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RESEARCH REPORT

Identification of *BMP2* and *BMP7* genes and association of their SNPs with growth traits in the hard clam (*Meretrix meretrix*)

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Abstract

The hard clam (*Meretrix meretrix*) is a commercially and ecologically important benthic mollusk in South and Southeast Asia. Growth is one of the most important and complex traits affecting clam breeding. Therefore, identification of key growth factor genes and their association with growth traits are the primary prerequisites for in-depth study of the mechanisms of growth regulation and molecular marker-assisted selection of the hard clam. In the present study, we generated the full-length cDNA sequences of the growth-related genes *BMP2* and *BMP7* in *M. meretrix* (*MmBMP2* and *MmBMP7*) and then investigated their gene expression patterns and associations with growth traits. The full-length cDNA of *MmBMP2* was 2641 base pairs (bp), with a 1395 bp open reading frame (ORF) encoding 464 amino acids. The full-length *MmBMP7* cDNA was 3161 bp, with a 1257 bp ORF encoding 418 amino acids. In adult clams, expression of *MmBMP2* and *MmBMP7* were significantly higher in the mantle than in the other five tested tissues ($p < 0.05$). Among the seven developmental stages of *M. meretrix*, expression of *MmBMP2* was the highest in juveniles ($p < 0.05$), and expression of *MmBMP7* was very low in 4-cell embryo and eyespot larva stages ($p < 0.05$). Two hundred and sixty-three individuals from two populations were screened for *MmBMP2* and *MmBMP7* polymorphisms, and single nucleotide polymorphisms (SNPs) associated with growth traits in *MmBMP2* was found in both populations. Subsequent analysis revealed that two SNPs in *MmBMP2* were shared between the populations. One was A726G, for which individuals with genotypes AG had significantly higher growth traits than those with genotype AA ($p < 0.05$). The other was A732T, for which individuals with genotype AT had significantly higher growth traits than those with genotype AA ($p < 0.05$). These results suggest that *BMP2* markers may have potential value in molecular selective breeding to improve clam growth.

Key Words: *Meretrix meretrix*; *BMP2*; *BMP7*; SNP; growth traits

Introduction

The transforming growth factor-beta (TGF-β) supergene family plays various roles in the regulation of cell growth, cell specialization, and morphogenesis (Massague, 1998; Derynck and Akhurst, 2007). More than 40 members of TGF-β have been identified in mammals (Li *et al.*, 2011), including bone morphogenetic proteins (BMPs), Smads, and activin. BMPs are multi-functional growth factors, and their major function is to induce the formation of both bone and cartilage in vertebrates (i.e. the process of biomineralization) (Chen *et al.*, 2004; Xiao *et al.*, 2007). Thus, most bone-related molecular studies focus on *BMP2*, *BMP4*, and *BMP7* (Salazar *et al.*, 2016). BMPs also play a role in cell growth and differentiation, embryogenesis, hematopoiesis, and neurogenesis (Chen *et al.*, 2004; Bragdon *et al.*, 2011). BMPs have been found in invertebrates, including echinoderms (Hwang *et al.*, 1994; Shih *et al.*, 2002), arthropods (Wharton *et al.*, 1991; Fritsch *et al.*, 2010), mollusks (Nederbragt *et al.*, 2002; Iijima *et al.*, 2008; Miyashita *et al.*, 2008), platyhelminths (Freitas *et al.*, 2009), cnidarians (Reinhardt *et al.*, 2004; Zoccola *et al.*, 2009), and poriferans (Müller *et al.*, 2011).

Among the members of the BMPs family, *BMP2* is the factor that can induce bone formation alone, inhibit myogenic differentiation, and promote skeletal cell differentiation (Musgrave *et al.*, 2001). During

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Table 1 Primers used for *MmBMP2* and *MmBMP7* genes in the experiments

Primer name	Sequence from 5'to 3'	Application
MmBMP2-F1	ATGGCGTGTCCTCTCAGTTTAC	3' RACE cloning
MmBMP2-R1	ATCACTCCCCAGCCTGTCCTTCAATG	5' RACE cloning
MmBMP2-F2	GTGGGCAGGATTTCACTGTAGG	Verifying the sequence of cDNA
MmBMP2-R2	GTAGCATTGAAGGACAGGCTGGGGAGT	Verifying the sequence of cDNA
MmBMP2-F3	CTAACAGCAGCGACAGTGTGT	SNP
MmBMP2-R3	CTGGACTTCATCTTCGTGGAA	SNP
MmBMP2-F4	CCAGTCTACCTACAGAACAGCC	SNP
MmBMP2-R4	TCCGTTTAGTCCGTGATAAGC	SNP
MmBMP2-F5	TATCACGGACTAAACGGAGCA	SNP
MmBMP2-R5	CGTAAACTACCTTGACTGGAAGCA	SNP
Real-2-F	TCAGTCGGGTGTATTTTAGCAGA	Real time PCR
Real-2-R	CATTTTTTCCAGCAGGTCAAGTG	Real time PCR
MmBMP7-F1	GTCAGGTGAGCGATGTAGGGTCGTTTC	3' RACE cloning
MmBMP7-R1	CAAGTGTCTGCTGGTTCCCGATTCC	5' RACE cloning
MmBMP7-F2	AAGTTGTGATAAAACTGCGCG	Verifying the sequence of cDNA
MmBMP7-R2	AACTATATATAGCTTGCATC	Verifying the sequence of cDNA
MmBMP7-F3	TTTAGGCAATTTCCCGAGAC	SNP
MmBMP7-R3	CCTTGTCGTGCCTAAGATGC	SNP
MmBMP7-F4	TTCCGCATCTTAGGCACGAC	SNP
MmBMP7-R4	GGGTAACCTTCAGGGGCGAT	SNP
MmBMP7-F5	ATTATCGCCCCTGAAGGTTAC	SNP
MmBMP7-R5	AAACGACCCTACATCGCTCAC	SNP
Real-7-F	GTTTTCGCCTGTTGTGGTG	Real time PCR
Real-7-R	GAGTGTGCGGTGTTCGTGTA	Real time PCR
18S-F	CTTTCAAATGTCTGCCCTATCAACT	Real time PCR
18S-R	TCCCGTATTGTTATTTTTCGTCACT	Real time PCR

