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Relationship Between Single Nucleotide Polymorphism of Glycogen Synthase Gene of Pacific Oyster *Crassostrea gigas* and Its Glycogen Content

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Abstract Glycogen is important not only for the energy supplementary of oysters, but also for human consumption. High glycogen content can improve the stress survival of oyster. A key enzyme in glycogenesis is glycogen synthase that is encoded by glycogen synthase gene *GYS*. In this study, the relationship between single nucleotide polymorphisms (SNPs) in coding regions of *Crassostrea gigas GYS* (*Cg-GYS*) and individual glycogen content was investigated with 321 individuals from five full-sib families. Single-strand conformation polymorphism (SSCP) procedure was combined with sequencing to confirm individual SNP genotypes of *Cg-GYS*. Least-square analysis of variance was performed to assess the relationship of variation in glycogen content of *C. gigas* with single SNP genotype and SNP haplotype. As a consequence, six SNPs were found in coding regions to be significantly associated with glycogen content ($P<0.01$), from which we constructed four main haplotypes due to linkage disequilibrium. Furthermore, the most effective haplotype H2 (GAGGAT) had extremely significant relationship with high glycogen content ($P<0.0001$). These findings revealed the potential influence of *Cg-GYS* polymorphism on the glycogen content and provided molecular biological information for the selective breeding of good quality traits of *C. gigas*.

Key words *Crassostrea gigas*; glycogen content; glycogen synthase gene; SNP

1 Introduction

The Pacific cupped oyster *Crassostrea gigas* is an important cultured aquatic species worldwide, with the global production reaching 0.66 million tons in 2010 (FAO 2012). To improve the productivity of *C. gigas*, genetic studies are mostly focused on the improvement of growth and survival to increase yields of cultured oysters via selective breeding schemes (Evans and Langdon 2006; Dégremont *et al*., 2010), while there has been little concern about meat quality traits of oysters. Meat quality traits usually have low heritability and can only be costly measured post-slaughter (Cinar *et al*., 2011), making progress via traditional breeding programs difficult. Marker-assisted selection (MAS) program can solve such problems (Dunham 2004), and has the potential to accelerate genetic improvement of meat quality. Thereby, the identification of genetic markers related to meat quality traits under selection can contribute to the selection response (Lo Presti *et al*., 2009).

In addition to amplified fragment length polymorphism analysis (AFLP) and microsatellite markers, single nucleotide polymorphisms (SNPs) can also be applied to screening markers linked to quantitative trait loci (QTLs) in genomes (Gibson and Muse 2004). SNP is, however, a bi-allele, co-dominant marker with the merit of the abundant polymorphisms spreading all over any organism (Liu and Cordes, 2004). Oysters are amongst the most diverse animal species with one SNP every 60 bp in coding regions (Sauvage *et al*., 2007). It is of great importance that some coding SNPs have the potential to influence phenotypic variation and then to provide a mean for application in breeding programs (Fuji *et al*., 2007). In recent years, SNP markers have been widely applied in molecular genetics for relationship analyses (He *et al*., 2008; Thanh *et al*., 2010; Kongchum *et al*., 2011). However, relationship of SNPs in candidate genes with meat quality was found in livestock (Zuo *et al*., 2005; Gill *et al*., 2010), and few report has been documented in aquaculture animals.

Regarding good meat quality, the fundamental means of food acceptability judged by most people are flavor and texture. Oyster-eating gourmets make the texture of 'fat' (high glycogen) as an important determinant in acceptance of raw oysters. Besides the consistency with the obesity of the soft body, high glycogen content could enhance the complexity and the sustainability of the flavor of oysters (Lin *et al*., 2002). In aquatic organisms, glycogen content is also associated with gametogenic

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cycle, as well as survival during summer and other stressful conditions (Berthelin *et al*., 2000; Fearman and Moltschaniwskyj, 2010; Zhou *et al*., 2011). Hence, individuals with high glycogen are able to gain more survival advantages, and, more importantly, could add texture and flavor to the meat (Stanley *et al*., 1981). Consequently, good meat quality improved by high glycogen content is an issue of concern for selective breeding.

