP02 | TT(0.0938) | TA(0.3490) | AA(0.5573) | 0.3490 | 0.3936 | 0.3155 | 0.1147 |
| SNP03 | GG(0.0990) | GA(0.5417) | AA(0.3594) | 0.5417 | 0.4673 | 0.3575 | 0.0270* |
| SNP04 | TT(0.3958) | TC(0.4948) | CC(0.1094) | 0.4948 | 0.4602 | 0.3536 | 0.2957 |
| SNP05 | GG(0.3021) | GC(0.5313) | CC(0.1667) | 0.5313 | 0.4921 | 0.3704 | 0.2692 |
| SNP06 | CC(0.3906) | CT(0.4740) | TT(0.1354) | 0.4740 | 0.4687 | 0.3582 | 0.8750 |
| SNP07 | CC(0.2344) | CA(0.5365) | AA(0.2292) | 0.5365 | 0.5013 | 0.3750 | 0.3298 |
| SNP08 | CC(0.5833) | CA(0.3802) | AA(0.0365) | 0.3802 | 0.3514 | 0.2891 | 0.2533 |

2 Note: * indicates significant differences ($P < 0.05$).

3

## Table 3(on next page)

Correlation analysis between eight SNPs and food habit domestication traits in *M. salmoides* fry

* and ** indicate significant ($P < 0.05$) and extremely significant differences ($P < 0.01$) , respectively. “PVE” represents the phenotypic variation explained.

**Table 3.** Correlation analysis between eight SNPs and food habit domestication traits in *M. salmoides* fry

| Locus | Genotype | Number (Domesticated) | Number (Non-domesticated) | Chi-square/Fisher | *P* Value | Cramér's V Correlation Coefficient | PVE(%) |
|---|---|---|---|---|---|---|---|
| SNP01 | GG | 59 | 35 | 13.670 | 0.001** | 0.267** | 7.08 |
| | GA | 33 | 48 | | | | |
| | AA | 4 | 13 | | | | |
| SNP02 | TT | 11 | 7 | 7.621 | 0.022* | 0.199* | 3.29 |
| | TA | 41 | 26 | | | | |
| | AA | 44 | 63 | | | | |
| SNP03 | GG | 14 | 5 | 4.740 | 0.093 | 0.157 | 1.00 |
| | GA | 49 | 55 | | | | |
| | AA | 33 | 36 | | | | |
| SNP04 | TT | 28 | 48 | 10.394 | 0.006** | 0.233** | 5.39 |
| | TC | 53 | 42 | | | | |
| | CC | 15 | 6 | | | | |
| SNP05 | GG | 34 | 24 | 6.901 | 0.032* | 0.190* | 0.02 |
| | GC | 42 | 60 | | | | |
| | CC | 20 | 12 | | | | |
| SNP06 | CC | 47 | 28 | 7.901 | 0.020* | 0.203* | 3.11 |
| | CT | 38 | 53 | | | | |
| | TT | 11 | 15 | | | | |
| SNP07 | CC | 19 | 26 | 4.604 | 0.100 | 0.155 | 2.11 |
| | CA | 49 | 54 | | | | |
| | AA | 28 | 16 | | | | |
| SNP08 | CC | 54 | 58 | 3.579 | 0.202 | 0.139 | 0.69 |
| | CA | 36 | 37 | | | | |
| | AA | 6 | 1 | | | | |

Note: * and ** indicate significant ($P < 0.05$) and extremely significant differences ($P < 0.01$), respectively. “PVE” represents the phenotypic variation explained.

## Table 4(on next page)

Correlation analysis between the different genotypes of eight SNPs and growth traits in *M. salmoides* fry

Different superscript letters in a column of each locus indicate significant a difference ($P < 0.05$), * and ** indicate significant ($P < 0.05$) and extremely significant differences ($P < 0.01$) , respectively. “PVE” represents the phenotypic variation explained.

**Table 4.** Correlation analysis between the different genotypes of eight SNPs and growth traits in *M. salmoides* fry

| Locus | Genotype | Number | Body Height(mm) | Pearson Correlation Coefficient | PVE(%) | Body Length(mm) | Pearson Correlation Coefficient | PVE(%) |
|---|---|---|---|---|---|---|---|---|
| SNP01 | GG | 94 | 5.647±0.720[a] | 0.291** | 8.45 | 20.643±1.936[a] | 0.172* | 2.95 |
| | GA | 81 | 5.393±0.574[b] | | | 20.113±1.604[b] | | |
| | AA | 17 | 4.971±0.607[c] | | | 19.774±1.478[ab] | | |
| SNP02 | TT | 18 | 5.423±0.702 | -0.019 | 0.04 | 20.288±1.831 | -0.074 | 0.55 |
| | TA | 67 | 5.534±0.680 | | | 20.623±1.709 | | |
| | AA | 107 | 5.456±0.678 | | | 20.176±1.816 | | |
| SNP03 | GG | 19 | 5.737±0.621 | 0.071 | 0.51 | 20.697±1.258 | 0.067 | 0.45 |
| | GA | 104 | 5.442±0.650 | | | 20.351±1.782 | | |
| | AA | 69 | 5.467±0.730 | | | 20.232±1.913 | | |
| SNP04 | TT | 76 | 5.397±0.701 | 0.074 | 0.54 | 20.452±1.731 | -0.082 | 0.67 |
| | TC | 95 | 5.548±0.678 | | | 20.359±1.849 | | |
| | CC | 21 | 5.475±0.595 | | | 19.871±1.675 | | |
| SNP05 | GG | 58 | 5.508±0.678 | 0.025 | 0.06 | 20.464±1.804 | 0.110 | 1.21 |
| | GC | 102 | 5.470±0.718 | | | 20.451±1.805 | | |
| | CC | 32 | 5.463±0.561 | | | 19.776±1.613 | | |
| SNP06 | CC | 75 | 5.472±0.717 | 0.042 | 0.18 | 20.087±1.843 | -0.074 | 0.55 |
| | CT | 91 | 5.535±0.647 | | | 20.575±1.736 | | |
| | TT | 26 | 5.312±0.671 | | | 20.265±1.733 | | |
| SNP07 | CC | 45 | 5.314±0.622[b] | 0.156* | 2.43 | 20.141±1.514 | 0.070 | 0.49 |
| | CA | 103 | 5.492±0.699[ab] | | | 20.360±1.833 | | |
| | AA | 44 | 5.621±0.663[a] | | | 20.507±1.936 | | |
| SNP08 | CC | 112 | 5.477±0.692 | 0.027 | 0.07 | 20.277±1.647 | 0.045 | 0.21 |
| | CA | 73 | 5.465±0678 | | | 20.422±1.995 | | |
| | AA | 7 | 5.703±0.506 | | | 20.554±1.759 | | |

Note: Different superscript letters in a column of each locus indicate significant a difference ($P < 0.05$), * and ** indicate significant ($P < 0.05$) and extremely significant differences ($P < 0.01$), respectively. “PVE” represents the phenotypic variation explained.