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Single Nucleotide Polymorphism Discovery of Molt Inhibiting hormone Gene 3 Exons and its Association with Growth Traits in White Shrimp (*Litopenaeus vannamei*)

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Abstract: Molt Inhibiting Hormone (MIH) is a neuropeptide member belonging to the eyestalk CHH family. It is a multifunctional protein, playing a major role in the molting activity of shrimp. *MIH* gene is one of the candidate genes for detecting polymorphisms associated with the growth traits. The present study was designed to investigate the effects of the *MIH* gene 3 exons on white shrimp growth traits. Primers for the 3 exons of *MIH* gene were designed from shrimp genomic sequences. Polymorphism was detected by DNA sequencing and the PCR-SSCP method was developed to genotype 340 samples.

Key words: *Litopenaeus vannamei*, MIH, SNPs, PCR-SSCP, polymorphism, China

INTRODUCTION

Litopenaeus vannamei has become a significant economic species as the development of shrimp culture industry worldwide. Enhancement of growth rate by genetic improvement is an important technique due to the wide development of breeding technology and resistant isolates which may decrease the length of grow-out cycles, resulting in the reduce of production cost and culture cycle of shrimp (http://www.shrimpnews.com/Species.html). The Molt-Inhibiting Hormone (MIH) and the Crustacean Hyperglycemic Hormone (CHH) belong to a large neuropeptide family which are involved in the regulation of the length of the intermolt period, keeping shrimp in the intermolt stage (Gu *et al*., 2000; Yodmuang *et al*., 2004). There are two kinds of MIH (MIH1 and MIH2) and one CHH. The *MIH1*, *MIH2* and *CHH* genes are candidate genes for detecting polymorphisms associated with the molting pathway and growth (Song *et al*., 2003). Therefore, the present study was conducted to detect the polymorphisms within *MIH* gene and then analyzed the association between each genotype and its growth traits. This will be helpful for conserving, utilizing and exploiting the genetic resources of shrimp.

MATERIALS AND METHODS

Animal source: Juvenile white shrimp (*L. vannamei*, n = 340, the mean body weight = 18.23±4.63 g) were obtained from Dongfang city of Hainan province. Before the experiment, the growth traits of shrimp were counted and weighed for statistical analysis.

DNA preparation and primer design: Genomic DNA was extracted from a pleopod of each shrimp using a phenol/chloroform isolation method (Klinbunga *et al*., 2001). The concentration of the extracted DNA was spectrophotometrically estimated. The DNA was stored at 4 EC until needed. The white shrimp MIH (Gene Bank accession: No.AF387485) gene sequence was used to design the three primers for SNP discovery:

Exon-1,19U21 5'-CTGCTGTCGTCCTCGTCGTCT-3',
203L17 5'-GCGGCTCTTACTTGCAC-3';
Exon-2,1419U18 5-TTCCTTCTCCCTTTACGA-3',
565L18 5-TACCATAGCCTTCACCCA-3';
Exon-3,1732U19 5'-GCTTTCCGCAACGATTATC-3',
990L22 5'-TGTTTCCTCCACATTAGCGTCC-3'.

PCR conditions: PCR reactions were carried out in a total volume of 15 μL with 40 ng of genomic DNA, 0.5 pmol of each of primers, 1.5 μL of 10 H buffers, 1.5 mM of $MgCl_2$, 0.25 mM of dNTP mixture and 1.5 U of Taq DNA polymerase (Fermentas, Canada).

Amplification was done under the following conditions: one cycle 94°C 5 min; 35 three step cycles (94°C 60 sec, 56°C 90 sec and 72°C 60 sec) followed by a final extension for 10 min at 72°C.

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SSCP: The amplification product was analyzed by SSCP (Orita *et al*., 1989). The PCR product (6 μL) was mixed with 6 μL of the loading dye (95% formamide, 0.25% bromophenol blue, 0.25% xylene cyanol and 10 mM NaOH), denatured in a boiling bath for 5 min and immediately cooled on ice for 3 min. The denatured products of MIH were electrophoretically analyzed (native 15.0% PAGE; 37.5:1 crosslink) at 200 V for 15 min and 110 V for 2 h at 4 EC. SSCP bands were visualized by silver staining.

