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RESEARCH REPORT

Characteristics of α-Amylase gene and its association with growth traits in *Meretrix meretrix*

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Abstract

α-Amylase is a major digestive enzyme in phytophagous animals that catalyzes the hydrolysis of starch into sugars so that it affects the carbohydrate metabolism and growth of many species. In the present study, the full-length cDNA and intron sequences of α-amylase (*MmAmy*) were investigated by rapid amplification of cDNA ends (RACE) method. The genomic DNA sequence of *MmAmy* is 6689 bp, which contains 7 exons and 6 introns. The cDNA of *MmAmy* is 1783 bp, with a 1569 bp of open reading frame (ORF) encoding 522 amino acids. No expression was detected in the early stages of unfertilized mature eggs, fertilized eggs, 4-cell embryo, blastula, gastrulae, trochophore, whereas it was detected after D-shaped larva showing the highest expression in umbo larvae. The quantitative expression of six different tissues in adults showed that expression of *MmAmy* was enriched in visceral mass, while no expression was detected in other tissues. Furthermore, gene expression and enzyme activity of *MmAmy* in visceral mass were highly correlated with the growth rates of four shell color strains. The positive correlation between *MmAmy* and growth traits suggested that α-amylase gene could be used as a candidate gene in the molecular breeding programs to improve clam growth.

Key Words: *Meretrix meretrix;* α-Amylase; cloning; expression; activity; growth traits

Introduction

α-Amylases are enzymes that catalyze the hydrolysis of the α-(1,4) glycosidic linkages in starch and related compounds. Because of their main functions in carbohydrate metabolism, α-Amylase enzymes are important for the utilization of energy sources and growth (Sellos *et al*., 2003). In marine bivalves, activities of digestive enzymes, especially α-Amylase, have been considered to control absorption efficiency for their corresponding substrates, which is one of the principal factors explaining growth variation (Prudence *et al*., 2006). Therefore molecular characterization of genes encoding α-Amylase is a prerequisite for further understanding their genetic contributions to growth of bivalves. So far, in mollusks, α-amylase genes have been reported in some species, such as *Pecten maximus* (Le Moine *et al*., 1997), and later from *Corbicula fluminea* (Da Lage *et al*., 2002), *Crassostrea gigas* (Sellos *et al*., 2003), *Pinctada maxima* (Pan, 2011), *Pteria penguin* (Pan, 2011), *Haliotis discus hannai* (Kumagai *et al*., 2013) and *Pinctada fucata* (Huang, *et al*., 2016). Furthermore, in the Pacific oyster *C. gigas*, two α-amylase genes (AMYA and AMYB) have been characterized (Sellos *et al*., 2003), and their associations between α-amylase gene polymorphism and growth have been established (Prudence *et al*., 2006; Huvet *et al*., 2008). In the razor clam *Sinonovacula constricta*, Liu *et al*. (2017) investigated the gene structure and expression of α-amylase gene, and identified one growth-associated single nucleotide polymorphism (SNP), suggesting the amylase markers was considerably valuable in selective breeding.

Although α-Amylases belong to the glycoside hydrolase superfamily, their gene structure was unique to the distinct species. For example, the number of introns is different in various species. No intron is identified in *Drosophila melanogaster* (Boer *et al*., 1986), but 14 introns in *Daphnia pulex* (Da Lage *et al*., 2011). Similarly, some differences were also found in the promoter region. No functional Pancreatic Transcription Factor-1 (PTF-1) binding site is identified in seabass (*Lates calcarifer*) amylase promoter, which is responsible for pancreas-specific transcription in higher vertebrates. Instead a Hepatocyte Nuclear Factor 3 (HNF-3) binding site is found to modulate the amylase

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Fig. 1 Phenotypes of four shell color strains in *M. meretrix.* DF: dark fringe, TC: thin checkered, WS: white shell, RS: red shell

promoter expression (Ma *et al*., 2004). Although α-amylases from diverse sources have different primary structures, they are homologous in three-dimensional structure (Maurus *et al*., 2005). The protein has been reported with 3 domains: A - a (beta/alpha) 8-barrel; B - a loop between the beta 3 strand and alpha 3 helix of A and C - the C-terminal extension characterized by a Greek key (Chen, 2007).

The hard clam (*Meretrix meretrix*) is extensively distributed along the eastern coastal area of Asia. It has been one of the most important farmed shellfishes in China. In recent years, several genes involved in growth, such as sulfotransferase-like gene (Wang *et al*., 2012a), *GRB*2 (Gao *et al*., 2015), *HDAC*1 (Gao *et al*., 2016), have been cloned and analyzed (Wang *et al*., 2012b; Dai *et al*., 2015; Zou *et al*., 2015). Moreover, several studies showed that the shell color of hard clam was varied and some of them were associated with their growth traits. In *Nodipecten nodosus* (Alfonsi and Perez, 1998; Jessica *et al*., 2012), *Argopecten purpuratus* (Wolff *et al*., 1991), and *P. fucata* (Zou *et al*., 2014), shell colors and decorative patterns have been used as genetic markers to assist selective breeding. Recently, the relationships linking α-amylase, shell color and growth have been reported. In *Chlamys nobilis*, growth traits and amylase activity are both associated with shell color strains (Deng *et al*., 2008). To investigate the regulation of *MmAmy*, the full-length cDNA and introns were cloned, and the expression profiles were detected during embryonic/larvae development stages, in adult tissues and in visceral mass of different shell color stains. And the activity of α-amylase was also tested. The results may contribute us to understand genetic mechanism in clam growth and offer candidate gene and markers for breeding programs in this species.

Materials and Methods

Experimental animals and sample collection

For cloning and gene expression of *MmAmy*, adult clams were obtained from the Danyan Nursery Field in Yinzhou District of Ningbo, China. Six tissues, including mantle, adductor muscle, visceral mass, foot, gill and siphon were dissected, immediately frozen in liquid nitrogen and then stored at -80 °C. Embryos/larvae of ten developmental stages (unfertilized mature eggs, fertilized eggs, 4-cell embryo, blastula, gastrulae, trochophore, D-shaped larva, umbo larva, eyespots larva, juvenile clams) were collected and preserved at -80 °C.