During the course of glycogen accumulation, glycogen synthase is a key enzyme in glycogenesis, which is involved in incorporating excess glucose residues one by one into a polymeric chain for storage as glycogen (Buschiazzo *et al*., 2004). In many studies on mammalian muscles, the enzyme possesses the rate-limiting function in glycogen synthesis (Fisher *et al*., 2002; Lai *et al*., 2007). In *C. gigas*, the glycogen synthase gene (*Cg-GYS*) has been identified and cloned. The expression of *Cg-GYS* gene corresponds to glycogen storage and resting period, reflecting the central role of the gene in glycogenesis (Bacca *et al*., 2005).

In this study, we used single-strand conformation polymorphism (SSCP) procedure (Bacca *et al*., 1989) to detect SNPs in *Cg-GYS*, and used this approach to examine their relationship with the glycogen content in *C. gigas*.

2 Materials and Methods

2.1 Animals

Experimental families were established by selective breeding of cultured *C. gigas* with mature gonadal development and great growth traits from Weihai, Shandong, China in 2009. Balanced nested mating design (each male was mated with three different females) was carried out to produce 36 full-sib families with artificial insemination techniques. Families 027, 028, 029, 032 and 034 were randomly chosen from them. In all, 321 oysters (shell height, 71.56 mm ± 10.51 mm; shell length, 45.08 mm ± 7.52 mm; total weight, 36.90 g ± 14.60 g) of the five full-sib families reared under the identical condition were collected randomly in March 2011 when they were about 650 days old. The tested samples of the five families included 69, 67, 61, 58 and 66 oysters, respectively.

2.2 Determination of Trait

The samples were transported to laboratory. Then the soft tissues were dissected to obtain gonad for glycogen content determination, and adductor muscles were collected for DNA extraction. The tissues were then frozen and stored at −80℃ until used. The glycogen content was determined with minor modifications of the anthrone, sulphuric acid analysis method described by Horikoshi (Horikoshi, 1958). Thirty mg of powdered, freeze-dried samples was suspended in 60 volumes of 30% KOH, and was heated to 100℃ for 30 min to be saponified. After cooling, a portion of the saponified mixture was treated with 5 mL cold 0.2% anthrone-sulphuric acid solution for 10 min. The absorbance of the resulting colored complex was measured at the wavelength of 620 nm.

2.3 Primer Design, DNA Extraction and PCR Amplification

According to the complete coding sequence of the glycogen synthase gene in *C. gigas* (GenBank accession number AY496064), nine primer sets (Table 1) were designed using Primer Premier 5.0 (http://www.premierbiosoft.com/). Genomic DNA was extracted from adductor muscles of experimental individuals based on phenol-chloroform method described by Li *et al*., (2006).

PCR amplification reactions were performed in 10 μL volumes. Each reaction contained 100 ng template DNA, 1 × PCR buffer, 2 mmol L^{-1} $MgCl_2$, 0.2 mmol L^{-1} dNTP (each kind), 1 μmol L^{-1} of each primer set, and 0.25 U *Taq* DNA polymerase. All PCRs were carried out as follows: One cycle of initial denaturation at 94℃ for 3 min; followed by 35 cycles of amplification and each cycle in-

Table 1 PCR primer sets designed for analysis of SNPs in the glycogen synthase gene in *Crassostrea gigas*

Name	Primer sequence (5'-3')	Annealing temperature (℃)	Product length (bp)	Location (bp)
Gb1F	ATTTACACTGTCATCAAGTCCAAG	61	183	182–357
Gb1R	TTGACTCACCTTGATACCTGC			
Gc1F	GAGGTTTATTTTGGTCGCTG	64	200	354–547
Gc1R	CTCACCTCACCAATAAACCAG			
Gd1F	GTTTCGGAAGAATCTGACT	61	180	546–725
Gd1R	TATTGTTGTAGAAGTCTGAGC			
Gg2F	GTTTATTCTGAAGGCACTATG	62	174	999–1120
Gg2R	CACCATCCTCCACAGAAC			
Gh1F	CTAACAGTGAGGCGACAG	65	171	1125–1286
Gh1R	GACTGTACCTCAGGCACTG			
Gk1F	TGTATTTCACCCCGAGT	56	112	1483–1594
Gk1R	CCAGGGCTCGTAGTAC			
Gl1F	AGCTGAGTGCACCGTGA	64	152	1606–1757
Gl1R	GCTGATGGATTGATTCGTC			
Gn1F	AAAGCCCGCCAGATCGC	62	119	1877–1995
Gn1R	GAGGGTGAGGAAGGTTCGG			
Gn2F	CGAACCTTCCTCACCCT	62	129	1978–2106
Gn2R	ATGTCGGACTCTGGGTTA			

cludes denaturation at 94℃ for 45 s, annealing at anoptimized temperature for 45 s, extension for 45 s at 72℃; and one cycle of final extension for 5 min at 72℃. Amplification productions were verified by electrophoresis on a 1.5% agarose gel stained with ethidium bromide.

