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RESEARCH REPORT

Association of α-amylase gene with growth traits in the razor clam *Sinonovacula constricta*

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Abstract

The razor clam *Sinonovacula constricta* is a commercially and ecologically important benthic mollusk. In the present study, we investigated the full-length cDNA sequence of the *S. constricta* α-amylase gene (*Scamy*) using expressed sequence tags and rapid amplification of cDNA ends. The genomic DNA sequence of *Scamy* is 5086 bp, which contains 6 exons and 5 introns. The full-length *Scamy* cDNA was 2196 bp, with a 2085 bp open reading frame encoding 694 amino acids. *Scamy* expression was very low before the D-shaped larvae stage, with the highest expression levels in juvenile clams. Expression levels of *Scamy* were significantly higher in the digestive gland compared with other tissues in adults ($p < 0.01$). Analysis of the *Scamy* gene in the digestive gland in starved clams indicated that both gene expression and enzyme activity increased before decreasing, reaching its highest expression on the second day. Gene expression and amylase activity both increased gradually after refeeding. These results demonstrated that starvation and refeeding increased amylase activity in razor clams. Association analysis identified one shared single nucleotide polymorphism, C1503T, for which individuals with genotypes TT and CT had significantly higher growth traits than those with genotype CC ($p < 0.05$). This study suggests the potential value of amylase markers in selective breeding to improve clam growth.

Key Words: *Sinonovacula constricta*; α-amylase; cloning and expression; SNP; enzyme activity; growth traits

Introduction

α-Amylase enzymes comprise a superfamily of structurally related proteins that hydrolyze α-1, 4-glycosidic bonds in starch to produce oligosaccharides and small dextrin molecules (Kuriki *et al.*, 1999; Yu *et al.*, 2013). Its role in controlling carbohydrate metabolism means that α-amylase is regarded as one of the main factors affecting the growth of many aquatic animals (Sellos *et al.*, 2003). Amylase is one of the most important digestive enzymes for phytophagous animals, especially in several bivalves. The α-amylase gene, *amy*, has been identified in several bivalves, including *Pecten maximus* (Le *et al.*, 1997), *Crassostrea gigas* and *Pinctada maxima* (Pan *et al.*, 2013). α-Amylase is the main digestive enzyme in shellfish, and thus has an important effect on growth. Amylase, lipase and cellulase activities in *Perna viridis* were shown to increase with the growth of individuals, but interestingly, activities of amylase, pepsin and lipase decreased with the growth of *Potamocorbula rubromuscula* (Huang *et al.*, 2003).

The razor clam *Sinonovacula constricta* is a benthic marine bivalve that is naturally distributed along the western Pacific coasts of China, Japan and Korea. It has been a popular seafood and important mariculture species in China for hundreds of years. Molecular research into growth-related genes in the clam is thus of great significance in relation to marker-assisted selection. Previous studies of this species have cloned and analyzed several genes associated with growth, including the genes for insulin-like peptide (Niu *et al.*, 2016), IGFBP-like (Xie *et al.*, 2013) and β-actin1 (Feng *et al.*, 2011).

In this study, we investigated the regulation of the *S. constricta* α-amylase gene (*Scamy*) by cloning the full-length cDNA and introns, examining its expression levels during embryonic development and in adult tissues. We also analyzed the gene expression and α-amylase activity in the digestive gland during starvation and refeeding. We additionally screened and analyzed associations

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between single nucleotide polymorphisms (SNP) of the *Scamy* gene and growth-related traits. The results of this study improve our understanding of the function of the *Scamy* gene, and provide a basis for molecular breeding programs of *S. constricta*.

Materials and Methods

Experimental animals and sample collection

Adult clams were collected from Danyan Farm, Yinzhou District of Ningbo, China. Mantle, adductor muscle, digestive gland, foot, gills, and siphon in adult clams were dissected, frozen immediately in liquid nitrogen and preserved at −80 °C. Unfertilized mature eggs, fertilized eggs, 2-cell embryos, 4-cell embryos, morulas, trochophores, D-shaped larvae and juvenile clams were obtained by independent spawning and artificial insemination and stored at −80 °C. A total of 215 individuals were randomly sampled at the same time from two razor clam populations: YL ("Yongle NO.1", a fast-growing strain, selected for four generations by our team from Changle population, Fujian province, China; n = 110) and SM (Wild population from Sanmen county, Zhejiang province, China; n = 105). Six growth index parameters, including body weight, soft-tissue weight, shell weight, shell width, shell length and shell height, were measured. Digestive glands were collected and stored at -80 °C. We explored the possible effects of starvation and refeeding on *Scamy* gene expression levels and enzyme activities using adult clams with an average shell length of 6.0 cm. Clams were kept in seawater at a salinity of 20 - 22 and temperature of 19 - 21 °C, and acclimated to the experimental conditions for 1 week before use. During this period, the clams were fed *Nannochloropsis oculata.* The clams were randomly allocated to experimental groups. Experiments were conducted in three replicates; each replicate included 60 clams. Groups were then starved for five days (S1 - S5) and then fed with *N. oculata* for 4 days, until satiety (F1 - F4). Four randomly selected clams from each replicates were sampled at 10 am every day.

Cloning of full-length Scamy *cDNA*

Total RNA was extracted from the digestive gland using TRIzol reagent (ComWin, Beijing, China). RNA integrity was examined by electrophoresis on a 1.0 % agarose gel and staining with ethidium bromide, and the quality and quantity of RNA were assessed by ultraviolet spectrophotometry. First-strand cDNA was synthesized using SMART rapid amplification of cDNA ends (RACE) reagents, according to the manufacturer's instructions (TaKaRa, Otsu, Shiga, Japan). We retrieved expressed sequence tag (EST) sequences of the *Scamy* gene from the 454 cDNA library of *S. constricta* (GenBank accession no. GALB00000000) and designed primers for 5′-RACE (*Scamy*-F1) and 3′-RACE (*Scamy*-R1) (Table 1).

Table 1 Primers and sequences used in this study

Primer	Sequence (5′→ 3′)	Application
Scamy-F1	ATTGGTATGGTGCCGAGGCTGGG	3′RACE
Scamy-R1	ATAGTTGCCCTGCCCAGCCTCGG	5′RACE
Scamy-F2	TTGCTATTCGTTTGCGGGG	Verifying the sequence of cDNA
Scamy-R2	GACCTCCCACAATAATCGCAAGTA	Verifying the sequence of cDNA
Scamy-F3	TGGAAGCACTGGATGATG	Cloning of intron
Scamy-R3	AACAGCCCGACACCTATT	Cloning of intron
Scamy-F4	AATAGGTGTCGGGCTGTT	Cloning of intron
Scamy-R4	GTCCACGAATACAAATGC	Cloning of intron
Scamy-F5	GTTCTATCACGAAGTCATC	Cloning of intron
Scamy-R5	CCTCCCACAATAATCGCAAGT	Cloning of intron
Scamy-F6	ACTTGCGATTATTGTGGGAGG	Cloning of intron
Scamy-R6	ACCTCCTGGTCTGTTAGTAGTCC	Cloning of intron
Scamy-F7	CCGTGTGGACTACTAACAGACC	Cloning of intron
Scamy-R7	CCACATCTATGGTATTGGGTTT	Cloning of intron
Scamy-F8	ATCAAGGCAGTGGTGAATGGC	Cloning of intron
Scamy-R8	CATCCACATCTATGGTATTGGGTTT	Cloning of intron
Scamy-F9	TTGCTATTCGTTTGCGGG	SNP
Scamy-R9	TCATTCCCCAGCCAAAGT	SNP
Scamy-F10	ACTTTGGCTGGGGAATGA	SNP
Scamy-R10	GCATTGTCTCGCATAGGGATA	SNP
Scamy-F11	TATCCCTATGCGAGACAATGC	SNP
Scamy-R11	ACCAGCCGTTGTCAGTCCGA	SNP
Real-A-F1	TGTTGACTTTGGCTGGGGAA	qRT-PCR
Real-A-R1	GATAAGCGGTTGCCATCCTGTA	qRT-PCR
18S-F	CTTTCAAATGTCTGCCCTATCAACT	qRT-PCR
18S-R	TCCCGTATTGTTATTTTTCGTCACT	qRT-PCR