In order to detect possible difference of *MmAmy* activity and gene expression in four shell color strains, thin checkered (TC), white shell (WS), dark fringe (DF) and red shell (RS) (Fig. 1), the parents of four strains were collected from Ningbo Yongsheng Shellfish Hatchery, Ningbo, China. The breeding offspring was cultured in the same growing environmental conditions and the main growth traits (shell length, shell width, shell height and total weight) were measured every six month. Samples of visceral mass of four strains were collected from 18-month clams, for the analysis of *MmAmy* activity and gene expression.

Cloning the full-length cDNA of MmAmy

Total RNA was extracted from the visceral mass using Trizol reagent (Sangon, China). RNA integrity was examined by electrophoresis on a formaldehyde-denatured 1.2% agarose gel staining with ethidium bromide, and the quality and quantity were assessed by ultraviolet spectrophotometry. The first-strand cDNA was synthesized according to the manufacturer's instruction of SMART RACE reagent (Clontech, USA).

On the basis of 454 cDNA library of *M. meretrix* (GenBank accession no. SRA021052), EST sequences of *MmAmy* gene were retrieved. Primers for 5′-RACE (MmAmy-F1) and 3′-RACE (MmAmy-R1) were designed (Table 1). The amplification was performed as follows: 5 cycles of 94 °C for 30s, 72 °C for 3 min; 5 cycles of 94 °C for 30 s, 70 °C for 30 s and 72 °C for 3 min; 25 cycles of 94 °C 30s, 68 °C 30s and a final extension at 72 °C for 3 min. The PCR products were purified using Gel Extraction Kit (TIANGEN, China) and then cloned into the pMD18-T vector (TaKaRa). The vector was transformed into DH5α cells (TIANGEN, China) according to manufacturer's protocols, and the positive clones were sequenced. The full-length cDNA sequence was determined by piecing together the sequences of the 3′ and 5′ RACE products.

To confirm the accuracy of cloning and sequencing, the full-length cDNA was re-amplified with high fidelity polymerase (TaKaRa), using a pair of gene specific primers, MmAmy-F2 and MmAmy-R2

Table 1 Oligo nucleotide primers used in the experiments

Primer name	Sequence from 5′ to 3′	Application
MmAmy-F1	GGGTGGGGAACCAATAAAAATGACTGA	3′ RACE
MmAmy-R1	AGCATACTCGCTCGCAGGGGAAACC	5′ RACE
MmAmy-F2	TAAAGCAGAGTGCGAGAG	Verifying the sequence of cDNA
MmAmy-R2	TGACGGAAGTCCAGTATT	Verifying the sequence of cDNA
MmAmy-F3	CCAACCCACGGCAAGCAGACAA	Cloning of intron
MmAmy-R3	ATAACTTACAGGCTGGTATCTCTC	Cloning of intron
MmAmy-F4	TAACCAATCCAAACCGCCC	Cloning of intron
MmAmy-R4	TGATTTCCCCCAGTCCCAA	Cloning of intron
MmAmy-F5	CTGGGTCTTACTCTGGCATTG	Cloning of intron
MmAmy-R5	TCAGTCATTTTTATTGGTTCCC	Cloning of intron
MmAmy-F6	TAGACATTGGTGTTGCTGGGAT	Cloning of intron
MmAmy-R6	ATCACCTCCCCCACCATCTC	Cloning of intron
MmAmy-F7	AAAGAGGAGATGGTGGGGGAG	Cloning of intron
MmAmy-R7	CACAGTAATCGCCTGACGGAA	Cloning of intron
MmAmy-F8	CTTGTTGGTGATGACAGTTCCTTA	Cloning of intron
MmAmy-R8	TAACAGATCAGTACTTGTCTTGCGT	Cloning of intron
Real-A-F	GGAAGGACCACCACATAACG	qRT-PCR
Real-A-R	ACAGACCCAATCACCGCTAC	qRT-PCR
18S-F	CTTTCAAATGTCTGCCCTATCAACT	qRT-PCR
18S-R	TCCCGTATTGTTATTTTTCGTCACT	qRT-PCR

(Table 1), which were designed based on the above mentioned cDNA sequence. PCR products were cloned and sequenced following the procedures described above.

Cloning the Introns of MmAmy

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, three primer pairs (MmAmy-F3, MmAmy-R3; MmAmy-F4, MmAmy-R4; MmAmy-F5, MmAmy-R5, Table 1) were designed to detect the introns. PCR conditions were as follows: an initial denaturation (94 °C , 5 min), followed by 35 cycles of amplification (94 °C, 30s; 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 and phylogenetic analysis

The sequences were spliced using BLAST algorithm at 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 location of the signal peptide and the cleavage sites in the amino acid sequence were predicted using SignalP 4.0 server (http://www.cbs.dtu. dk/services/SignalP/). The multiple alignment of α-amylase proteins of *M. meretrix* and other species was performed using the ClustalW2 multiple alignment program (http://www.ebi.ac.uk/Tools/msa/clustalw2/). A phylogenetic tree was constructed using the neighbor-joining (NJ) method with MEGA 6.0.

Quantitative analysis of MmAmy

The expression profiles of *MmAmy* at different developmental stages (n>500, 3 sets of samples for each stage), in different adult tissues (4 sets of samples for each tissue) from four randomly sampled clams were analyzed using real-time quantitative reverse transcription PCR (qRT-PCR), with three technical repeats for each PCR reaction. Total RNA was extracted from the samples using the same method described above.