2.4 SNP Genotyping

Three hundred and twenty one oysters from five families were scanned for the SNPs confirmation. PCR products of the Pacific oyster glycogen synthase gene were genotyped by SSCP analysis. First, 5 μL of each PCR product was mixed with 10 μL denaturing buffer (98% formamide, 0.09% xylene cyanole FF, and 0.09% bromophenol blue). Then, the samples were denatured at 98℃ for 5 min and placed on ice for 10 min. The denatured samples were run on 8% to 12% native polyacrylamide gel electrophoresis (PAGE) at 4℃ for 15 to 19 h at 120 V. SSCP patterns on the gels were finally visualized by silver staining (Qu *et al*., 2005). For the confirmation of the genotypes, PCR products with the identical SSCP pattern of more than three different individuals were sequenced for both directions using an ABI 3730 sequencer (Applied Biosystems).

2.5 Statistical Analysis

2.5.1 Glycogen content between families

One-way analysis of variance (ANOVA) followed by mean comparisons post hoc Tukey test was used in the analysis of glycogen content data to estimate significant differences ($P<0.05$) among different families.

2.5.2 Relationship analysis between single SNP locus and trait

Least-square analysis of variance was performed for the dominance model (Model 1) to assess the relationship of single SNP genotype with individual glycogen content.

$$y_{ijk} = \mu + F_i + G_j + e_{ijk}, \quad \text{(Model 1)}$$

where y_{ijk} is the trait value of the kth individual; μ is the mean of trait values; F_i and G_j are the fixed effects of the family i and the genotype j, respectively; and e_{ijk} is the random residual effect of the nth individual. Due to the sampling strategy and the same rearing condition, other effects such as age, generation and sampling site were not taken into account.

2.5.3 Relationship analysis between multi-locus haplotype and trait

Haplotypes of SNPs associated with glycogen content above were constructed with software PHASE 2.1 (Stephens *et al*., 2001). Without the effect from variance with family, the processing model was changed into Model 2 to deal with the relationship between multi-locus haplotype and trait.

$$y_{ij} = \mu + H_i + e_{ij}, \quad \text{(Model 2)}$$

where y_{ij} is the trait value of the jth individual; μ is the mean of trait values; H_i is the fixed effects of haplotype i; and e_{ij} is the random residual effect of the jth individual.

The One-way ANOVA and the dominance models above were processed by the GLM (General Linear Model) procedure in SAS statistical package version 9.1 (SAS Institute Inc 2003). Significant differences of least-squares means of SNP genotypes were calculated at the $P<0.01$ level, as well as haplotypes at the $P<0.05$ level, and then followed by the Bonferroni's multiple range test for correction of false positive.

3 Results

3.1 Glycogen Content Among Different Families

There were significantly different glycogen content levels among the five full-sib families of *C. gigas* ($P<0.05$, Fig.1). The relatively high levels of glycogen content reached 36.10% and 37.01% in families 027 and 034, respectively. By contrast, individuals of families 028, 029 and 032 possessed the low glycogen content with the means of 30.26%, 30.17% and 29.35%, respectively.

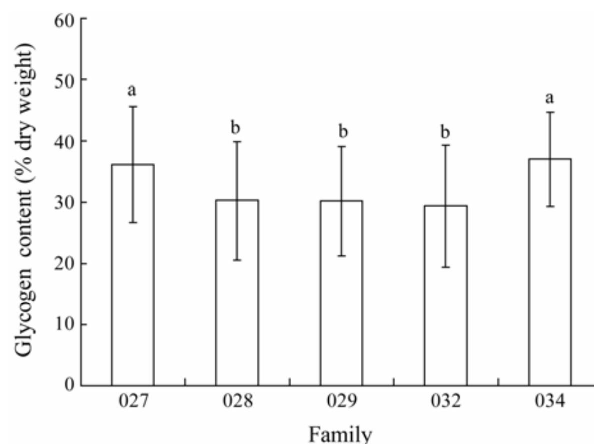

Fig.1 The different glycogen contents among the five full-sib families of *C. gigas*.