Polymerase chain reaction (PCR) amplification was performed as follows: five cycles of 94 °C for 30 s and 72 °C for 3 min; five cycles of 94 °C for 30 s, 70 °C for 30 s and 72 °C for 3 min; 25 cycles of 94 °C for 30 s and 68 °C for 30 s and a final extension at 72 °C for 3 min. The PCR products were purified using a Gel Extraction Kit (TianGen, Beijing, China), cloned into the pEASY-T1 vector (TransGen, Beijing, China), and transformed into Trans1-T1 Phage-resistant cells (TransGen, Beijing, China) according to the manufacturer's protocols. Positive clones were selected and sequenced, and the full-length cDNA sequence was determined by piecing together the sequences of the 3′ and 5′ RACE products. To confirm the accuracy of the cloning and sequencing, the full-length cDNA was re-amplified with high-fidelity polymerase (TaKaRa, Otsu, Shiga, Japan), using a pair of gene-specific primers, *Scamy*-F2 and *Scamy*-R2 (Table 1), designed based on the above-mentioned cDNA sequence. PCR products were cloned and sequenced following the procedures described above.

Cloning the introns of Scamy

Genomic DNA from adductor muscle tissue was extracted with phenol/chloroform/isoamyl alcohol (25:24:1), and then re-extracted by chloroform/isoamyl alcohol (24:1). The other steps were the same as the procedure described by Sambrook *et al.* (2001). According to the full-length cDNA, six primer pairs (F3, R3, F4, R4, F5, R5, F6, R6, F7, R7, F8 and R8) were designed to detect the introns (Table1). PCR conditions were as follows: an initial denaturation (94 °C, 5 min), followed by 35 cycles of amplification (94 °C, 30 s; 55 °C, 30 s; 72 °C, 2 min), and a final extension (72 °C, 10 min). PCR products were cloned and sequenced following the procedures described above.

Sequence analysis of Scamy

The sequences were spliced using the BLAST algorithm in the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/blast/). The deduced amino acid sequence was analyzed using the simple modular architecture research tool (SMART) (http://smart.embl-heidelberg.de) to predict conserved domains. The presence and locations of the signal peptide and cleavage sites in the amino acid sequence were predicted using the Signal P 4.0 server (http://www.cbs.dtu.dk/services/SignalP/). Multiple alignments of α-amylase proteins from *S. constricta* and other species were performed using the ClustalW2 multiple alignment program (http://www.ebi.ac. uk/Tools/msa/clustalw2/). A phylogenetic tree was constructed by the neighbor-joining method with MEGA 6.0.

Quantitative expression analysis of Scamy

Expression levels of *Scamy* were determined at different developmental stages (unfertilized mature eggs, fertilized eggs 2-cell embryos, 4-cell embryos, morulas, trochophores, D-shaped larvae and juvenile clams; n > 500, three sets of samples for each stage), and in different adult tissues (mantle, adductor muscle, digestive gland, foot, gills and siphon; four sets of samples for each tissue), using real-time quantitative reverse transcription PCR (qRT-PCR), with three technical repeats for each PCR reaction. Total RNA was extracted from the samples as described above. A 142-bp fragment of *Scamy* was amplified from the cDNA template using the primers Real-A-F and Real-A-R (Table 1), and 186-bp products of 18S rRNA were amplified as an internal control for qRT-PCR using primers 18S-F and 18S-R (Table 1). PCR amplification was performed in a 20-μl volume containing 10 μl iTaq Universal SYBR Green Supermix (Bio-Rad, CA, USA), 7.2 μl deionized water, 0.8 μl first-strand cDNA and 1 μl forward and reverse primers. Amplification was performed using the following thermal cycling conditions: incubation at 94 °C for 20 s, 40 cycles of 94 °C for 3 s, 60 °C for 15 s and 72 °C for 10 s. We compared *Scamy* expression levels between starved and refed clams based on four individuals from each replicates selected at random every day throughout the experiments. Total RNA was extracted from the digestive glands of 120 clams, and qRT-PCR was performed as described above.

α-Amylase assay

We assayed α-amylase activity in digestive glands extracted from starved and refed clams (twelve samples per day). After homogenization for 10 min in an ice bath at 4 °C, samples were centrifuged for 10 min at 10,000*g*, and the supernatant was used as the crude enzyme extract. All samples were analyzed within 24 h. α-Amylase activity and total protein content were measured using kits from Shanghai Yuanye (China) and Nanjing Jiancheng (China), respectively, according to the manufacturers' instructions. α-Amylase activity was measured using the microplate iodine starch method and total protein content was measured using Coomassie Blue staining. One unit of α-amylase activity was defined as the amount necessary to hydrolyze 10 mg starch per milligram of protein at 37 °C for 30 min.

SNP identification and association analysis of Scamy *gene exons*

RNA samples were extracted from 215 clams, as described above. According to the cDNA sequence of the *Scamy* gene, three pairs of (F9, R9, F10, R10, F11 and R11) primer sets were selected (Table 1). PCR amplification was performed in 50-μl volumes containing 2 μl template cDNA, 19 μl dH_2O, 25 μl PCR Mix and 2 μl of each primer. The PCR conditions were as follows: 3 min at 94 °C; 35 cycles of 30 s at 94 °C, 1 min at 58 °C and 2 min at 72 °C; and a final 5 min extension at 72 °C. Each amplification product was verified by electrophoresis on a 1.0 % agarose gel and staining with ethidium bromide. The amplicons representing unique banding patterns were sent to Beijing Genomics Institute (Beijing, China) for sequencing in both directions. The sequence was aligned using MEGA6.0 software. Mutation sites were named according to the position of the initiation codon. Univariate analysis was performed according to the general linear model procedure in SPSS 20.0 software.

Statistical analysis

The results of qRT-PCR analysis were based on the CT values of the PCR products and the expression levels of *Scamy* were analyzed using the comparative CT method. Statistical analysis was performed using SPSS 19.0 (Chicago, IL, USA). Differences in relative *Scamy* mRNA expression levels among different developmental stages and different adult tissues, and between starved and refed clams were compared by one-way analysis of variance (ANOVA). Multiple comparisons were conducted using the Student-Newman-Keuls test. Growth traits and α-amylase activities in starved and refed clams were compared as above. Differences were considered significant at $p < 0.05$. Body weight, soft weight, shell width, shell length and shell height of the clams were analyzed by one-way ANOVA using SPSS 20.0. The effects of SNPs on growth traits in the two populations were analyzed using the above method. SNP markers with genotypes that showed a significant correlation with growth traits were studied by post hoc multiple comparison (Duncan method).