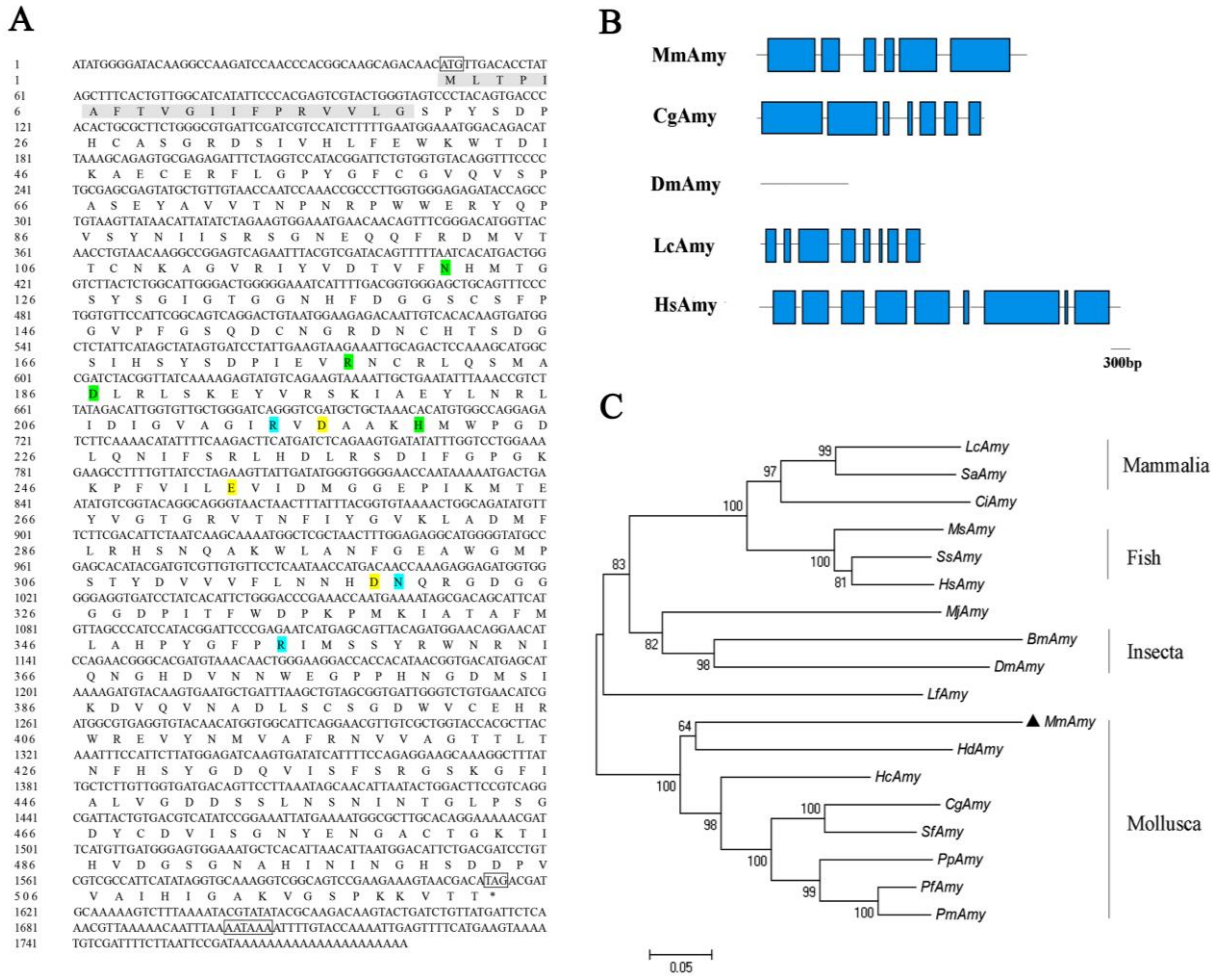

Fig. 2 Analysis of sequence and structure of *MmAmy* gene. (A) The full length cDNA and deduced amino acid sequence of *MmAmy*. The letters boxes are the start codon, the stop codon and the polyadenylation signal sequence, the * represents the end of the protein translation, shaded gray part is the signal peptide, green letters are calcium binding residues, cyan letters are chloride binding sites, yellow letters are active site residues. (B) Structure of *MmAmy* and selected *Amy* genes from model species. Blue boxes are introns and lines are exons. The number of introns in the *Amy*s is significantly different in different species, no introns in *DmAmy* and 9 introns in *HsAmy*. (C) Neighbor-joining phylogenetic tree of Amy between *M. meretrix* and other species using MEGA6.0 software. The abbreviation of Amys and the GenBank accession numbers used to construct phylogenetic tree are given in Table 2.

The primers Real-A-F and Real-A-R (Table 1) located in the exon 4 and exon 5, respectively, were used to amplify a 104bp fragment of *MmAmy* from cDNA template. Primers 18S-F and 18S-R (Table 1) were used to amplify 186 bp products of 18SrRNA, as the internal control for qRT-PCR. PCR amplification was performed in a 20 μl reaction volume containing 10 μl of iTaq Universal SYBR Green Supermix (Bio-Rad), 7.2 μl deionized water, 0.8 μl of the first strand cDNA and 1 μl of forward and reverse primers. Amplification was performed using the following thermal cycling conditions: incubation at 94 °C for 20s, 40 cycles of 94 °C for 3 s, 60 °C for 15s and 72 °C for 10s. All qRT-PCR was performed in three biological replicates with one replicate for each clam, and negative controls were set with each primer pair.

To compare the *MmAmy* expression levels in the viscera of clams with the different shell colors, six individuals of each shell color strains were selected. Total RNA was extracted from the viscera of 24 clams and qRT-PCR was performed as described above.