3.2 SNP Identification

Nine sets of specific primers were designed for DNA sequence amplification of sampled oysters (Table 1). In total, 1420 bp DNA fragments were amplified from the genome of *C. gigas* and they covered the majority of the coding regions within the complete coding sequence of the *C. gigas* glycogen synthase gene (*Cg-GYS*) characterized by Bacca *et al*. (2005). To efficiently capture common functional variation in transcribed sequences, the relationship strategy on coding SNPs was adopted. All 321 individuals of five full-sib families were genotyped at 41 coding SNPs (Fig.2). However, 40 SNPs were synonymous mutations and only one (G2057A) of them caused an amino acid change from Asp^{675} to Asn^{675} (GenBank accession no. AY496064). In view of complete linkage disequilibrium, fifteen SNPs were divided into six groups (data not shown). The SNPs in each group displayed complete dependency to each other. Different SNP

alleles shared the identical frequencies, and the least-squares means of the trait were also identical among genotypes at A277G, G280A, G295A and A328T loci (shown in Table 2).

Fig.2 Distribution of 41 SNPs in the cDNA of *Cg-GYS*. 3' untranslated region and 5' untranslated region are shown in lowercase, and coding sequences are in uppercase letters. SNPs are underlined, and the corresponding types are below.

3.3 Relationship Between Single SNP Locus and Trait

Six SNPs in *Cg-GYS* coding region exhibited significant relationship with glycogen content ($P<0.01$, Table 2). Least-squares means from dominance model indicated the significant differences ($P=0.0100$) of glycogen content at G211A locus between the genotype AG and the genotype GG. With the identical statistical significant value of $P=0.0043$, homozygotes at A277G locus con-

tained higher glycogen content than heterozygotes, while it was reversal at G280A locus. Individuals with the genotype CC at C493T locus had relatively lower glycogen content compared with the genotype CT ($P=0.0089$).

Table 2 SNP genotypes of the *C. gigas* glycogen synthase gene and the effect on glycogen content

SNP	Genotype	Number	Glycogen content least-squares (%)	*P*-value
Gb1 G211A	AG	36	38.01 ± 2.16^{a}	0.0100
	GG	285	31.85 ± 0.58^{b}	
Gb1 A277G	AA	286	33.21 ± 0.56^{a}	0.0043
	AG	35	27.75 ± 1.75^{b}	
Gb1 G280A	AA	35	27.75 ± 1.75^{a}	0.0043
	AG	286	33.21 ± 0.56^{b}	
Gb1 G295A	AG	35	27.75 ± 1.75^{a}	0.0043
	GG	286	33.21 ± 0.56^{b}	
Gb1 A328T	AA	286	33.21 ± 0.56^{a}	0.0043
	AT	35	27.75 ± 1.75^{b}	
Gc1 C493T	CC	246	31.59 ± 0.63^{a}	0.0089
	CT	75	35.86 ± 1.35^{b}	

Notes: SE = standard error. Glycogen content within a column followed by different letters is significantly different after sequential Bonferroni correction ($P<0.01$, means ± SE).

3.4 Relationship Between Haplotype and Trait

Five haplotypes were constructed using the maximum likelihood estimation of linkage disequilibrium across six SNP markers, which had been associated with glycogen content at the $P<0.01$ level. Four of the five haplotypes were main haplotypes with frequencies over 0.01 (Table 3). The most common haplotype was H1 (GAGGAC) with a frequency of 0.829. While the other three haplotypes H2 (GAGGAT), H3 (GGAATC) and H4 (AAG GAT) exhibited much lower frequencies of 0.061, 0.053 and 0.056 than H1.

Table 3 SNP haplotype frequencies of the *C. gigas* glycogen synthase gene and the effect on glycogen content

Haplotype	211	277	280	295	328	493	Frequency	Glycogen content least-squares (%)
H1	G	A	G	G	A	C	0.829	32.81 ± 0.41^{a}
H2	G	A	G	G	A	T	0.061	37.23 ± 1.53^{b}
H3	G	G	A	A	T	C	0.053	26.07 ± 1.64^{c}
H4	A	A	G	G	A	T	0.056	32.69 ± 1.59^{ab}

Notes: SE = standard error. Glycogen content within a column followed by different letters is significantly different after sequential Bonferroni correction ($P<0.05$, means ± SE).