embryonic development, *BMP2* not only promotes embryonic muscle growth by inducing the expression of myogenic genes, but also inhibits muscle growth by inducing apoptosis (Amthor *et al.*, 1998). *BMP7* is a key factor in bone homeostasis and spinal development (Cook and Rueger, 1996). Previous studies indicated that *BMP7* is abundantly expressed in articular cartilage and synovial membrane obtained from human patients undergoing autologous chondrocyte implantation (Schmal *et al.*, 2012). Furthermore, researchers discovered that *BMP7* in mouse was expressed in a wide variety of tissues during development (Lyons *et al.*, 1995) and was strongly expressed in the developing myocardium of embryos (Dudley and Robertson, 2010). *BMP7*-deficient mice were found to exhibit defects in eye development, renal development, and skeletal patterning. The deletion of *BMP7* gene results in lens development defects, affects the development of ribs and skulls, and causes polydactyly of hind limbs. (Dudley *et al.*, 1995; Luo *et al.*, 1995).

Because of their important roles in growth and development, *BMP2* and *BMP7* have become widely studied genes in mollusks. Several researchers reported that BMPs and their homologs are involved in the regulation of morphogenesis in the shells of mollusks (Herpin *et al.*, 2010; Lelong *et al.*, 2010). Furthermore, *dpp-BMP2/4* is expressed around the shell gland of the embryo in gastropods such as *Lymnaea stagnalis* and *Patella vulgata*, which suggests that it may be involved in determination of the boundary of the embryonic shell region and in shell formation (Nederbragt *et al.*, 2002). The high-level expression of *Pinctada fucata BMP2* in the inner layer of mantle tissue suggests that it plays an important role in the formation of nacre (Miyashita *et al.*, 2008). In *Pinctada martensii*, the microstructure observed using a scanning electron microscope indicated a disordered growth status in the nacre and obvious holes in the prismatic layer in the dsRNA-*Pm-BMP7*-injected group, which suggests that *BMP7* plays a crucial role in the nacre and prismatic layer formation process of the shell (Yan *et al.*, 2014).

The hard clam (*Meretrix meretrix*) is a commercially important bivalve species that is distributed in the intertidal zone of South and

Southeast Asia (Liu *et al.*, 2006; Tang *et al.*, 2006). With the success of artificial breeding, clam culture has become an economically important aquaculture industry in China. Although a selective breeding program for genetic improvement of growth traits of *M. meretrix* has begun (Wang *et al.*, 2011), association analysis between phenotype and genotype has still been relatively rare to date. However, molecular marker-assisted selection of growth traits would probably further increase selection efficiency and lead to rapid progress in improving the process of clam breeding (Lande and Thompson, 1990; Hospital *et al.*, 1997; Moreau *et al.*, 1998; Xie and Xu, 2010). Therefore, identification of growth-related markers for further development of phenotype-selective breeding is very necessary. Studies of growth-related functional genes of *M. meretrix*, including growth factor receptor-bound protein 2 (Gao *et al.*, 2015), cathepsin B (Yao *et al.*, 2011), sulfotransferase (Wang *et al.*, 2012), and fructose-1, 6-diphosphate aldolase 9 (Wang *et al.*, 2012), have been conducted. However, the *M. meretrix BMP2* and *BMP7* genes (*MmBMP2* and *MmBMP7*) have not been studied to date.

In the current study, we investigated *MmBMP2* and *MmBMP7* by cloning their full-length cDNAs and examining their expression levels during embryonic development and in adult tissues. We also screened and analyzed associations between single nucleotide polymorphisms (SNPs) and growth traits. The results of this study will improve our understanding of the functions of *MmBMP2* and *MmBMP7* and provide a basis for molecular breeding programs for *M. meretrix*.

Materials and methods

Experimental materials and sample collection

For cloning and gene expression analysis of *MmBMP2* and *MmBMP7*, adult clams were obtained from the Ningbo Danyan Aquaculture Company, Zhejiang, China. Six tissues (digestive gland, mantle, adductor muscle, foot, siphon, and gills) were dissected, immediately frozen in liquid nitrogen, and stored at -80 °C. Samples from seven developmental stages (unfertilized mature eggs, 4-cell embryos, blastulae, gastrulae, trochophores, eyespot larvae, and juvenile clams) were collected and preserved at -80 °C.

A total of 263 individuals were randomly sampled at the same time from two populations: 136 individuals from the Jiangsu population (JS) and 127 individuals from Wanli No. 2 strain (WL2). JS was collected from the wild population located in Jiangsu province, China. WL2, a new fast-growing variety with a dark gray shell color and zigzag patterns, had been selected for five generations by our research team. The following six growth index parameters, total weight, soft weight, shell weight, shell width, shell length, and shell height, were measured for each clam. The mantles of each clam were collected and stored at -80 °C.

Cloning the full-length cDNA of MmBMP2 *and* MmBMP7

Total RNA was extracted from the mantle using TRIzol reagent (Sangon, Shanghai, China). RNA integrity was examined by electrophoresis on a formaldehyde-denatured 1.2% agarose gel and staining with ethidium bromide, while the quality and quantity were assessed by ultraviolet spectrophotometry. First-strand cDNA was synthesized using the simple modular architecture research tool (SMART) rapid amplification of cDNA ends (RACE) reagents (Clontech, USA) according to the manufacturer's instructions.