Assay for α-amylase activities

To assay the activities of α-amylase, viscera of four shell color strains (6 sets of samples for each shell color strain) were homogenized 10min in ice bath. The homogenate was centrifuged at 11000 r/min for 10 min at 4 °C. The supernatant of enzyme crude extracts was preserved at 0~4 °C and analyzed within 24h. α-amylase activities were measured using the colorimetric iodine starch and total protein contents were measured

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

Species	Abbreviation type	GenBank no.	Size
Meretrix meretrix	*MmAmy*	KP250879	522
Crassostrea gigas	*CgAmy*	CAA69658.1	520
Pinctada fucata	*PfAmy*	AGN55419.1	522
Hyriopsis cumingii	*HcAmy*	AGW45296.1	523
Pinctada maxima	*PmAmy*	AEI58897.1	518
Pteria penguin	*PpAmy*	AEI58894.1	523
Haliotis discus hannai	*HdAmy*	BAM74656.1	511
Saccostrea forskali	*SfAmy*	AHA11414.1	520
Colobus angolensis	*CaAmy*	ABW02886.1	511
Ctenopharyngodon idella	*CiAmy*	ACX35465.1	512
Marsupenaeus japonicus	*MjAmy*	AHN91844.1	512
Sus scrofa	*SsAmy*	AAF02828.1	511
Bombyx mori	*BmAmy*	ACT64133.1	500
Salmo salar	*SaAmy*	ABD13895.1	505
Drosophila melanogaster	*DmAmy*	CAA28238.1	494
Lithobius forficatus	*LfAmy*	ACA34374.1	539
Homo sapiens	*HsAmy*	AAA52280.1	511
Mus musculus	*MsAmy*	CAA24099.1	511
Lates calcarifer	*LcAmy*	AAL84163.1	509

using coomassie blue staining according to the manufacturer's instruction of kits (Nanjing Jiancheng, China). One unit of α-amylase activity is defined as: per milligram of protein in tissues at 37 °C and substrate for 30 min, the amount necessary to hydrolyze 10 mg starch.

Statistical analysis

The results of qRT-PCR analyses were based on the CT values of the PCR products and the comparative CT method was used to analyze the expression level of *MmAmy*. Statistical analysis was performed using software SPSS 19.0. One way analysis of variance (ANOVA) was used to compare the differences of relative *MmAmy* mRNA expression in different developmental stages, in various adult tissues and in different shell color strains. Multiple comparisons were conducted using the Student Newman Keuls test. The comparisons of growth traits and α-amylase activities of four shell color strains were performed as above. The significance level of difference is $P < 0.05$.

Results

cDNA and intron sequence analysis of MmAmy

The full-length *MmAmy* cDNA was comprised of 1783 bp (GenBank accession no. KP250879) and contained a 1569 bp open reading frame (ORF) that encoded 522 amino acids. The cDNA also contained a 5' untranslated region (UTR) of 49 nucleotides, a 3'UTR of 117 nucleotides that included a stop codon (TAG), a putative polyadenylation consensus signal (AATAAA) and a polyA tail (Fig. 2A). The calculated molecular mass of the deduced mature MmAmy was 57.58 kDa, and the theoretical isoelectric point was 6.72.

Sequences analysis indicated that MmAmy has a signal peptide of 19 amino acids (MLTPIAFTVGIIFPRVVLG), 11 cysteine residues, 3 active site residues (Asp216, Glu252, Asp318), 4 calcium binding residues (Asn121, Arg177, Asp186, His220) and 3 chloride binding sites (Arg214, Asn319, Arg354) (Fig. 2A). Analysis with SMART revealed that the deduced amino acid sequence contained 8 stranded alpha/beta barrel that contains the active site, also known as domain A (Asp32-Arg416). The domain A was interrupted by a 70 amino acid calcium-binding domain B protruding between beta strand 3 and alpha helix 3, and a carboxyl-terminal Greek key beta-barrel domain C (Asn425-Lys512).

Three pairs of primers PCR amplification produced fragments of 1555 bp, 682 bp, 424 bp, 367 bp, 2041 bp and 1850 bp, respectively. After assembly, seven exons and six introns were revealed. All six introns were located within the ORF (Fig. 2B). The maximum length of MmAmy intron is 1563 bp, while the minimum intron length is 272 bp. The other introns ranged from 311 bp to 1268 bp. All exon–intron junctions follow the consensus rule of the splice acceptor –AG/GT– splice donor for splicing.

A phylogenetic tree was constructed with the deduced amino acid sequences of all mollusk Amys