The relationships between the haplotypes and glycogen content were shown in Table 3. The haplotype H3 was detected to cause the lowest glycogen content (26.07%) among the four haplotypes after Bonferroni correction ($P<0.05$). Conversely, no significant difference in glycogen content was observed between the oysters with H1 and H4, as well as those with H2 and H4. Nevertheless, individuals with H2 possessed the extremely significant ($P<0.0001$) high glycogen content compared with those with H3. As a result, H2 was likely to be the most effective haplotype associated with high glycogen content.

4 Discussion

The main aim of this study was to investigate whether there were significant relationships between single nucleotide polymorphisms in the glycogen synthase gene and glycogen content of *C. gigas*. The majority of *Cg-GYS* coding sequences were successfully amplified from 321 individuals belonging to five full-sib families, and finally 41 SNPs were found in coding regions from the 1420-bp amplicons. The average density of SNPs in coding region reached one SNP in every 35 bp, which was higher than one SNP in every 60 bp estimated by Sauvage *et al*., (2007). Interestingly, we found a couple of SNPs linked together but were divided into different groups due to linkage disequilibrium. All of these results might be the proof of high polymorphism of oysters.

Among these SNPs, 40 exonic SNPs were synonymous polymorphisms and six of these synonymous SNPs showed significant relationship with the glycogen content. It was in accordance with the hypothesis that synonymous polymorphisms could affect mRNA splicing, stability, and structure as well as protein folding to consequently influence the function of proteins (Hunt *et al*., 2009). On the other hand, only one SNP was non-synonymous mutation which caused an amino acid change in the coding region from Asp^{675} to Asn^{675} (GenBank accession no. AY496064). However, this SNP was not significantly associated with glycogen content. The variant might be a neutral mutation on the basis of the neutral theory (Kimura, 1985) due to the fact that neutral changes are often happened to a chemically similar amino acid that works just as well. After all, the molecular biological mechanism of the *Cg-GYS* gene expression is still ambiguous.

Performing haplotype estimations over several SNPs from a locus was especially effective to study the relationship between phenotypic traits and candidate allelic polymorphisms (Vignal *et al*., 2002). Therefore, the assessment of glycogen storage capacity associated with haplotypes of candidate gene is meaningful for the improvement of good quality traits of oysters for human consumption. Finally, we found that one haplotype H2 (GAGGAT) was probably responsible for the extremely significant high level of glycogen content at 37.23% of dry weight ($P<0.05$). Selecting for this haplotype would result in the abundant accumulation of glycogen content in *C. gigas*. However, in terms of the validity of DNA markers in this relationship analysis, the further verification in unbiased and independent populations are necessary.

There are many enzymes during the glycogen metabolism to influence glycogen content in organisms and these enzymes could affect the glycogen content in a coordinated way (Lai *et al*., 2009). Nevertheless, the expression level of *Cg-GYS* was strongly and seasonally implicated in the regulation of the glycogen content (Bacca *et al*., 2005). This intimate connection between the expression level of *Cg-GYS* and glycogen content was also proved by the results in this study that the SNP polymorphisms in

Cg-GYS significantly affected the glycogen content between different oysters. Thereby, the haplotype H2 (GAGGAT) of *Cg-GYS* possibly is a candidate marker for oyster breeding on high glycogen content.

The determination of glycogen content is not able to realize directly via biochemical methods during the larval stages. However larval DNA information is accessible, and exploiting the molecular information to forecast the glycogen content level of adults is possible to shorten the breeding cycle and improve breeding efficiency in bivalves. In view of glycogen content in *C. gigas*, more correlated molecular information was expected to implement MAS for improving texture and flavor of oysters during long-term studies in the future.

5 Conclusion

The assessment of the relationship between single nucleotide polymorphisms in coding regions of the *C. gigas* glycogen synthase gene *Cg-GYS* and individual glycogen content was investigated in 321 individuals from five full-sib families. The most effective haplotype H2 (GAGGAT) had the extremely significant relationship with high glycogen content ($P<0.0001$). These findings revealed the potential influence of *Cg-GYS* gene polymorphisms on the glycogen content in *C. gigas*, and provided molecular biological markers for identifying *C. Gigas* with high quality traits.

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