Based on the 454 cDNA library of *M. meretrix* (GenBank accession no. SRA021052), the expressed sequence tags of *BMP2* and *BMP7* genes were retrieved. The primers for 3'-RACE (MmBMP2-F1, MmBMP7-F1) and 5'-RACE (MmBMP2-R1, MmBMP7-R1) were designed (Table 1). The amplification was conducted as follows: 5 cycles of 94 °C for 30 s, 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 for 30 s, 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, Sichuan, China). The purified PCR products were cloned into the pMD18-T vector (Takara), which was transformed into DH5α cells (Tiangen) according to the 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 gene-specific primers (MmBMP2-F2 and MmBMP2-R2, MmBMP7-F2 and MmBMP7-R2) (Table 1) that were designed based on the above-mentioned cDNA sequences. PCR products were cloned and sequenced following the procedures described above.

Sequence analysis

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 sequences were analyzed using the SMART tool (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/). Protein transmembrane were analyzed using the TMHMM server (http://www.cbs.dtu.dk/services/TMHMM). Multiple alignments of *BMP2* and *BMP7* domains structures from *M. meretrix* and other species were performed using the ClustalW2 multiple alignment program (http://www.ebi.ac.uk/Tools/msa/clustalw2/). A phylogenetic tree was constructed using the neighbor-joining method with MEGA 6.0.

Quantitative expression analysis of MmBMP2 *and* MmBMP7

The expression levels of *MmBMP2* and *MmBMP7* at seven developmental stages (n > 500, three sets of samples for each stage) and in six tissues from adults (four sets of samples for each tissue) 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 method described above.

The primers for *MmBMP2* and *MmBMP7*, Real-2-F and Real-2-R, and Real-7-F and Real-7-R (Table 1) were designed. Primers 18S-F and 18S-R (Table 1) were used as the internal reference for qRT-PCR. PCR amplification was performed in a 20 μl reaction volume containing 10 μl of iTaq Universal SYBR Green Supermix (Bio-Rad, CA, USA), 7.2 μl of 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 20 s, 40 cycles of 94 °C for 3 s, 60 °C for 15 s, and 72 °C for 10 s. All qRT-PCR analyses were performed using three biological replicates, and negative controls were run with each primer pair.

The results of qRT-PCR analysis were based on the CT values of the PCR products, and the expression levels of *MmBMP2* and *MmBMP7* were analyzed using the comparative CT method. Statistical analysis was performed using SPSS 20.0 (Chicago, IL, USA). Differences in relative *MmBMP2* and *MmBMP7* mRNA expression levels among different developmental stages and different adult tissues were compared by one-way analysis of variance (ANOVA). Multiple comparisons were conducted using the Student-Newman-Keuls test. Differences were considered significant at $P < 0.05$ and extremely significant at $P < 0.01$.

SNP identification and association analysis

RNA samples were extracted from all 263 clams. Based on the cDNA sequences of *MmBMP2* and *MmBMP7*, six pairs of primers (2-F3, 2-R3, 2-F4, 2-R4, 2-F5, 2-R5, 7-F3, 7-R3, 7-F4, 7-R4, 7-F5 and 7-R5) were selected (Table 1). PCR amplification was performed in 50 μl reaction volumes containing 2 μl of template cDNA, 19 μl of dH2O, 25 μl of PCR Mix, and 2 μl of each primer. The PCR conditions were as follows: 94 °C for 3 min; 35 cycles at 94 °C for 30 s, 58 °C for 1 min, and 72 °C for 2 min; and a final extension at 72 °C for 5 min. 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 Sangon Biotech (Shanghai, China) for sequencing in both directions.

The sequences were aligned using MEGA6.0 software. Mutation sites were named according to the position of the initiation codon. Total weight, soft weight, shell width, shell length, shell height, and shell weight of the clams were analyzed by one-way ANOVA using SPSS 20.0. The association of SNPs with growth traits in the two populations was 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

The full-length *MmBMP2* cDNA was 2641 bp (GenBank accession no. KP250876) and contained a 1395-bp open reading frame (ORF) encoding 464 amino acids. The calculated molecular mass of the deduced mature MmBMP2 was 52.906 kDa, and the theoretical isoelectric point was 9.33. Analysis with SignalP revealed that the deduced amino acid sequence contained a signal peptide of 25 amino acids (MTPSYRVSILVLAVLLSELITSTYT). The full-length *MmBMP7* cDNA was 3161 bp (GenBank accession no. KP250875) and contained a 1254-bp ORF encoding 418 amino acids. The calculated molecular mass of the deduced mature MmBMP7 was 47.54 kDa, and the theoretical isoelectric point was 7.81. Analysis with TMHMM showed that the MmBMP7 protein contained a potential transmembrane domain that was located between amino acid residues 12 and 31. SMART analysis showed that the deduced amino acid sequence of them both contained a TGF-β superfamily domain (Table 2).