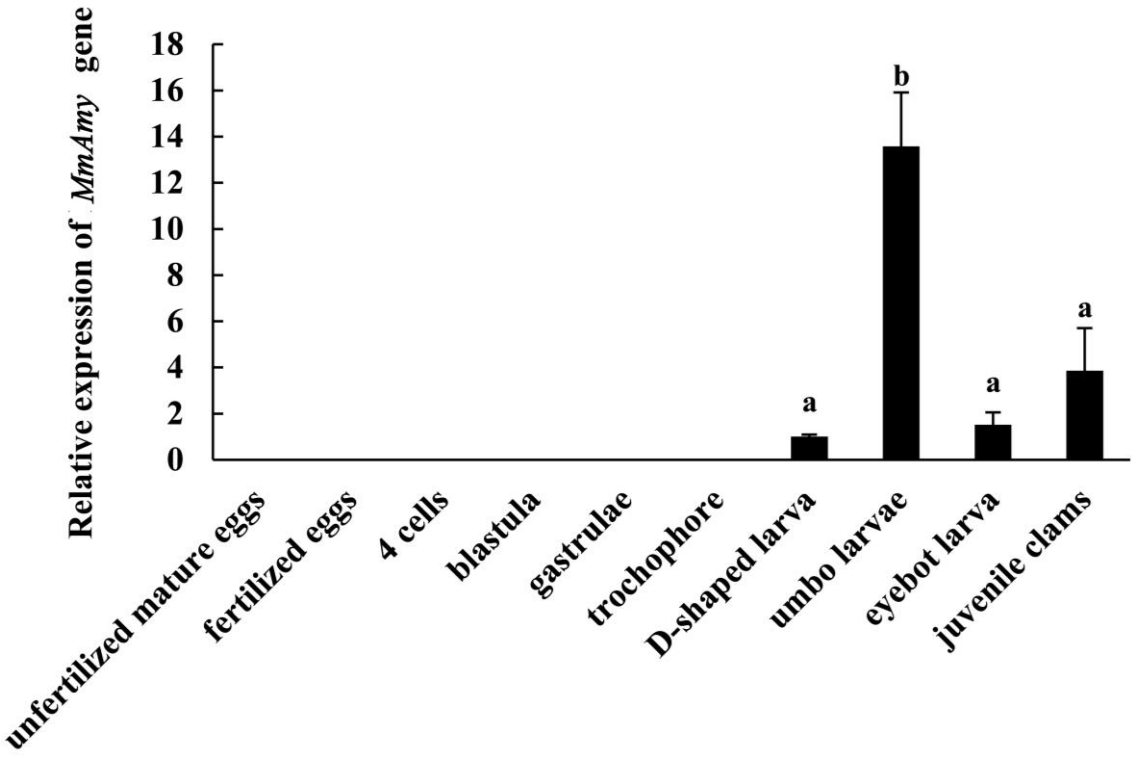

Fig. 3 Analysis of expression difference of *MmAmy* gene in different developmental stages of *M. meretrix*. The same letters indicate no difference in the level of expression, whereas different letters indicate significant differences in expression levels among various developmental stages ($p < 0.05$, based on ANOVA, $n>500$).

and some model animals' Amys available from the NCBI database using the Neighbor-joining method (Fig. 2C). The abbreviation of Amys and the GenBank accession numbers used to construct phylogenetic tree are shown in Table 2. The obtained NJ tree showed that Amys were divided into two major groups. One group is comprised of all mollusk Amys, while the other group contains mammals, fish and insects. In the mollusk group, MmAmy first clusters with Hd-Amy, then with the others mollusk. Multiple alignment indicated that MmAmy shared the highest identity (73.6%) with *C. gigas* and 57%~72.3% with other species.

Quantitative expression analysis of MmAmy

For the different tissues of adults, *MmAmy* was found to express at moderated level in visceral mass, but no expression was detected in other five tissues.

For the developmental stages, *MmAmy* was not expressed in the early stages, such as unfertilized mature eggs, fertilized eggs, 4 cells, blastula, gastrulae, and trochophore. In contrast, it was expressed after the stage of D-shaped larva, showing the highest expression in the umbo larvae. No significant difference was found among D-shaped larva, eyespots larva and juvenile clams (Fig. 3).

mRNA and enzyme activity of MmAmy *associated with growth traits of four shell color strains*

The results of growth traits, such as shell length, shell width and shell height, showed that strain DF had a significantly higher growth rates than strain RS. Consistently, the comparison in total weight between the four lines showed the same result as size measurements, where the highest mean value was observed in strain DF, and the lowest mean is in strain RS (Fig. 4).

The expression of *MmAmy* in the visceral mass of four shell color strains indicated that the highest expression was observed in stain DF and the lowest expression was found in RS. However, no significant difference was detected between strain DF and WS (Fig. 5A).

The α-amylase activity of four shell color strains indicated that the highest activity was observed in strain DF and lowest in strain RS (Fig. 5B). No significant difference among other color strains. This result is similar to that of growth traits and the expression in four shell color strains of *M. meretrix*, which suggest the potential connection among α-amylase activity and growth traits.

Discussion

Analysis of genomic structure

In this study, the full-length *MmAmy* cDNA sequence is 1,783 bp, with an ORF of 1,569 bp encoding 522 amino acid, which was within the range of Amys length in most animals. The calculated molecular mass of the mature *MmAmy* protein was 55.948 kDa, and the range of animal Amys was 45–67 kDa (Zółtowska, 2001; Sellos *et al.*, 2003).