Results

cDNA sequence analysis of Scamy

The full-length *Scamy* cDNA comprised 2,196 bp (GenBank accession no. KX197931.1) and contained a 2,085 bp open reading frame encoding 694 amino acids. The cDNA contained a 5′ untranslated region of 41 nucleotides, a 3′ untranslated region of 73 nucleotides including a terminator codon (TAG), a putative polyadenylation consensus signal (AATAA) and a poly(A) tail (Fig. 1). The calculated molecular mass of the deduced mature protein was 76.02 kDa, and its theoretical isoelectric point was 5.64. Sequence analysis suggested that the protein contained a signal peptide but had no transmembrane region and was a hydrophilic protein. SMART analysis revealed that the deduced amino acid sequence contained an A domain (27-386) and a C domain (395-473).

A phylogenetic tree of α-amylase was constructed using the neighbor-joining method, based on the deduced amino acid sequences of α-amylase from molluscs and some other animals for which the sequences were available in the NCBI database (Fig. 2). The obtained tree showed that α-amylases were divided into two major groups: one group comprised mollusc and crustacean α-amylases, and the other contained mammal and fish α-amylases. In the mollusc group, *S. constricta* α-amylase clustered with α-amylases from *Cerastoderma edule* and *Corbicula fluminea*, and then with others molluscs. Multiple alignment indicated that the *S. constricta* α-amylase shared the highest sequence identity (78.6 %) with *C. fluminea* α-amylase and 41.4 % - 71.6 % identity with other species (Fig. 3, Table 2).

Introns analysis of Scamy

Six pairs of primers PCR amplification produced fragments of 432 bp, 408 bp, 534 bp, 594 bp and 922 bp, respectively. After assembly six exons and five introns were obtained. All five introns were located within the ORF (Fig. 4). The maximum intron

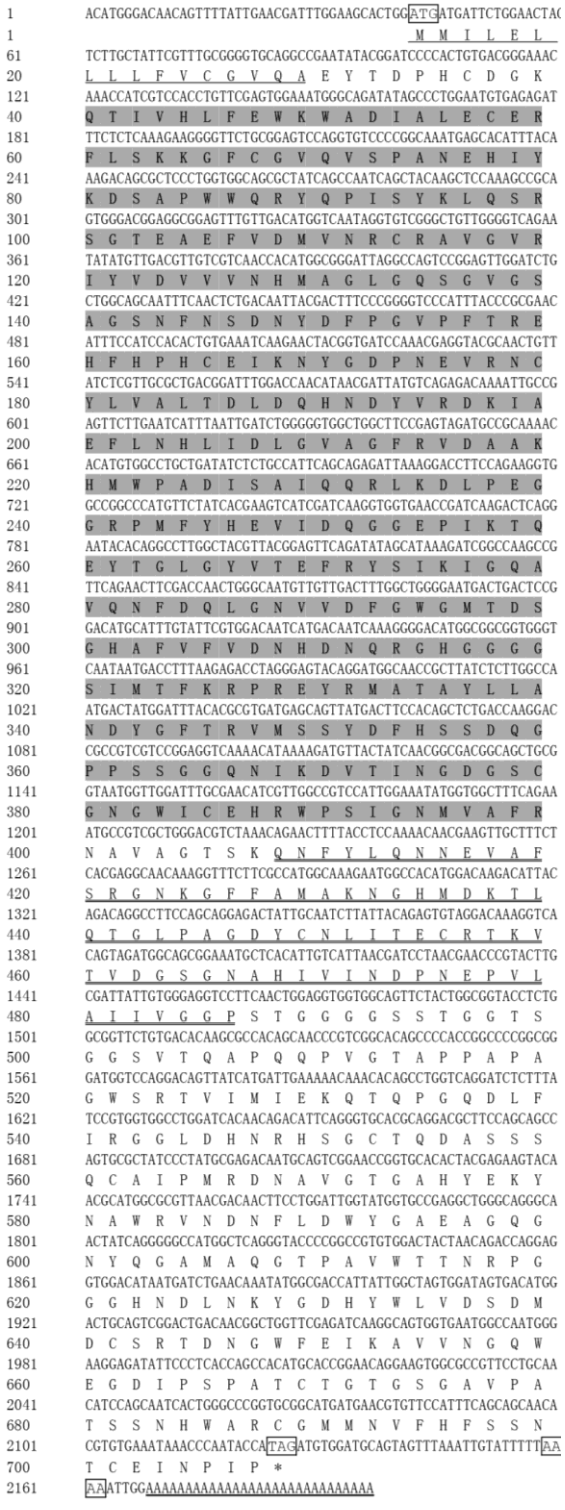

Fig. 1 Full-length cDNA sequence and deduced amino acid sequence of the *Scamy.* The three letters boxes are the initiation codon, the terminator codon and polyadenylation signal sequence, the * represents the end of the protein translation, the single underlined is the signal peptide of protein, the bold shaded part is A Domain, the double underlined part is C Domain and the Coarse underlined part is polyA.