A phylogenetic tree was constructed using the neighbor-joining method and the deduced amino acid sequences of *MmBMP2* and *MmBMP7* and BMPs from some selected animals available in the NCBI database (Fig. 1). The tree obtained showed that *BMP2* and *BMP7* were divided into two major groups. One group contained all mollusks, whereas the other group contained vertebrates. In the *BMP2* mollusk group, *Azumapecten farreri* clustered with

Table 2 Comparison of cDNA sequence between *MmBMP2* and *MmBMP7*

cDNA sequence information	*MmBMP2*	*MmBMP7*
full-length	2641bp	3161bp
ORF	1395bp	1257bp
number of amino acids	464aa	418aa
5'UTR	546bp	431bp
3'UTR	703bp	1476bp
protein molecular mass	52.906kDa	47.54kDa
TGF-β superfamily domain	363Cys-464Arg	317Cys-418His

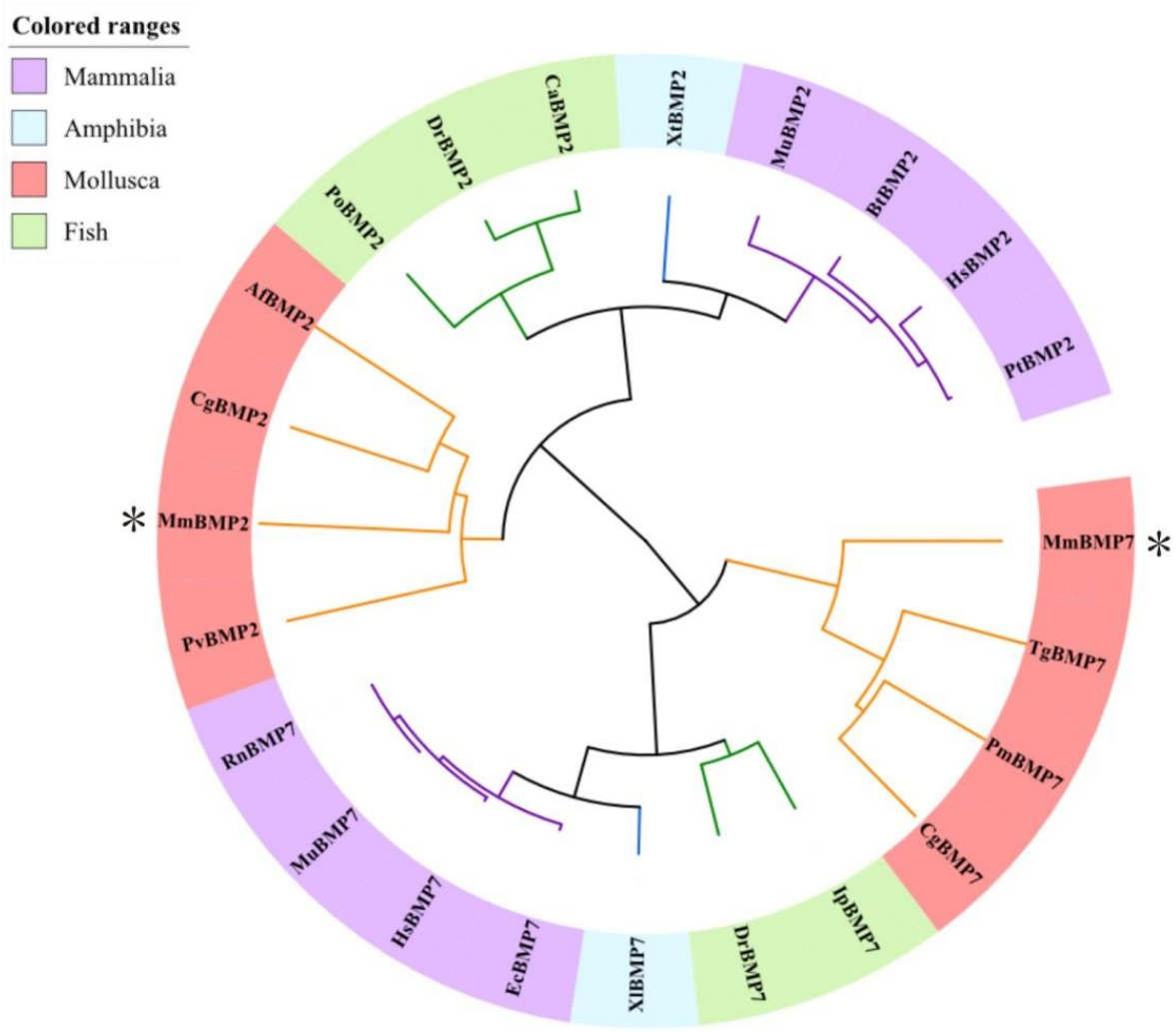

Fig. 1 Neighbor-joining phylogenetic tree of *BMP2* and *BMP7* between *M. meretrix* and other selected species using MEGA6.0 software. The abbreviation and the GenBank accession numbers used to construct phylogenetic tree are given in Table 3

Table 3 Species and GenBank accession numbers of BMPs used for multiple alignment and phylogenetic analysis

Species	Abbreviation	GenBank no.	Species	Abbreviation	GenBank no.
Meretrix meretrix	*MmBMP2*	ALG64479	*Meretrix meretrix*	*MmBMP7*	ALG64478
Azumapecten farreri	*AfBMP2*	AGF68558.1	*Tegillarca granosa*	*TgBMP7*	AFP57673.1
Crassostrea gigas	*CgBMP2*	EKC18750.1	*Crassostrea gigas*	*CgBMP7*	EKC34211.1
Patella vulgata	*PvBMP2*	AAM33143.1	*Pinctada martensii*	*PmBMP7*	AGS32053.1
Carassius auratus	*CaBMP2*	BAN17326.1	*Danio rerio*	*DrBMP7*	AAF17558.1
Danio rerio	*DrBMP2*	AAC25595.1	*Ictalurus punctatus*	*IpBMP7*	AHH42433.1
Paralichthys olivaceus	*PoBMP2*	BAD16743.1	*Xenopus laevis*	*XlBMP7*	AAI08478.1
Xenopus tropicalis	*XtBMP2*	AAI70888.1	*Rattus norvegicus*	*RnBMP7*	EDL85136.1
Bos taurus	*BtBMP2*	AAI42130.1	*Equus caballus*	*EcBMP7*	ADK93983.1
Mus musculus	*MuBMP2*	EDL28371.1	*Mus musculus*	*MuBMP7*	EDL06610.1
Pan troglodytes	*PtBMP2*	JAA30020.1	*Homo sapiens*	*HsBMP7*	AAH08584.1
Homo sapiens	*HsBMP2*	ACV32596.1			

