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Crassostrea gigas

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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*.

Crassostrea gigas; glycogen content; glycogen synthase gene; SNP

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 abun-

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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*.

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

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.

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

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).

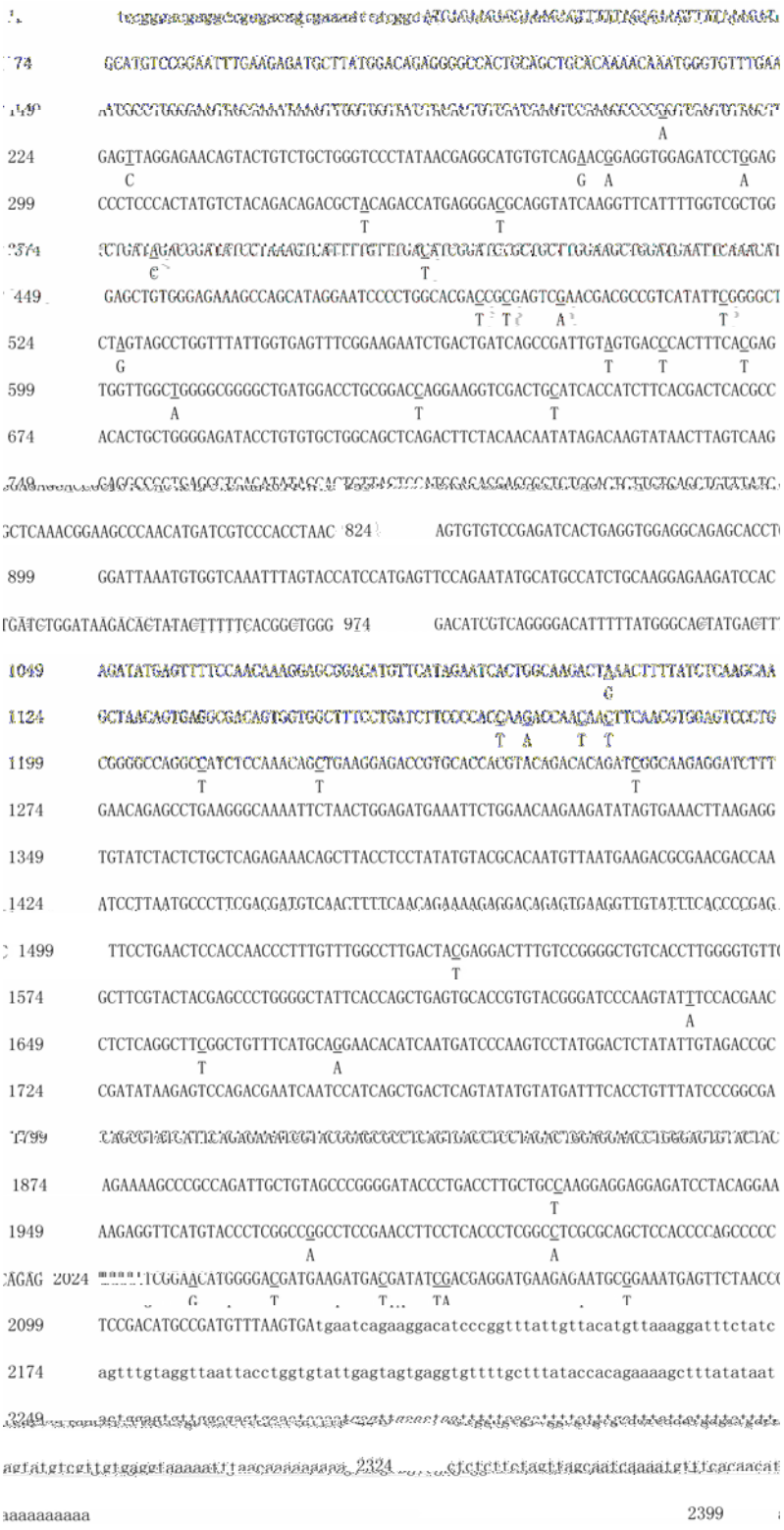

Fig.2 Distribution of 41 SNPs in the cDNA of *Cg-GYS*. 3' untranslated region and 5' untranslated region are shown in lowercase, and coding sequences are in uppercase letters. SNPs are underlined, and the corresponding types are below.

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).

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.

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.

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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