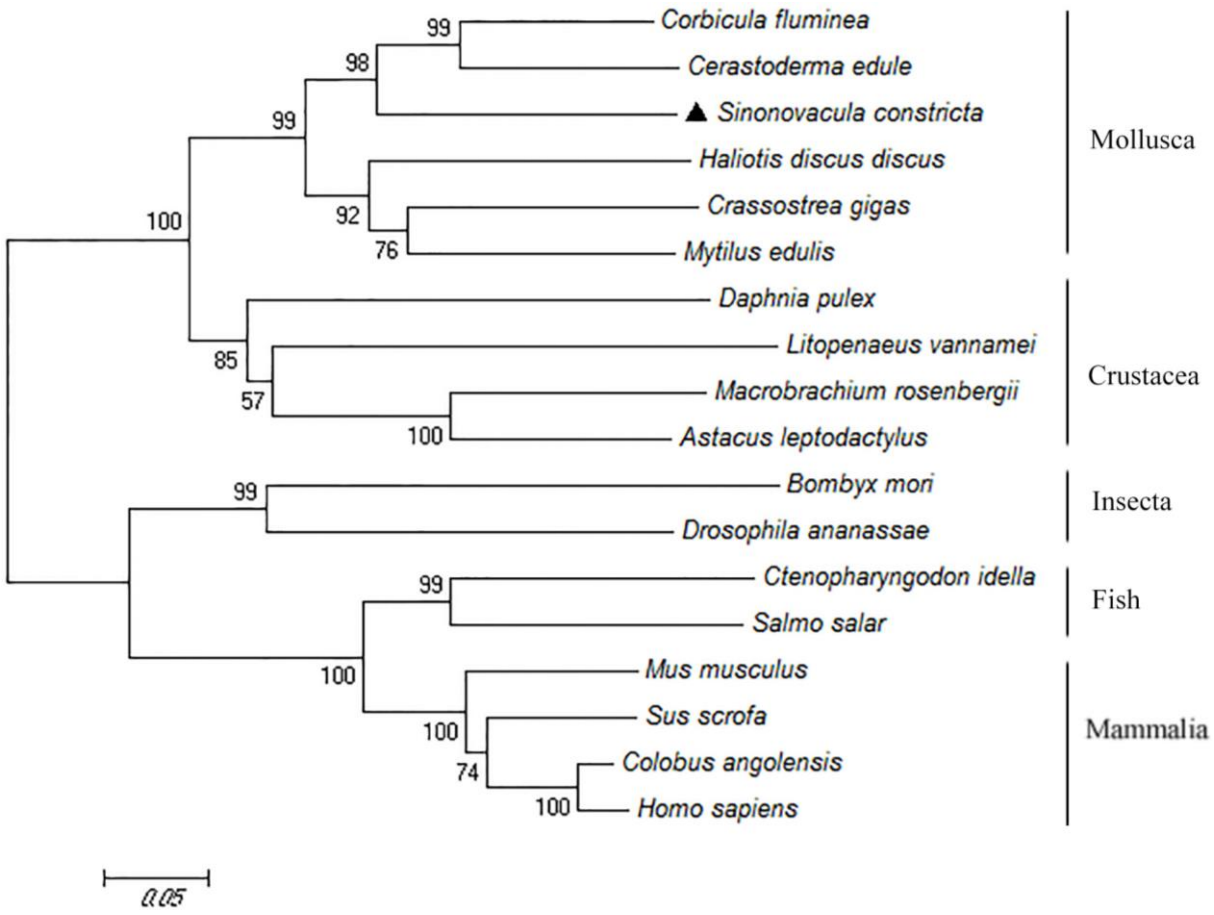

Fig. 2 Phylogenetic analysis of *Scamy* and other known α-amylases. The neighbor-joining tree was generated with MEGA6.0. Species and the accession number of each species are shown in Table 2.

Table 2 Species and GenBank accession numbers of AMYs sequence used for multiple alignment and phylogenetic analysis

Species	GenBank no.	Size (bp)	Identity (%)
Sinonovacula constricta	KX197931.1	694	-
Crassostrea gigas	EKC28393.1	697	64.2
Corbicula fluminea	AAO17927.2	699	78.6
Litopenaeus vannamei	AIJ02080.1	719	61.4
Macrobrachium rosenbergii	AKL71614.1	706	65.5
Mytilus edulis	ACA34372.1	660	64.9
Haliotis discus discus	ABO26611.1	694	71.6
Colobus angolensis	ABW02886.1	511	41.2
Ctenopharyngodon idella	ACX35465.1	512	39.3
Astacus leptodactylus	AIW65942.1	696	65.0
Sus scrofa	AAF02828.1	511	40.7
Bombyx mori	ACT64133.1	500	41.0
Salmo salar	ABD13895.1	505	40.9
Drosophila ananassae	AAC79122.1	494	41.1
Homo sapiens	AAA52280.1	511	41.4
Mus musculus	CAA24099.1	511	41.0
Cerastoderma edule	ACA34380.1	460	54.4

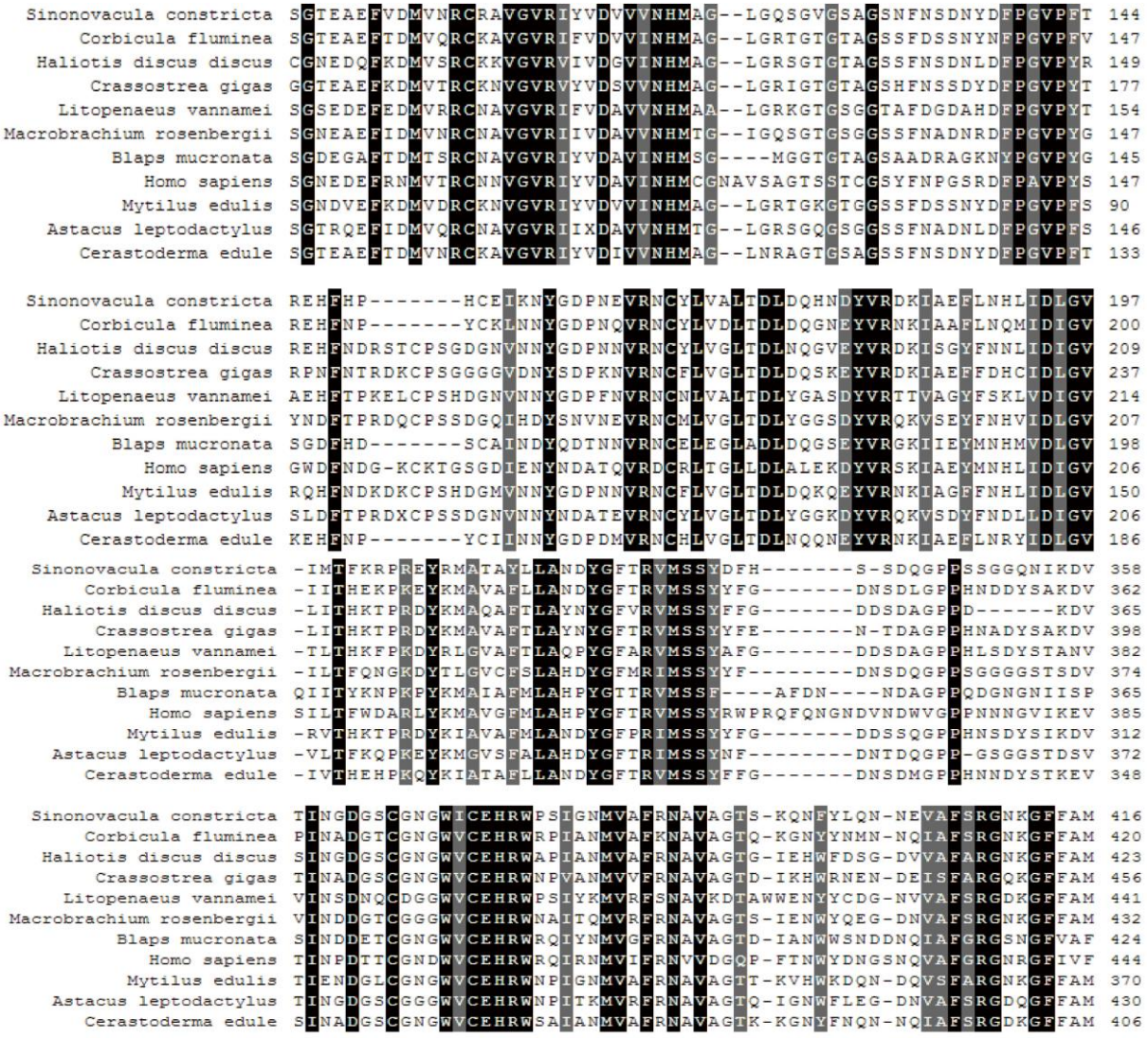

Fig. 3 Amino acid sequence alignments of Amy between *S. constricta* and other species. Identities are shaded dark and similarities are shaded gray.

was found to have the length of 922 bp, compared to the minimum length of 408 bp. The other introns ranged from 432 bp to 594 bp. All exon-intron junctions followed the consensus rule of the splice acceptor -AG/GT- splice donor for splicing.