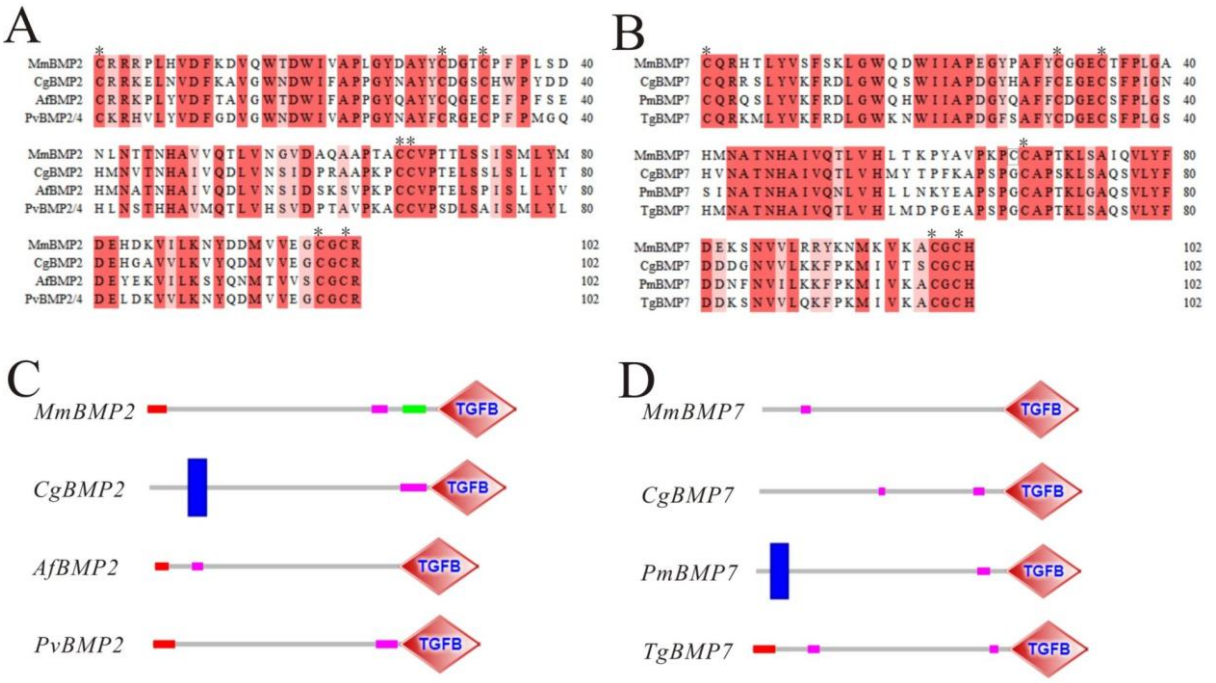

Fig. 2 (A) Multiple alignment of the *MmBMP2* TGF-β domain structures peptide sequence with other mollusc species, (B) Multiple alignment of the *MmBMP7* TGF-β domain structures peptide sequence with other mollusc species. Identities are shaded very light red and similarities are shaded lightest red. The * indicate the shared cysteine residues in compared species, the letter box is additional cysteine residue in *MmBMP7*. (C) Prediction of *BMP2* TGF-β domains in mollusc species, (D) Prediction of *BMP7* TGF-β domains in mollusc species. Blue boxes indicate transmembrane region, green box indicates coiled coil region, and pink and red boxes mean low complexity region

Crassostrea gigas and then with *M. meretrix* and *P. vulgata*. In the *BMP7* mollusk group, *P. martensii* first clustered with *C. gigas* and then with *Tegillarca granosa* and *M. meretrix*. *MmBMP2* shared the highest identity (53.1%) with *A. farreri*, and *MmBMP7* shared the highest identity (58.7%) with *P. martensii*. For the mature BMP2 protein of the compared mollusks, the TGF-β domain structure peptide sequences shared seven cysteine residues and the spacing between cysteine residues was well conserved (Fig. 2). The BMP7 protein had seven cysteine residues in *M. meretrix* but six common cysteines in *T. granosa*, *P. martensii* and *C. gigas* (Fig. 2).

Quantitative expression analysis

The expression patterns of *MmBMP2* and *MmBMP7* in the embryos/larvae and adult tissues were analyzed using qRT-PCR. In adult clams, the expression of *MmBMP2* in the mantle was extremely significantly higher than that in the other five tissues ($p < 0.01$) (Fig. 3), suggesting that it might be involved in shell growth. Among the seven developmental stages, *MmBMP2* transcript levels were the highest in juvenile clams ($p < 0.05$), followed by unfertilized mature eggs, blastulae, gastrulae, and eyespot larvae, with the lowest expression in 4-cell embryos and trochophores (Fig. 3). This result may be related to shell formation in the juvenile clams. In adults, *MmBMP7* expression was also significantly higher in the mantle than in the other five tissues ($p < 0.05$) (Fig. 3). The mantle is a shell-forming tissue, and its high expression of BMPs is in agreement with its role in bone formation. Among the developmental stages, *MmBMP7* expression was significantly higher in unfertilized mature eggs, blastulae, gastrulae, trochophores, and juvenile clams than in the other two stages ($p < 0.05$) (Fig. 3), which suggests that it may be involved in morphogenesis and formation of the hard shell. *MmBMP2* and *MmBMP7* were expressed to different degrees during development, which indicates that BMPs might play a role in ontogeny.

