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Identification of SNPs in *NPY* and *LEP* and the association with food habit domestication traits in mandarin fish

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Introduction

Mandarin fish (*Siniperca chuatsi*), a typical carnivorous fish, is traditionally cultured freshwater fish with high commercial value in China. However, the mandarin fish has very peculiar food habits. They exclusively feed on live fish as soon as they start feeding (Liu and Cui 1989). And due to the special feeding habit of mandarin fish, its aquaculture industry is still restricted. So, Liang *et al.* (2001) designed a specific training procedure and found that most of the mandarin fish could feed minced prey fish successfully while some fish still refused. This suggested that food habit domestication traits of mandarin fish may vary among individuals. In fact, there is growing evidence that inherited differences are closely linked to food habits, as demonstrated in stickleback (Purnell *et al.* 2007) and in humans (Beaver *et al.* 2012). Selective breeding of mandarin fish for aquaculture may take advantage of this phenotypic difference to address the challenge that mandarin fish usually cannot feed on artificial diets.

Single nucleotide polymorphisms (SNPs) are highly abundant markers and believed to be often linked to genes impacting phenotypes (Barreiro *et al.* 2008). They represent the most frequent type of genetic variation in populations and have been widely used as candidate gene association studies aimed at identifying alleles potentially affecting important traits in aquaculture species (Sánchez *et al.* 2009). Genetic variation is the genetic basic of adaptation to dietary environments in the evolution of natural populations and is of great interest in animal breeding. The knowledge of genes associated to behavioural traits is increasing. Associations between genetic polymorphisms and food habit domestication traits in mandarin fish are an important step in understanding the genetics of complex traits that are commercially important. Therefore, study of the relationship between SNPs and food habit domestication traits in mandarin fish is of great significance.

Various studies indicated that genetic differences in food intake are associated with appetite (Silverstein *et al.* 2001; Boujard *et al.* 2006; Kause *et al.* 2006; Lõhmus *et al.* 2008). In mammals, the two well-known and widely studied hormones and neurotransmitters which are implicated in the control of appetite are neuropeptide Y (NPY) and leptin (LEP) (Volkoff 2006). NPY, a 36 amino acid peptide, is widely expressed in the central and autonomic nervous systems (White 1993) and implicated in the regulation of food intake (Wyss *et al.* 1998; Volkoff 2006). LEP is produced in adipose tissue and has a number of functions (Robertson *et al.* 2008). Several of the functions of LEP in fish seem to be similar to those reported in mammals, one of which is to inhibit feeding (Volkoff *et al.* 2005; De Pedro *et al.* 2006; Volkoff 2006).

Some genetic markers in the *NPY* and *LEP* genes may be associated with feeding behaviour (van der Lende *et al.* 2005; Sherman *et al.* 2008). However, there has been no report on SNPs of *NPY* and *LEP* genes associated with behavioural traits in fish as yet. The objective of this study was to identify SNPs in the *NPY* and *LEP* genes and examine associations of such polymorphisms with food habit domestication traits in mandarin fish.

Materials and methods

Fish and DNA samples

The fingerlings of *S. chuatsi* were obtained from Xinrong Fry Breeding Farm (Foshan, Guangdong, China) by artificial breeding techniques. Domestication of food habit was performed by following the methods of Liang *et al.* (2001) using net-cages as the experimental culture in Guangdong Freshwater Fish Farm (Panyu, Guangdong, China). In this study,

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fry of Indian mrigal, *Cirrhina mrigola*, was used as the live prey fish for mandarin fish and the dead prey fish were prepared by freezing. During the training period, the fish were visually separated into feeders and nonfeeders on the basis of plumpness or emaciation, respectively. After two weeks, two groups were successfully obtained: 152 feeders and 115 nonfeeders. Genomic DNA was extracted from the caudal fin ray using the TIANamp Genomic DNA kit (Tiangen Biotech, Beijing, China) according to manufacturer's directions.