Quantitative expression of Scamy

The expression profiles of *Scamy* in embryos/larvae and in adult tissues were analyzed by qRT-PCR. In adult clams, *Scamy* was mainly expressed in the digestive gland and was barely expressed in the other tested tissues (mantle, adductor muscle, foot, gills and siphon) ($p < 0.01$) (Fig. 5). Among the eight developmental stages, *Scamy* transcripts were expressed at low levels before the trochophore stage, and increased from the D-shaped larva stage, with the highest expression levels in juvenile clams. There were significant differences in *Scamy* expression levels between juvenile clams and other developmental stages ($p < 0.01$) (Fig. 6).

Quantitative expression of Scamy *in starved and refed clams*

We also analyzed the expression patterns of *Scamy* in starved and refed clams by qRT-PCR. During starvation, *Scamy* expression initially increased up to the second day (S2), but then fell sharply on S3. *Scamy* mRNA levels on the last two days of starvation (S4 - S5) were lower than the level before starvation. *Scamy* expression then increased after refeeding, and was slightly reduced on the fourth day after refeeding (F4), but remained higher than before starvation (Fig. 7).

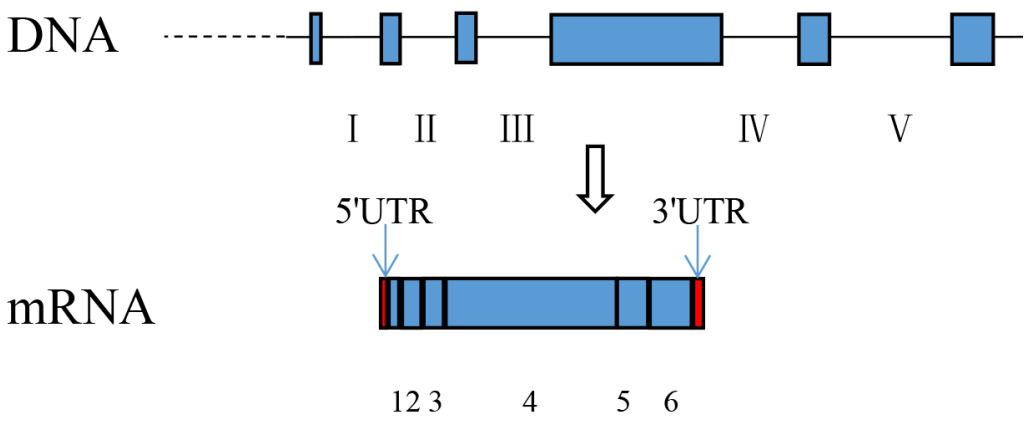

Fig. 4 The structures of *Scamy* gene. Ⅰ-Ⅴ are the five introns of *Scamy* gene, 1-6 are the six exons of *Scamy* gene. They are shown relative to their lengths in the cDNA sequences obtained.

Amylase activity assay

Amylase-specific activity initially increased and then decreased significantly during starvation of *S. constricta*. Activity increased rapidly on S2 and decreased sharply on S3, and then stabilized during late starvation. Amylase activity rose progressively after refeeding, and reached a higher level than before starvation, with a peak at F4 (Fig. 8).

Association between Scamy *SNPs and growth traits*

Twenty-one SNPs were found in the YL population, including seven associated with growth traits. Nineteen SNPs in exons of *Scamy* were identified in the SM population, including three potentially associated with *S. constricta* growth traits.

YL individuals with genotype AC at position C952A grew faster than those with the CC genotype in terms of all of the measured growth traits ($p < 0.05$). Clams with genotype CT at C963T had faster growth in all the growth traits compared with TT individuals ($p < 0.05$). SNP T1371C CT genotype was also associated with significantly higher growth traits than the TT genotype ($p < 0.05$). In addition, genotype TT at position C1503T had a significant positive effect on all the growth traits compared with genotype CC ($p < 0.05$) (Table 3).

T1527C was significantly associated with shell width and shell length in the SM population SM, but not in the YL population ($p < 0.05$). Multiple comparison analyses showed that individuals with C1503T TT genotype grew faster than those with CC genotype in terms of shell width, shell height, body weight and soft weight ($p < 0.05$) (Table 4).

Comparing the loci in the two populations identified one shared SNP at C1503T in the amylase coding region (Fig. 9). On further analysis, it was found that the SNP C1503T was synonymous. Association analysis showed that individuals with the TT genotype at locus 1503 had significantly higher growth traits than those with the CC genotype in both populations ($p < 0.05$).

Discussion

α-Amylases from various species have been crystallized and analyzed by X-ray diffraction. Their structure comprises three domains: a TIM barrel (domain A); a long loop region inserted between βA3 and αA3 (third β-strand and α-helix in the A domain), known as domain B; and a C domain at the end of the sequence (Gerard *et al*., 2001). The predicted *S. constricta* amino acid sequence aligned well with these conserved regions, suggesting that its primary structure had features typical of other α-amylases. The deduced amino acid sequence of *S. constricta* α-amylase shared 41.4 % - 71.6 % identity with α-amylases from other animals. It was thus confirmed to belong to the α-amylase family and to have similar biological functions to α-amylases in other species. Phylogenetic analysis showed distinct

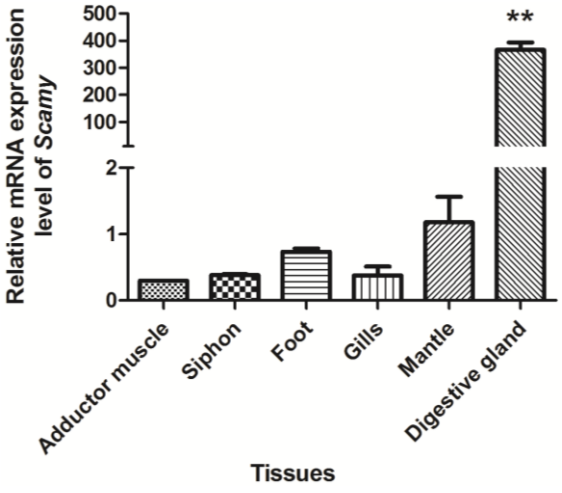

Fig. 5 mRNA expression levels of *Scamy* gene in different tissues (adductor muscle, siphon, foot, gills, mantle, digestive gland,). $^{**}p < 0.01$.

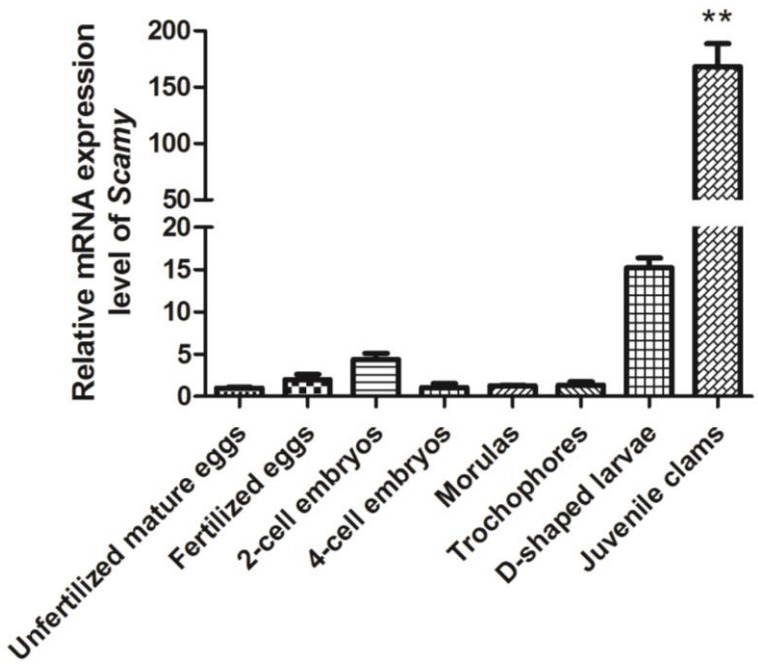

Fig. 6 mRNA expression levels of *Scamy* gene in different developmental stages(unfertilized mature eggs, fertilized eggs, 2-cell embryos, 4-cell embryos, morulas, trochophores, D-shaped larvae, juvenile clams). $^{**}p < 0.01$.