Association of SNPs in MmBMP2 *and* MmBMP7 *with growth traits*

The SNP analysis revealed that eight *MmBMP2* SNPs (T134C, A476G, C570T, T585C, A715T, A726G, A732T, and T786C) were present in the JS population, of which three (T585C, A726G, and A732T) were associated with growth traits. Five SNPs (G390A, C570T, A726G, A732T, and T786C) in exons of *MmBMP2* were identified in the WL2 population, including two (A726G and A732T) that were potentially associated with growth traits. JS individuals with genotype TC at position T585C grew faster than those with the TT genotype in terms of shell length, shell width, and shell height ($p < 0.05$).

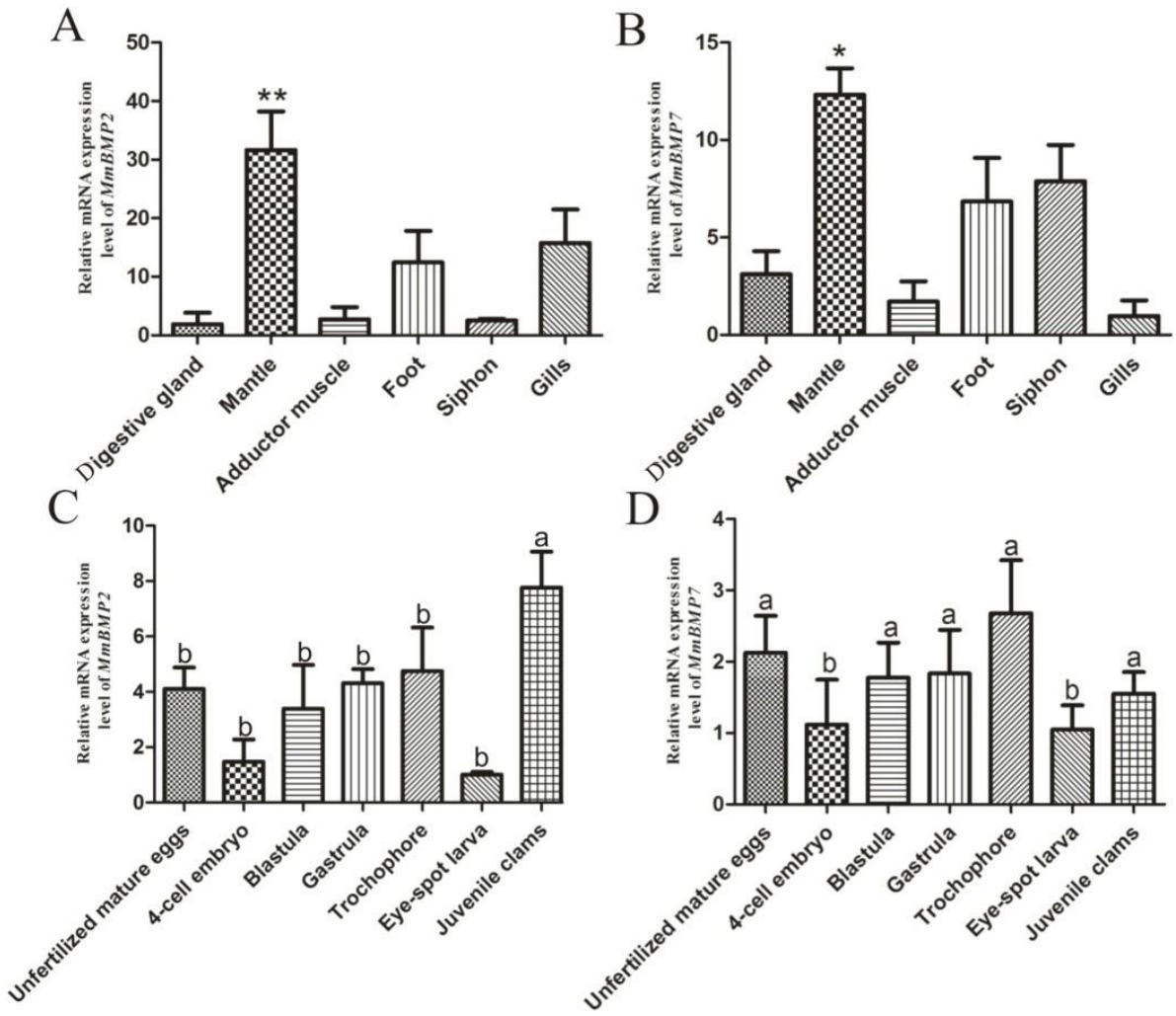

Fig. 3 Analysis of expression differences of *MmBMP2* and *MmBMP7*. (A) Analysis of expression difference of *MmBMP2* in six tissues, (B) Analysis of expression difference of *MmBMP7* in six tissues, (C) Analysis of expression difference of *MmBMP2* in seven developmental stages, (D) Analysis of expression difference of *MmBMP7* in seven developmental stages. The same letters indicate no difference in the level of expression, whereas different letters indicate significant differences in expression levels among various developmental stages ($p < 0.05$). **$p < 0.01$, *$p < 0.05$

Clams with genotype AG at A726G exhibited faster growth in all growth traits except shell weight compared with AA individuals ($p < 0.05$). The SNP A732T AT genotype was also associated with significantly higher growth traits than the AA genotype ($p < 0.05$) (Table 4). WL2 individuals with genotype AG at position A726G had significantly better growth traits compared with genotype AA ($p < 0.05$). Multiple comparison analysis showed that individuals with the A732T AT genotype grew faster than those with the AA genotype in all growth traits measured ($p < 0.05$) (Table 4).