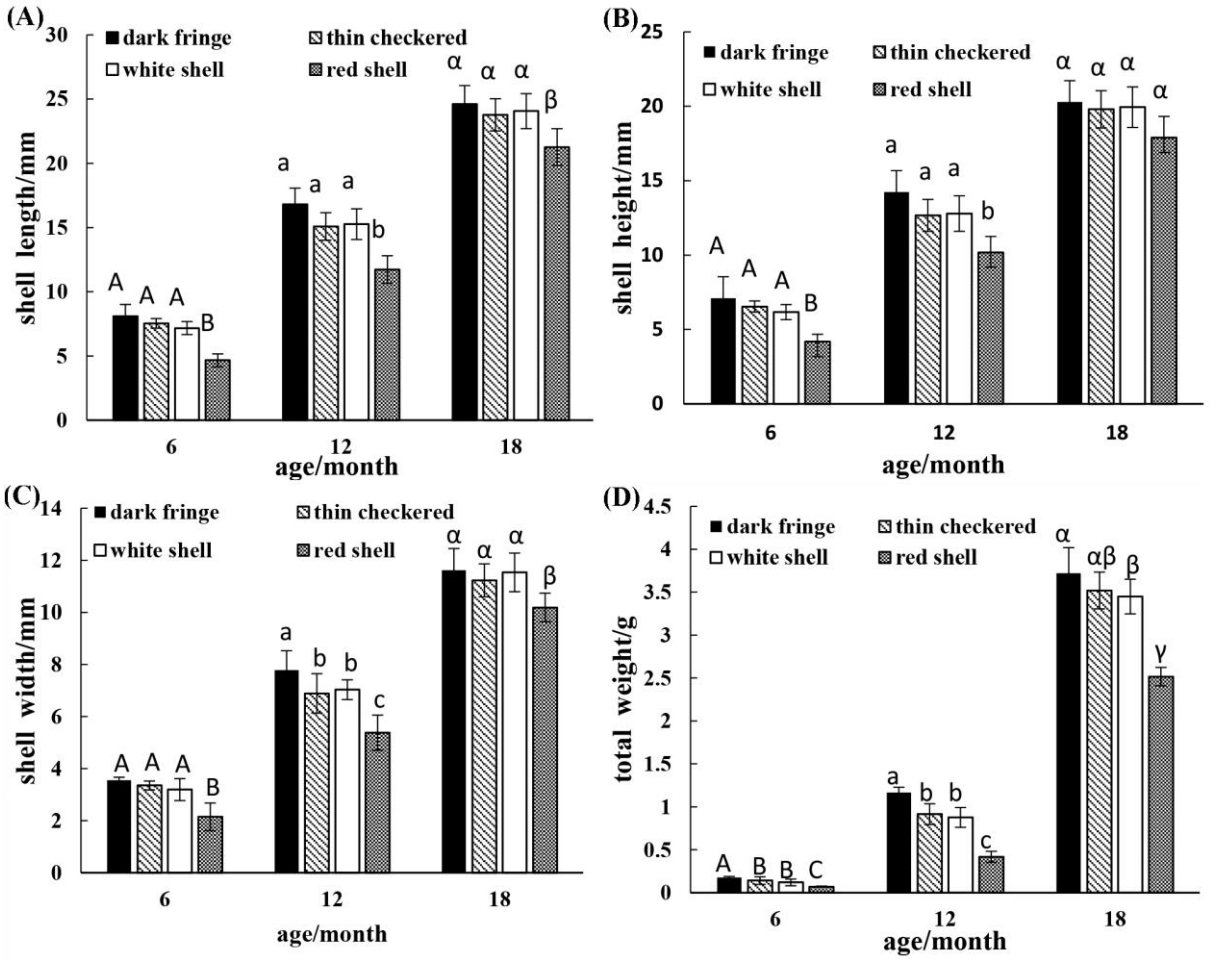

Fig. 4 Comparison of growth parameters (A. shell length; B. shell height; C. shell width; D. total weight) in four shell color strains. The same letters indicate no difference in the growth trait, whereas different letters indicate significant differences in the growth trait among four shell color strains ($p < 0.05$, based on ANOVA, n>500).

Like other animals' Amys, *MmAmy* had all the conserved motifs, such as the active sites, calcium biding sites and chloride binding sites (Janecek, 1997). Amys of all animals are chloride-dependent enzymes that require chloride for full activity (Strobl *et al*., 1997; Qian *et al*., 2005). Like most animals, the three amino acid residues in the hard clam were involved in the chloride binding of AMYs, including two arginine (R) residues and an asparagine (N) residue. All animal Amys contain eight conserved cysteine residues that form four disulfide bridges (Janecek, 1993). However, the number of cysteine residues is not usually the same among different species. For example, 12 residues of Amys were found in silkworm *Bombyx mori* (Ngernyuang *et al*., 2011), whereas 10 residues were observed in the shrimp *Penaeus vannamei* (Wormhoudt and Sellos, 1996). In mollusk, 10 and 11 residues of Amy were detected in Indian rock oyster *Saccostrea forskali* (Thongsaiklaing, 2014) and pearl oyster *P. maxima* (Pan, 2011), respectively, which are comparable to 11 cysteine residues of *MmAmy* in this study.

The genomic sequence of *MmAmy* contains 7 exons and 6 introns, and their junctions obey the rule of "GT ... AG". It is well known that the number of intron-extron in the Amys is highly variable between taxa (Da Lage *et al*., 2002). For example, no intron was found in *D. melanogaster*, while 14 introns were observed in *D. pulex.* By comparing *MmAmy* with other 6 mollusks (*Patella vulgate, P. maximus, C. gigas*, *S. constricta*, *P. fucata* and *Lottia gigantea*), the maximum number of 8 introns occurred in *P. fucata* (Huang, *et al*., 2016), 7 introns in *C. gigas* (Sellos and Van Wormhoudt, 2002) and the minimum of 5 introns in *L. gigantean* and *S. constricta* (Liu *et al*., 2017). This may be more likely related to factors linked to splicing mechanisms and requirements, and to recognition of introns and exons, or to more extrinsic factors (Da Lage *et al*., 2011).

Quantitative expression analysis of MmAmy

No expression of *MmAmy* was detected at embryonic stages probably because they have yolk as nutrients and don't live on food from surroundings. Though trochophore started into the larval stage they

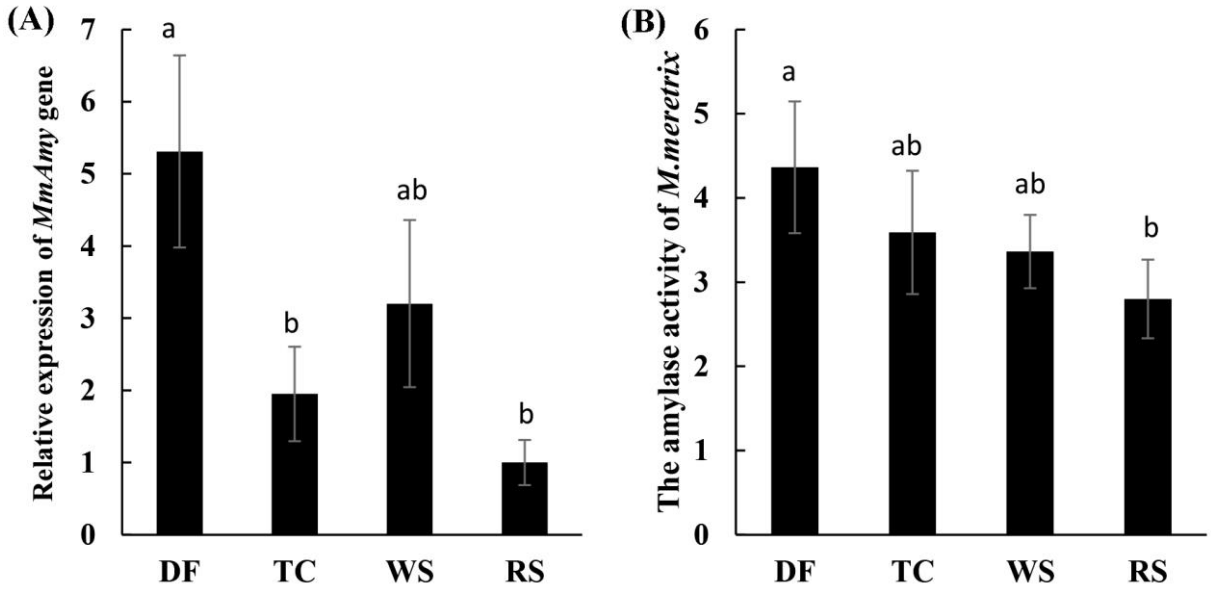

Fig. 5 Analysis of expression difference of *MmAmy* gene (A) and α-amylase activity difference (B) in different shell color strains of *M. meretrix.* The same letters indicate no difference in the level of gene expression or enzyme activity, whereas different letters indicate significant differences in the level of gene expression level or enzyme activity among four shell color strains ($p < 0.05$, based on ANOVA, n=6). DF: dark fringe, TC: thin checkered, WS: white shell, RS: red shell.