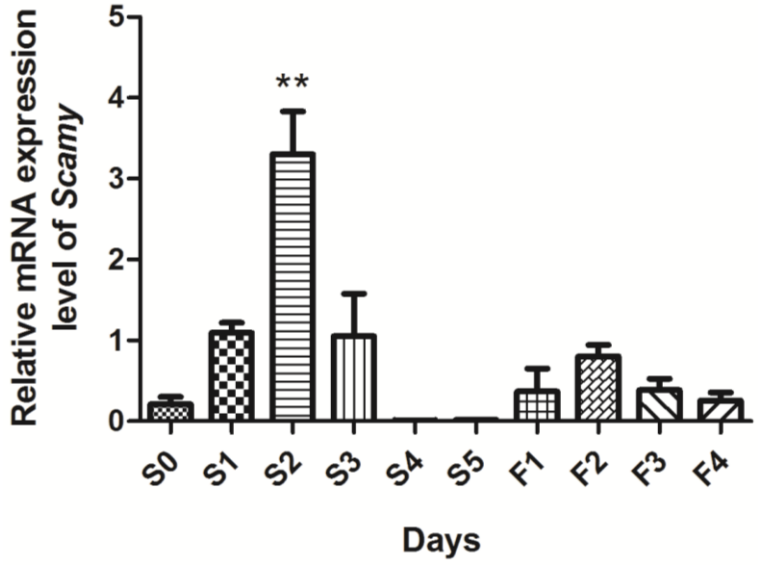

Fig. 7 Analysis of expression difference of *Scamy* gene in starvation and refeeding (S0.before starvation, S1-S5.starvation, F1-F4.refeeding). $^{**}p < 0.01$.

boundaries in terms of α-amylase structures among crustaceans insects, mammals, fish and molluscs, with *S. constricta* α-amylase grouped in a subcluster with molluscs, forming a branch with *C. fluminea*, suggesting that *S. constricta* and *C. fluminea* are closely related.

Scamy mRNA was mainly expressed in the digestive gland, as demonstrated by qRT-PCR, consistent with its function. Similar results have been observed in *C. gigas* (Huvet *et al*., 2003) and *P. fucata* (Huang *et al*., 2016), and α-amylase was also expressed only in the digestive gland in *Saccostrea forskali* (Thongsaiklaing *et al*., 2014). *Scamy* mRNA levels were higher in the digestive gland than in the mantle, adductor muscle, foot, gills or siphon, indicating that the digestive gland is the main digestive organ in bivalves, given that α-amylase is the main digestive enzyme (Le Moine *et al*., 1997). Further studies of the α-amylase gene are needed to elucidate the key factors regulating its expression.

Table 3 Effect of seven SNPs in the *Scamy* gene on growth traits in population YL

SNP	Geno type	N*	Frequency (%)	Shell length (mm)	Shell width (mm)	Shell height (mm)	Body weight (g)	Soft weight (g)
420	AA	12	11.11	**51.20±4.26^{a}**	**12.85±0.90^{a}**	**17.45±1.61^{a}**	**7.91±2.12^{a}**	**5.79±1.87^{a}**
	GA	32	29.63	**54.51±3.67^{b}**	**14.41±1.62^{b}**	**18.81±1.23^{b}**	**10.19±2.62^{b}**	**7.28±2.11^{b}**
	GG	64	59.26	**54.10±5.03^{b}**	13.87±1.85	**18.52±1.71^{b}**	9.54±2.84	6.96±2.29
952	CC	16	15.24	**51.76±4.22^{a}**	**13.13±1.15^{b}**	**17.72±1.48^{a}**	**8.40±2.17^{a}**	**6.14±1.84^{a}**
	AC	40	38.10	**55.21±4.53^{b}**	**14.60±1.65^{a}**	**19.03±1.43^{b}**	**10.58±2.72^{b}**	**7.63±2.24^{b}**
	AA	49	46.67	53.67±4.71	**13.63±1.80^{b}**	**18.33±1.66^{a}**	**9.14±2.77^{a}**	**6.64±2.22^{a}**
963	CC	44	43.56	53.56±4.84	**13.58±1.77a**	**18.31±1.72^{a}**	**9.14±2.78^{a}**	**6.62±2.21^{a}**
	CT	40	38.10	**55.14±4.58^{a}**	**14.56±1.72^{b}**	**19.01±1.44^{b}**	**10.54±2.79^{b}**	**7.60±2.28^{b}**
	TT	17	16.19	**51.83±4.10^{b}**	**13.12±1.11^{a}**	**17.78±1.45^{a}**	**8.37±2.10^{a}**	**6.08±1.80^{a}**
1287	CC	59	56.73	**54.43±4.85^{a}**	13.89±1.82	**18.61±1.67^{a}**	9.57±2.81	6.96±2.27
	CT	34	32.69	**54.55±3.40^{a}**	**14.40±1.61^{a}**	**18.76±1.26^{a}**	**10.24±2.61^{a}**	7.33±2.12
	TT	11	10.58	**50.86±4.34^{b}**	**12.81±0.84^{b}**	**17.52±1.67^{b}**	**8.03±2.23^{b}**	5.96±1.99
1371	CC	60	54.55	**54.20±5.11^{a}**	13.86±1.84	**18.55±1.72^{a}**	9.48±2.87	6.90±2.30
	CT	34	30.91	**54.56±3.60^{a}**	**14.40±1.61^{a}**	**18.76±1.26^{a}**	**10.24±2.61^{a}**	**7.33±2.12^{a}**
	TT	16	14.55	**50.83±3.51^{b}**	**12.92±1.31^{b}**	**17.64±1.38^{b}**	**8.08±2.10^{b}**	**5.94±1.83^{b}**
1503	CC	24	23.53	**51.98±3.84^{a}**	**13.23±1.34^{a}**	**17.97±1.38^{a}**	**8.42±2.08^{a}**	**6.09±1.75^{b}**
	CT	54	52.94	53.30±5.17	14.41±1.97	18.20±1.71	9.97±2.70	6.47±2.10
	TT	24	23.53	**55.13±4.57^{b}**	**14.45±1.58^{b}**	**18.96±1.55^{b}**	**10.36±2.86^{b}**	**7.50±2.33^{a}**
1737	TT	49	45.37	**53.28±4.53^{b}**	**13.65±1.92^{a}**	**18.26±1.60^{b}**	**9.12±2.73^{b}**	**6.65±2.17^{b}**
	CT	45	41.67	**55.13±4.61^{a}**	**14.44±1.68^{b}**	**18.97±1.55^{a}**	**10.46±2.81^{a}**	**7.58±2.33^{a}**
	CC	14	12.96	**51.34±3.68^{b}**	**13.11±1.20^{a}**	**17.49±1.35^{b}**	**8.23±2.09^{b}**	**6.00±1.80^{b}**

Note: bold parts are the SNPs associated with growth traits, $p < 0.05$; underline parts are the SNPs potentially associated with growth traits, $p < 0.01$.