Statistical methods and analysis: After the preliminary screening, polymorphism of MIH were tested against a larger sample set of juvenile *L. vannamei* (n = 340) collected from a commercial farm in Hainan. Relationships between the frequencies of identified genotypes and the body weight of shrimp were statistically analyzed using one way Analysis of Variance (ANOVA) and Duncan's new multiple range test ($p<0.05$). Association analysis between the SNPs and traits in 340 shrimp were performed using SPSS 16.0 by the following model:

$$y_{ij} = \mu+S_i+M_j + e_{ij}$$

Where:

y_{ij} = The dependent variable (analyzed traits)
μ = The overall mean; Genotype (G) of MIH exon-1 (AA, BB and AB), the shrimp population (L), interactions between genotype and shrimp population (GHL) were the fixed effects
e = The random error

Significant differences between least squares means of the 3 genotypes were analyzed using a contrast test.

RESULTS AND DISCUSSION

The candidate gene approach is a powerful method to investigate associations of gene polymorphisms with economically important traits in farm animals (Rothschild and Soller, 1997). Many studies reported that the *MIH* gene was associated with the individual growth performance in crustaceans (El-Haj, 1996; Chang *et al*., 2001; Bocking *et al*., 2002; Lyons *et al*., 2007; De-Santis and Jerry, 2007). To the best knowledge, there have no polymorphisms of *MIH* gene of *L. Vannamei* were reported. In this study, the *MIH* gene was selected as a candidate gene to investigate associations of gene polymorphisms with some growth traits in *L. vannamei*.

The comparisons among sequencing results in the study showed two mutations in exon-1 and exon-2 but no mutation was found in exon-3. The A>G mutation in the 171th nucleotide of the exon-1 and T>G mutation in the 480th nucleotide of the exon-2 were identified. The association analyses revealed that significant association of exon-1 (A>G171) with body weight existed in the present population. The mutation of the exon-2, T>G 480 caused a synonymous mutation but it was no associated with body weight existed in the present population. The A>G171th caused a synonymous mutation and differentiated the big bodyweight shrimp (genotypes AA and AB) from the small body weight shrimp (genotypes BB) communally cultured in the same pond. Individuals with genotype AA were significantly higher than those of individuals with genotype BB ($p<0.05$, Table 1).

Genotypic and allele frequencies of *MIH* gene in *L. vannamei* were shown in Table 2. The frequencies of allele A in exon-1 was 0.8238, B haplotype was predominant in *L. vannamei*. The frequency of allele A in exon-2 was 0.5676, the frequency of allele B in exon-1 was 0.4323. The obtainded results showed that A was the preponderant alleles in *MIH* gene.

SSCP is a method for distinguishing between similar sized DNA fragments according to the mobility of single stranded DNA under polyacrylamide gel electrophoresis (Orita *et al*., 1989). It is favored for examining genetic diversity of various species owing to its convenience and cost effectiveness. The PCR-SSCP results in the present study suggested that the exon-1 of the *MIH* gene showed polymorphism patterns in the shrimp. The comparisons among sequencing results showed two

Table 1: Exon-1and exon-2 genetype and association analysis with bodyweight of *Litopenaeus vannamei*

	Genetype		
Exon	AA	AB	BB
Exon-1	14.6698±4.0887[a]	13.5485±3.1355[a]	11.8964±4.7736[b]
Exon-2	14.7147±3.8416	13.2938±3.2210	14.2291±4.4481

[a]Different lower case letters indicate significant ($p<0.05$), [b]Different lower case letters indicate significant ($p<0.01$)

Table 2: Exon-1 and exon-2 genotypic frequency and gene frequency of *Litopenaeus vannamei*

		Genotypic frequency			Gene frequency	
Exon	Sample size	AA	AB	BB	A	B
Exon-1	340	0.6912 (235)	0.2647 (90)	0.0441 (15)	0.8238	0.1767
Exon-2	340	0.4529 (154)	0.2294 (78)	0.3176 (108)	0.5676	0.4323

mutations in exon-1. The mutation of the exon-1 was strongly associated with body weight existed in the present population. Therefore, the *MIH* gene may be a major candidate gene or linked to a major candidate gene that impact body weight. The SNP could be used in molecular Marker-Assistant Selection (MAS) as a genetic marker for shrimp growth traits. Future studies should examine larger data sets to better determine if an association exists between candidate genes and growth.

CONCLUSION

It is conclude that two novel polymorphism, synonymous mutation in A>G171 of 1st exon and T>G480 of 2nd exon *MIH* gene were found. The mutation in exon-1 of MIH may be linked with potential major loci or genes affecting some growth traits but no significant associations of exon-2 were detected.

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