still retained the yolk for nutrients. D-shaped larva began to filter and digest food resulted in the increased expression levels of *MmAmy* after this stage, which is similar to the situation in *S. constricta* (Liu *et al*., 2017). The highest expression level occurred in umbo larvae was possibly connected with the increased food intake and fast growth. In contrast, eyespot larva, an important stage of metamorphosis and attachment for bivalve molluscs, showed the decreased expression, probably due to the declined amount of food intake (Wang *et al*., 2008). A similar profile of *Amy* mRNA expression was observed in *H. discus hannai*, with the lowest or even no expression in early embryos and higher expression during the larval stages (He *et al*., 2011). These similar results suggest that the nature of α-amylase may be served as the main digestive enzyme in mollusks. In addition, amylase expression also had a similar trend of profiles that transcripts were first detected at 5 dph, peaked at 20 dph and then decreased during metamorphosis in the winter flounder, *Pleuronectes americanus* (Susan *et al*., 2000).

There were mainly salivary amylase and pancreatic amylase in mammals, and the two types of enzymes were expressed in tissue specific pattern (Nishide *et al*., 1986). Since there was no salivary gland in fish, the amylase were main expressed in hepatopancreas, and also intestine, even with high activity (Tomita, 1987). For mollusks, in *C. gigas*, the main expression of the two amylase gene was in digestive gland leading to high amylase activities, in accordance with the digestive function, however, trace mRNA was observed in all five tissues (Huvet *et al*., 2003). In this study, the specific expression of *MmAmy* in visceral mass showed a similar profile with that in *H. discus hannai*, in which amylase was only expressed in digestive gland (Kumagai *et al*., 2013).

Correlation analysis of the activity of amylase and growth traits

The shell color of marine bivalves may have been previously dismissed as mere excretory products (Cain *et al*., 1988). As evidenced, shell color is not only related to ecology and habits (Beukema and Meehan., 1985), but also associated with phenotypic traits such as growth and survival in some species (Alfonsi and Perez, 1998; Zhang *et al*., 2009). Digestive enzyme activity was an important index to reflect the physiological function of the digestive system, which determined the ability of the nutrient digestion and absorption, and the speed of the growth and development (Pan *et al*., 2006).To accommodate their fast growth, larger clams need more nutrients to maintain their metabolic activity, which required higher activity of digestive enzyme. In this study, the positive correlation among growth traits, shell color, α-amylase activity in different strains were also observed in some other shellfish species such as *P. fucata* (Zou *et al*., 2014) and *C. nobilis* (Deng *et al*., 2008). This might be explained by pleiotropic effects, which inferred that the expression of shell color genes may closely link with genes determining growth traits (Sokolova and Berger, 2000). However, further study is necessary to shed light on their molecular mechanisms.

Acknowledgments

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References

Alfonsi C, Perez J E. Growth and survival in the scallop *Nodipecten nodosus* as related to self-fertilization and shell colour. Biol. Inst. Oceanology Venez. 37: 69-73, 1998.

Beukema JJ, Meehan BW. Latitudinal variation in linear growth and other shell characteristics of *Macoma balthica*. Mar. Biol. 90: 27-33, 1985.

Boer P, Hickey D. The alpha-amylase gene in *Drosophila mdanogaster*: nucleotide sequence, gene structure and expression motifs. Nucleic Acids Res. 14: 8399-8411, 1986.

Cain AJ. The colours of marine bivalve shells with special reference to *Macoma balthica*. Malacologia 28: 289-318, 1988.

Da Lage LJ, Van WA, Cariou ML. Diversity and evolution of the α-amylase genes in animals. Biologia 57: 181–189, 2002.

Da Lage LJ, Maczkowiak F, Cariou ML. Phylogenetic distribution of intron positions in alpha-amylase genes of bilateria suggests numerous gains and losses. Plos One. 6: e19673, 2011.

Dai P, Huan P, Wang H, Lu X, Liu B. Characterization of a long-chain fatty acid-CoA ligase 1 gene and association between its SNPs and growth traits in the clam *Meretrix meretrix*. Gene 566: 194-200, 2015.

Deng YW, Zhang LF, Du XD. A comparison of growth and gigestive enzymes between two shell color stocks of Huagui scallop *Chlamys nobili*. J. Guangdong Ocean Univ. 28: 20-23, 2008.

Gao XY, Dong YH, Yao HH, Lin ZH. Cloning, spatiotemporal expression and SNPs identification of HDAC1 gene in hard clam *Meretrix meretrix*. Acta hydrobiologica sinica 40: 601-608, 2016.

Gao XY, Dong YH, Shi SJ, Yao HH, Ruan WB, Zhao JX, *et al*. Cloning, spatiotemporal expression and SNPs identification of GRB2 gene in hard clam *Meretrix meretrix*. J. Fish. China 39: 1324-1332, 2015.

He QG. Researches on mRNA expression of four polysaccharidelyase genes in Paeific abalone, *Haliotis discus hannai* Ino. Chinese academy of science, 2011.

Huang G, Guo Y, Li L, Fan S, Yu Z., Yu D. 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.

Huvet A, Daniel JY, Quéré C, Dubois S, Prudence M, Van Wormhoudt A, *et al*. Tissue expression of two α-amylase genes in the Pacific oyster *Crassostrea gigas*. Effect of two different food rations. Aquaculture 228: 321-333, 2003.

Huvet A, Jeffroy F, Fabioux C, Daniel JY, Quillien V, Van Wormhoudt A, *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.