In this study, *Scamy* mRNA appeared at the beginning of embryonic development, and its expression level was increased in D-shaped larvae, by which stage the digestive gland had formed. This is similar to the situation in grass carp, in which amylase gene expression was shown to increase obviously in line with the development of the hepatopancreas and digestive tract (Tang *et al*., 2015). Among mollusks, low expression of α-amylase has been observed in early embryos and higher expression during the larval stages in *Haliotis discus hannai* (He *et al*., 2015) and *Meretrix meretrix* (unpublished data), consistent with its role as the main digestive enzymes in mollusks. Amylase gene expression peaked in juvenile clams, suggesting that expression of the *Scamy* gene may have been promoted by increased ingestion in juvenile clams.

We analyzed the effects of starvation and refeeding on *Scamy* expression, and showed that expression initially increased and then decreased during starvation, followed by an increase during refeeding. This was in accord with another study that demonstrated decreased amylase mRNA levels from day 29 of starvation in *Dicentrarchus labrax* (Péres *et al*., 1998). The results of the current study suggested that starvation stress may enhance amylase activity to boost energy, indicating that the body can improve the metabolic activity of various enzymes to meet the energy requirements imposed by different physiological situations. However, the ability to respond to starvation stress decreased with prolonged starvation, and amylase activity decreased and then stabilized during late starvation. Similar phenomena have been found in other aquatic animals, such as *Litopenaeus vannamei* (Meng *et al*., 2006), *Megalobrama pellegrini* (Zheng *et al*., 2015) and *Ruditapes philippinarum* (Li *et al*., 2016). Previous studies showed that refeeding significantly improved digestive enzyme activity in aquatic animals, but that the degree of recovery differed among different species. Amylase activity increased in *Rutilus rutilus caspicus* after refeeding (Abolfathi *et al*., 2012), while amylase and cellulose activities in *R. philippinarum* improved to different degrees after refeeding, and reached the levels seen

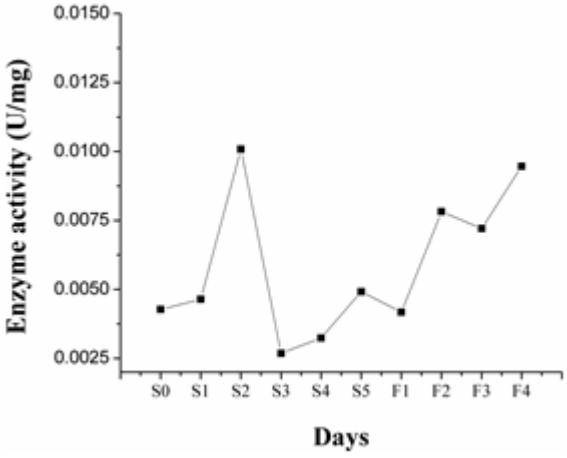

Fig. 8 mRNA expression levels of *Scamy* gene in starvation and refeeding (n = 4). S0 = before starvation, S1-S5 = starvation, F1-F4 = refeeding.

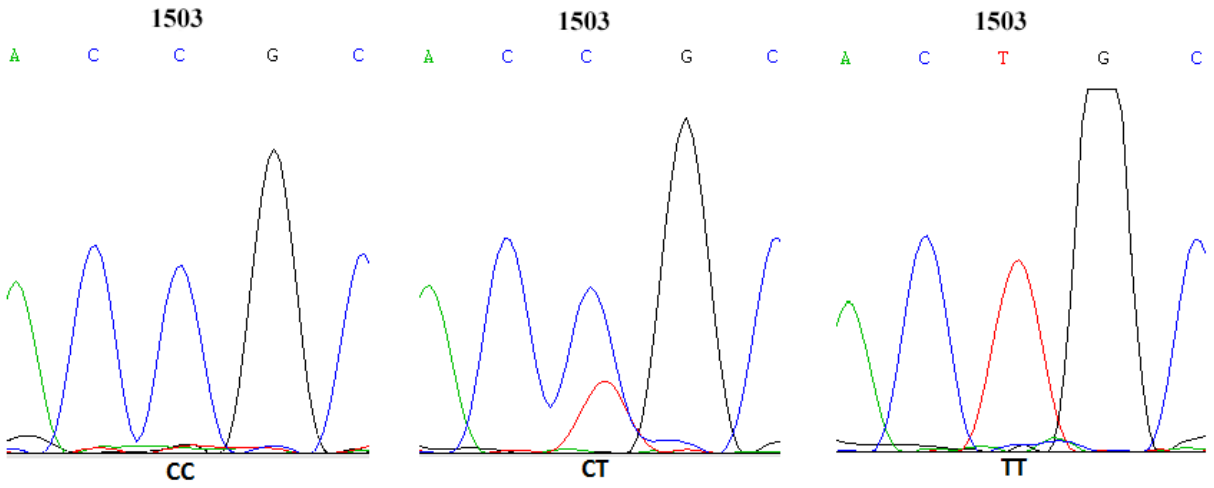

Fig. 9 The sequencing maps of SNP for *Scamy* gene at the position of C1503T

on the first and second days, respectively, within 3 days (Li *et al*., 2016). Amylase activity in *Macrobrachium nipponense* increased slightly and then decreased upon refeeding (Li *et al*., 2007). Our results showed a progressive increase in amylase activity to a much higher level than that before starvation, with a peak after refeeding for 4 days. These results indicated that starvation and refeeding could increase amylase activity, thus providing a physiological basis for compensatory growth in razor clams (Zhang *et al*., 2010). Further studies are needed to develop the optimal starvation and refeeding model to maximize growth.

Recent research has focused on correlations between gene polymorphisms and growth of animals. Two α-amylase gene SNPs were highly correlated with growth traits in *Haliotis diversicolor supertexta* (unpublished data), and one site was found in *Litopenaeus vannamei* (Glenn *et al*., 2015), but its relationship with growth was not significant, probably because of the small sample size. In the current study, we identified seven SNPs potentially associated with growth traits in the YL *S. constricta* population and three in the SM population, including one SNP in the *Scamy* exon that was common to both populations and potentially associated with clam growth. *S. constricta* individuals with the TT genotype of SNP C1503T grew significantly faster than CC individuals in both populations. We therefore hypothesize that clams with the C1503T TT or CT genotype are favorable for breeding. Furthermore, this *Scamy* SNP could influence growth performance and may be a suitable marker for marker-assisted selection in this species.