SNP discovery

Thirty feeders and 30 nonfeeders were randomly selected from each group. The full length of the *NPY* and *LEP* genes were directly sequenced in *S. chuatsi* genomic DNA samples using an ABI PRISM 3700 DNA analyser (Applied Biosystems, Foster City, USA). Primer sets used in the amplification and sequencing analyses were designed on the basis of the reference genome sequence for *NPY* (EF554595) and *LEP* (FJ588588). Information concerning the primers for the amplification and sequencing of the *NPY* and *LEP* genes are provided in table 1. Polymerase chain reaction (PCR) conditions were optimized for each pair of primers. PCRs were performed in 25 μL reaction volumes containing 2.5 μL of 10× PCR buffer, 1.0–3.0 mM $MgCl_2$, 50 μM dNTPs, 0.4 μM of each primer, 1 U *Taq* polymerase (Takara, Shiga, Japan) and 50 ng genomic DNA. PCR conditions were as follows: initial denaturation at 94°C for 3 min followed by 30 cycles at 94°C for 30 s, the optimized annealing temperature (table 1) for 30 s, 72°C for 30 s, and then a final extension step at 72°C for 10 min. The PCR products were purified using the TIANquick Midi Purification kit (Tiangen Biotech, Beijing, China) for direct sequencing. Sequences were analysed using DNASTAR software, Madison, USA.

Genotyping of SNPs

One SNP in *NPY* gene was genotyped by the PCR-based RFLP. Two other SNPs used the SNaPshot Multiplex System (Applied Biosystems) to genotype. The primers for multiplex PCR and SNP genotyping probe sequences are provided in table 2.

PCR-RFLP

PCR amplification was performed as described in SNP discovery using the primers: F: 5′-GAAGCACTTAGTG ATACAG-3′, R: 5′-TACATGAACCCTTCTTTGACAAGC-3′. The conditions used for PCR amplification included an initial denaturation at 94°C for 3 min, followed by 30 cycles at 94°C for 30 s, 60°C for 30 s, 72°C for 1 min and a final elongation at 72°C for 10 min. A 15 μL aliquot of the PCR product mixture was completely digested with 5 U of restriction enzyme (*Hin*dIII) at 37°C for 100 min and then separated on a 8% nondenaturing polyacrylamide gel electrophoresis (PAGE) and visualized by silver staining. A denatured pBR322 DNA/*Msp*I molecular weight marker (Tiangen, Beijing, China) was used as a size standard to identify alleles.

Multiplex PCR of loci for SNP genotyping

The multiplex PCR reactions were carried out in an EDC 810 (Dongsheng International Trade, Guangzhou, China) in a total volume of 15 μL which included 10 ng genomic DNA, 1.5 μL 10× PCR buffer, 0.3 mM dNTPs, 2 μM each primer and 1 U *Taq* DNA polymerase (TaKaRa, Japan). Cycling parameters were as follows: 95°C for 3 min; 35 cycles of 94°C for 15 s; 54°C for 15 s; 72°C for 30 s and a final extension step at 72°C for 3 min. Following amplification, 4 μL of a solution containing 2.9 μL H_2O, 4 U exonuclease I (New England Biolabs, USA), 0.7 μL of exonuclease I buffer (New England Biolabs, Ipswich, USA) and 2 U Shrimp alkaline phosphatase (New England Biolabs) was added to 3 μL PCR product. Samples were then incubated for 45 min at 37°C followed by 15 min at 80°C.

Single base extension

SNaPshot® (Applied Biosystems) reactions included 2 μL of exonuclease I treated PCR product, 1 μL of SNaPshot

Table 1. Primers used to amplify and sequence the genomic DNA sequence of the *S. chuatsi NPY* and *LEP* genes.

Primer pair	Primer sequence	Position[a] (bp)	Annealing temperature (°C)	Product size (bp)	Amplified region
NPY-01F	GTCACCTGTGTCTAAGATA	−1933 ∼ −1914	55	1582	5′ flanking
NPY-01R	TGGCGTCTCTTCTGCTC	−368 ∼ −351			
NPY-02F	TAATTGTAGCAGCTGGATTG	−669 ∼ −649	55	758	5′ flanking–exon 2
NPY-02R	GGTTTCACCGGGTATCCTT	79 ∼ 89			
NPY-03F	GAGGAGCTGGCCAAGTACTA	126 ∼ 146	64	1443	Exon 2–exon 4
NPY-03R	GAGGTCAGAATGTCAGCAGC	1549 ∼ 1569			
LEP-01F	TAACAGCTTTTTGGAAAGTTTG	−526 ∼ −505	55	513	5′ flanking
LEP-01R	CCCCGGATGTACAGAATTCA	−33 ∼ −13			
LEP-02F	ATGCCTGCTGTCTTCAT	−171 ∼ −154	60	1204	Exon 1–exon 2
LEP-02R	CATCAAACCAGTTTTCAGGA	1031 ∼ 1033			

[a]Numbers indicate the nucleotide position relative to transcription start codon (+1).