Comparison of the loci between the JS and WL2 groups revealed that the populations shared two SNPs at A726G and A732T in the *MmBMP2* coding region. Association analysis showed that individuals with the AG genotype at locus 726 had significantly higher growth traits than those with the AA genotype in both groups ($p < 0.05$). Additionally, individuals with the AT genotype at locus 732 had significantly higher growth traits than those with the AA genotype in both groups ($p < 0.05$). SNP A732T was significantly associated with better outcomes for all growth traits tested in both the JS and WL2 populations ($p < 0.05$).

The SNP analysis revealed the presence of three *MmBMP7* SNPs (C32T, C126G, and G144A) in the JS population, including two (C32T and G144A) associated with growth traits. JS individuals with genotype CT at position C32T grew faster than those with the CC genotype in terms of shell height, shell weight, soft weight, and shell weight ($p < 0.05$). Clams with genotype GA at G144A exhibited faster growth in terms of shell width,

Table 4 Correlation analysis of SNPs in the *MmBMP2* with growth traits in two groups

Group	Locus	Genotype	Sample	Percent (%)	Shell length (cm)	Shell width (cm)	Shell height (cm)	Total weight (g)	Soft weight (g)	Shell weight (g)
Jiangsu	585 T>C	TT	121	88.97	**4.11±0.61^{a}**	**2.05±0.32^{a}**	**3.32±0.48^{a}**	17.28±6.68	8.38±3.37	8.89±3.42
		TC	15	11.03	**4.52±0.57^{b}**	**2.27±0.33^{b}**	**3.65±0.47^{b}**	20.51±5.86	9.83±2.70	10.68±3.39
		AA	63	46.32	**3.97±0.62^{a}**	**1.97±0.33^{a}**	**3.20±0.48^{a}**	**15.50±6.32^{a}**	**7.49±3.25^{a}**	**8.01±3.16^{a}**
	726 A>G	AG	50	36.76	**4.26±0.60^{b}**	**2.14±0.32^{b}**	**3.42±0.48^{b}**	**18.35±6.21^{b}**	**8.92±3.05^{b}**	**9.43±3.29^{b}**
		GG	23	16.91	**4.47±0.45^{b}**	**2.22±0.24^{b}**	**3.64±0.40^{b}**	**21.92±6.34^{c}**	**10.61±3.08**	**11.31±3.45^{c}**
	732 A>T	AA	70	51.47	**4.01±0.63^{a}**	**2.00±0.34^{a}**	**3.24±0.49^{a}**	**15.91±6.39^{a}**	**7.68±3.25^{a}**	**8.23±3.22^{a}**
		TA	50	36.76	**4.29±0.59^{b}**	**2.15±0.32^{b}**	**3.44±0.47^{b}**	**18.95±6.47^{b}**	**9.27±3.19^{b}**	**9.68±3.43^{b}**
		TT	16	11.76	**4.40±0.47^{b}**	2.17±0.24	**3.60±0.42^{b}**	**21.04±6.52^{b}**	**10.05±3.20**	**10.99±3.50^{b}**
Wanli 2	726 A>G	AA	41	32.28	**35.95±2.56^{a}**	**17.28±1.34^{a}**	**30.04±2.23^{a}**	**11.35±2.70^{a}**	**5.57±1.31^{a}**	5.78±1.44
		AG	59	46.46	**37.36±3.14^{b}**	**17.96±1.68^{b}**	**31.07±2.51^{b}**	**12.71±3.43^{b}**	**6.30±1.60^{b}**	6.42±1.88
		GG	27	21.26	36.18±3.27	17.34±1.59	30.29±2.77	11.64±3.60	5.70±1.67	5.94±1.96
	732 A>T	AA	51	40.16	**36.25±2.63^{a}**	**17.40±1.38^{a}**	30.28±2.32	**11.56±2.83^{a}**	**5.70±1.33^{a}**	**5.86±1.55^{a}**
		AT	52	40.94	**37.51±3.22^{b}**	**18.09±1.70^{b}**	**31.17±2.52^{a}**	**12.95±3.51^{b}**	**6.38±1.64^{b}**	**6.57±1.91^{b}**
		TT	24	18.90	**35.70±3.11^{a}**	**17.05±1.46^{a}**	**29.94±2.69^{b}**	**11.15±3.36^{a}**	**5.50±1.62^{a}**	**5.65±1.77^{a}**

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

total weight, soft weight, and shell weight compared with GG individuals ($p < 0.05$) (Table 5). Three SNPs (C32T, C126G, and G306A) were found in exons of *MmBMP7* from the WL2 population, but none was associated with growth traits.

Discussion

In vertebrates, every mature BMP shares seven conserved cysteines (Bragdon *et al.*, 2011), six of which build a cystine knot by forming three intrachain disulfide bonds to resist heat, denaturants, and extreme pH levels. The remaining cysteine residue forms an interchain disulfide bond that links two monomers into active hetero- or homodimers (Kingsley, 1994). This unique cysteine is absent in some mollusks (*T. granosa*, *C. gigas*, and *P. martensii*) (Yan *et al.*, 2014). This absence raises the possibility that *BMP7* in some mollusks might not form dimers or might form noncovalently linked dimers between the subunits, which could be dynamic and subject to regulation. However, both *BMP2* and *BMP7* in *M. meretrix* contained all seven cysteine residues.