Huvet A, Jeffroy F, Daniel JY, Quéré C, Le Souchu P, Van Wormhoudt A, *et al*. Starch supplementation modulates amylase enzymatic properties and amylase B mRNA level in the digestive gland of the Pacific oyster *Crassostrea gigas*. Comp. Bioch. Physiol. B 163: 96-100, 2012.

Janecek S. Sequence similarities in (alpha/beta) 8-barrel enzymes revealed by conserved regions of alpha-amylase. Febs. Lett. 316: 23-26, 1993.

Janecek S. Alpha-amylase family: molecular biology and evolution. Prog. Biophys. Mol. Biol. 67: 67-97, 1997.

Jessica L, Melinda R, Ana M, May B. A first-generation linkage map of the Pacific lion-paw scallop (*Nodipecten subnodosus*): Initial evidence of QTL for size traits and markers linked to orange shell color. Aquaculture 350-353: 200-209, 2012.

Kumagai Y, Satoh T, Inoue A, Ojima T. Enzymatic properties and primary structures of two α-amylase isozymes from the Pacific abalone *Haliotis discus hannai*. Comp Biochem Physiol B Biochem Mol. Biol. 164: 80-88, 2013.

Le Moine S, Sellos D, Moal J, Daniel JY, San JSF, 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.

Liu CS, Lin DH, Dong YH, Xue QG, Yao HH, Lin ZH. Association of α-amylase gene with growth traits in the razor clam *Sinonovacula constricta*. Invert. Surviv. J., 14: 494-504, 2017.

Ma P, Liu Y, Reddy KP, Chan WK, Lam TJ. Characterization of the seabass pancreatic alpha-amylase gene and promoter. Gen. Comp. Endocr. 137: 78-88, 2004.

Maurus R, Begum A, Kuo HH, Racaza A, Numao S, Andersen C, *et al*. Structural and mechanistic studies of chloride induced activation of human pancreatic alpha-amylase. Protein Sci. 14: 743-755, 2005.

Ngernyuang N, Kobayashi I, Promboon A, Ratanapo S, Tamura T, Ngernsiri L. Cloning and expression analysis of the Bombyx mori α-amylase gene (Amy) from the indigenous Thai silkworm strain, Nanglai. J. Insect. Sci. 11: 38, 2011.

Nishide T, Nakamura Y, Emi M, Yamamoto T, Ogawa M, Mori T, *et al*. Primary structure of human salivary α-amylas gene. Gene 41: 299-304, 1986.

Pan LL. Cloning and expression analysis of cathepsin D and alpha amylase gene in pearl oyster *Pinctada maxima* and *Peteria penguin*. Shanghai: Shanghai Ocean University, 2011.

Pan LQ, Liu H, Xiao GQ. A review on digestive enzyme of crustacean larvae. J. Fish. China 13: 492-501, 2006.

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.

Qian M, Ajandouz el H, Payan F, Nahoum V. Molecular basis of the effects of chloride ion on the acid-base catalyst in the mechanism of pancreatic alpha-amylase. Biochemistry 44: 3194-3201, 2005.

Sambrook J. Molecular cloning: a laboratory manual, third edition. New York: cold spring harbor laboratory press, 2001.

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.

Sellos D and Van Wormhoudt A. Structure of the ofα-amylase genes in crustaceans and molluscs: evolution of the exon/intron organization. Biologia Bratislava 57/Suppl. 11: 191-196, 2002.

Sokolova IM, Berger VJ. Physiological variation related to shell color polymorphism in white sea *Littorina saxatilis*. J. Exp. Mar. Biol. Ecol. 245: 1-23, 2000.

Strobl S, Gomis-Rüth FX, Maskos K, Frank G, Huber R, Glockshuber R. The alpha-amylase from the yellow mealworm: complete primary structure, crystallization and preliminary X-ray analysis. Febs. Lett. 409: 109-114, 1997.

Susan ED, Suzan M, Jeffrey WG. Molecular analysis of the amylase gene and its expression during development in the winter flounder, *Pleuronectes americanus*. Aquaculture 190: 247-260, 2000.

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*. Fisheries. Sci. 80: 589-601, 2014.

Tomita N. Primary structure of human Panceatic α-amylase gene: its comparison with human salivary alpha- amylase gene. Gene 60: 57-64, 1987.

Wormhoudt AV, Sellos D. Cloning and sequencing analysis of three amylase cDNAs in the shrimp *Penaeus vannamei* (Crustacea: Decapoda): evolutionary aspects. J. Mol. Evol. 42: 543-551, 1996.

Wang C, You Y, Wang H, Liu B. Genetic diversity of the sulfotransferase-like gene and one nonsynonymous SNP associated with growth traits of clam, *Meretrix meretrix*. Mol. Biol. Rep. 39: 1323-1331, 2012a.

Wang C, Wang H, Li Y, Liu B. Identification of a fructose-1,6-bisphosphate aldolase gene and association of the single nucleotide polymorphisms with growth traits in the clam *Meretrix meretrix*. Mol. Biol. Rep. 39: 5017-5024, 2012b.

Wang RC, Wang ZP. Science of marine shellfish culture. Qingdao: China Ocean University Press, 2008.

Wolff M, Garrido J. Comparative study of growth and survival of two color morp Hs of the chilean scallop *Argopecten purpuratus* (Lamarck) in suspended culture. J. Shellfish Res. 10: 47-53, 1991.

Zhang GF, Zheng HP. Breeding genetics of bay scallop. Beijing: Science and Technology Press, 2009.

Zółtowska K. Purification and characterization of alpha-amylases from the intestine and muscle of *Ascaris suum* (Nematoda). Acta. Biochim. Pol. 48: 763-774, 2001.

Zou K, Zhang D, Guo H, Zhu C, Li M, Jiang S. A preliminary study for identification of candidate AFLP markers under artificial selection for shell color in pearl oyster *Pinctada fucata*. Gene 542: 8, 2014.

Zou L, Liu BZ. Identification of a serum amyloid A gene and the association of SNPs with Vibrio-resistance and growth traits in the clam *Meretrix meretrix*. Fish Shellfish Immun. 42: 301-309, 2015.