Table 2. Primer sets for multiplex PCR amplification and genotyping of each SNPs located in the *NPY* gene.

SNPs locus		Multiplex PCR amplification primer	Genotyping primer
−1258A/C	Forward	TGGGTTTCTTGGAGATTGGGC	TTTTTTTTTTTTTTTTTTTTTTTTTGAACCCTTCTTTGACATGCT
	Reverse	TATGACGACGCTTCGGGTGAT	
−622T/C	Forward	GGACATTCGTTTTTTGGTTAG	TTTTTTTATGAGACCAAGAGGGATTAGG
	Reverse	GTCACTGCTGATGCTGTCATT	
1490G/A	Forward	GACACGATTCCACAGTCAAGG	TTTTTTTTTTAGCATCACCACAATGATGGGT
	Reverse	ATAGAGGTAAAAGGGGCAGAG	

multiplex ready reaction mix (Applied Biosystems), 1 μL H_2O and 2 μL probe mix. Thermocycling conditions consisted of 96°C for 1 min followed by 30 cycles at 96°C for 10 s, 52°C for 5 s, 60°C for 30 s.

Electrophoresis and scoring

One μL of the SNaPshot product was added to 8 μL loading buffer and the mixture was then denatured at 95°C for 3 min, chilled rapidly in ice water and analysed on an ABI 3730XL (Applied Biosystems). Genemapper ver. 4.0 (IBM, New York, USA) was used to interpret the genetic profiles.

Statistical analysis

Allelic frequencies, genotype frequencies, Hardy–Weinberg equilibrium and observed heterozygosity (H_e) were statistically analysed in the feeders and nonfeeders separately using the PopGene ver. 1.31 software (http://www.ualberta.ca/~fyeh/popgene_download.html). Polymorphism information content (PIC) was computed according to the following formula:

$$\mathrm{PIC} = 1 - \left(\sum_{i=1}^{n} q_i^2\right) - \left(\sum_{i=1}^{n-1}\sum_{j=i+1}^{n} 2q_i^2 q_j^2\right),$$

q_i and q_j are the frequencies of the ith and jth alleles at one locus and n is the number of alleles at one locus. Associations between genotypes and food habit domestication traits were examined using the chi-squared test. Results were considered to be statistically significant if bilateral P values were <0.05. Statistical analyses were carried out using SPSS ver. 17.0 software.

Results and discussion

Appetite is one of the signals that control food intake in fish and plays a key role in feeding process. Liang *et al.* (1998) found that mandarin fish relied on sense organs in feeding. The individual differences in accepting dead prey fish may be attributed to the appetite stimulated by sense organs. NPY and LEP are very important regulators in the regulation of appetite (Volkoff 2006). Hence, in this study, *NPY* and *LEP* were selected as candidate genes. SNPs of the two genes were examined for effects on food habit domestication traits in mandarin fish.

After direct sequencing by scanning the complete genomic sequence of the *NPY* and *LEP* genes, three SNPs (−1258A/C, −622T/C, 1490G/A) were found in *NPY* but none in *LEP*. Two SNPs were located in the 5′-flanking region and the

Table 3. Genotypic frequencies and genetic diversity parameter at each SNPs located in the *NPY* gene.