Table 4 Effect of three SNPs in the *Scamy* gene on growth traits in population SM

SNP	Geno type	N*	Frequency (%)	Shell length (mm)	Shell width (mm)	Shell height (mm)	Body weight (g)	Soft weight (g)
1098	CC	85	82.52	**35.97±3.06**[a]	8.23±0.94	12.02±1.09	2.36±0.67	1.84±2.35
	CT	16	15.53	**34.27±2.63**[b]	7.91±0.91	11.49±1.06	2.10±0.57	1.65±0.44
	TT	2	1.94	35.03±3.97	7.73±0.35	11.86±1.19	2.18±0.66	1.71±0.51
1503	CC	36	35.29	35.23±3.25	**7.91±0.92**[a]	**11.69±1.09**[a]	**2.16±0.61**[a]	**1.68±0.45**[a]
	CT	56	54.90	35.67±2.89	8.22±0.88	11.95±1.10	2.34±0.67	1.84±0.53
	TT	10	9.80	36.99±3.61	**8.80±1.08**[b]	**12.53±1.17**[b]	**2.63±0.68**[b]	**2.08±0.55**[b]
1527	CC	95	92.23	35.84±3.04	**8.23±0.92**[a]	**12.01±1.09**[a]	2.35±0.65	1.83±0.50
	CT	6	5.83	33.91±2.71	**7.41±0.70**[b]	**10.89±0.80**[b]	1.86±0.54	1.48±0.44
	TT	2	1.94	33.74±3.82	7.88±1.73	11.52±0.88	2.09±0.92	1.68±0.70

Note: bold parts are the SNPs associated with growth traits, $p < 0.05$; underline parts are the SNPs potentially associated with growth traits, $p < 0.01$.

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References

Abolfathi M, Hajimoradloo A, Ghorbani R, Zamani A. Effect of starvation and refeeding on digestive enzyme activities in juvenile roach, *Rutilus rutilus caspicus*. J. Comp. Biochem. Physiol. A 161: 166-173, 2012.

Feng BB, Zhong YM, Niu DH, Chen H, Lin GW, Li JL. Molecular characteristics and expression analysis of β-actin 1 gene from *Sinonovacula constricta*. J. Fish. China 35: 650-659, 2011.

Pujadas G, Palau J. Evolution of α-amylases: architectural features and key residues in the stabilization of the (β/α)8 scaffold. Mol. Biol. Evol. 18: 38-54, 2001.

Glenn KL, Grapes L, Suwanasopee T, Harris DL, Li Y, Wilson K, *et al*. SNP analysis of AMY2 and CTSL genes in *Litopenaeus vannamei* and Penaeus monodon shrimp. Anim. Genet. 36: 235-236, 2005.

He QG, Wang SW, Li JQ, Liu X. Expression of polysaccharidase enzymes in the f_4 adults of a mass selected pacific abalone strain. Mar. Sci. 39: 7-12, 2015.

Huvet A, Daniel JY, Quéré C. Dubois S, Prudence M, Wormhoudt AV, *et al.* Tissue expression of two α-amylase genes in the pacific oyster *Crassostrea gigas*. Effects of two different food rations. Aquaculture 228: 321-333, 2015.

Huvet A, Jeffroy F, Fabioux C, Daniel JY, Quillien V, Van WA, *et al*. Association among growth, food consumption-related traits and amylase gene polymorphism in the pacific oyster *Crassostrea gigas*. Anim. Genet. 39: 662-665, 2008.

Huang BL, Lei XG, Wang LG. Studies on digestive enzymes of *Perna viridis Potamocorbula rubromuscula* and *Sipunculus nudus* in dongzhai harbor. Mar. Fish Res. 24, 2003.

Huang GJ, Guo YH, Li L, Fan SG, Yu ZN, Yu DH. Genomic structure of the α-amylase gene in the pearl oyster *Pinctada fucata* and its expression in response to salinity and food concentration. Gene 587: 98-105, 2016.

Kuriki T, Imanaka T. The concept of the alpha-amylase family: structural similarity and common catalytic mechanism. J. Biosci. Bioeng. 87: 557-565, 1999.

Le Moine S, Sellos D, Moal J, Daniel JY, San Juan Serrano F, Samain JF, *et al*. Amylase on *Pecten maximus* (Mollusca, bivalves): protein and cDNA characterization; quantification of the expression in the digestive gland. Mol. Mar. Biol. Biotechnol. 6: 228-237, 1997.

Li LM, Wang CH, He LH. Effect of starvation on digestive enzyme activity and antioxidant capacity of *Ruditapes philippinarum*. Fujian Agricultural Science & Technology, 2016.

Li ZH, Xie S, Wang JX, Chen DQ. Effects of intermittent starvation on growth and some digestive enzymes in the shrimp *Macrobrachium nipponense*. J. Fish. China 31: 456-462, 2007.

Meng QW. Effects of starvation on feeding behaviour and digestive enzyme activities of *Litopenaeus vannamei postlarvae*. Mar. Fish Res., 2006.

Niu D, Wang F, Zhao H, Wang Z, Xie S, Li J. Identification, expression, and innate immune responses of two insulin-like peptide genes in the razor clam *Sinonovacula constricta*. Fish. Shellfish. Immunol. 51: 401-404, 2016.

Pan LL, Huang GJ, Cheng SY, Wang XN, Yu DH. Cloning and characterization of alpha amylase cDNA and its introns in the pearl oyster *Pinctada maxima*. J. Trop. Oceanogr. 32: 52-58, 2013.

Prudence M, Moal J, Boudry P, Daniel JY, Quéré C, Jeffroy F, *et al*. An amylase gene polymorphism is associated with growth differences in the Pacific cupped oyster *Crassostrea gigas*. Anim. Genet. 37: 348-351, 2006.

Péres A, Infante JLZ, Cahu C. Dietary regulation of activities and mRNA levels of trypsin and amylase in sea bass (*Dicentrarchus labrax*) larvae. Fish Physiol. Biochem. 19: 145-152, 1998.

Sellos D, Moal J, Degremont L, Huvet A, Daniel JY, Nicoulaud S, *et al*. Structure of amylase genes in populations of Pacific cupped oyster (*Crassostrea gigas*): tissue expression and allelic polymorphism. Mar. Biotechnol. 5: 360-372, 2003.

Thongsaiklaing T, Sehawong W, Kubera A, Ngernsiri L. Analysis of the α-amylase gene sequence and the enzyme activity of Indian rock oyster *Saccostrea forskali*. Fish. Sci. 80: 589-601, 2014.

Tang XH, Fan, JJ, Bai JJ. Expression analysis of α-amylase gene in various tissues and early development of *Ctenopharyngodon idellus*. Mar. Fish 37: 31-37, 2014.

Xie SM, Niu DH, Ruan HD, Wang Z, Wang F, Chen S, *et al*. Molecular characterization of *IGFBP* and association analysis with growth traits in the razor clam *Sinonovacula constricta*. J. Fish. China 39: 799-809, 2015.

Yu JG, Wang CL, Hu YJ, Dong YQ, Wang Y, Tu XM, *et al*. Purification, crystallization and preliminary crystallographic analysis of the marine α-amylase AmyP. Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun. 69: 263-266, 2013.

Zhang CJ, Liu J, Chen JH, Wu JH, Li JL, Wang L, *et al.* Effects of starvation and refeeding on digestive enzyme activity and antioxidative capacity of razor clam (*Sinonovacula constricta*). J. Fish. China 34: 1106-1112, 2010.