Gene expression analysis showed that expression of *MmBMP2* and *MmBMP7* was the highest in the mantle, which suggests that they might be involved in shell growth in *M. meretrix*. In *P. martensii*, expression of *BMP2* and *BMP7* was also the highest in the mantle (Miyashita *et al.*, 2008; Yan *et al.*, 2014). Previous studies showed that BMPs of mollusks may play an important role in formation of the shell boundary. Using northern blot and in situ hybridization assays, Miyashita *et al.* (2008) found that expression of *BMP2* occurred predominantly in the mantle, suggesting that *BMP2* played a key role in nacre formation. Zhou *et al.* (2010) reported that *P. martensii BMP2* was involved in repair after shell incision. Furthermore, the expression of *BMP7b* increased significantly 24 h after shell incision in *P. martensii*. In the current study, both *MmBMP7* and *MmBMP2* were expressed in all six tissues tested, which suggests that they may be involved in a wide range of biological processes. For example, mGDF, which has high similarity to BMP2, in *C. gigas*, along with other growth factors, is involved in differentiation of the digestive gland from stem cells into functional cells (Lelong *et al.*, 2010).

Expression of *MmBMP2* and *MmBMP7* was detected at every embryonic stage of *M. meretrix* during development, suggesting that they may be involved in differentiation of early tissues and organs. Bivalves have a common process of shell formation that occurs at the very early stage of larval development. During the gastrula stage, the shell formative region sinks, evaginates and enlarges until

Table 5 Correlation analysis of SNPs in the *MmBMP7* with growth traits in two groups

Group	Locus	Genotype	Sample	Percent (%)	Shell length (cm)	Shell width (cm)	Shell height (cm)	Total weight (g)	Soft weight (g)	Shell weight (g)
Jiangsu	32 C>T	CC	90	66.67	4.12±0.60	2.06±0.32	**3.33±0.47^{a}**	**17.03±6.36^{a}**	**8.19±3.14^{a}**	**8.83±3.34^{a}**
		CT	27	20.00	4.42±0.53	2.21±0.28	**3.58±0.46^{b}**	**20.98±6.08^{b}**	**10.43±3.0^{b}**	**10.55±3.20^{b}**
		TT	18	13.33	4.08±0.69	2.05±0.37	3.37±0.49	**16.67±7.34^{a}**	**7.97±3.69^{a}**	**8.86±3.68^{a}**
	144 G>A	GG	78	57.78	**4.08±0.60^{a}**	**2.03±0.31^{a}**	**3.29±0.47^{a}**	**16.56±6.25^{a}**	**8.03±3.17^{a}**	**8.52±3.21^{a}**
		GA	38	28.15	**4.33±0.59^{b}**	**2.15±0.31^{b}**	**3.49±0.48^{b}**	**19.91±6.91^{b}**	**9.63±3.41^{b}**	**10.28±3.65^{b}**
		AA	19	14.07	4.31±0.61	**2.20±0.37^{b}**	3.49±0.52	18.48±6.42	8.93±3.23	8.82±3.34

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

the whole embryo is encapsulated (Kin *et al.*, 2009). Afterward, the conchiolin is secreted and calcified, and simultaneously the shell forms a hinged part in the back and divides into two pieces until bivalve stage I (prodissoconch I) is reached. During formation of the velum, the phase II embryo shell (prodissoconch II) continues to form (Kakoi *et al.*, 2008). Miyazaki *et al.* (2010) reported that the shell of *P. martensii* begins to form prodissoconch I at the trochophore stage and then forms prodissoconch II at the D-shaped larvae stage. In the current study, *MmBMP2* and *MmBMP7* were highly expressed at the trochophore stage, which suggests that they might play an important role in the formation of the protegulum. Moreover, expression of *MmBMP2* was the highest in the juvenile clam stage, which indicates that organs and shells grew fast and developed rapidly at this stage.

Molecular marker-assisted breeding can be used to select new varieties with certain traits at the molecular level and thereby greatly improve breeding efficiency. SNPs, as the third generation of molecular markers, are a powerful tool for assessing genetic diversity and conducting association analysis. In marine mollusks, association analysis between SNPs of candidate genes and economic traits such as growth and stress tolerance has been conducted. For example, Guo *et al.* (2012) identified a growth-related SNP locus in the 3'UTR region of TGF-β receptor type 1 in *Chlamys farreri*. In the exon region of myostatin in *Argopecten irradians*, two SNP loci associated with growth were identified (Guo *et al.*, 2011). Bao *et al.* (2013) characterized three immune-related SNP loci in the hemoglobin of *T. granosa*. In the current study, we identified three SNPs potentially associated with growth traits in *MmBMP2* of the JS population and two in the WL2 population, and two of them were common to the two populations. For *MmBMP7*, we found two SNPs potentially associated with growth traits in the JS population and none in the WL2 population. The individuals with the AG genotype of SNP A726G grew significantly faster than those with the AA genotype, and individuals with the AT genotype of SNP A732T grew significantly faster than those with the AA genotype in both populations. Furthermore, these two SNPs were located within the coding region of *MmBMP2* without amino acid changes (A726G encoding threonine, A732T encoding valine). Many studies have shown that synonymous mutations regulate gene transcription and translation. Greenwood and Kelsoe (2003) found that the synonymous mutation of many genes in promoters and introns could affect transcriptional efficiency. Kimchi-Sarfaty *et al.* (2007) reported that the synonymous mutation of the *MDR1* gene could change the spatial structure of the protein. Capon *et al.* (2004) demonstrated that synonymous mutations of the corneodesmosin gene could improve the stability of mRNA molecules. Therefore, we hypothesize that clams with the A726G AG genotype or the A732T AT genotype are favorable for breeding. Furthermore, the *MmBMP2* SNP could influence growth performance and may be a suitable marker for marker-assisted selection in these *M. meretrix* populations.

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