Group	Sample size	Genotypic frequencies			Allelic frequencies		HWE	PIC	H_e
−1258A/C		AA	AC	CC	A	C			
Feeders	152	0.1974 (30)	0.5789 (88)	0.2237 (34)	0.4868	0.5132	$\chi^2 = 3.6706$ $P = 0.0554$	0.3748	0.4997
Nonfeeders	115	0.3217 (37)	0.4174 (48)	0.2609 (30)	0.5304	0.4696	$\chi^2 = 3.1876$ $P = 0.0742$	0.3741	0.4981
−622T/C		CC	CT	TT	C	T			
Feeders	152	0.5000 (76)	0.4145 (63)	0.0855 (13)	0.7072	0.2928	$\chi^2 = 0.0009$ $P = 0.9762$	0.3284	0.4141
Nonfeeders	115	0.5565 (64)	0.4000 (46)	0.0435 (5)	0.7565	0.2435	$\chi^2 = 0.7674$ $P = 0.3810$	0.3006	0.3684
1490G/A		AA	AG	GG	A	G			
Feeders	152	0.2566 (39)	0.5197 (79)	0.2237 (34)	0.5164	0.4836	$\chi^2 = 0.2115$ $P = 0.6456$	0.3747	0.4995
Nonfeeders	115	0.2696 (31)	0.5130 (59)	0.2174 (25)	0.5261	0.4739	$\chi^2 = 0.0692$ $P = 0.7926$	0.3743	0.4986

HWE, Hardy–Weinberg equilibrium; H_e, gene heterozygosity; PIC, polymorphism information content.

third one was sited in intron 4. Two alleles and three genotypes were detected in every locus. The results of the genotypic frequencies, allele frequencies and genetic diversity parameters are provided in table 3. The major alleles for SNP −622T/C are C allele and for SNP 1490G/A are A allele in two groups. For SNP −1258A/C, the major alleles are different in two groups. The major allele is C in feeders whereas it is A in nonfeeders. The Hardy–Weinberg and chi-square which were tested separately showed that the two groups were in genetic equilibrium ($P > 0.05$). As shown in table 4, the association analysis showed that only the −1258A/C SNP in 5′-flanking region was strongest, associated with food habit domestication traits in mandarin fish ($P = 0.007$).

Mutations in the regulatory and coding regions of *NPY* gene can potentially affect the neuronal regulation of appetite and feeding behaviour in cattle (Lagonigro *et al.* 2003). A study in humans also concluded that a polymorphism in *NPY* affected obesity (van Rossum *et al.* 2006). Similarly, in our result, this is consistent with the role of NPY as appetite stimulant. Mutation in *LEP* gene was associated with feed intake, such as in cattle (Lagonigro *et al.* 2003); however, we found no SNPs in *LEP*. The mandarin fish feeding behaviour is closely related to the mechanism of a regulatory SNP in *NPY*. *LEP* is regulated by glucose after feeding (Mueller *et al.* 1998). This indicates that the peculiar food habit of mandarin fish might not be associated with the satiation and glucose mechanism which is regulated by leptin. In the regulation of food intake, NPY is a strong orexigenic neurotransmitter but LEP inhibits feeding. Thus, no SNPs were found in LEP may be an attribute to the role of LEP in the regulation of food intake.

The PIC value is commonly used in genetics as a measure of polymorphism for a marker (Shete *et al.* 2000). Bostein *et al.* (1980) described that the locus was low polymorphic when the PIC value is <0.25, average polymorphic when the value is between 0.25 and 0.5 and highly polymorphic when the value is higher than 0.5. In two groups, average expected heterozygosity (H_e) ranged from 0.3684 to 0.4997 and PIC was between 0.3006 and 0.3748. PIC of the two groups was between 0.25 and 0.5. So the mutation in *NPY* gene belonged to middle genetic variation. Higher PIC values indicate more genetic variation and more selection potential (table 3) PIC of feeders was higher than nonfeeders.

In conclusion, we first identified three SNPs in *NPY* and none in *LEP*. Association analysis showed that the heterozygous AC genotype of −1258A/C SNP in 5′-flanking region of *NPY* may be associated with food habit domestication traits. *NPY* could be the potential candidate gene which affect food habit domestication traits in mandarin fish and it would be useful in selection breeding of mandarin fish in the future.

Table 4. Association analysis between genotypes of three SNPs and feed habit domestication traits in studied population.

Genotypes		Feeders	Nonfeeders	*P* value
−1258A/C	AC	88	48	
	AA	30	37	0.007
	CC	34	30	0.117
−622T/C	CC	76	64	
	CT	63	46	0.580
	TT	13	5	0.247
1490G/A	AG	79	59	
	AA	39	31	0.833
	GG	34	25	0.961

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